INVESTIGATION INTO THE MECHANISMS OF BETA-CELL SPECIFIC OVEREXPRESSION OF PEROXISOME PROLIFERATOR ACTIVATED RECEPTORS IN OBESITY-INDUCED TYPE 2 DIABETES

by

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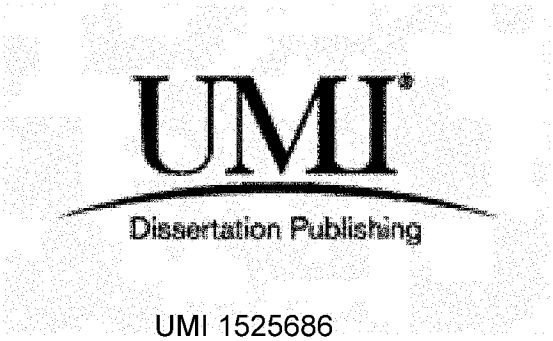

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Abstract

In obesity, adipose tissue expandability and triglyceride storage is challenged resulting in lipid accumulation in ectopic tissues, including pancreatic β-cells. It is hypothesized that accumulation of toxic lipid derivatives induces β-cell failure, causing dysregulated glucose homeostasis in obesity.

I am interested in understanding if manipulating expression of peroxisome proliferator activated receptors (PPARs), mediators of lipid sensing and metabolism, in β-cells during development of obesity will affect lipotoxic β-cell failure. Activation of endogenous PPARδ *in vitro* has been shown to protect against lipotoxic β-cell failure. I report that PPARδ overexpression in β-cells under lipotoxic conditions diminishes this protective effect. Furthermore, *in vivo* models of β-cell specific PPARγ2 overexpression exhibit impaired carbohydrate metabolism. I report that islets from these obese mice demonstrate reductions in lipids promoting signalling of insulin release and upregulation of genes regulating recruitment of fatty acids and lipid oxidation, which may explain carbohydrate metabolism impairments in these mice.

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LIST OF ABBREVIATIONS

ABCa1	ATP-binding cassette transporter 1
AOX	Alternative oxidase
cAMP	Cyclic adenosine monophosphate
CD36	Cluster of differentiation-36
Cer	Ceramide
CHOP	C/EBP homologous protein
CMV	Cytomegalovirus
CPT	Carnitine palmitoyl transferase
DAG	Diacylglycerol
DGAT	Diacylglycerol acyl transferase
DMSO	Dimethyl sulfoxide
dsAAV8	Doublestranded adeno-associated virus serotype 8
ECM	Extracellular matrix
ER	Endoplasmic reticulum
FAO	Fatty acid oxidation
FFA	Free fatty acid
eGFP	Enhanced green fluorescent protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GIPR	Gastric inhibitory polypeptide receptor
GLUT	Glucose transporter
GPR	G-protein coupled receptor
GusB	β-glucuronidase
HDL	High-density lipoproteins

HSL	Hormone sensitive lipase
IAPP	Islet amyloid polypeptide
iPLA2	Phospholipase A2
IRS	Insulin receptor substrate family
LCAD	Long chain acyl-CoA dehydrogenase
LC/MS	Liquid chromatography/Mass spectrometry
LPL	Lipoprotein lipase
LysoPC	Lysophosphatidylcholine
LysoPE	Lysophosphatidylethanolamine
MAG	Monoacylglycerol
NEFA	Non-esterified fatty acid
Npc1l1	Niemann-Pick C1-like 1
PC	Phosphatidylcholine
PDE3B	Phosphodiesterase 3B
PDK	Pyruvate dehydrogenase kinase
PDX1	Pancreatic duodenal homeobox 1
PE	Phosphatidylethanolamine
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1-α
PIP_2	Phosphatidyl inositol-4,5-biphosphate
PI-3-K	Phosphatidyl inositol 3-kinase
PI-3,4,5-P_3	Phosphatidyl inositol-3,4,5-triphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C

PKD	Protein kinase D
PPAR	Peroxisome proliferator activated receptor
PPRE	Peroxisome proliferator response element
PUMA	p53 upregulated modulator of apoptosis
RIP	Rat insulin promoter
ROS	Reactive oxygen species
Rpl19	60S ribosomal protein L19
SM	Sphingomyelin
SREBP1c	Sterol regulatory element-binding protein 1c
TAG	Triacylglycerol
TBP	TATA-Binding protein
TNF-α	Tumor necrosis factor-α
TXNIP	Thioredoxin interacting protein
TZD	Thiazolidinedione
T2D	Type 2 diabetes
UCP	Uncoupling protein
VLDL	Very low density lipoprotein
XBP1s	X-box binding protein-1 spliced

DEDICATION

The completion of this thesis would not have been possible without the amazing people in my life and I would like to take a moment to dedicate this achievement to some of those people.

First and foremost, I would like to recognize my dad and mom, Noel and Helen. You guys have made me the person I am today and I am truly thankful to have been blessed with such amazing parents. Your unwavering support in all aspects of my life have taught me that it doesn't matter what you do, as long as you can be happy in your life. I really could not have done this without either of you.

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Finally, I need to dedicate this work to my incredible group of friends. There are too many of you to name individually but please know that I am thinking of each and every one of you right now. It's been a long haul these last couple of years and I want to just finish by saying thank you to everyone who has helped me stay focussed and especially those that have motivated me to just finish writing this thesis in these final days.

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CHAPTER 1

Lipotoxic mechanisms in the pathogenesis of obesity-induced type 2 diabetes

1.1 Lipotoxicity

Recent estimates suggest that 347 million people worldwide are affected by type 2 diabetes (T2D) and that this figure will rise to 439 million by the year 2030 (Danaei et al., 2011; Shaw et al., 2010), placing an enormous strain on global healthcare systems. Prevalence of obesity exceeds these figures and has been established as the main risk factor for the development of T2D, with 80% of people diagnosed with T2D being overweight or obese (Smyth and Heron, 2006). T2D occurs when the regulatory actions of insulin are ineffective at target tissues or the mechanisms by which insulin is secreted from β-cells of the pancreas are impaired. These impairments result in dyslipidemia and the inability to maintain blood glucose levels within the normal physiological range in patients with the disease. Patients with T2D commonly demonstrate characteristics of the metabolic syndrome (Chavez and Summers, 2010; Cusi, 2010; Del Prato, 2009; Somwar et al., 2005), which is defined by the World Health Organization as elevated fasting blood glucose, insulin resistance, elevated blood pressure, elevated triglycerides or high-density lipoprotein (HDL) cholesterol, and central obesity with a body mass index greater than $30kg/m^2$ (Alberti and Zimmet, 1998; Stumvoll et al., 2005).

What remains unclear is whether impaired insulin action in T2D arises first, leading to dyslipidemia and associated symptoms of the metabolic syndrome, or if symptoms associated with the metabolic syndrome arise first, causing impairments in insulin action and the progression of T2D. One of the prevailing hypotheses linking obesity to progression of T2D is lipotoxicity, where limitations of adipose tissue expandability result in the inability of adipose tissue to accommodate the chronic oversupply of energy in obese patients (Ahima, 2006; Chavez and Summers, 2010; Gray and Vidal-Puig, 2007; Kershaw and Flier, 2004).

Adipose tissue is a complex endocrine organ composed of adipocytes, connective tissue, nervous tissue, vascular cells, and immune cells (Abedini and Shoelson, 2007; Ahima, 2006; Kershaw and Flier, 2004). Adipose tissue plays an important role in the regulation of energy homeostasis through secretion of regulatory molecules called adipokines, such as leptin and adiponectin, that play a role in appetite regulation and act as insulin sensitizers respectively (Abedini and Shoelson, 2007; Ahima, 2006; Myers et al., 2008). In addition to its endocrine role, adipose tissue also acts as an energy reserve in which excess fuel is stored for later use as triglycerides (Gray and Vidal-Puig, 2007; Virtue and Vidal-Puig, 2010).

In order for adipose tissue to store excess lipids, preadipocytes must first undergo differentiation into mature adipocytes (Lefterova and Lazar, 2009; MacDougald and Mandrup, 2002). In instances where there is an influx of nutrients, mature adipocytes will recruit pre-adipocytes and transcriptional regulators, such as peroxisome proliferator activated receptor-γ (PPARγ), will facilitate the transformation of preadipocytes into their mature cell type (Barak et al., 1999; Tontonoz et al., 1994).

Under conditions of chronic overnutrition it has been suggested that adipose tissue is unable to expand to an infinite capacity (Gray and Vidal-Puig, 2007) resulting in a deficit of storage space. Some studies have suggested that physical space for expansion in cell proliferation may be responsible for this finite limit of storage in adipose tissue. When adipocytes proliferate, there is a requirement for the extracellular matrix (ECM) surrounding the cells to be remodelled to make room for the newly formed adipocytes. For this to occur, proteases must be secreted by the cells to break down the ECM, which is a complex process that requires time (Khan et al., 2009). The delay in ECM remodelling places physical stress on the hypertrophic adipocytes resulting in rupture of the adipocytes and the release of lipids which causes an immune response and resulting recruitment of macrophages (Shi et al.,

2006). Macrophage recruitment begins a cycle in which adipose tissue expandability is thwarted and mature adipocytes are unable to recruit additional preadipocytes. Macrophage-dependent inhibition of preadipocyte differentiation results in a decrease in mature adipocyte formation and an even larger deficit of lipid storage space, causing an even further increase in circulating lipid levels and a further increase in the immune response and production of macrophages (Khan et al., 2009; Shi et al., 2006). The result is entry of excess lipids into the circulatory system and storage of lipids in ectopic tissues.

The term lipotoxicity has been used to describe the process of impaired cellular function and apoptosis due to the accumulation of lipid species in non-adipose tissue (Chavez and Summers, 2010; Imrie et al., 2010; Schrauwen et al., 2010; Unger et al., 2010). A proposed mechanism includes the accumulation of non-esterified fatty acids resulting in increased production of lipid species such as ceramides and diacylglycerols (Boden, 1997; Kahn et al., 2006; Reaven et al., 1988), lipid species that can promote apoptosis as well as mediate an immune response and production of cytokines (Dbaibo et al., 2001; de la Monte et al., 2010; Lecour et al., 2006; Summers, 2006). Taken together, these outcomes promote activation of apoptotic pathways and induce oxidative stress in cells resulting in impairments in endoplasmic reticulum (ER) and mitochondrial function (Carobbio et al., 2011; Chavez and Summers, 2010; Kusminski et al., 2009; Schrauwen et al., 2010; Summers, 2006). In obesity, it has been demonstrated that accumulation of toxic lipid species in non-adipose tissues, such as skeletal muscle or the liver, may be responsible for impairments in insulin sensitivity at these sites, potentiating the progression towards dyslipidemia and hyperglycemia in T2D (Chavez and Summers, 2010; Summers, 2006). Another site of ectopic lipid deposition is the pancreatic islet itself and thus lipotoxic β-cell failure is a proposed mechanism in obesity induced T2D.

To understand obesity-induced pathogenesis of T2D, it is first useful to understand the mechanisms by which the pancreas regulates glucose and lipid metabolism. Insulin released from β-cells of the pancreas travels through the blood and binds insulin receptors on target tissues in the periphery. Upon binding of insulin to its receptor, a self-phosphphorylating tyrosine kinase, a series of phosphorylation events occurs within the cytoplasm. An example of this phosphorylation cascade in glucose uptake is the activation of the insulin receptor substrate family-1 (IRS-1), which contains phosphatidyl inositol 3-kinase (PI-3-K) (Boron and Boulpaep, 2005). The active form of PI-3-K then causes an activation of phosphatidyl inositol-4,5,-biphosphate (PIP_2) to form phosphatidyl inositol-3,4,5-triphosphate (PI-3,4,5-P_3), which will in turn cause the activation of phosphatidyl inositol-dependent kinase (PDK). A classic example of how insulin-activated PDK causes uptake of glucose from the blood is via the subsequent activation of protein kinase B (PKB) which will act on the GLUT4 transporter causing its insertion into the plasma membrane so that glucose may be transported into the cell (Boron and Boulpaep, 2005). Insulin receptor activation promotes many anabolic glucose and lipid pathways while inhibiting catabolic pathways. Insulin also plays a considerable role in cell growth and cell survival through increasing DNA replication and protein synthesis (Costa et al., 2012; Zheng et al., 1997).

1.2 Lipotoxicity and peripheral insulin resistance in type 2 diabetes

Ectopic deposition of toxic lipid species in obesity inhibits insulin signalling contributing to insulin resistance and impaired glucose tolerance in T2D. The effects of insulin insensitivity can be seen in many tissue types throughout the body, including adipose, liver, and skeletal muscle.

Insulin insensitivity in adipose tissue disrupts the activation of lipogenic pathways (Lewis et al., 2002) and inhibition of lipolytic pathways (Holm et al., 2000) normally observed during the fed state. As seen in Figure 1.1, presence of insulin normally activates lipogenesis by increasing the activity of lipoprotein lipase (LPL), causing an increase in free fatty acid (FFA) production for transport into adipocytes for storage. Furthermore, once inside the adipocytes, conversion of FFAs to triacylglycerols (TAGs) will be promoted by insulin's activation of diacylglycerol acyl transferase (DGAT). However, as a result of insulin insensitivity LPL remains inactivated causing a decrease in FFA cleavage resulting in elevated levels of TAG-rich very low density lipoproteins (VLDLs) and chylomicrons. Furthermore, due to the inability to activate DGAT, insulin insensitivity prevents the formation of TAGs from diacylglycerols (DAGs) and FFA precursors (Lewis et al., 2002; Liu et al., 2007). The net result of inactive DGAT in adipose tissue is the accumulation of toxic lipid species further exacerbating the lipotoxic state in adipocytes (Cusi, 2010).

In adipose tissue during the fed state, insulin also acts as an inhibitor of lipolysis to prevent the mobilization of FFAs from TAG stores. This inhibitory effect is achieved through binding of the insulin receptor on adipocytes, causing the phosphorylation of phosphodiesterase 3B (PDE3B) (Fig. 1.2), ultimately decreasing the activity of hormone sensitive lipase (HSL). However, under lipotoxic conditions when insulin binding is disrupted, HSL remains in its active form, allowing for uncontrolled hydrolysis of FFAs and glycerol from TAGs or perilipins (Holm et al., 2000). Elevated levels of circulating FFAs will be transported to the liver causing an increase in gluconeogenesis and elevations in blood glucose levels (Chen et al., 1999) further contributing to the impaired ability to regulate blood glucose levels in T2D.

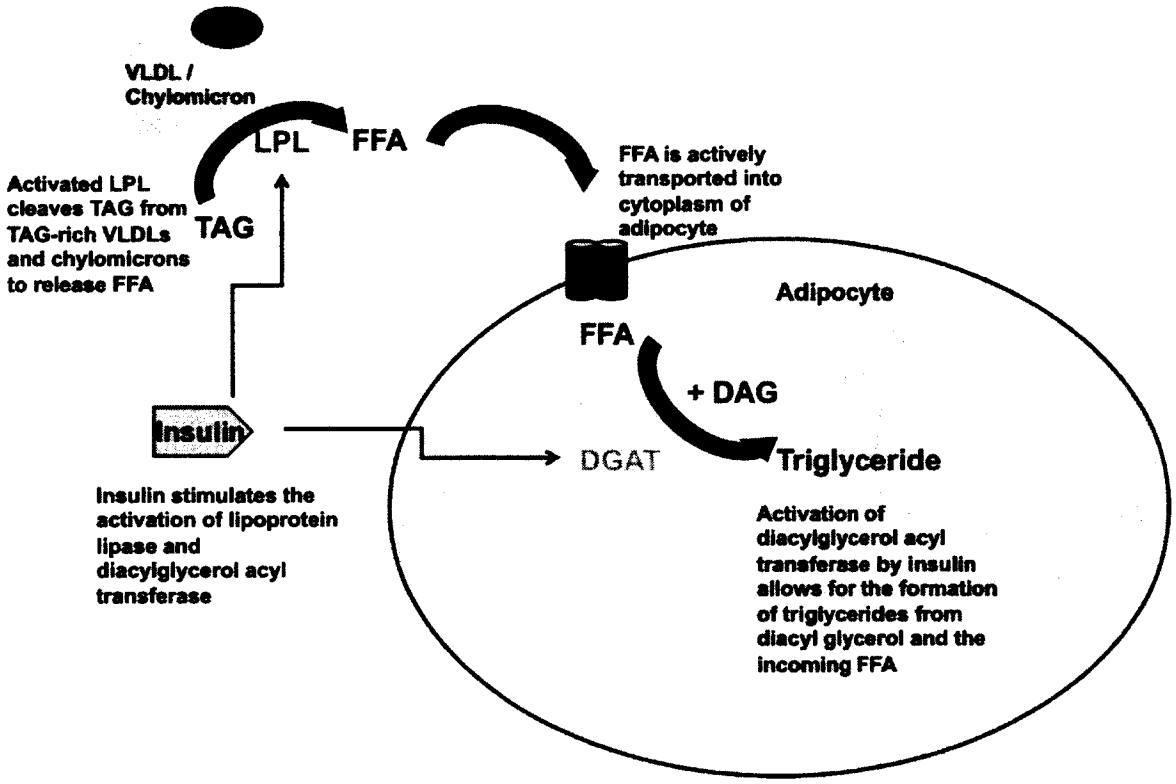

Figure 1.1 Mechanisms of insulin activation on lipogenesis in adipocytes. Insulin will cause activation of lipoprotein lipase, allowing FFAs to be cleaved from VLDLs and chylomicrons arriving from the periphery. FFAs can then enter the adipocyte where they will combine with DAGs to become triglycerides. This conversion from FFAs and DAGs is achieved by insulin's activation of diacylglycerol acyl transferase.

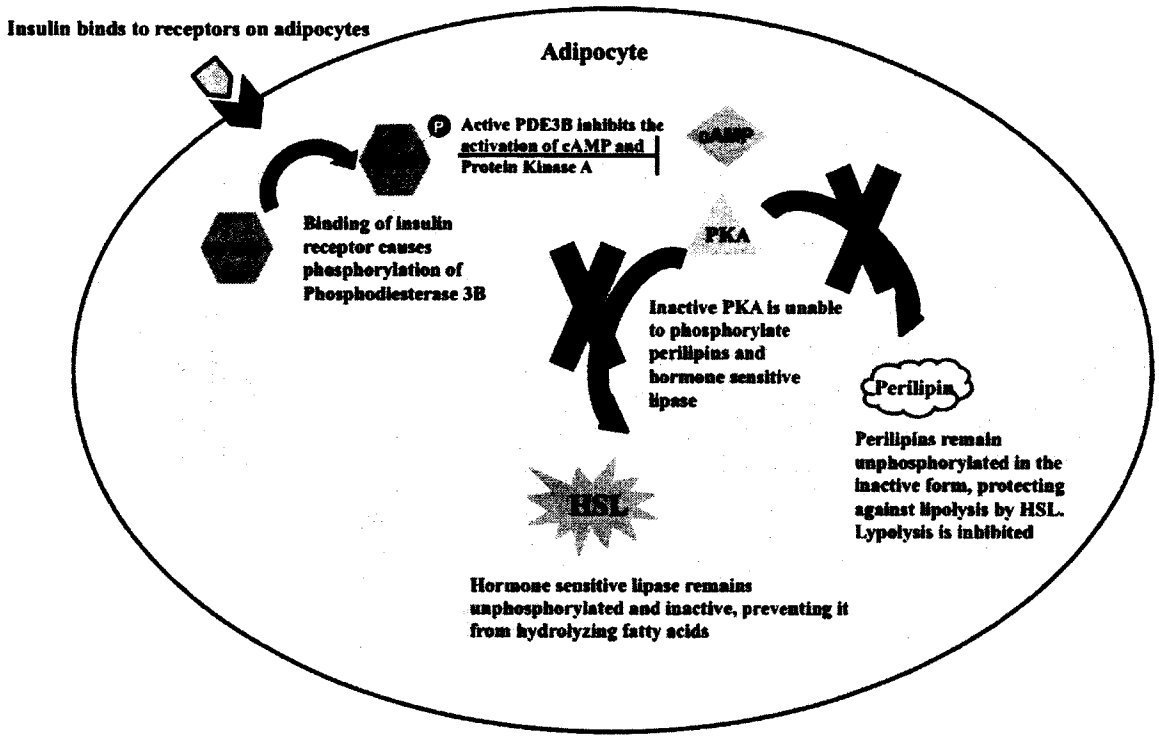

Figure 1.2 Mechanisms of insulin inhibitory action on lipolysis in adipocytes. Binding of insulin receptors causes phosphorylation of PDE3B which can then inhibit the activation of cAMP and PKA. Due to inactive PKA and cAMP, HSL and perilipins remain unphosphorylated in their inactive form. The net result of this inactivation is inhibited lipolysis and a decrease in the hydrolysis of TAGs.

The liver is also an important organ regulated by insulin signalling. It has been demonstrated that lipid accumulation in hepatocytes due to high fat feeding causes a decrease in the expression of Insulin Receptor Substrate-2 (IRS-2) as well as increased markers of inflammation, resulting in insulin resistance and impaired carbohydrate metabolism (Xing et al., 2011). In the fed state when the supply of energy is high, insulin promotes the storage and utilization of glucose and lipids. When insulin sensitivity is impaired, insulin is unable to up regulate the transcription of glucokinase and glycogen synthase (Fig. 1.3) resulting in the inability to store glucose in the form of glycogen (Boron and Boulpaep, 2005). Furthermore, insulin insensitivity will result in uninhibited activity of glycogen phosphorylase and glucose-6-phosphatase allowing for the uncontrolled breakdown of glycogen despite already elevated blood glucose levels. Insulin insensitivity also prevents glycolysis and the oxidation of pyruvate through impairments in the activity of glucokinase, phosphofructokinase, and pyruvate dehydrogenase resulting in the inability to break down glucose and the conversion to pyruvate. Inhibition of gluconeogenesis by the liver also becomes disrupted in cases of insulin insensitivity by preventing the inhibition of phosphoenolpyruvate carboxykinase, fructose-1,6-bisphosphatase, and glucose-6-phosphatase (Boron and Boulpaep, 2005) allowing for synthesis of glucose from pyruvate and further contribution to the chronic elevations in blood glucose levels in patients with T2D.

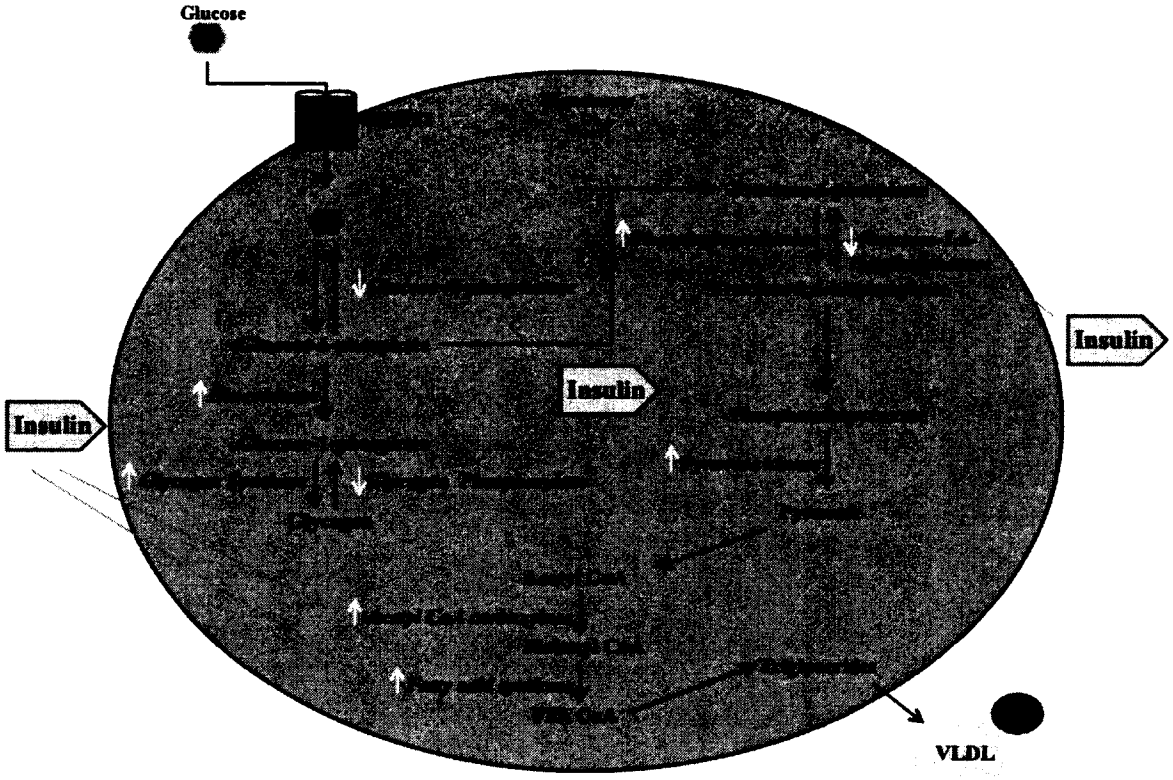

Figure 1.3 Mechanisms of insulin action in liver cells. In the liver, insulin plays a regulatory role in both glucose and lipid metabolism. Insulin promotes the utilization of glucose through activation of enzymes involved in glycolysis and the storage of glucose through activation of enzymes involved in glycogen synthesis. Insulin also prevents the *de novo* synthesis of glucose through inhibition of enzymes involved in glycogenolysis and gluconeogenesis. Regulation of lipid metabolism in the liver is achieved through activation of enzymes promoting the conversion of FFAs to TAGs for transport to adipose tissue in VLDLs.

In addition to its role in glucose regulation, insulin is an important regulator of lipid metabolism in the liver. In the fed state, this regulation of lipid metabolism ultimately results in the formation of TAGs from FFAs which are then stored in the form of VLDLs for transport in the blood plasma to adipose tissue (Boron and Boulpaep, 2005). Impairments in insulin sensitivity prevent the activity of acetyl CoA carboxylase and fatty acid synthase, disrupting the conversion of acetyl CoA to FFA CoA. By impairing the formation of FFA CoA, insulin insensitivity ultimately causes the accumulation of FFAs in hepatocytes and prevents their transport to adipose tissue for storage.

In skeletal muscle, it has been suggested that mitochondrial dysfunction in muscle tissue may play a role in the development of insulin resistance in T2D (Schrauwen et al., 2010). One culprit for the mitochondrial dysfunction seen in muscle tissue is the accumulation of reactive oxygen species (ROS) leading to peroxidation of lipid species that can have detrimental effects on mitochondrial RNA and proteins resulting in impaired mitochondrial function (Schrauwen et al., 2010). It has also been shown that presence of toxic lipid species such as ceramides can prevent the translocation of the GLUT4 transporter into the plasma membrane (Hoehn et al., 2008; JeBailey et al., 2007), as well as disrupt the activation of the insulin receptor through promotion of phosphorylation at serine residues in place of tyrosine residues on IRS-1 (Yu et al., 2002).

1.3 Lipotoxicity and β-cell failure in type 2 diabetes

Type 2 diabetes is thought to be initiated by the previously described impairments in insulin sensitivity associated with the lipotoxic effects in peripheral tissues; however, it has also been suggested that direct lipotoxic effects on β-cell function may contribute to the impairments in insulin secretion that accompany insulin resistance, resulting in

hyperglycemia. A predominant theory in the literature for β-cell failure is β-cell exhaustion and disruption of the insulin secretatory pathways seen in Figure 1.4 due to lipotoxic accumulation in the β-cell (Kahn et al., 2006; Poitout, 2004; Robertson et al., 2004).

Under normal circumstances, glucose is acquired exogenously from a meal or synthesized endogenously in the liver through the processes of gluconeogenesis and glycogenolysis to drive the production of the ATP required to carry out normal cellular processes. In response to elevated blood glucose levels and uptake of glucose by the β-cells of the pancreas, insulin is secreted and binds cell receptors on target tissues causing an uptake of glucose in the periphery. There are several mechanisms by which the β-cell can be stimulated to secrete insulin, the primary mechanism being elevation in blood glucose levels (Fig. 1.4). Other mechanisms that act on β-cells to secrete insulin include an influx of amino acids into the β-cell, binding of FFAs to G-protein coupled receptors -40 and -119 (GPR40/119), binding of incretin hormones released by the stomach to induce insulin secretion, and nerve activation from the parasympathetic nervous system (Boron and Boulpaep, 2005; Grassiolli et al., 2007; Kahn et al., 2006; Overton et al., 2008; Prentki and Nolan, 2006).

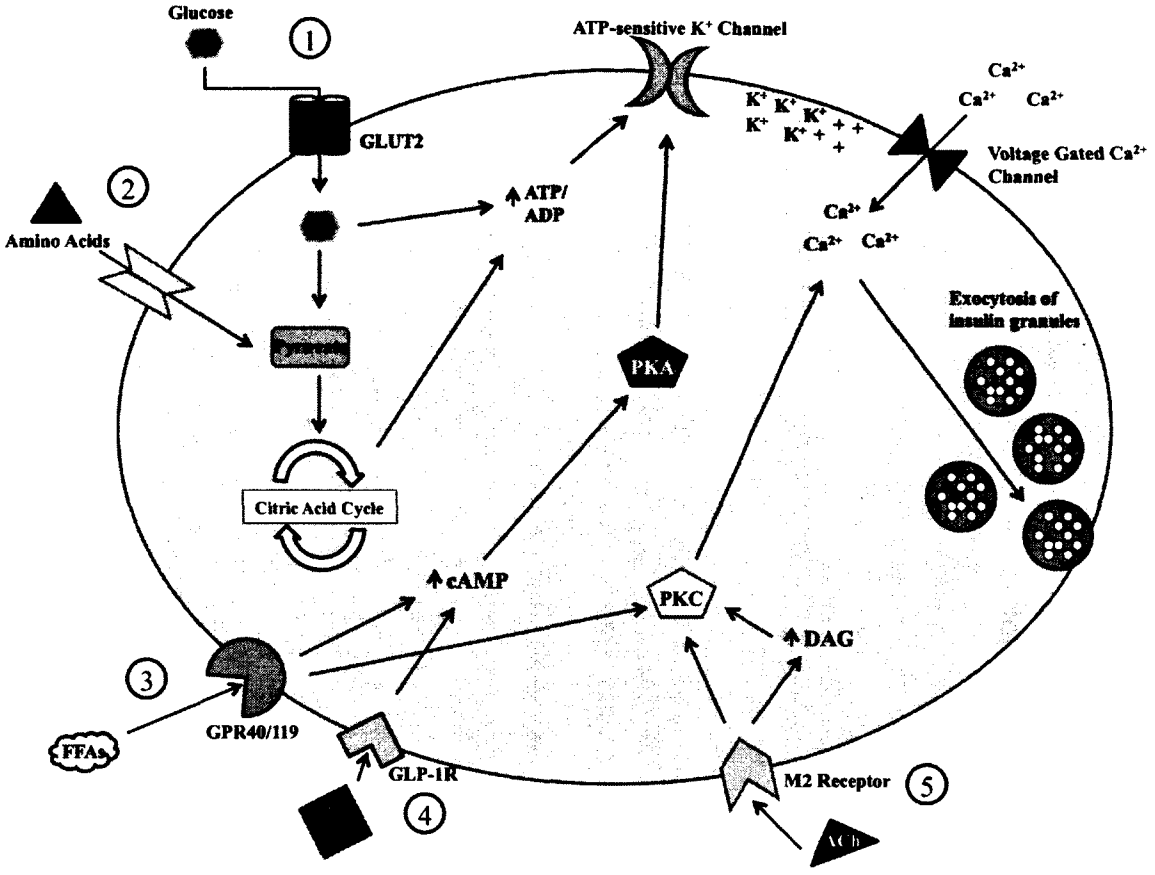

Figure 1.4 Mechanisms of action for the potentiation of insulin secretion from pancreatic β-cells. External stimuli promote the secretion of insulin through five primary mechanisms. Binding of glucose (1) or amino acids (2) will increase pyruvate production from the citric acid cycle causing an increase in the ratio of ATP to ADP resulting in closure of ATP-dependent K^+ channels and membrane depolarization. Depolarization causes an increase in intracellular Ca^{2+} concentrations which promotes exocytosis of insulin granules. Binding of FFAs to G-coupled protein receptors (3) or incretin hormones to their respective receptors (4) will cause either closure of K^+ channels through protein kinase A (PKA) activity or have direct effects on increasing intracellular Ca^{2+} levels through protein kinase C (PKC) activity. Activation of PKC can also be achieved by innervation from the parasympathetic nervous system (5) resulting in elevations in intracellular Ca^{2+} and the exocytosis of insulin granules. Modified from Boron and Boulpaep, 2005.

As T2D progresses, tissues become more resistant to insulin, and β-cells increase insulin secretion to compensate for the decrease in insulin sensitivity. In many patients, the compensatory increase in insulin is not sustained as β-cells ultimately fail, resulting in impaired insulin secretion in conjunction with insulin insensitivity in peripheral tissues and induction of hyperglycemia in T2D (Poitout, 2004; Robertson et al., 2004). Lipotoxic effects of ectopic lipid accumulation described as a mechanism for insulin resistance in skeletal muscle, liver, and adipose tissue above, have also been associated with similar impairments on β-cell function. Although acutely FFAs stimulate insulin secretion, prolonged exposure of β-cells to FFAs impairs insulin secretion (Carpentier et al., 2000; Lee et al., 1994; Poitout, 2004; Robertson et al., 2004; van Herpen and Schrauwen-Hinderling, 2008). The specific mechanisms by which ectopic lipid accumulation inhibits insulin secretion remains unclear. Ceramides and diacylglycerols have been shown to induce oxidative stress inducing apoptosis of β-cells (Summers, 2006; van Herpen and Schrauwen-Hinderling, 2008) while other studies suggest that changes in the expression of genes related to insulin secretion and other related signalling pathways may be the cause of impaired β-cell function due to elevations in the levels of FFAs (Lameloise et al., 2001; Shao et al., 2013; van Herpen and Schrauwen-Hinderling, 2008).

The lipotoxic hypothesis shows the progression of obesity-induced T2D as a confounding cycle in which oversupply of nutrients leads to lipotoxic accumulation of cytotoxic lipid species, resulting in insulin insensitivity in peripheral tissue and impaired insulin secretion from β-cells. Together, impairments in insulin sensitivity and secretion will lead to further impairments in lipid storage and alterations in immune response, lipid profiles, and adipokine profiles, which further exacerbate dyslipidemia in diabetic patients.

At the heart of this entire lipotoxic dilemma is the characterization and quantitation of lipid species disrupting the function of various cell types. As such, it would be wise to examine the regulation of lipid pathways to gain a better understanding of the specific lipid species involved in lipotoxic impairment of non-adipose tissue function. One such regulatory element that is known to play a primary role in the regulation of lipid metabolism is a family of nuclear transcription factors known as peroxisome proliferator activated receptors (PPARs) (Lee et al., 2003).

1.4 Peroxisome proliferator activated receptors: master regulators of lipid metabolism

PPARs are a family of nuclear transcription factors that have been shown to have key regulatory effects on lipid metabolism (Barrera et al., 2008; Filip-Ciubotaru et al., 2011). The PPAR family consists of four isoforms in humans; PPARα, PPARβ/δ, PPARγ1, and PPARγ2, all of which are variably expressed throughout the body and have varied functions with respect to lipid metabolism (Barrera et al., 2008).

More specifically, PPARs are a member of the steroid hormone nuclear receptor family (Barrera et al., 2008). Upon binding of a FFA ligand to the ligand binding domain, the transactivating domain will undergo a conformational change and allow for heterodimerization with the retinoic X receptor and allow for binding to DNA at the peroxisome proliferator response elements (PPREs) within the promoter region of target genes (Palmer et al., 1995).

PPARα is highly expressed in liver and skeletal muscle in which activation will induce transcription of genes involved in fatty acid oxidation (Evans et al., 2004). It has been seen that PPARα activation is a result of a fasting response in which pro-oxidative effects would be desirable in times of fasting when exogenous nutrient supplies are low. It has also

been shown recently that the pro-oxidative effects of PPARα activation specifically in β-cells of obese mice are able to preserve β-cell function despite exposure to a high fat diet (Hogh et al., 2013).

PPARβ/δ, commonly termed PPARδ, is also a pro-oxidative member of the PPAR family. PPARδ has been shown to be expressed in similar levels across several tissue types (Barrera et al., 2008) and is an important mediator in lipid metabolism specifically in skeletal muscle and adipose tissue (Barish et al., 2006; Evans et al., 2004). In these tissues PPARδ has been shown to play an important role in energy uncoupling and inhibition of macrophages in the immune response (Barish et al., 2006). More recently, a study has shown that PPARδ may also play a role in the mechanical machinery required for the secretion of insulin in pancreatic β-cells (Iglesias et al., 2012).

PPARγ1 and PPARγ2 have been established as primarily lipogenic and adipogenic transcription factors within the PPAR family. Being expressed most highly in adipose tissue, PPARγ1 and PPARγ2 activation has been shown to increase lipogenesis and lipid storage as well as adipogenesis and adipokine production in adipose tissue (Evans et al., 2004; Tontonoz et al., 1995; Tontonoz et al., 1994).

With their potent effects on lipid metabolism, PPARs are the molecular targets of pharmacological therapies for metabolic syndrome and T2D. Most notably, activation of PPARγ1 and PPARγ2 is the mechanism of action for the thiazolidinediones (TZDs), a class of anti-diabetic drug (Forman et al., 1995; Lehmann et al., 1995) that potently improves insulin sensitivity and thus normalizes hyperglycemia in patients with T2D. Furthermore, synthetic PPARα agonists, the fibrates, are a class of drug that have been designed to selectively target PPARα to effectively improve hypercholesterolemia and improve lipid

profiles of patients with dyslipidemia (Lalloyer et al., 2006; Reifel-Miller et al., 2005; Staels et al., 2008). More recent studies have also attempted to couple the effect of PPARδ or PPARα activation with activation of PPARγ to combine the pro-oxidative benefits of PPARα or PPARδ with the lipogenic characteristics of PPARγ (Fievet et al., 2006; Gathiaka et al., 2013).

1.5 A novel study for the characterization of PPARδ and PPARγ in pancreatic β-cell lipid metabolism

Many studies have investigated the impact of lipotoxicity causing impaired insulin sensitivity in peripheral tissue as well as impaired insulin secretion from β-cells of the pancreas. What still remains unclear are the precise mechanisms by which the accumulation of lipid in β-cells disrupt the insulin secratory mechanisms described previously in this chapter. While a number of studies have explored the regulatory role of PPARs on lipid metabolism, immune response, and proliferation in insulin sensitive tissues, the impact of PPARs on lipid metabolism in pancreatic β-cells remains largely unknown.

Previously, Dr. Gray's laboratory has shown that PPAR overexpression in pancreatic β-cells of obese mice alters carbohydrate metabolism. Specifically overexpression of pro-oxidative PPARα in pancreatic β-cells improves carbohydrate metabolism in obese mice (Hogh et al., 2013), while β-cell overexpression of PPARγ1 or PPARγ2 worsens obesity induced impairment of carbohydrate metabolism in obese mice (Hogh et al., 2014).

I hypothesize that β-cell specific overexpression of PPARs in a setting of obesity will alter β-cell lipid metabolism impacting β-cell function. This hypothesis will be explored through two studies; **study 1** will examine if overexpression of PPARδ in pancreatic β-cells under lipotoxic conditions affects glucose stimulated insulin secretion with

associated changes in gene expression (Chapter 2), and **study** 2 will utilize a mouse model of obesity to examine if β-cell specific overexpression of PPARγ2 will induce changes in islet lipid and gene expression profiles associated with impairments in carbohydrate metabolism in these mice (Chapter 3).

These studies may provide evidence to support an important role for PPARs in the regulation of islet cell lipid metabolism and perhaps provide evidence to support the development of PPAR targeted treatment strategies for β-cell dysfunction seen in patients with obesity induced T2D. Additionally, these studies will provide further insight into the types of lipid species contributing to lipotoxic β-cell failure in obesity.

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CHAPTER 2

Characterization of β-cell specific overexpression of PPARδ on glucose stimulated insulin secretion *in vitro*

2.1 INTRODUCTION

Peroxisome proliferator activated receptor-δ (PPARδ) belongs to the PPAR nuclear transcription factor family, which regulates lipid metabolic pathways (Barrera et al., 2008; Filip-Ciubotaru et al., 2011). Previously PPARγ and PPARα have been the primary members of the PPAR family targeted for improving insulin sensitivity and dyslipidemia respectively (Ravnskjaer et al., 2005; Reifel-Miller et al., 2005); however, in recent years PPARδ has emerged as an alternative target that, when activated, may improve both of these states in patients suffering from the metabolic syndrome and T2D (Barish et al., 2006).

Being expressed in several tissues throughout the body, including heart, muscle, adipose, liver, and intestines, PPARδ function has been shown to play important roles in β-oxidative pathways as well as in inflammation, and regulation of glucose metabolism. Collectively, these tissue specific effects of PPARδ activation have been shown to help regulate body weight, improve dyslipidemia, and improve peripheral insulin sensitivity (Barish et al., 2006; Gathiaka et al., 2013).

Congestive heart failure is one of the risks associated with the metabolic syndrome and is associated with decreased fatty acid oxidation (FAO) and a shift to a dependence on glucose metabolism (Stanley et al., 2005). A study in which PPARδ expression was knocked out in cardiac tissue demonstrated that decreased contraction and relaxation of cardiac muscle was associated with decreased cardiac output and failure (Cheng et al., 2004). Furthermore, it was found that failure resulting from deletion of PPARδ was due to suppression of oxidative genes and lipid accumulation.

Muscle tissue is a highly active metabolic tissue and expresses PPARδ at fifty times that of PPARγ and 10 times that of PPARα (Braissant et al., 1996). One study has suggested that PPARδ activation in muscle is an effective means of reducing lipid strain on adipocytes

to prevent lipotoxicity through increasing β-oxidation during high fat feeding (Tanaka et al., 2003). Other studies have shown that PPARδ mRNA is increased in response to fasting and exercise in muscle tissue and have suggested a role for PPARδ in increasing FAO and insulin-dependent glucose uptake in these cells (Holst et al., 2003; Luquet et al., 2003).

Another tissue in which PPARδ has been shown to play an important role in dyslipidemia and lipotoxicity through its regulation of β-oxidation and inflammation is adipose tissue. Overexpression of PPARδ specifically in adipose tissue has been shown to play a protective role against obesity. Improvements in these transgenic mice are associated with a 20% reduction in body weight and a 40% lower inguinal fat pad mass (Wang et al., 2003). These authors also noted a decrease in adipose tissue triglyceride content with reductions in circulating triglycerides and FFAs. Furthermore, the overexpression of PPARδ in these mice caused an increase in the pro-oxidative genes uncoupling protein 1 and 3 (UCP1 and 3). In contrast, these authors also report that in an adipose specific knockout of PPARδ, mice had significantly higher body mass with a reduction in the expression of UCP1 and 3 (Wang et al., 2003). In summary, these authors suggest that PPARδ improves dyslipidemia in adipose tissue by promoting fatty acid oxidation and increasing energy uncoupling, thereby improving insulin resistance in T2D.

Finally, PPARδ has also been implicated in several metabolic pathways in the liver which may be implicated in the maintenance and treatment of the metabolic syndrome. The net result of PPARδ activation in the liver is a reduction in glucose output (Barish et al., 2006). It is speculated that the PPARδ dependent decrease in glucose output is due to an increased flux of glucose into the pentose phosphate shunt resulting in an enhancement of fatty acid synthesis (Lee et al., 2006).

Studies examining the targeted activation of PPARδ may prove useful in the maintenance of symptoms associated with T2D and the metabolic syndrome. Administration of GW501516, a synthetic PPARδ ligand, improves dyslipidemia by raising levels of high density lipoproteins (HDL) while reducing circulating triglyceride levels (Oliver et al., 2001). It has been speculated that improvements in serum cholesterol levels are due to the increased expression of the ATP-binding cassette transporter 1 (ABCA1), a cholesterol transport protein, and decreased cholesterol absorption in the intestines due to a reduction in Niemann-Pick C1-like 1 (Npc1l1), a protein involved in mediating the binding of cholesterol (van der Veen et al., 2005). PPARδ activation has also been shown to mitigate the inflammatory immune response in macrophages (Barish et al., 2006), providing a possible target for improvements in adipose tissue expandability as a treatment for lipotoxicity and dyslipidemia.

Systemic administration of PPAR agonists have proven to be effective treatments for symptoms associated with T2D and the metabolic syndrome; however, it has been suggested that these treatments should be approached with caution as activation in all tissues may cause undesirable side effects. An example of such side effects occurs with systemic Rosiglitazone administration, in which PPARγ improves peripheral insulin sensitivity at the cost of increased risk of cardiac failure (Nissen and Wolski, 2007). It is outlined here that activation of PPARδ has varying tissue specific effects and therefore its role should be considered in all metabolic tissues before systemic administration of PPARδ ligands is considered as a therapeutic approach to manage metabolic disease. Therefore, I am interested in the role of PPARδ in pancreatic β-cells, a cell type where the role of PPARδ has still not been fully elucidated.

Studies investigating the role of PPARδ on lipid metabolism *in vitro* have suggested that PPARδ activation in HIT-15 cells, a hamster-derived β-cell line, causes an upregulation of genes involved in mitochondrial FAO and helps to alleviate the impairments on glucose-stimulated insulin secretion induced by palmitate (Wan et al., 2010). Specifically, the authors report increased expression of FAO genes uncoupling protein 2 (UCP2), long chain acyl-CoA dehydrogenase (LCAD), pyruvate dehydrogenase kinase 4 (PDK4) and carnitine palmitoyl transferase 1 (CPT1). Further studies in INS1 cells, a rat-derived β-cell line, report similar findings suggesting PPARδ restores glucose-stimulated insulin secretion in conditions of lipotoxicity and that PPARδ is the most highly expressed member of the PPAR family in β-cells (Ravnskjaer et al., 2010).

In addition to its role in lipid metabolism in the β-cell, a recent *in vivo* model of β-cell specific PPARδ knockout suggests a role for PPARδ in the regulation of the cellular machinery involved in secretion of insulin granules (Iglesias et al., 2012). Here the authors report that removal of PPARδ from the β-cell causes enhanced second phase insulin secretion associated with increased disassembly of filamentous actin, allowing for uninhibited secretion of insulin granules. These authors also report alterations in Golgi organization due to increased levels of protein kinase D (PKD).

The primary goal of this study will be to further characterize the role of PPARδ on β-cell function in a model of obesity. Chronic exposure to high levels of FFAs has been demonstrated to induce lipotoxicity in β-cells and impair glucose stimulated insulin secretion and induce apoptosis (Carpentier et al., 2000; Lee et al., 1994; Poitout, 2004; Robertson et al., 2004; van Herpen and Schrauwen-Hinderling, 2008).

I hypothesize that overexpression of PPARδ in a β-cell line will restore glucose stimulated insulin secretion to levels seen in a non-lipotoxic settings due to the pro-oxidative potential of PPARδ. This hypothesis will be tested through two experimental aims:

In **aim 1** I will measure oxidative gene expression in response to overexpression and activation of PPARδ in β-cells.

In **aim 2** I will assess the impact of PPARδ overexpression on β-cell function by measuring glucose stimulated insulin secretion in normal and lipotoxic conditions.

Results from these studies will set the ground work for future *in vivo* studies evaluating the effects of β-cell specific overexpression of PPARδ on whole animal glucose homeostasis in obesity induced T2D. Additionally, the use of palmitate will allow us to assess the suitability of long chain FAs as a natural PPARδ ligand, an area of research that remains yet unaddressed in the literature.

2.2 MATERIALS AND METHODS

Preparation of PPARδ construct

A plasmid (dsAAV8) containing the *Mus musculus*-derived PPARδ gene (GenBank ID: NC_000083.6) insert under control of the rat insulin promoter (dsAAV8-RIP-PPARδ, 5931bp) was transformed into XL1-Blue competent *Escherichia coli* cells for cloning. Cells were lysed and plasmid DNA was purified (Plasmid *Plus* Giga Kit, Qiagen, Valencia CA, USA). A restriction enzyme digest was performed using MluI endonuclease (New England Biolabs, Whitby ON, Canada) and plasmid DNA sequenced to ensure plasmid contained the RIP-PPARδ inserted region which can be expressed only in the presence of insulin.

Cell culture

Several insulinoma cell lines have been generated for the *in vivo* examination of β-cell function. Two of the most widely used of these cell lines are MIN6, derived from mouse pancreatic islets, and INS1, derived from rat pancreatic islets (Skelin et al., 2010). For the purpose of this study, MIN6 cells were used for their ability to more closely mimic β-cells *in vitro* with respect to insulin secretion in response to glucose stimulation. Due to their robust nature and ease of culturing, INS1 cells were used in luciferase assays in which transfection of multiple constructs was required.

MIN6 cells were cultured using Dulbecco's Modified Eagles Medium (DMEM) (D5671, Sigma, Oakville ON, Canada) at 37°C and 5% CO_2. Culture media was supplemented with 10% fetal bovine serum (vol/vol) (F6178, Sigma, Oakville ON, Canada), 200mM L-glutamine (G7513, Sigma, Oakville ON, Canada), 50μmol/L β-mercaptoethanol (M3148, Sigma, Oakville ON, Canada), and 100U/mL penicillin-streptomycin (P4333, Sigma, Oakville ON, Canada).

INS1 cells were cultured using RPMI-1640 Medium (R8758, Sigma, Oakville ON, Canada) at 37°C and 5% CO_2. Culture media was supplemented with 10% fetal bovine serum (vol/vol) (F6178, Sigma, Oakville ON, Canada), 10mM HEPES (G7513, Sigma, Oakville ON, Canada), 25μmol/L β-mercaptoethanol (M3148, Sigma, Oakville ON, Canada), and 100U/mL penicillin-streptomycin (P4333, Sigma, Oakville ON, Canada).

For overexpression of enhanced Green Fluorescent Protein (eGFP) and PPARδ, MIN6 (Passage 18-24) and INS1 (Passage 85-95) cells were cultured at 50% confluency in media containing no antibiotic. Cells were transfected with 800ng of plasmid DNA (24-well plate) or 200ng of plasmid DNA (96-well plate) using 1μL per well of Lipofectamine 2000 (11668019, Invitrogen, Burlington ON, Canada) and 100μL per well of OPTI-MEM

(11058021, Invitrogen, Burlington ON, Canada). Transfection efficiency of plasmid DNA was assessed by visualization of eGFP using 70% efficiency as an acceptable cut-off (Fig. S2.2).

Upon successful transfection of plasmid DNA, cells were incubated for either 24 or 48 hours in cell culture media containing no treatment, 250μM palmitate, 500μM palmitate, DMSO vehicle (1:2000), or GW501516 in DMSO. Palmitate media was prepared (500μM stock concentration) by dissolving 56mg palmitic acid (P5585, Sigma, Oakville ON, Canada) in 10mL of water containing 1mL of 1M NaOH. This solution was then heated to 70°C and 0.5mL combined with 1.65mL of 20% bovine serum albumin (BSA) (A6003, Sigma, Oakville ON, Canada) preheated to 37°C. Complexed BSA and palmitic acid (6:1 molar ratio) were then added to 17.85mL of cell culture media preheated to 37°C. GW501516 media was initially prepared by dissolving 1mg GW501516 (ALX-420-032-M001, Enzo Life Sciences, Farmingdale NY, USA) in DMSO to create a 50μM stock solution; however, upon review of the luciferase assay results reported below, where it was observed that high concentrations of DMSO were not appropriate as a vehicle control, GW501516 was re-prepared as a 2000μM stock solution in DMSO. Final GW501516 working solutions were created at a concentration of 1μM in cell culture media after optimization of treatment conditions with qPCR (outlined in results).

Gene expression optimization

A recent publication regarding the use of qPCR suggests that several factors must be considered before one can publish robust data generated using this method (The MIQE guidelines) (Bustin et al., 2009). Some of these factors include proper handling of RNA and generation of cDNA, optimization of PCR reaction efficiencies, and the use of stably expressed endogenous control genes with which to compare relative gene expression levels.

Relative gene expression is calculated taking into consideration the amplification efficiencies of primers and probes. Previously it has been acceptable to simply assume an amplification efficiency of 2, representing a perfect doubling of PCR product with each cycle of amplification. The MIQE guidelines suggests that expression levels should be calculated based on the actual efficiency for a given primer and probe set (Bustin et al., 2009), as this value will not be exactly 2 in all cases. For this reason, primer and probe sets for all genes evaluated in this study were designed and the optimal annealing temperature was established using a temperature gradient. Specificity of primer and probe sets were evaluated on an agarose gel or using a melt curve with the Bio-Rad iQ5 software. After establishing specificity, standard curves for each primer and probe set were constructed to ensure efficiencies fell within the range of 1.8-2.2 (Table 2.1). Gene expression levels were then calculated using these calculated efficiency values.

Table 2.1. Efficiency values (E) with slope and correlation coefficients (R^2) for primer and probe sets optimized for gene expression analysis using optimal annealing temperature (T_A).

Gene	Slope	E	R^2	T_A (°C)
Reference Targets				
GAPDH	3.3481	1.99	0.996	65.0
β-actin	3.0145	2.09	0.990	67.7
GUSb	3.7727	1.84	0.998	61.7
TBP	3.483	1.94	0.989	61.7
Rpl19	3.5598	1.91	0.990	61.7
Targets of Interest				
PPARδ	3.219	2.04	0.999	55.9
CPT1	3.236	2.04	0.994	57.0
UCP2	3.346	1.99	0.999	57.0
LCAD	3.125	2.09	0.999	57.0
PDK4	3.685	1.87	0.999	62.2

Comparison of gene expression levels using endogenously expressed housekeeping genes reduces variability in the qPCR reaction and should always be used according to the MIQE guidelines. To ensure suitable endogenous control genes were used in this study, five housekeeping genes were evaluated across conditions in which RNA was extracted from MIN6 cells overexpressing PPARδ or eGFP and treated with 250μM palmitate or untreated cell culture media and the three most stably expressed genes were used in the experimental gene expression analysis. Using GeNorm's algorithm for establishing variability and an M-value cut off of 1, it was found that β-glucuronidase (GusB), TATA-Binding protein (TBP), and β-actin were more stably expressed in β-cells than glyceraldehyde 3-phosphate (GAPDH) and 60S ribosomal protein L19 (Rpl19) (Fig. S2.3). As such, all future PPARδ gene expression experiments were carried out with GusB, TBP, and β-actin as endogenous controls.

Gene expression Analysis

RNA was extracted from MIN6 cell cultures using RNeasy Mini Kit spin columns (74104, Qiagen, Valencia CA, USA). Total RNA samples were treated with Ambion TURBO-DNA free (AM1907, Invitrogen, Burlington ON, Canada) to remove potential genomic DNA contamination. Concentration of RNA was determined using the Qubit RNA Broad Range Assay (Q10211, Invitrogen, Burlington ON, Canada) and purity assessed using 260nm and 280nm absorbance readings from an ND-1000 spectrophotometer. Integrity of RNA samples was evaluated by visualizing intact 18S and 28S ribosomal RNA subunits separated on a 1.5% agarose gel (Supplemental Fig. 2.2). Reverse transcription was then carried out to generate cDNA using 1μg of input RNA with an iScript cDNA synthesis kit

utilizing both oligo(dT) and random hexamer primers (170-8891, BioRad Laboratories, Hercules CA, USA).

cDNA was then used to evaluate gene expression levels relative to β-actin, TBP, and GusB. All qPCR reactions were carried out in triplicate using master mix containing 12.5μL iQ Supermix with Taqman probe (2.55μM), 2.5μL forward and reverse primers (10μM), 2.5μL RNAse free H_2O, and 2.5μL cDNA (BioRad Laboratories; Sigma, Oakville, ON, Canada; IDT, Coralville, IA, USA; Ambion, Austin, TX, USA). Primer and probe sequences for these genes can be found in Table 2.2. Reactions were conducted on a BioRad iQ5 qPCR machine (BioRad iQ5 Multicolor RT-PCR Detection System, BioRad Laboratories, Hercules CA,

USA) using the following temperatures, times, and number of cycles: one cycle at 95°C for 3min followed by 40 cycles alternating between 95°C for 10 seconds and primer-specific annealing temperature for 30 seconds.

Relative gene expression levels of PPARδ, carnitine palmitoyltransferase 1 (CPT1), uncoupling protein 2 (UCP2), long chain acyl-CoA dehydrogenase (LCAD), and pyruvate dehydrogenase kinase 4 (PDK4) were calculated with the delta-delta Ct method using Biogazelle's qbasePlus qPCR software (Zwijnaarde, Belgium). Primer and probe sequences for all endogenous control genes and experimental genes can be found in Table 2.2.

Table 2.2 Primer and probe sequences used for real-time qPCR analysis of RNA extracted from MIN6 cells.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Probe (5'-3')
Endogenous Controls			
β-actin	GCTCTGGCTCCTAGCACCAT	GCCACCGATCCACACAGAGT	GATCAAGATCATTGCTCCTCCTGAGCG
TBP	CACCAATGACTCCTATGA	CCAAGATTCACGGTAGATA	CCTATCACTCCTGCCACACCA
GusB	CTCATCTGGAATTTCGCCGA	GGCGAGTGAAGATCCCCTTC	CGAACCAGTCACCGCTGAGAGTAATC
GAPDH	TGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTC	CAGAAGACTGTGGATGGCCCCTC
Rpl19	GAAGCTGATCAAGGATGG	CTTCCCTATGCCCATATG	CATCCGCAAGCCTGTGACTG
Target Genes			
PPARδ	CAACGAGATCAGTGTGCA	TCACCTGGTCATTGAGGAA	TCTACCGCTGCCAGTCCACCACA
CPT1	GCGTGCCAGCCACAATTC	TCCATGCGGTAATATGCTTCAT	CCGGTACTTGGATTCTGTGCGGCC
UCP2	GATCTCATCACTTTCCCTCTGGATA	CCCTTGACTCTCCCCTTGG	CGCCAAGGTCCGGCTGCAGA
LCAD	GCATGAAACCAAACGTCTGGA	TGTTTTGTAATTCAGATGCCCAGT	TCCGGTTCTGCTTCCATGGCAAAA
PDK4	GACACGCTGGTCAAAGTTC	GGACTACTGCTACCACATCA	ATGTGGTCCCTACAATGGCTCAAGGCA

Luciferase assay

To assess the ability to overexpress functional PPARδ protein, a dual-luciferase reporter assay was used. The PPAR reporter assay (CCS-3026L, Qiagen, Valencia CA, USA) contains plasmid DNA with a firefly luciferase gene insert under control of the peroxisome proliferator response element (PPRE-Luc). Expression of Firefly luciferase is driven by the binding of the PPRE in the promoter region of the Firefly luciferase gene (Fig. 2.1). As such, using a luminometer to measure quantity of light and the activity of the luciferase enzyme in the conversion of luciferin to oxyluciferin can be used as measure of active PPAR transcription factors. The PPAR reporter assay also contains a plasmid with a constitutively active Renilla luciferase (CMV-Luc) to normalize for transfection variability of firefly luciferase.

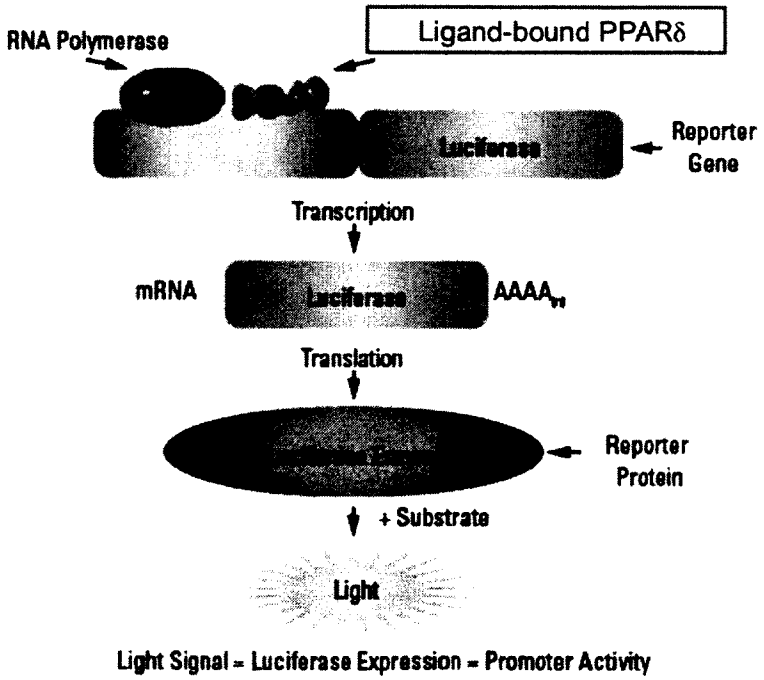

Figure 2.1 Ligand binding of PPARδ will induce PPRE binding in the promoter region of the luciferase gene. Luciferase activity can then be used as a measure of functional PPAR activity in cell culture. Figure modified from Snider, 2014.

Experimental groups were established by co-transfecting INS1 cells with either RIP-eGFP, PPRE-Luc, and CMV-Luc constructs; or RIP-PPARδ, PPRE-Luc, and CMV-Luc constructs. Negative controls were established by transfecting cells with a construct containing a non-inducible luciferase gene and positive controls were established by transfecting cells with a construct containing a constitutively active Firefly luciferase gene. Cells were transfected and transfection efficiency assessed using fluorescent microscopy to visualize eGFP. Upon successful transfection, cells were incubated with untreated cell culture media, 250μM palmitate, DMSO vehicle (1:50 or 1:2000 v/v), or 1μM GW501516 media.

Firefly and Renilla luciferase activity was measured using a Dual-Glo Luciferase Assay System (E2920, Promega, Madison WI, USA) with a luminometer to measure light intensity (Synergy 2 Multi-Mode Microplate Reader, BioTek, Winooski VT, USA). Results are expressed as a ratio of Firefly luminescence/Renilla luminescence.

Glucose stimulated insulin secretion assays

To assess the effect of PPARδ activation and overexpression on insulin secretion in both non-obese and obese settings in MIN6, cells underwent a glucose stimulated insulin secretion and insulin content of the media was measured.

Cells initially underwent two 1-hour glucose starvation periods in which they were incubated in glucose-free KRH buffer containing 5M NaCl, 1M KCl, 1M Mg_2SO_4, 1M $NaHCO_3$, 1M $CaCl_2$, 0.5M KH_2PO_4, 1M HEPES, and 0.5g BSA (Sigma, Oakville ON, Canada). Cells were then incubated for 1 hour in KRH buffer containing either basal (2.8mM) or stimulated (20mM) levels of D-glucose (G8270, Sigma, Oakville ON, Canada) after which the buffer was collected for measurement of insulin.

Insulin content was measured in triplicate using a Mouse Ultrasensitive Insulin ELISA (80-INSMSU-E01, Alpco, Salem NH, USA). Protein from cell culture wells was extracted using 400µL per well of RIPA buffer (R0278, Sigma, Oakville ON, Canada) containing 1X HALT protease inhibitor and 1X EDTA (78430, Thermo Scientific, Rockford Illinois, USA). Cells were incubated on ice for 5 minutes and the cell lysates collected and centrifuged for 15 minutes at 14,000xg. Supernatant was collected and total protein content measured using a Pierce BCA Protein Assay Kit (23225, Thermo Scientific, Rockford Illinois, USA). Insulin content from each sample was then normalized to total protein and expressed as ng insulin/mg protein.

Statistical analysis

Results are expressed as mean ± standard error of the mean. Data were assumed to be normally distributed as tests for normality are difficult to conduct when working with small sample sizes such as cell culture biological replicates. One of the benefits of working with cell culture models is the high degree of reproducibility between samples, which is often not the case when working with more heterogeneous human populations that require large sample sizes. For this reason, assuming a normal distribution and using student's t-tests is acceptable here and most consistent with the literature. Comparisons were performed using student's 1-tailed or 2-tailed t-tests with Graphpad Prism 6.0 software (La Jolla CA, USA). Significance was declared if p-values were less than 0.05.

2.3 RESULTS

Preparation of PPARδ construct

Restriction enzyme digest of purified plasmid DNA demonstrates successful cloning of dsAAV8 plasmid containing the RIP-PPARδ gene insert (Fig. 2.2).

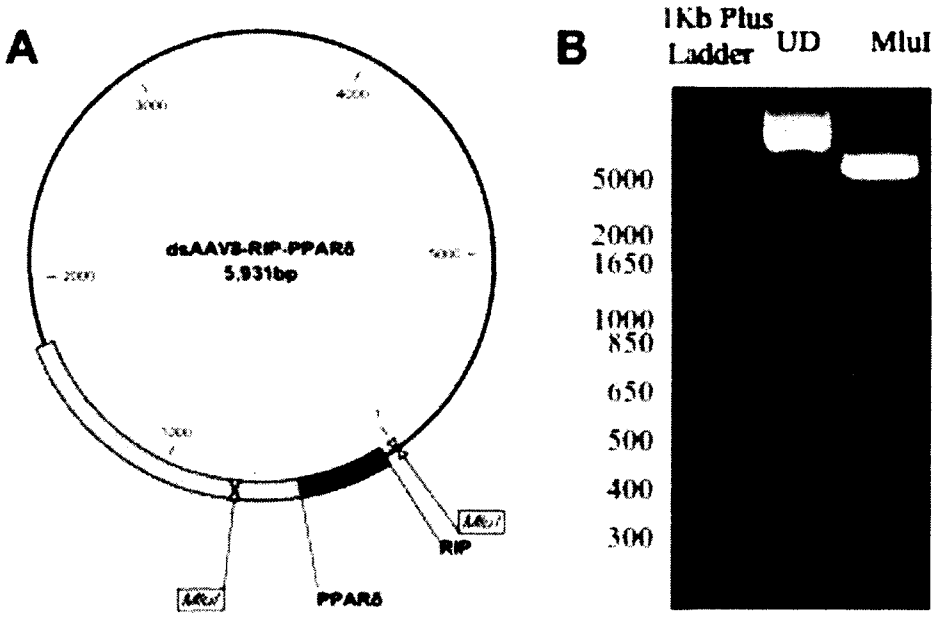

Figure 2.2 Plasmid map demonstrating the location of MluI restriction sites and RIP-PPARδ insert in dsAAV8 construct (a). Restriction enzyme digest shows successful cloning of dAAV8-RIP-PPARδ construct (b). (UD=Undigested)

Successful overexpression of PPARδ mRNA in MIN6 transfected with RIP-PPARδ

Overexpression of PPARδ induced by transfection of the RIP-PPARδ plasmid was assessed in MIN6 cells using real-time PCR. It was found that MIN6 cells transfected with RIP-PPARδ had a 95.08-fold increase in PPARδ mRNA expression compared to non-transfected MIN6 cells and a 130.7-fold change compared to cells overexpressing RIP-eGFP (Fig. 2.3). No significant difference in PPARδ mRNA expression was observed between non-transfected and RIP-eGFP cells.

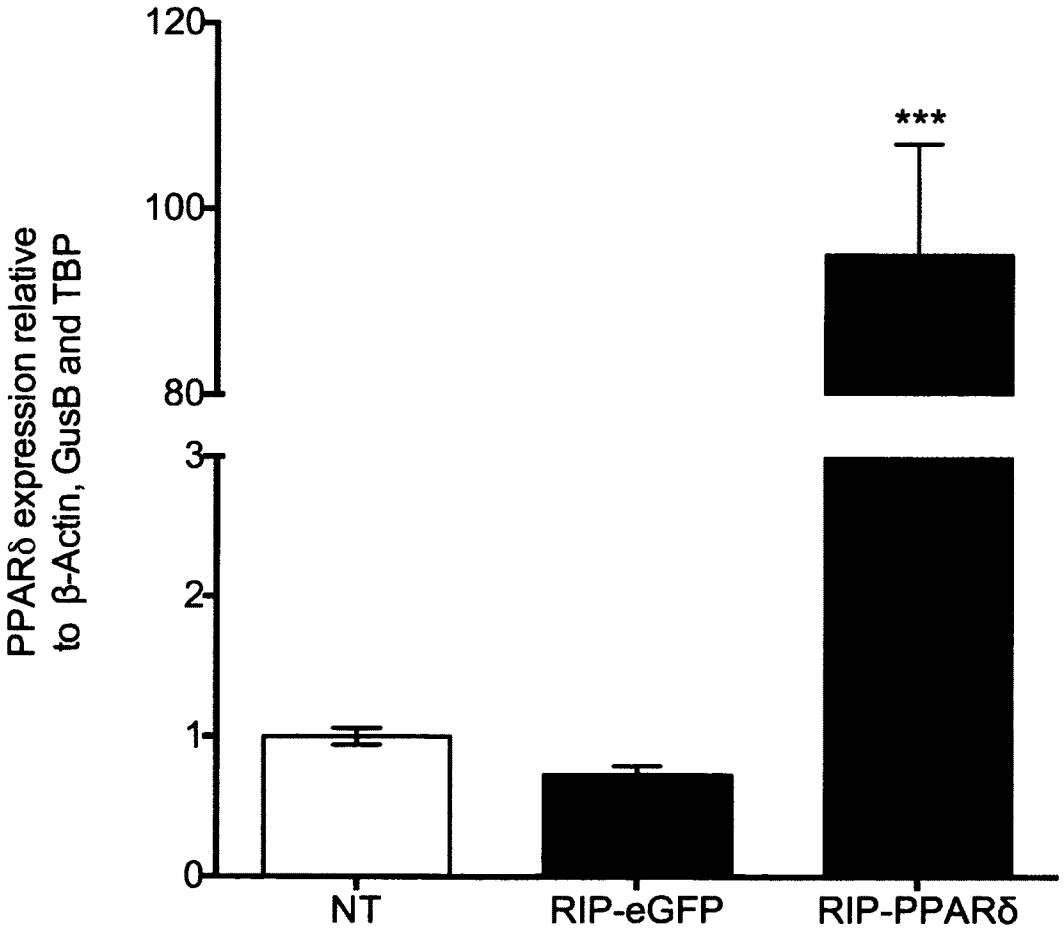

Figure 2.3 Transfection of MIN6 cells with RIP-PPARδ plasmid resulted in a 95.08-fold increase in PPARδ mRNA expression compared to non-transfected (NT) cells. *1-tailed t-test* ***$p < .001$

Gene expression analysis of MIN6 overexpressing PPARδ

Initial evaluation of gene expression resulted in no change to expression levels of PPARδ target genes; CPT1, LCAD, and UCP2, after treatment with 250μM palmitate or 100nM GW501516 for 48 hours (results not shown), while expression of PDK4 was undetectable in MIN6 mRNA samples. However, changes in gene transcription can occur within a specific window of time ranging from hours to days. It may be that changes in target gene expression are not detected when RNA samples are collected outside of one of these windows making it a possibility to observe no change in expression when these changes actually do exist. For this reason, optimal treatment times and optimal treatment concentrations to detect any changes in target gene expression were established in non-transfected MIN6 cells so that target gene expression analysis could be repeated.

MIN6 cells treated with 1μM GW501516 (1:50 v/v DMSO vehicle concentration) for 6, 12, 24, or 48 hours revealed significant increases in UCP2 ($P = .05$) after 24 hours of treatment and LCAD expression after treatment for 24 hours ($P = .017$) and 48 hours ($P = .014$) (Fig. 2.4). MIN6 cells treated with 250μM palmitate revealed a significant increase in expression of UCP2 ($P = .037$) and LCAD ($P = .005$); while treatment with 500μM palmitate caused a significant increase only in UCP2 ($P = .042$). Furthermore, the DMSO vehicle induced a significant increase in LCAD expression ($P = .004$), however, when treated with 1μM GW501516 expression of LCAD was significantly increased above the DMSO vehicle control ($P = .027$) (Fig. 2.5). Taken together, it was concluded that the experimental procedure should be duplicated with optimized parameters of 24 hours incubation time with 250μM palmitate, and 1μM GW501516.

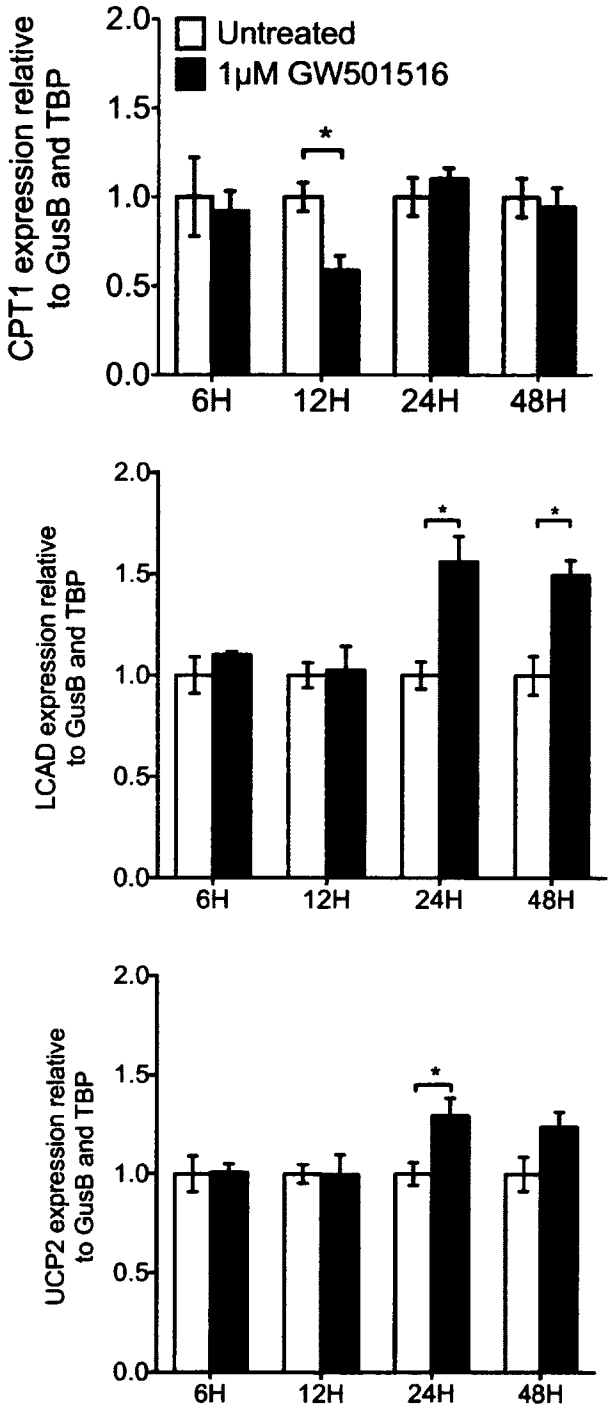

Figure 2.4 Optimization of treatment time for the induction of PPARδ target genes in MIN6 cells revealed that 24 hours of treatment with 1μM GW501516 (1:50 v/v DMSO vehicle) was optimal and caused increases in two out of three PPARδ target genes. *2-tailed t-test*
$*p < .05$

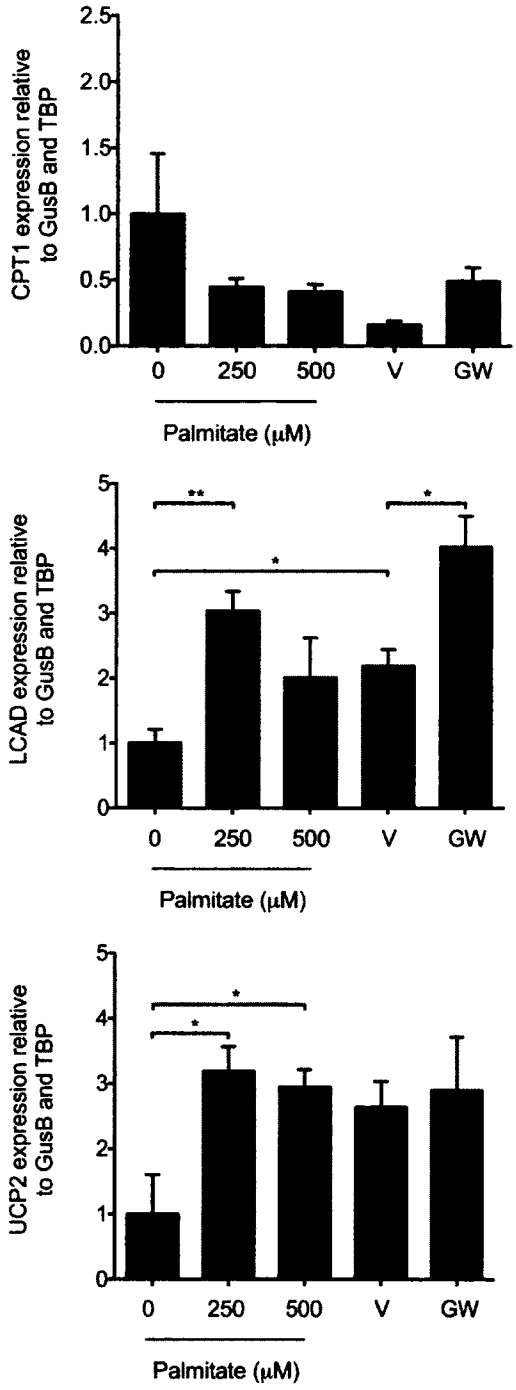

Figure 2.5 Optimization of ligand concentrations in MIN6 cells revealed that 250μM palmitate, and 1μM GW501516 (1:50 v/v DMSO vehicle) were optimal for inducing increases in PPARδ target genes LCAD and UCP2, and that the DMSO vehicle control caused an increase in expression of LCAD. *2-tailed t-test* $*p < .05$, $**p < .01$ (V = DMSO vehicle control, GW = GW501516).

Upon optimization of treatment times and ligand concentrations, the gene expression analysis of MIN6 cells overexpressing PPARδ was repeated and it was found that treatment with 250μM palmitate or 1μM GW501516 (1:50 v/v DMSO vehicle) both caused no increase in PPARδ target genes UCP2 or LCAD (Fig. 2.6). These results are difficult to interpret, as both previous studies (Wan et al., 2010) and optimization of treatment conditions both show that activation with the PPARδ ligand GW501516 can cause upregulation of UCP2 and LCAD; however, upon repeating the experiment in which PPARδ was overexpressed in MIN6 cells, no induction of these target genes was observed even when looking at RIP-eGFP control cells. Based on the inability to reproduce the upregulation of these PPARδ target genes, it was decided that examination into ability of the PPARδ construct to overexpress functional protein needed to be evaluated in a more controlled system before re-evaluating the effects of PPARδ overexpression on upregulation of downstream target genes. For this reason, a PPAR-response element reporter system was utilized to look at the effect of PPARδ overexpression in the presence of GW501516, a high affinity PPARδ ligand.

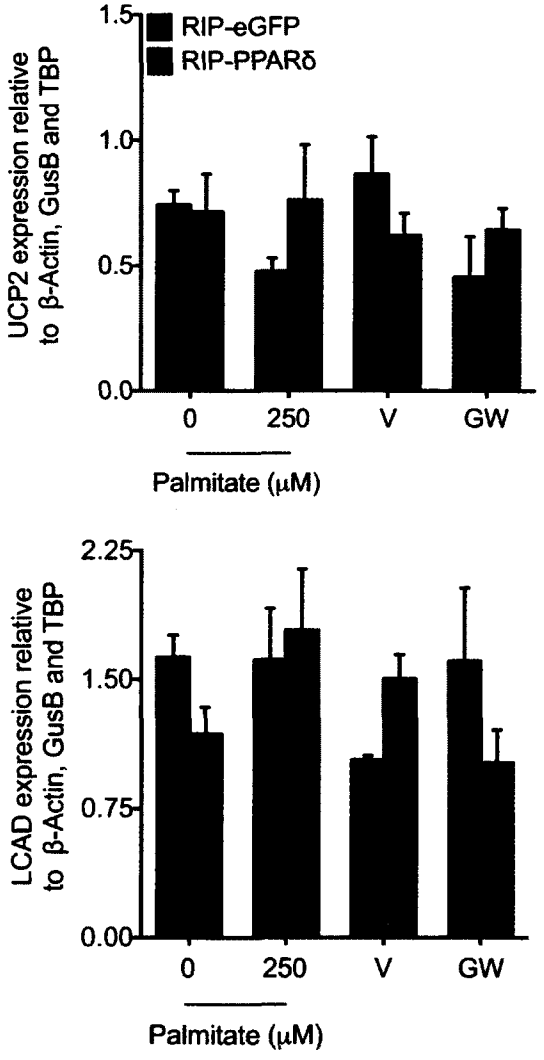

Figure 2.6 Overexpression of PPARδ in MIN6 cells treated with 250μM palmitate or 1μM GW501516 (1:50 v/v DMSO) caused no change in expression of PPARδ target genes UCP2 or LCAD. *2-tailed t-test* (V = DMSO vehicle control, GW = GW501516).

Overexpression of PPARδ in INS1 yields functional PPARδ protein

After target gene expression analysis in MIN6 cells overexpressing PPARδ resulted in inconclusive findings, a PPAR-response element luciferase reporter assay was utilized as a more controlled system in which it was possible to assess the ability of the RIP-PPARδ construct to overexpress functional PPARδ protein capable of binding to the peroxisome proliferator response element (PPRE) promoter region (Fig. 2.1). Initially it was found that INS1 cells treated with 1μM GW501516 caused significantly increased luciferase activity; however, a vehicle treatment with 1:50 (v/v) DMSO also induced a significant increase in luciferase activity (results not shown). These findings highlighted an error in the original experimental design, which was likely impacting the results of previously conducted gene expression studies examining the effects of PPARδ overexpression on regulation of target gene expression. The high concentration of DMSO (1:50 v/v) was itself inducing changes in target gene expression making it difficult to decipher the effects of PPARδ agonism from DMSO effects in cell culture media. It was decided that robust conclusions could not be drawn from studies assessing the effects of PPARδ activation with 1μM GW501516 where the ligand was being delivered to cell culture media with such a high concentration of DMSO (1:50 v/v) and a new GW501516 stock solution was prepared in which the ligand could be added to cell culture media with a DMSO concentration of 1:2000 (v/v) while maintaining a final GW501516 concentration of 1μM in cell culture media.

Upon preparation of a new 1μM GW501516 stock solution (1:2000 v/v DMSO), luciferase activity was re-assessed and it was observed in the RIP-eGFP control group that palmitate induced a significant increase in luciferase activity ($P < .001$), while treatment of RIP-eGFP cells with DMSO or the synthetic PPARδ ligand GW501516 caused no increase in luciferase activity (Fig. 2.7). When examining the effect of palmitate treatment on RIP-

PPARδ cells, it can be seen that luciferase activity is not significantly different than 250μM palmitate treated RIP-eGFP cells.

When comparing RIP-eGFP and RIP-PPARδ INS1 cells without palmitate or GW501516, it was found that there was no difference in luciferase activity. Furthermore, RIP-PPARδ cells showed no activation of luciferase when treated with DMSO (1:2000 v/v), indicating that DMSO at the lower concentration is not causing activation of the PPRE. Results showed that treatment of RIP-PPARδ cells with specific ligand (1μM GW501516) induces a significant increase ($P < .001$) in luciferase activity not observed in the RIP-eGFP control cells treated with 1μM GW501516.

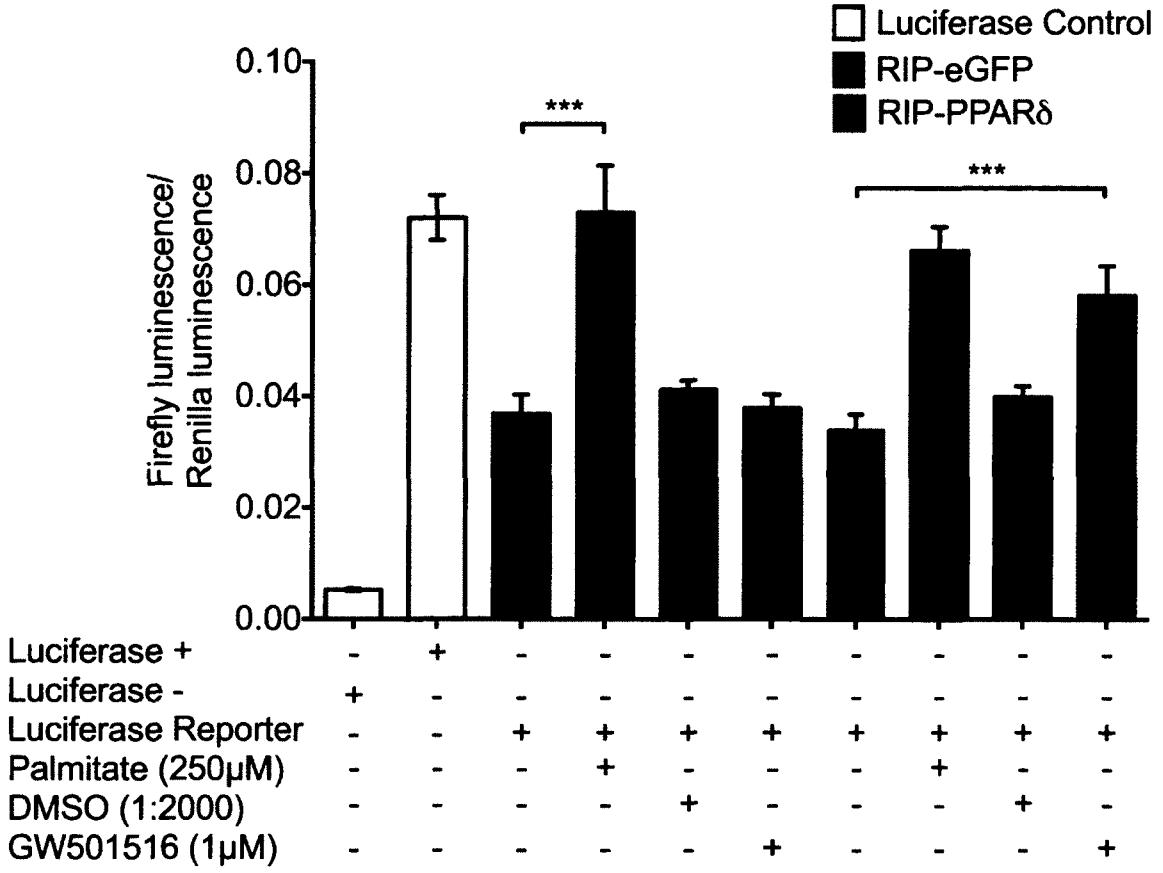

Figure 2.7 Treatment with GW501516 (1:2000 v/v DMSO), a synthetic PPARδ ligand, reveals that overexpression with the RIP-PPARδ plasmid in INS1 cells yields overexpression of functional PPARδ protein and that palmitate may not be a preferential ligand for PPARδ. Luciferase activity is reported as a ratio of Firefly luciferase/Renilla luciferase activity. *2-tailed t-test* $^{**}p < .01$, $^{***}p < .001$

Glucose stimulated insulin secretion in MIN6

Treatment of control MIN6 cells (RIP-eGFP) with 250μM palmitate was found to decrease insulin secretion as expected due to the known lipotoxic effects of palmitate exposure (Fig. 2.8) (P = .001) (Wan et al., 2010). Upon treatment of RIP-eGFP cells with the PPARδ ligand, 1μM GW501516 (1:2000 v/v DMSO), it was found that stimulated insulin secretion levels were restored to levels of control cells treated with DMSO (P = .04), and that cells treated with 250μM palmitate were able to maintain stimulated insulin secretion levels in the presence of 1μM GW501516.

When observing the effects of PPARδ overexpression on insulin secretion in MIN6 cells, it was found that increased PPARδ levels in the presence of 1μM GW501516 had no effect compared to cells overexpressing eGFP treated with 1μM GW501516. Interestingly, in a lipotoxic setting in which cells were overexpressing PPARδ and treated with 1μM GW501516, decreased stimulated levels of insulin secretion were observed (P = .015) (Fig. 2.8).

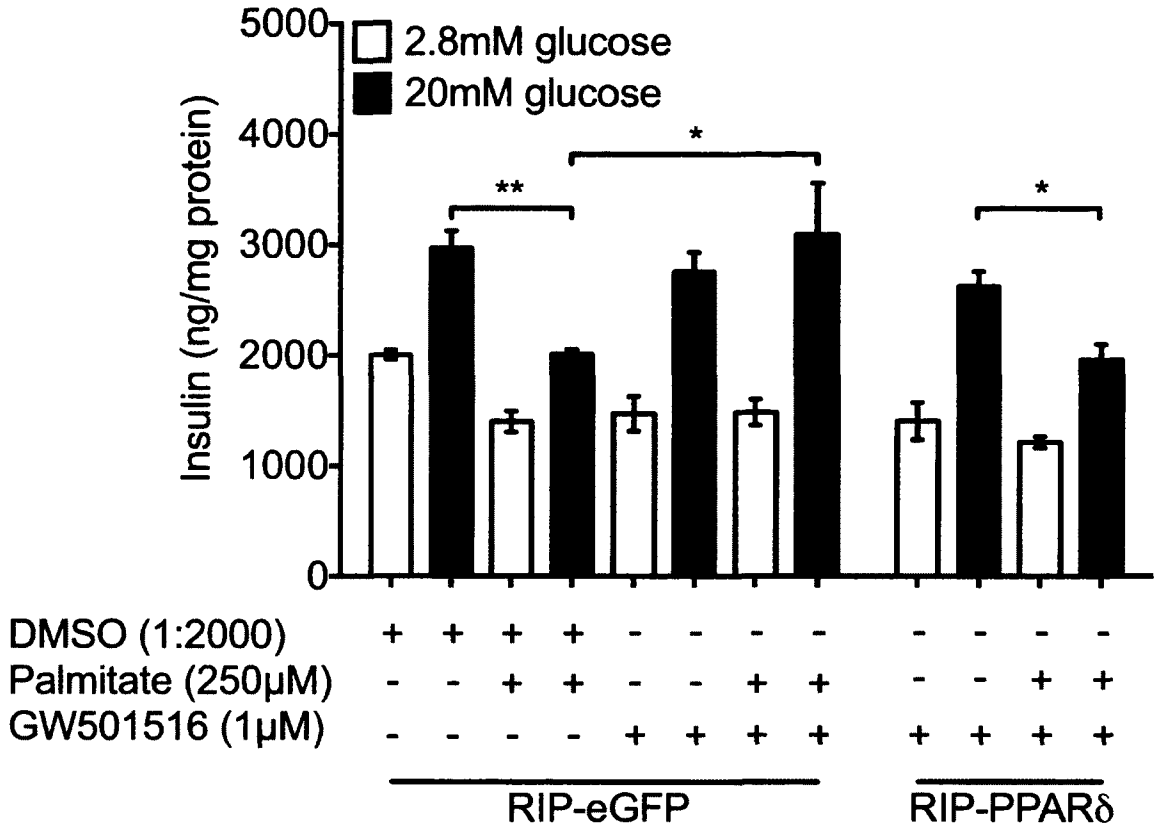

Figure 2.8 Glucose stimulated insulin secretion revealed that activation of endogenous PPARδ in MIN6 cells preserved β-cell function in a lipotoxic environment. Overexpression and activation of PPARδ alone had no effect on glucose stimulated insulin secretion while in a lipotoxic environment PPARδ overexpression and activation impaired glucose stimulated insulin secretion. *2-tailed t-test* $*p < .05$

2.4 DISCUSSION

Targeted activation of PPARδ is emerging as a promising treatment for dyslipidemia and insulin resistance associated with T2D and the metabolic syndrome (Barish et al., 2006). PPARδ activation with synthetic agonists has shown to improve serum cholesterol levels while restoring peripheral insulin sensitivity in rodent models of obesity and T2D (Oliver et al., 2001; van der Veen et al., 2005); however, tissue specific effects of systemic PPARδ activation need to be considered before a widely used treatment strategy can be adopted. The metabolic role of PPARδ in pancreatic β-cells is still not clearly defined in the literature and should be further elucidated.

Previous *in vitro* studies have suggested PPARδ activation plays a protective role against obesity-induced β-cell failure (Ravnskjaer et al., 2005; Ravnskjaer et al., 2010; Wan et al., 2010) while other *in vivo* models suggest that PPARδ is responsible for inhibiting insulin secretion through changes in cellular machinery (Iglesias et al., 2012). The aim of this study was to further explore the role of PPARδ in pancreatic β-cells in an obese setting and to examine the suitability of palmitate as a natural FA ligand for PPARδ. This was carried out using an *in vitro* model in which PPARδ was overexpressed and subjected to a lipotoxic environment characteristic of obesity.

Gene expression analysis confirmed that transfected MIN6 cells exhibited significant overexpression of PPARδ mRNA (Fig. 2.3), after which it was decided that PPARδ target gene expression would be assessed to explore the functional effects of PPARδ overexpression on downstream target genes. Treatment conditions including treatment time (Fig. 2.4) and concentration of agonists (Fig. 2.5) were first optimized to detect changes in target gene expression. Literature review of previous studies resulted in varied concentrations of GW501516 used to activate PPARδ in cell culture ranging from 100nM to 1μM (Cohen et

al., 2011; Oliver et al., 2001; Tanaka et al., 2003; Wan et al., 2010). Additionally, concentrations of palmitate used to induce lipotoxicity vary from 250μM to 500μM in previously published studies (Chen et al., 2013; Hogh et al., 2013; Lalloyer et al., 2006). Treatment time was also considered due to variability that can occur in genetic activity and transcriptional pathways (McAdams and Arkin, 1997).

The effect of 250μM palmitate or 1μM GW501516 on target gene expression was assessed after these parameters were determined to be the optimal conditions for detecting any changes in target gene expression levels. It was found that, for both UCP2 and LCAD, neither 250μM palmitate nor 1μM GW501516 (1:50 v/v DMSO vehicle) induced changes in gene expression levels when comparing RIP-eGFP and RIP-PPARδ cells (Fig. 2.6). Furthermore, despite optimization of treatment conditions, I was unable to reproduce the upregulation of UCP2 and LCAD even in RIP-eGFP control cells that was previously observed during optimization of treatment conditions, and therefore unable to draw robust conclusions regarding the effects of PPARδ overexpression on regulation of downstream target gene expression. At this time, it was decided that it was necessary to examine the ability to overexpress functional PPARδ protein in a more controlled setting using a luciferase reporter assay before considering functional changes on target gene expression associated with PPARδ overexpression.

Gene expression analysis determined that dsAAV8-RIP-PPARδ was able to increase PPARδ expression 130.7-fold in MIN6 cells. The next step was to confirm that functional protein was translated from the transcripts generated from the construct. In order to assess the presence and levels of functional protein a PPRE-luciferase reporter system was utilized to measure the activity of PPARδ induced with dsAAV8-RIP-PPARδ in response to palmitate and a PPARδ specific ligand (GW501516) in INS1 cells.

Initially, the experimental design of the luciferase reporter assay included treatment of INS1 cells with GW501516 at a concentration of 1μM in a DMSO vehicle at a concentration of 1:50 (v/v) in cell culture media. After initial examination of luciferase activity it was found that this high concentration of DMSO was inducing luciferase activity, suggesting that DMSO was capable of binding PPAR transcription factors and activating the PPRE. This finding was interesting as this provides a reason for the inconclusive results regarding PPARδ target gene expression analysis discussed previously. For this reason, a new GW501516 stock solution (1:2000 v/v DMSO) was prepared and the luciferase assay conducted on a second subset of INS1 cells in an attempt to abolish the effects of the DMSO vehicle. Future experiments to determine the effect of PPARδ overexpression in MIN6 cells will use a lower concentration of DMSO (1:2000 v/v) to evaluate the PPARδ-specific changes on target gene expression.

After re-establishing the luciferase assay, it was found that a lower concentration of DMSO (1:2000 v/v) provided a more suitable vehicle for delivery of GW501516 to cell culture media, as this lower level caused no significant induction of luciferase activity (Fig. 2.7). Furthermore, observation of RIP-eGFP cells expressing the luciferase construct revealed that treatment with palmitate caused an increase in luciferase activity (Fig. 2.7). Being that luciferase is under control of PPRE, which can bind any of the PPAR isoforms, these findings suggest that treatment of INS1 cells with palmitate induces activation of endogenously expressed PPARs. Despite being the most highly expressed member of the PPAR family in pancreatic β-cells (Ravnskjaer et al., 2010), treatment of RIP-eGFP control cells with 1μM GW501516, a PPARδ-specific agonist, caused no increase in luciferase expression. It may be the case here that endogenous expression and activation solely of the

PPARδ isoform in the β-cell is insufficient to induce significant changes in luciferase expression despite its high expression relative to the other PPAR isoforms.

Examination of PPARδ overexpression revealed that RIP-PPARδ cells had no significant increase in luciferase activity compared to RIP-eGFP cells, suggesting that presence of non-activated PPARδ protein without a ligand is not sufficient to bind the PPRE and induce expression of luciferase. Upon the addition of 1μM GW501516 to RIP-PPARδ cells, I note a significant increase in luciferase activity not observed in the RIP-eGFP control group, suggesting that transfection of the RIP-PPARδ plasmid induces overexpression of functional PPARδ protein capable of binding a ligand and subsequent binding of the PPRE in the promoter region.

When observing the effect of palmitate treatment on RIP-PPARδ INS1 cells, it can be seen that there is no significant difference between RIP-eGFP and RIP-PPARδ cells treated with palmitate, suggesting that palmitate is not causing an increase in luciferase activity despite having excess PPARδ available. These findings imply that palmitate may not be the most suitable fatty acid ligand for PPARδ; however, from figure 2.7, it can also be seen that the palmitate-treated cells in both the RIP-eGFP and RIP-PPARδ groups exhibit similar luciferase activity compared to the luciferase assay positive control. The positive control represents INS1 cells transfected with a plasmid containing the luciferase reporter gene under control of a constitutively active promoter, cytomegalovirus (CMV). Based on these findings, it could be that the reliability of the luciferase reporter assay above the threshold of the positive control cannot distinguish differences between experimental groups; therefore, it may be that palmitate is causing an activation of PPARδ to induce luciferase activity but the sensitivity is outside the range of this assay. For this reason, further evaluation of palmitate as a natural PPARδ ligand is required.

It has been reported here that transfection with the RIP-PPARδ plasmid results in overexpression of PPARδ mRNA and functional PPARδ protein (Fig. 2.3 and 2.7 respectively). For this reason it was suitable to look at the effect of PPARδ overexpression on β-cell function in MIN6 cells by measuring changes in glucose stimulated insulin secretion.

Analysis of glucose stimulated insulin secretion revealed that, upon treatment with 250μM palmitate, MIN6 cells exhibited impaired insulin secretion in response to glucose as expected (Fig. 2.8). Furthermore, administration of 1μM GW501516 restored insulin secretion in RIP-eGFP cells, supporting previous *in vitro* studies suggesting that activation of endogenous levels of PPARδ plays a protective role against lipotoxicity (Ravnskjaer et al., 2010; Wan et al., 2010).

Based on previous *in vivo* work, where it was reported that PPARδ plays an inhibitory role on insulin secretion through changes in cellular machinery (Iglesias et al., 2012), I also examined the effect of PPARδ activation on β-cell function in non-lipotoxic conditions. Interestingly, I report here that in both RIP-eGFP and RIP-PPARδ cells in the absence of palmitate, that PPARδ activation causes no significant change in insulin secretion.

To further examine the pro-oxidative potential of PPARδ in the β-cell, lipotoxicity was induced in RIP-PPARδ cells by palmitate exposure and 1μM GW501516 was administered. Interestingly, it was seen that overexpression and activation of PPARδ in the absence of lipotoxicity caused no change in insulin secretion, while under lipotoxic conditions, activation of PPARδ in RIP-PPARδ cells caused a significant decrease in insulin secretion. The decrease in β-cell function observed in lipotoxic RIP-PPARδ cells may be explained by an increase in peroxisomal lipid oxidation leading to the formation of cytotoxic peroxide species, or perhaps an increase in reactive oxygen species resulting from an increase in mitochondrial β-oxidation. It has been previously demonstrated that pancreatic β-cells are

not able to compensate for elevated levels of cytotoxic peroxides, resulting from reduced catalase activity inherent to β-cells, contributing to apoptosis (Elsner et al., 2011). It has also been shown that PPARδ is involved in the upregulation of genes involved in peroxisomal lipid oxidation such as peroxisomal acyl-coenzyme A oxidase 1 (Cheng et al., 2004; Wang et al., 2003), which catalyzes the desaturation of acyl-CoAs producing hydrogen peroxide as a bi-product. An increase in peroxisomal oxidative genes accompanied by an inability to compensate for the rise in cytotoxic peroxide species may explain the reduced insulin secretion observed in RIP-PPARδ cells exposed to 250μM palmitate. Furthermore, these effects of lipid oxidation may not have been observed in RIP-eGFP cells exposed to 250μM palmitate with PPARδ activation by 1μM GW501516 as a result of dosing. Activation of endogenous levels of PPARδ might not be sufficient to induce accumulation of cytotoxic peroxide or reactive oxygen species, while the increased PPARδ expression in RIP-PPARδ cells may have a more pronounced effects on β-oxidation.

I have confirmed here that at endogenous levels of PPARδ expression in the β-cell, administration of the PPARδ agonist GW501516 restores insulin secretion in a lipotoxic setting. In the case of PPARδ overexpression in a lipotoxic setting, it was observed that β-cell function once again deteriorated, suggesting PPARδ may impair β-cell function by upregulating both peroxisomal and mitochondrial β-oxidative pathways. In the future, it will be useful to repeat gene expression studies using a more robust approach in which effects of DMSO have now been removed from experimental conditions. This will allow for the exploration of potential mechanisms, such as upregulation of mitochondrial and peroxisomal β-oxidation, into the impairments on β-cell function induced by PPARδ overexpression under conditions of lipotoxicity.

Furthermore, exploration into the role of PPARδ in pancreatic β-cells will be required to assess the role of PPARδ on whole animal glucose homeostasis. This will include the generation of a transgenic mouse model in which PPARδ is overexpressed specifically in pancreatic β-cells of C57Bl6 mice where changes in carbohydrate metabolism will be measured. Finally, examination of β-oxidative pathways both *in vitro* and *in vivo* using qPCR and lipidomic analysis may reveal novel mechanisms for the role of PPARδ on β-cell function and insulin secretion.

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2.6 APPENDIX

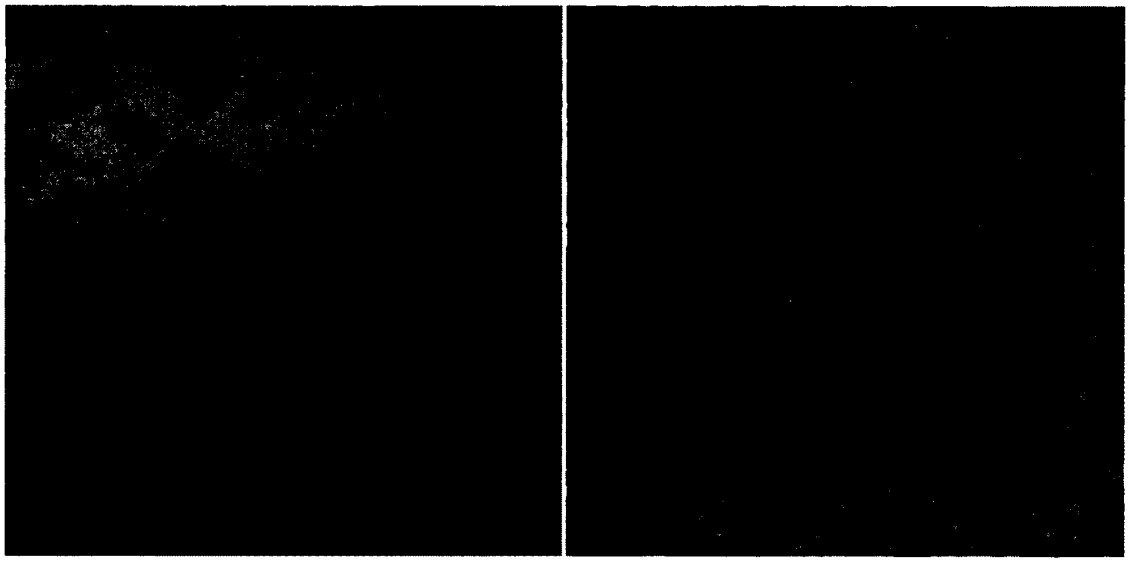

Figure S2.1. Confocal microscopy of cell cultures revealed that transfection of MIN6 cells with dsAAV8-RIP-eGFP construct resulted in successful overexpression of eGFP.

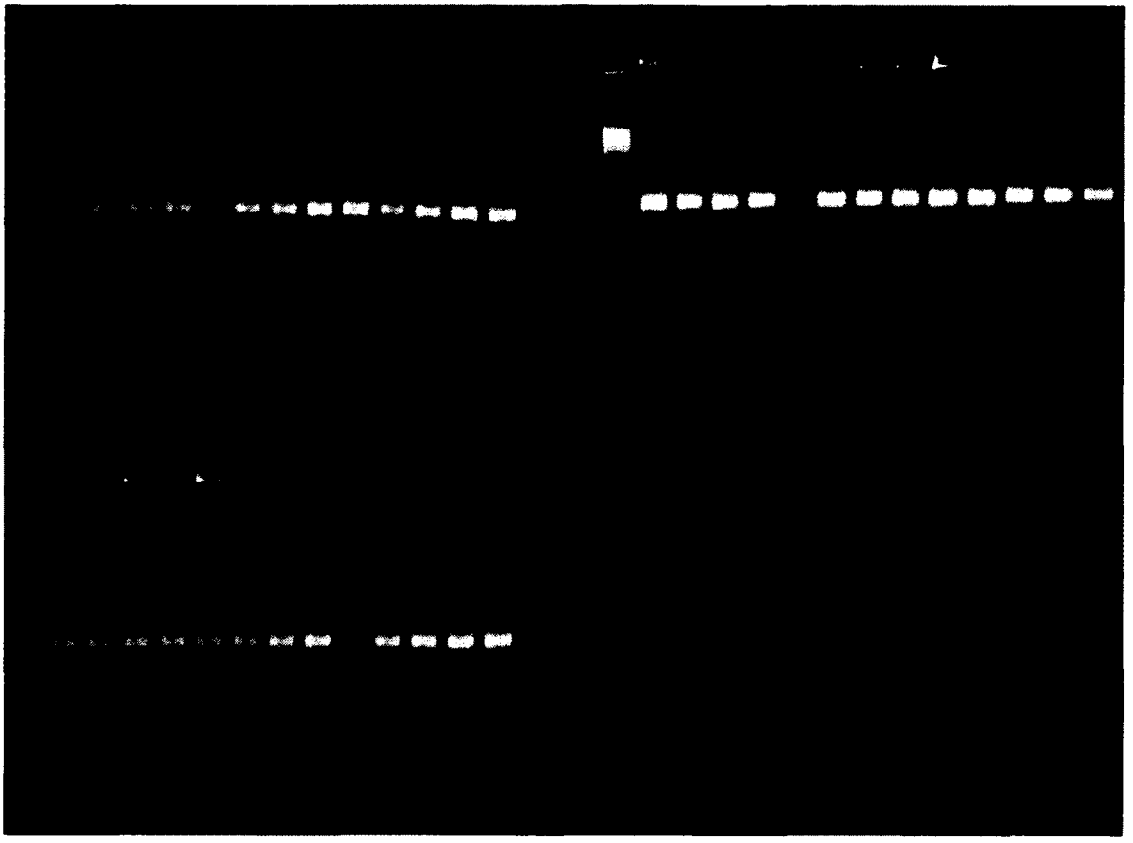

Figure S2.2. Total RNA extracted from MIN6 cells run on a 1.5% agarose gel demonstrated intact 18S and 28S ribosomal subunits indicating successful extraction of non-degraded RNA.

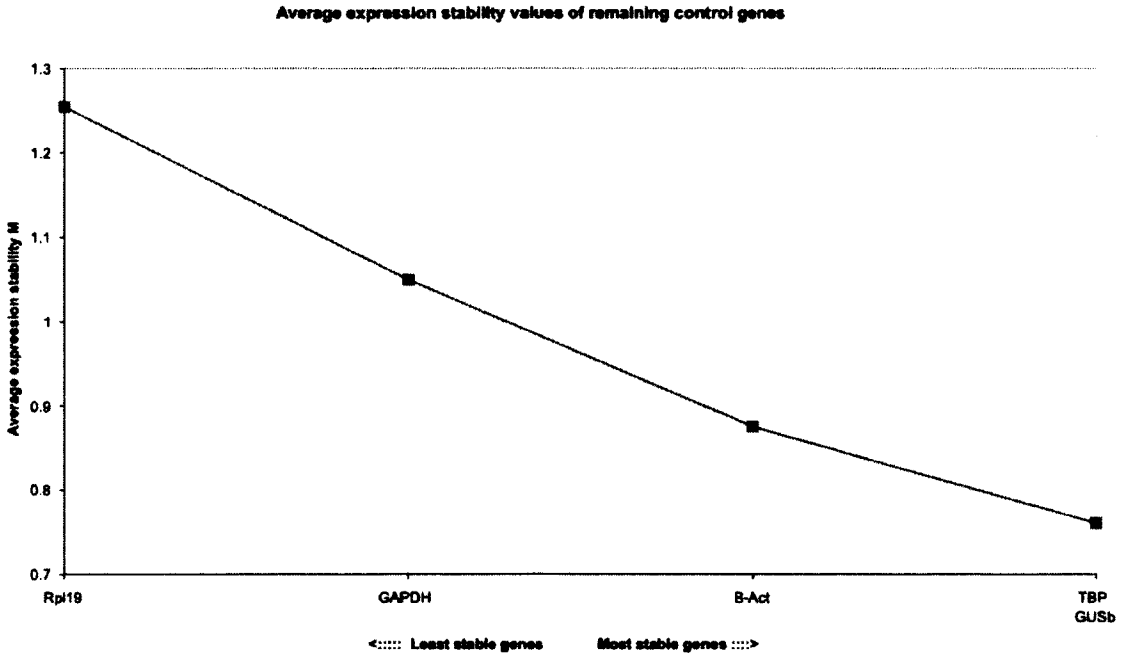

Figure S2.3. Assessment of endogenous control stability using GeNorm algorithm revealed β-actin, GusB, and TBP to be the most stable endogenous control genes.

CHAPTER 3

Characterization of lipid and gene expression profiles in islets isolated from mice overexpressing PPARγ2 specifically in pancreatic β-cells

A version of this chapter has been submitted for publication:

Hogh, K. N., Craig, M. N., Uy, C. E., Nygren, H., Asadi, A., Speck, M., Fraser, J. D., Rudecki, A. P., Baker, R. K., Oresic, M., Gray, S. 2013. Overexpression of PPARγ specifically in pancreatic β-cells exacerbates obesity-induced glucose intolerance, reduces β-cell mass and alters islet lipid metabolism. *Submitted for publication.* Year of Submission: 2014.

3.1 INTRODUCTION

Peroxisome proliferator activated receptors (PPARs) are a family of nuclear transcription factors that play a major role in the regulation of lipid metabolism (Barrera et al., 2008; Filip-Ciubotaru et al., 2011). PPARγ has been established as a primarily lipogenic and adipogenic transcription factor within the PPAR family. Being expressed most highly in adipose tissue, PPARγ activation in adipose tissue has been shown to increase lipogenesis and lipid storage, to induce adipogenesis and influence adipokine production, such as adiponectin (Evans et al., 2004; Tontonoz et al., 1995; Tontonoz et al., 1994).

Activation of PPARγ has been a successful target for the treatment of type 2 diabetes (T2D) and symptoms of the metabolic syndrome. Studies have shown that systemic administration of drugs known as Thiazolidinediones (TZDs), which specifically activate PPARγ, have potent anti-diabetic effects through improving insulin sensitivity in peripheral tissues (Day, 1999; Hauner, 2002). It is speculated that improvements seen in these patients may be a result of the regulatory role PPARγ plays on the expression of factors released from adipose tissue, including adiponectin, leptin, and tumor necrosis factor-α (TNF-α) (Ahmadian et al., 2013). It is also observed that these patients exhibit lower circulating levels of triglycerides and non-esterified fatty acids (NEFAs), suggesting that improvements in the lipotoxic state of peripheral tissues is likely due to the effects of PPARγ activation on lipid storage and adipogenesis leading to the improvements seen on impaired glucose tolerance in T2D (Kanda et al., 2010). Unfortunately, systemic administration of TZDs has been linked to harmful side effects and contraindications such as heart failure and cardiovascular risks in these already at-risk patients (Lago et al., 2007; Nissen and Wolski, 2007). For this reason, further exploration into the tissue specific effects of PPARγ activation by TZDs has been a popular topic of research.

Interestingly, associated with improvements in peripheral insulin sensitivity, an improvement in β-cell function can also be seen upon systemic administration of TZDs in patients with T2D (Kanda et al., 2010). However, what remains unclear is whether TZDs play a direct role on improving β-cell function or if these improvements are a secondary result of improvements to insulin sensitivity and the lipotoxic state. Within the literature there has been much debate about the effect of PPARγ activation in pancreatic β-cells on insulin secretion.

Studies in clonal β-cell lines have shown that activation of PPARγ causes an upregulation of genes involved in insulin gene transcription and secretion as well as glucose sensing (Gupta et al., 2010). It has also been shown that PPARγ activation in the β-cell preserves β-cell function through inhibition of pro-apoptotic pathways resulting from high levels of glucose, lipids, cytokines, and islet amyloid polypeptide (IAPP) (Chung et al., 2011; Gupta et al., 2010). Conversely, other studies have demonstrated that activation or overexpression of PPARγ and its co-receptors in clonal β-cell lines is unable to preserve β-cell function and that PPARγ actually plays an inhibitory role in the secretion of insulin under lipotoxic conditions (Ravnskjaer et al., 2005; Welters et al., 2004).

This ambiguity in the literature can also be seen when reviewing the role of PPARγ on β-cell function *in vivo*. The normal β-cell response to high fat feeding is to increase β-cell mass to compensate for the state of overnutrition (Terauchi et al., 2007). One group has suggested that β-cell specific knockout of the PPARγ gene impairs islet hyperplasia in obesity (Rosen et al., 2003). The net result of this impairment was that animals administered a high fat diet were unable to activate islet proliferative mechanisms resulting in reduced β-cell function and impairments in glucose homeostasis. However, other *in vivo* studies investigating the role of PPARγ in the β-cell have reported that β-cell specific knockout of

PPARγ has no effect on the insulin sensitizing effects of TZD administration to mice fed a high fat diet (Welters et al., 2012).

Previously in Dr. Sarah Gray's lab, it has been shown that obese mice overexpressing PPARγ1 (β-PPARγ1-HFD) or PPARγ2 (β-PPARγ2-HFD) specifically in β-cells of the pancreas have a worsened ability to maintain normal blood glucose levels compared to control mice overexpressing eGFP (β-eGFP-HFD) (Fig. 3.1a). It is of note that the impairments on glucose homeostasis in β-PPARγ1-HFD and β-PPARγ2-HFD mice are not associated with changes in body weight or peripheral insulin sensitivity in these animals (Fig. 3.1b,c), suggesting changes in glucose homeostasis are likely a result of alterations in β-cell function. Indeed, β-PPARγ1-HFD and β-PPARγ2-HFD mice exhibit a decrease in β-cell mass and an increase in β-cell apoptosis (Fig. 3.2a,b). Furthermore, β-PPARγ1-HFD mice also had significantly lower plasma insulin levels after an overnight fast, while β-PPARγ2-HFD mice had significantly lower plasma insulin in the fed state (Table 3.1), which also supports PPARγ overexpression playing a role in β-cell function in obesity.

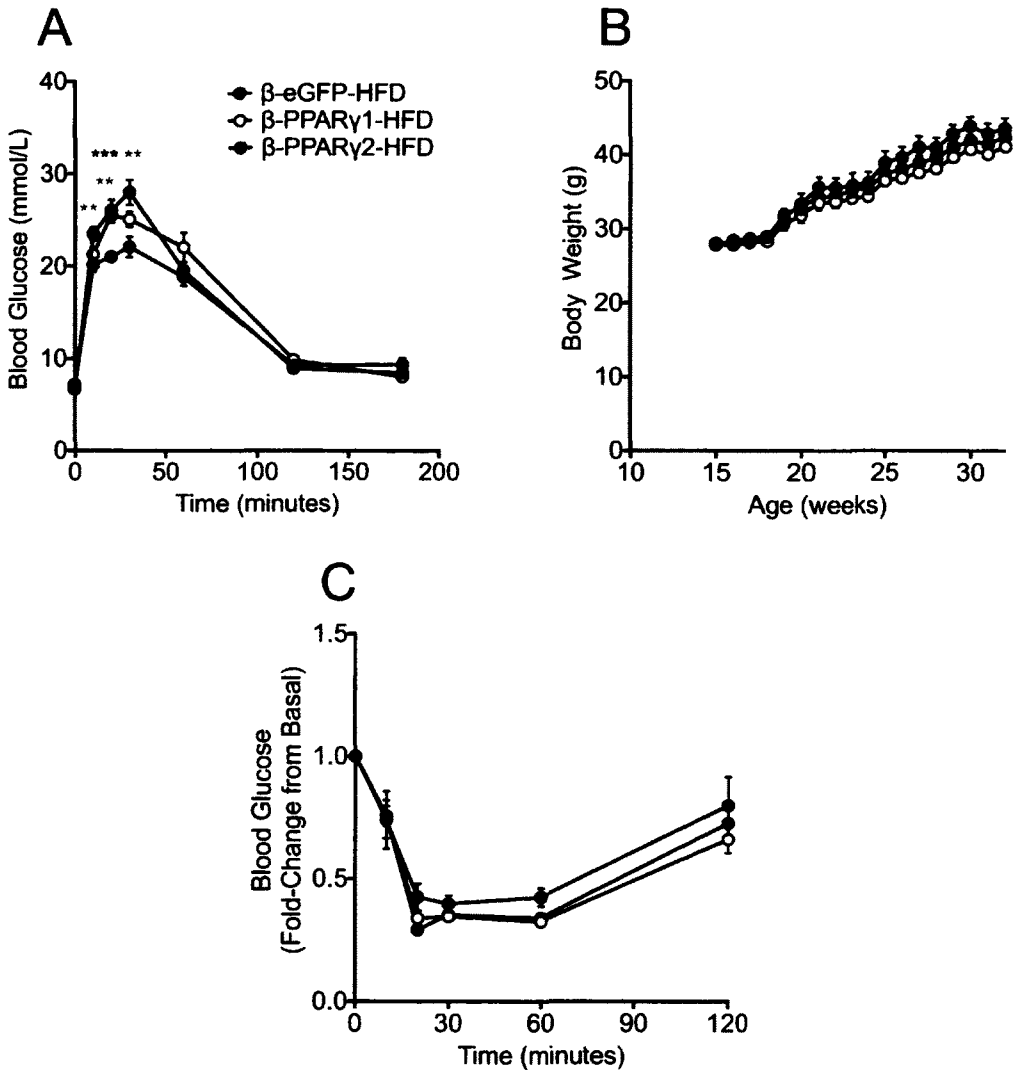

Figure 3.1 β-PPARγ1-HFD and β-PPARγ2-HFD mice demonstrate impaired glucose tolerance when challenged with an oral glucose tolerance test compared to β-eGFP-HFD control mice (a). Impairments in oral glucose tolerance are not associated with changes in body weight (b) or insulin sensitivity (c) in β-PPARγ-HFD and β-PPARγ2-HFD mice. *2-tailed t-test* $^{**}p < .01$, $^{***}p < .001$. β-eGFP-HFD (n=6), β-PPARγ-HFD (n=8) and β-PPARγ2-HFD (n=8).

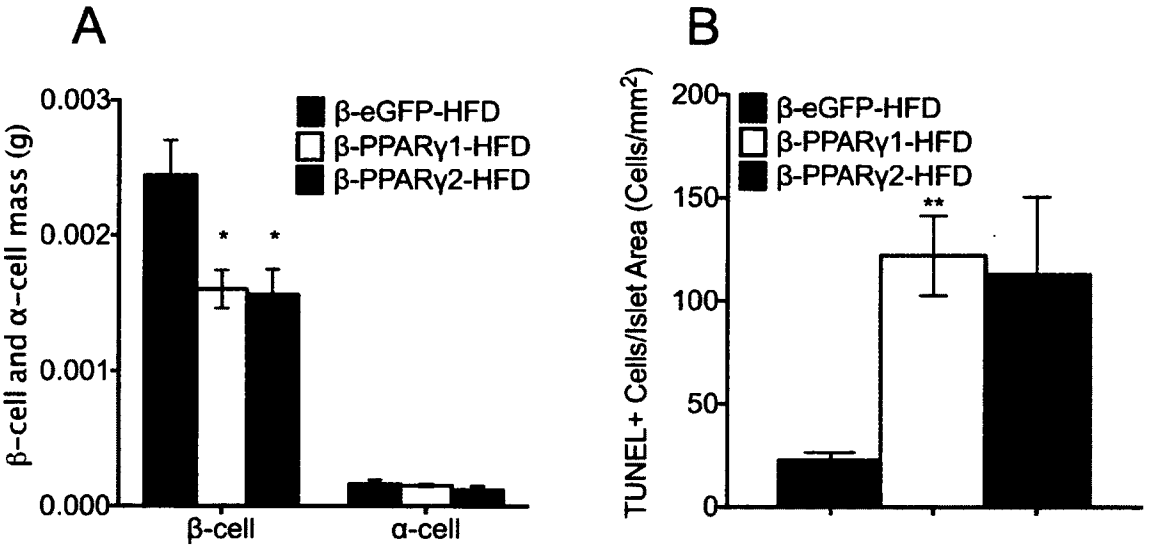

Figure 3.2. β-PPARγ1-HFD and β-PPARγ2-HFD mice demonstrate decreased β-cell mass (a) associated with increased apoptosis in β-PPARγ-HFD islets (b) compared to β-eGFP-HFD control mice. *2-tailed t-test* $*p < .05$, $**p < .01$

Table 3.1 Compared to β-eGFP-HFD control mice, β-PPARγ-HFD mice demonstrated significantly lower plasma insulin levels after an overnight fast while β-PPARγ2-HFD mice demonstrated significantly lower plasma insulin levels after random feeding.
2-tailed t-test $*p < .05$, $***p < .001$

Plasma Insulin (ng/ml)	**β-eGFP-HFD**	**β-PPARγ1-HFD**	**β-PPARγ2-HFD**
Overnight Fast	1.13 ± 0.42	0.28 ± 0.01 *	0.61 ± 0.22
2hr Refeed	3.68 ± 1.25	3.47 ± 1.09	3.00 ± 0.90
Random Fed	4.01 ± 0.30	4.01 ± 0.47	2.55 ± 0.26 ***
5min post glucose (GSIS)	1.64 ± 0.63	1.44 ± 0.24	1.14 ± 0.35
10min post glucose (GSIS)	1.75 ± 0.47	1.40 ± 0.54	1.23 ± 0.47

The objective of this study is to further elucidate the role of PPARγ in β-cells of the pancreas in an obese setting with the goal of identifying mechanisms causing the impaired β-cell function in β-PPARγ2-HFD mice. As described in Chapter 1, prolonged exposure of β-cells to free fatty acids (FFAs) *in vitro* and *in vivo* causes impaired insulin secretion and β-cell failure (Carpentier et al., 2000; Lee et al., 1994; Poitout, 2004; Robertson et al., 2004; van Herpen and Schrauwen-Hinderling, 2008). Furthermore, it has been suggested that exposure of β-cells to specific lipid species such as ceramides or diacylglycerols (DAGs) may induce oxidative stress promoting the activation of apoptotic pathways (Summers, 2006; van Herpen and Schrauwen-Hinderling, 2008). Alterations in lipid profiles have been suggested to cause changes in gene transcription or disruption of insulin signalling pathways thus contributing to β-cell failure (Lameloise et al., 2001; Shao et al., 2013; van Herpen and Schrauwen-Hinderling, 2008).

The primary theme of this chapter will be to identify changes in specific genes or lipid species that may be linked to the impairments in glucose homeostasis seen in β-PPARγ2-HFD mice. **I hypothesize that mice overexpressing PPARγ2 specifically in pancreatic β-cells will exhibit increased levels of lipid species previously determined to be lipotoxic to β-cells, with associated changes in lipogenic genes known to be regulated by PPARγ2.** This hypothesis will be tested through two experimental aims:
In **aim 1** I will measure mRNA expression of key target genes (insulin production, lipogenic pathways, ER stress, oxidative stress, mitochondrial lipid oxidation, peroxisomal lipid oxidation, and insulin signalling) in islets isolated from β-PPARγ2-HFD mice using real-time PCR.

In **aim** 2 I will quantify lipid species in pancreatic islets isolated from β-PPARγ2-HFD mice using LC/MS.

Results from this study may provide insight into which lipid species are responsible for lipotoxic β-cell failure in obesity-induced T2D as well as help to further characterize the role that PPARγ plays in pancreatic β-cell physiology.

3.2 MATERIALS AND METHODS

β-PPARγ1-HFD and β-PPARγ2-HFD transgenic mouse models

Transgenic mice overexpressing eGFP (β-eGFP-HFD), *Mus musculus*-derived PPARγ1 (β-PPARγ1-HFD, GenBank ID: NM_011146), or *Mus musculus*-derived PPARγ2 (β-PPARγ2-HFD, GenBank ID: NM_011146) specifically in β-cells of the pancreas were previously characterized in Dr. Gray's laboratory to assess the role of PPARγ in the β-cell on carbohydrate metabolism. A second cohort of mice was generated for the current study using intraperitoneal injection of double stranded adeno-associated virus serotype 8 (dsAAV8) ($5x10^{12}$ vg/ms) coupled with the rat insulin promoter driving the expression of eGFP or PPARγ2 (dsAAV8-RIP-eGFP and dsAAV8-RIP-PPARγ2) (Children's Hospital of Philadelphia (CHOP) Research Vector Core Services Philadelphia PA, USA) specifically in pancreatic β-cells. Post-infection, mice were administered a high fat diet containing 45% kcal as fat (D12451, Research Diets, New Brunswick NJ, USA). Mice were monitored for 16 weeks and the same trends in carbohydrate metabolism were observed in the second cohort, at which time mice were sacrificed for islet lipidomic and gene expression analysis.

Pancreatic islet isolation

Mice were anaesthetized and then euthanized by cardiac puncture followed by cervical dislocation. Pancreata were perfused using collagenase XI (1mg/ml) (C7657, Sigma) in Hanks balanced salt-solution (HBSS) (14185, Invitrogen) as previously described (Beith et al., 2008; Salvalaggio et al., 2002). Isolated islets were then either fixed in formalin for histological staining or stored at -80°C for qRT-PCR and lipidomic analysis.

Gene expression optimization

RNA extracted from pancreatic islets yields low concentrations of RNA. As such, RNA quantity for use in the generation of cDNA was optimized. cDNA was generated from 30ng, 50ng, 100ng, 300ng, or 1000ng of total MIN6 RNA and the expression levels of four different genes (PPARγ1, PPARγ2, β-actin, and Rpl19) was assessed as a measure for reverse transcription efficiency. It was found that 50ng of input RNA was sufficient to provide qPCR threshold cycles (Ct) within the acceptable range, while still allowing for conservation of islet RNA (Appendix Fig. S3.1).

Primer and probe sets for all genes evaluated in this study were designed and the optimal annealing temperature was established using a temperature gradient. Specificity of primer and probe sets were evaluated on an agarose gel or using a melt curve with the Bio-Rad iQ5 software. After establishing specificity, standard curves for each primer and probe set were constructed to ensure efficiencies fell within the range of 1.8-2.2 (Table 3.2). Gene expression levels were then calculated using these calculated efficiency values.

Table 3.2. Efficiency values (E) with slope and correlation coefficients (R^2) for primer and probe sets optimized for gene expression analysis using optimal annealing temperature (T_A).

Gene	Slope	E	R^2	T_A (°C)
Reference Targets				
GAPDH	3.3481	1.99	0.996	65.0
β-actin	3.0145	2.09	0.990	67.7
GUSb	3.7727	1.84	0.998	61.7
TBP	3.483	1.94	0.989	61.7
Rpl19	3.5598	1.91	0.990	61.7
Targets of Interest				
PPARγ	3.5535	1.91	0.985	65.8
PPARγ$_2$	3.3055	2.01	0.999	54.8
CPT1	3.236	2.04	0.994	57.0
UCP2	3.346	1.99	0.999	57.0
ABC1	3.3277	2.00	0.995	54.8
PGC1α	3.365	1.98	0.989	54.0
CD36	3.3155	2.00	0.996	53.0
AOX	3.3354	1.99	0.991	61.9
Insulin	3.420	1.96	0.999	57.4
XBP1s	3.4994	1.93	0.986	52.2
CHOP	3.5452	1.91	0.996	52.2
TXNIP	3.0588	2.12	0.996	54.0
PDX1	3.5209	1.92	0.998	60.8
GPR40	3.3893	1.97	0.997	60.8
SREPB1c	3.2963	2.01	0.999	53.8
GIPR	3.1446	2.08	0.995	54.0

Comparison of gene expression levels using endogenously expressed housekeeping genes reduces variability in the qPCR reaction and should always be used according to the MIQE guidelines (Bustin et al., 2009). To ensure suitable endogenous control genes were used in this study, five housekeeping genes were evaluated across conditions in which RNA was extracted from MIN6 cells overexpressing PPARγ2 or eGFP and treated with 250μM palmitate or untreated cell culture media and the two most stably expressed genes were used in the islet gene expression analysis. Using GeNorm's algorithm for establishing variability and an M-value cut off of 1, it was found that Rpl19 and β-actin were more stably expressed in β-cells than GusB, GAPDH, and TBP (Appendix Fig. S3.2). As such, all future analyses of islet gene expression were carried out with Rpl19 and β-actin endogenous controls.

Pancreatic islet gene expression

RNA from β-PPARγ2-HFD and β-eGFP-HFD islets (120 islets per mouse) was extracted using RNeasy micro RNA kit (Qiagen, Valencia, CA, USA). Concentration was measured using Qubit RNA BR Assay Kit (Invitrogen, Burlington ON, Canada), purity assessed using spectrophotometry (nanodrop ND-1000, Thermo Scientific, Rockford, IL, USA) and integrity assessed by visualizing intact 18s and 28s rRNA bands using the Experion RNA StdSens chips (BioRad Laboratories, Hercules, CA USA). mRNA (50ng) was reverse transcribed to cDNA using random hexamers (Superscript III, Invitrogen) as per manufacturers protocol. Gene expression levels were evaluated as per the MIQE guidelines (Bustin et al., 2009) using two candidate reference genes. Genes used for reference included β-actin and 60S ribosomal protein L19 (Rpl19), established to be the most stable for PPARγ1 and PPARγ2 manipulation by the Genorm algorithm in qbase+ as described above (Biogazelle, Zwijnaarde, Belgium). Relative expression levels of several genes (PPARγ,

PPARγ2, ATP binding cassette 1 (ABCA1), Cluster of differentiation-36 (CD36), Sterol regulatory element-binding protein 1c (SREBP1c), Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), pancreatic duodenal homeobox 1(PDX1), gastric inhibitory polypeptide receptor (GIPR), FFA receptor GPR40, insulin, C/EBP homologous protein (CHOP), X-box binding protein-1 spliced (XBP1s), thioredoxin-interacting protein (TXNIP), Uncoupling protein 2 (UCP2), Carnitine palmitoyltransferase 1 (CPT1), and Alternative oxidase (AOX)) were assessed in islets isolated from β-PPARγ2-HFD and β-eGFP-HFD mice. qPCR master mix contained: iQ Supermix with Taqman probes (2.55μM) or iQ SYBR Green Supermix, Taqman forward and reverse primers (10μM), RNAse free H_2O, cDNA (BioRad Laboratories; Sigma, Oakville, ON, Canada; IDT, Coralville, IA, USA; Ambion, Austin, TX, USA). Primer and probe sequences for these genes can be found in Table 3.1. Reactions were conducted on a BioRad iQ5 qPCR machine (BioRad iQ5 Multicolor RT-PCR Detection System, BioRad Laboratories, Hercules CA, USA) using the following temperatures, times, and number of cycles: one cycle at 95°C for 3min followed by 40 cycles alternating between 95°C for 10 seconds and primer-specific annealing temperature for 30 seconds.

Table 3.3 Primer and probe sequences used for real-time qPCR analysis of islets isolated from β-eGFP-HFD and β-PPARγ2-HFD mice.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Probe (5'-3')
Reference Targets			
β-actin	GCTCTGGCTCCTAGCACCAT	GCCACCGATCCACACAGAGT	GATCAAGATCATTGCTCCTCCTGAGCG
Rpl19	GAAGCTGATCAAGGATGG	CTTCCCTATGCCCATATG	CATCCGCAAGCCTGTGACTG
Target of Interest			
PPARγ	GCCTAAGTTTGAGTTTGCTGT	CAGCAGGTTGTCTTGGATGT	CTCAGTGGAGACCGCCCAGG
PPARγ2	GGAGATTCTCCTGTTGAC	CATGGTAATTTCTTGTGAAG	CATGGTGCCTTCGCTGATGC
ABCA1	CTGTGTATACCTGGAGAG	CATGGACTTGTTGATGAG	CAATGAGACCAACCAGGCAATCC
CD36	GCCAAGCTATTGCGACATGA	TCTCAATGTCCGAGACTTTTCA	CACAGACGCAGCCTCCTTTCCACCT
SREBP1c	CGGAAGCTGTCGGGGTAG	GTTGTTGATGAGCTGGAGCA	*
PGC1α	CCTCTTCAAGATCCTGTTA	TGCTCATAGGCTTCATAG	AAGCCACTACAGACACCGCA
PDX1	ACATCTCCCCATACGAAGTGC	GTAAGCACCTCCTGCCCACT	*
GIPR	TCAACAAAGAGGTGCAGTCG	GGGGTCCCTTTACCTAGCAG	TTGTGTGGGAGCCAATTACA
GPR40	GGCTTCCTAGCTGCTCTC	ACTGTTGTCCAGCCAGCTTC	*
CHOP	CTGCCTTTCACCTTGGAGAC	CGTTTCCTGGGGATGAGATA	*
XBP1s	GAGTCCGCAGCAGGTG	GTGTCAGAGTCCATGGGA	*
TXNIP	CATGAGGCCTGGAAACAAAT	ACTGGTGCCATTAGGTCAGG	*
Insulin	GGAGCGTGGCTTCTTCTACA	TTCATTGCAAAGGGGTGGGG	*
UCP2	GATCTCATCACTTTCCCTCTGGATA	CCCTTGACTCTCCCCTTGGG	CGCCAAGGTCCGGCTGCAGA
CPT1	GCGTGCCAGCCACAATTC	TCCATGCGGTAATATGCTTCAT	CCGGTACTTGGATTCTGTGCGGCC
AOX	AATTGGCACCTACGCCCAG	AGTGGTTTCCAAGCCTCGAA	CGGAGATGGGCCACGGAACTCA

*SYBR Green master mix used in place of Taqman probes

Measurement of islet lipid content with liquid chromatography/mass spectrometry

Isolated islet samples from β-eGFP-HFD and β-PPARγ2-HFD mice were sent to Espoo, Finland where Dr. Matej Oresic and Heli Nygren from the VTT Technical Institute performed the islet lipid quantification. To each of the isolated islet samples, 50μl of 0.9% NaCl was added and tubes sonicated at 40kHz for 5 minutes at 5°C. Lipidomic analysis was conducted using 30μl of this suspension. Samples were spiked with 20μl of an internal standard solution composed of lysophosphatidylcholine LysoPC(17:0), phosphatidylcholine PC(17:0/17:0), phosphatidylethanolamine PE(17:0/17:0), ceramide Cer(d18:1/17:0), phosphatidylglycerol PG(17:0/17:0), cholesteryl ester CE(19:0), phosphatidic acid PA(17:0/17:0), monoacylglycerol MG(17:0/0:0/0:0), diacylglycerol DG(17:0/17:0/0:0), and triacylglycerol TG(17:0/17:0/17:0). 100μl of Chloroform:Methanol solvent (2:1) was then added to each sample and the lower phase (60μl) was collected. To the lower phase, 10μl of a standard solution composed of three stable isotope-labeled reference compounds was added. Liquid chromatography/Mass spectrometry (LC/MS) was then conducted using an injection of 2μl of each sample solution. Ordering for analysis of samples was established by randomization. Lipid content was analyzed using a Q-ToF Premier mass spectrometer (Waters) in positive ion mode (ESI+) in combination with an Acquity Ultra Performance Liquid Chromatography system. Raw data was processed using MZmine to identify lipid species based on retention time (RT) and mass to charge ratio (m/z) in comparison to an in house lipid species database. Lipid species were quantified through normalization to internal standards. Total islet protein content was measured using a micro-BCA-assay kit (Pierce).

Analysis of lipidomic data from β-eGFP-HFD and β-PPARγ2-HFD islets

LC/MS was used to determine the quantity of 416 lipid species. Lipid levels were normalized to total islet protein content and were expressed as μmoles of lipid per gram of protein. Each of the 416 lipid species were then analyzed individually or grouped into lipid classes (lysophosphatidylethanolamine (LysoPE), triacylglycerol (TG), lysophosphatidylcholine (LysoPC), ceramide (Cer), sphingomyelin (SM), phosphatidylethanolamine (PE), or phosphatidylcholine (PC)) for statistical comparison between β-eGFP-HFD and β-PPARγ2-HFD islets.

Statistical analysis

Results are expressed as mean ± standard error of the mean. Data were assumed to be normally distributed as tests for normality are difficult to conduct when working with small sample sizes such as cell culture biological replicates and inbred transgenic animal models. One of the benefits of working with cell culture and inbred animal models is the high degree of reproducibility between samples, which is often not the case when working with more heterogeneous human populations that require large sample sizes. For this reason, assuming a normal distribution and using student's t-tests is acceptable here and most consistent with the literature. Comparisons were performed using student's 1-tailed or 2-tailed t-tests with Graphpad Prism 6.0 software (La Jolla CA, USA). Significance was declared if p-values were less than 0.05.

3.3 RESULTS

Successful overexpression of PPARγ mRNA in β-cells of whole pancreas using adeno-associated virus (dsAAV8)

Overexpression of PPARγ1 and PPARγ2 induced using the dsAAV8-RIP construct was assessed in islets isolated from β-eGFP-HFD and β-PPARγ2-HFD mice using real-time PCR. PPARγ2 contains an additional 30 amino acids at the N-terminal end of the PPARγ transcript (Spiegelman, 1998). A primer/probe set that detected total PPARγ expression showed transfection of MIN6 cells with dsAAV8-RIP-PPARγ1 induced overexpression of PPARγ by 36-fold and transfection of MIN6 cells with dsAAV8-RIP-PPARγ2 induced overexpression of PPARγ by 5-fold compared to MIN6 cells expressing a control plasmid dsAAV8-RIP-eGFP (Fig. 3.3a). When using a primer/probe that specifically detects PPARγ2 to show the induction of PPARγ expression by dsAAV8-RIP-PPARγ1 was due to an increase in PPARγ1 mRNA only, MIN6 cells overexpressing dsAAV-RIP-PPARγ2 showed a significant 17-fold increase in PPARγ2 expression compared to cells expressing either dsAAV8-RIP-PPARγ1 or dsAAV8-RIP-eGFP, with no difference in PPARγ2 expression between cells overexpressing dsAAV8-RIP-PPARγ1 and dsAAV8-RIP-eGFP. Together these results suggest that the primer/probe sets are effectively able to distinguish between total PPARγ and specifically PPARγ2 mRNA expression.

Using these primer/probe sets that detect total PPARγ and PPARγ2, β-PPARγ2-HFD islets displayed a 25-fold increase in total PPARγ mRNA compared β-eGFP-HFD islets (Fig. 3.3b). Additionally, a 3168-fold increase was observed in PPARγ2 mRNA in islets isolated from β-PPARγ2-HFD mice compared to β-eGFP-HFD control mice.

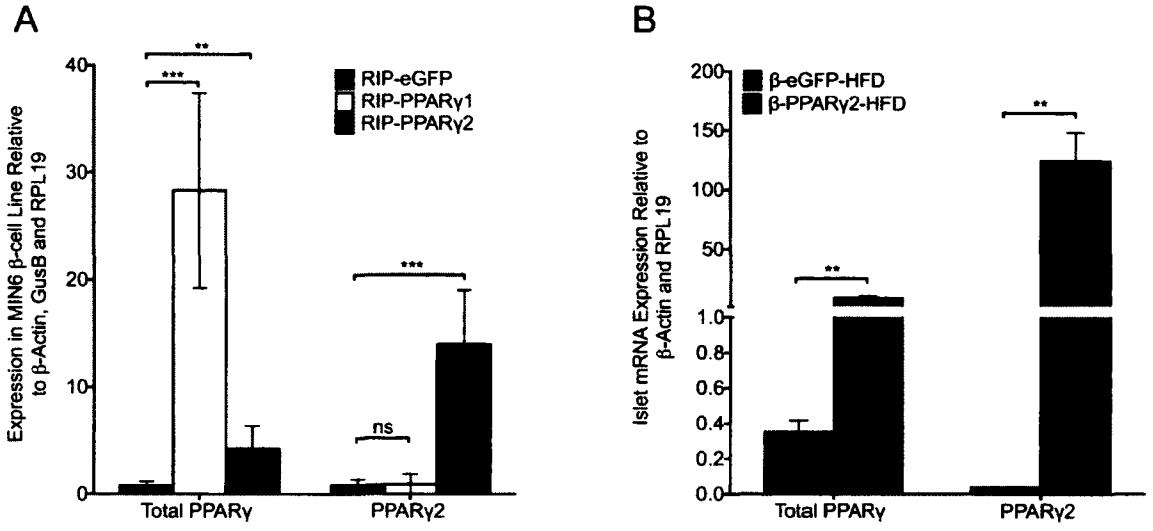

Figure 3.3 MIN6 cells overexpressing PPARγ or PPARγ2 under control of the rat insulin promoter demonstrate primer specificity for PPARγ and PPARγ2 mRNA (a). Isolated islets from β-PPARγ2-HFD mice demonstrate significantly increased expression of PPARγ2 mRNA compared to β-eGFP-HFD control islets. *1-tailed t-test* $^{**}p < .01$, $^{***}p < .001$

Lipid profiles of islets isolated from β-PPARγ2-HFD mice

Lipidomics is a relatively new field, and as such the standard for reporting lipid data has not yet been established in the literature. For this reason, lipid measurements were normalized to total islet number as well as total islet protein concentration for consideration. It was found that there were no significant differences between total islet number or islet protein concentration when comparing β-PPARγ2-HFD and β-eGFP-HFD islets (Fig. 3.4a,b). It was also seen that strategies for normalization of lipid data resulted in similar trends when observing differences in total lipid classes between β-eGFP-HFD and β-PPARγ2-HFD islets (Fig. 3.4c,d). Due to the similarities in results between normalization strategies, further analysis of lipid data was carried out normalizing lipid content to total protein only.

Lipidomic analysis of pancreatic islets showed a significant decrease in total lysophophotidylcholines (LysoPCs) in islets isolated from β-PPARγ2-HFD mice when compared to islets isolated from β-eGFP-HFD control mice (Fig. 3.4c). There were no significant changes in total lipids of the other lipid classes measured (Lysophosphatidylethanolamines (LysoPE), triacylglycerols (TG), ceramides (Cer), sphingomyelins (SM), phosphatidylethanolamines (PE), phosphatidylcholines (PC)). In comparing quantities of specific lipid species measured in islets isolated from β-PPARγ2-HFD and β-eGFP-HFD control mice, it was found that β-PPARγ2-HFD islets had significantly lower levels of LysoPC(18:0) and LysoPC(16:0) than β-eGFP-HFD control islets ($P = 0.030$ and $P = 0.013$ respectively) (Fig. 3.5a). It was also found that β-PPARγ2-HFD islets had significantly higher levels of PC(32:1) (Fig. 3.5b) with only a single ceramide species, Cer(d18:1/23:0) being increased in β-PPARγ2-HFD islets compared to β-eGFP-HFD control islets (Fig. 3.5c). Detection of monoacylglycerols (MAGs) and diacylglycerols (DAGs) were also included in the analysis, however due to low levels and sensitivity of the assay, detection of these species was unreliable and it was decided that a more targeted approach is required for reliable measures of these lipid classes.

Lipidomics is based on comparison of mass spectrometry data to library databases. Due to the novelty of this field, it was expected that species would be measured of which the identity has not been established in any database. Interestingly, β-PPARγ2-HFD islets had significantly higher levels of 10 unknown lipid species compared to β-eGFP-HFD control islets. Due to the novelty of lipidomic profiling, the identity of these species is still uncertain and will require future re-analysis once the identity of these unknown species are determined.

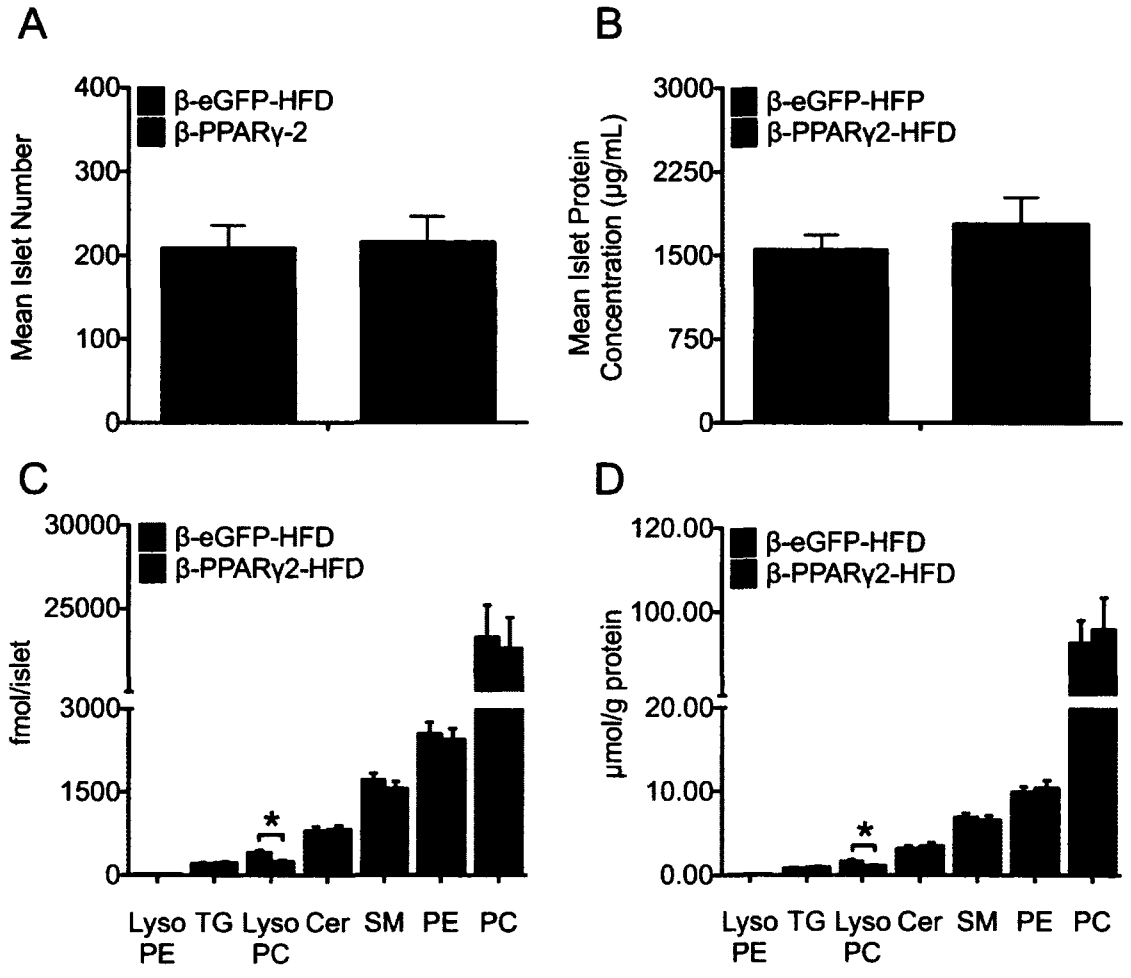

Figure 3.4 Number of islets isolated per mouse for lipidomic analysis (a) and total protein content of isolated islets (b) was not significantly different between β-eGFP-HFD and β-PPARγ2-HFD mice. Normalization of islet lipid species content as fmol/islet (c) and µmol/gram of protein (d) show similar trends in total lipid classes between β-eGFP-HFD and β-PPARγ2-HFD islets with significant decreases in total lysophosphatidylcholines.
2-tailed t-test $*p < .05$

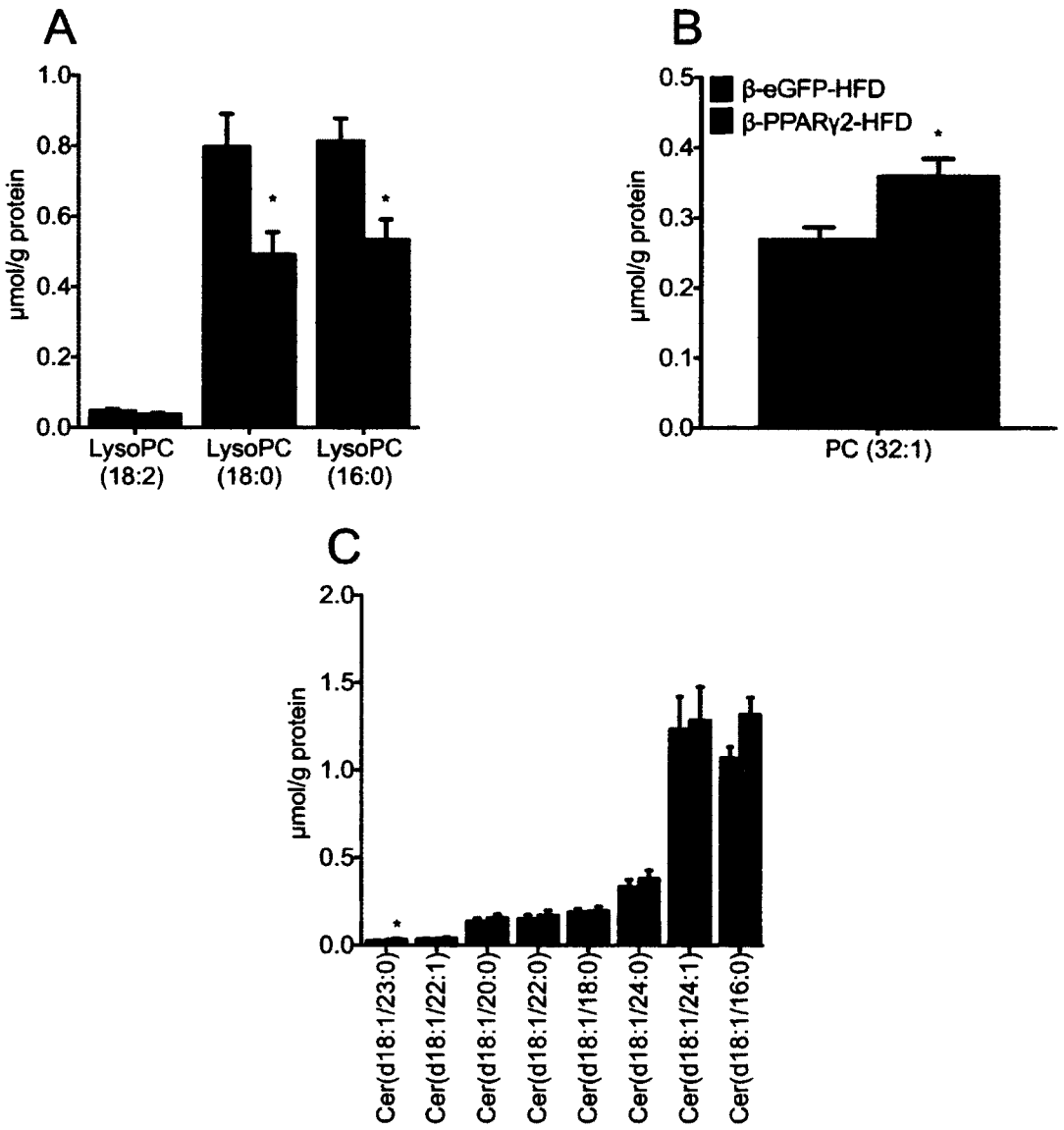

Figure 3.5 Islets isolated from β-PPARγ2-HFD mice had significantly lower levels of two specific lysophosphatidylcholines (a) and significantly increased levels of a single phosphatidylcholine (b) and ceramide (c) species when compared to β-eGFP-HFD control islets. *2-tailed t-test* $*p < .05$

Gene expression analysis of isolated islets from PPARγ2-HFD mice

Gene expression analysis of islet mRNA revealed that β-PPARγ2-HFD islets had significantly increased expression of the fatty acid transporter CD36, the fatty acid receptor GPR40, the acyltransferase CPT1, the mitochondrial membrane protein UCP2, and the electron transport chain protein AOX compared to β-eGFP-HFD control islets (Fig. 3.6). Other genes measured were not significantly changed compared to control islets.

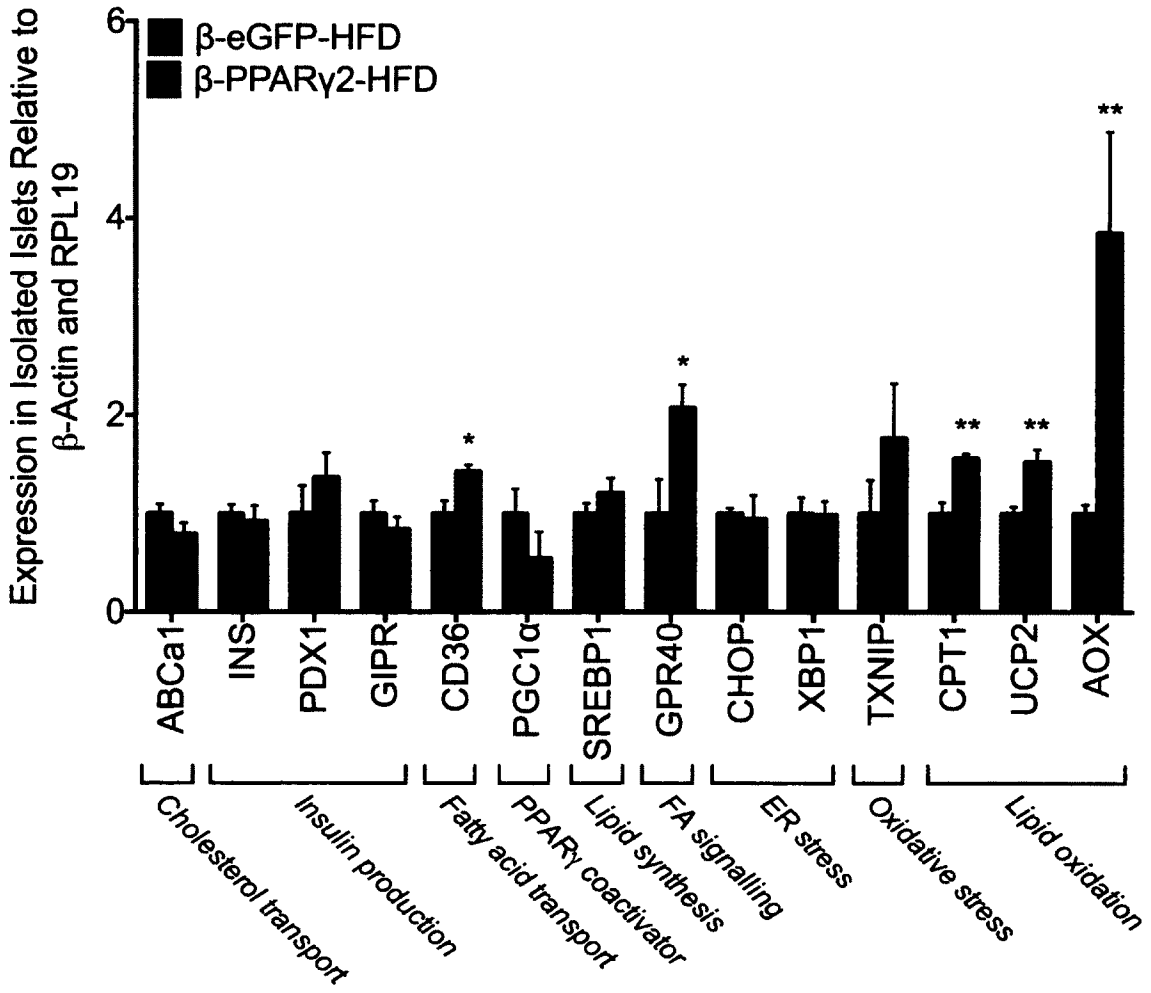

Figure 3.6 Islets isolated from β-PPARγ2-HFD mice demonstrated significantly increased mRNA expression of CD36, GPR40, CPT1, UCP2, and AOX. *2-tailed t-test* $*p < .05$, $**p < .01$

3.4 DISCUSSION

Activation of PPARγ by systemic administration of TZDs has been used as an effective means for improving insulin sensitivity in patients with T2D. However, due to the potential cardiovascular side effects and other contraindications associated with this class of drugs, their use has become limited. These findings have led to further questions as to what effect PPARγ plays in tissues other than adipose tissue, with β-cell function being of particular interest in the field of T2D.

Previously, Dr. Gray's laboratory has reported that obese mice overexpressing PPARγ1 or PPARγ2 specifically in β-cells of the pancreas exhibit impaired glucose tolerance when compared to obese control mice (Fig. 3.1a). It is also important to note that these impairments in glucose intolerance are not associated with changes in body weight between experimental groups or changes in insulin sensitivity at peripheral tissues (Fig 3.1b and c). As such, it is speculated that the impairments in glucose tolerance seen in β-PPARγ1-HFD and β-PPARγ2-HFD mice is related to changes mediated by PPARγ1 or PPARγ2 in the β-cell.

To support this claim, islet morphology and function were assessed and it was found that β-PPARγ1-HFD and β-PPARγ2-HFD islets had a decrease in β-cell mass with increased apoptosis (Fig. 3.2a,b), with significantly lower plasma insulin levels (Table 3.1) compared to β-eGFP-HFD control mice. From these results it is clear that PPARγ1 and PPARγ2 overexpression in the β-cell has a significant effect on whole animal glucose homeostasis; however, the mechanisms by which these transcription factors are inducing impairments in islet function remains unclear.

One potential mechanism could be related to the known proliferative effects of PPARγ on islet morphology and β-cell mass. Previous studies have shown that activation of

PPARγ in response to high fat feeding stimulates islet hyperplasia, allowing for an increase in β-cell mass as a compensatory mechanism in obesity (Rosen et al., 2003). Furthermore, these authors report that a β-cell specific knockout of PPARγ shows a blunting of this hyperplasia effect resulting in impaired glucose homeostasis in these animals. Other β-cell specific knockout studies have suggested that PPARγ activation by TZDs in pancreatic β-cells has no effect on islet hyperplasia and ultimately has no effect on glucose homeostasis in these animals (Welters et al., 2012). Based on the findings in the PPARγ knockout mice generated by Rosen et al., an enhancement in islet hyperplasia in obese mice overexpressing PPARγ in the β-cell might have been expected (2003); however, a model of β-cell specific PPARγ overexpression shows the opposite effect. Upon β-cell specific overexpression of PPARγ1 and PPARγ2, there is a decrease in β-cell mass associated with increased apoptosis and impaired glucose homeostasis.

Upon initial evaluation, the impairments in glucose homeostasis associated with β-cell specific overexpression of PPARγ may seem counterintuitive to previously published work (Rosen et al., 2003); however, it may be possible to explain these discrepancies with the question of dosage. PPARγ1 and PPARγ2 are known to be highly expressed in adipose tissue, while in the β-cell, their expression is relatively low. When considering transgenic studies in which these transcription factors are knocked out, one must consider that this may have a drastically different effect, and not always completely opposite, to one in which the same gene is over expressed to pharmacological levels. It could be possible that cells overexpressing the PPARγ1 or PPARγ2 transcription factors become overloaded with these proteins disrupting their function, or that perhaps secondary inhibitory effects of PPARγ1 or PPARγ2 transcriptional regulation override the pro-transcriptional activity of these proteins.

A second possible mechanism, and the focus of this thesis chapter, for the impairments in β-cell function seen in β-PPARγ1-HFD and β-PPARγ2-HFD mice may be related to the known potent lipogenic effect of PPARγ. The goal of this study was to further explore the role that PPARγ plays on lipid metabolism in pancreatic β-cells in an obese setting and to seek potential mechanisms for the changes observed in these animals. This goal was achieved by measuring changes in pancreatic islet lipid content and gene expression profiles in mice overexpressing PPARγ2 specifically in β-cells.

I hypothesized that due to its lipogenic potential, overexpression of PPARγ2 in pancreatic β-cells may cause alterations in lipid profiles resulting in accumulation of lipotoxic lipid species causing an impairment in β-cell function or increased activation of apoptotic pathways in these cells. β-PPARγ2-HFD mice exhibited a more severe impairment in glucose tolerance than β-PPARγ1-HFD mice and additionally PPARγ2 is known to be the more lipogenic isoform of PPARγ (Ahmadian et al., 2013). As such, lipidomic analysis was conducted on islets isolated from β-PPARγ2-HFD and compared to lipid profiles in islets from β-eGFP-HFD control mice.

I hypothesized that islets isolated from β-PPARγ2-HFD mice would yield increased levels of lipotoxic species known to induce oxidative stress and apoptosis, such as ceramides and diacylglycerols (DAGs), and that there may be an increase in total triglycerides (TAGs) due to the lipogenic role of PPARγ2 on TAG synthesis in adipose tissue. Surprisingly, no increase in either total triglycerides or ceramides was observed. Interestingly, a decrease in total Lysophosphatidylcholines (LysoPCs) was the only observable difference in total lipid classes between β-PPARγ2-HFD and β-eGFP-HFD islets. Furthermore, when observing specific lipid species within these lipid classes, it was found that β-PPARγ2-HFD islets exhibited a modest increase in only one single ceramide species, with DAG levels being so

low in islet samples that sensitivity of the assay was unable to measure these species. Due to the established lipotoxic effects of ceramides and DAGs, it was surprising that increased apoptosis and decreased β-cell mass was not associated with increased accumulation of these lipotoxic species.

Despite seeing only modest changes in lipid species widely known to be lipotoxic, lipidomic analysis of islets led to further interesting findings with respect to changes in lipid profiles. It was found that β-PPARγ2-HFD islets had significant decreases in total LysoPCs compared to β-eGFP-HFD control islets. Upon review of the literature, changes in LysoPCs have been implicated in regulating β-cell function in many different tissue types in obesity and T2D.

It has been suggested by two groups that an accumulation of LysoPCs in liver and heart tissue carries a lipotoxic effect on these cell types by induction of ER stress and upregulation of pro-apoptotic p53 upregulated modulator of apoptosis (PUMA) (Gurung et al., 2011; Kakisaka et al., 2012). Here I report that a decrease of LysoPCs is associated with impaired β-cell function. These findings suggest that there may be different mechanisms at play involving the metabolism of LysoPCs depending on tissue type.

Indeed, further review of the literature has revealed that in patients with obesity and T2D, there is a significant decrease in circulating plasma LysoPC levels (Barber et al., 2012). In adipose tissue, it has been shown that increases in specific LysoPC species 12:0, 16:0, and 18:0 caused an increase in the glucose 4 transporter and therefore increased glucose uptake in a dose dependant manner (Yea et al., 2009). These findings provide another example of how changes in lipid profiles of adipose tissue could result in impaired glucose uptake and therefore lead to hyperglycemia in patients with T2D. Interestingly, the LysoPC species

changes observed in adipose tissue during conditions of obesity are the same species that have been reported to change in this chapter (Fig. 3.5a).

Looking specifically at the role of LysoPCs in pancreatic β-cells, it has been shown that LysoPCs may play an important role in mediating the secretion of insulin granules. One study has shown that uptake of glucose by the β-cell may stimulate the conversion of phosphatidylcholines (PCs) to LysoPCs and arachidonic acid through the activity of Phospholipase A2 (iPLA2) (Fig. 3.7) (Ramanadham et al., 1999). This, as well as other studies, have shown that accumulation of arachidonic acid as well as LysoPCs causes an increase in insulin secretion and that inhibition of the formation of either of these two lipids will cause a decrease in insulin secretion (Ramanadham et al., 1993; Ramanadham et al., 1999; Wolf et al., 1991). Further studies have suggested that LysoPCs potentiate the secretion of insulin by binding to the G-coupled protein receptor-119 (GPR119) causing an increase in cyclic adenosine monophosphate (cAMP) levels (Overton et al., 2008; Soga et al., 2005) ultimately leading to increased mobilization of intracellular calcium stores and increased secretion of insulin granules (Fig 1.4) (Metz, 1988).

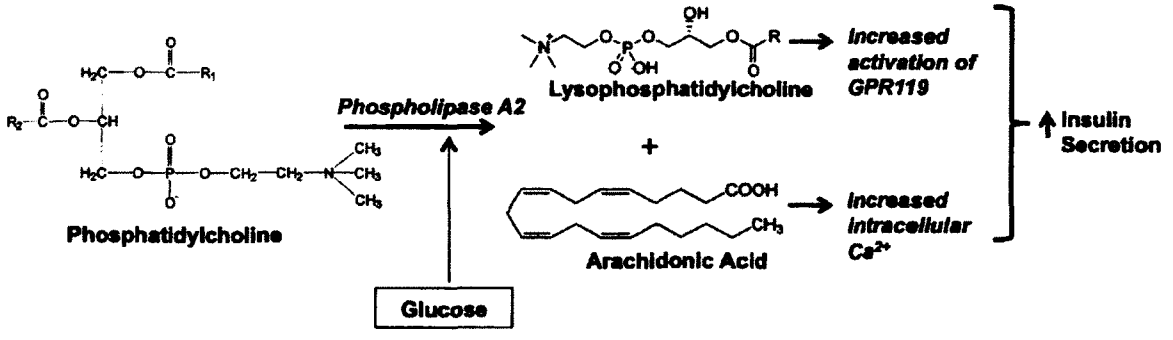

Figure 3.7. Glucose stimulates the activity of phospholipase A2 (iPLA2) causing the conversion of phosphatidylcholines to arachidonic acid and lysophosphatidylcholines potentiating the secretion of insulin in pancreatic β-cells.

The suggested mechanisms for LysoPCs on insulin secretion correlate well with the findings in this chapter. In reviewing the literature, it seems that in β-cells LysoPCs are able to enhance insulin secretion. Here I have reported that β-PPARγ2-HFD mice exhibit an increase in a specific PC, which is a LysoPC precursor, with a decrease in LysoPCs, which could be responsible for the observed impairment in β-cell function and whole animal glucose metabolism. Further studies will be required to assess how PPARγ regulates the conversion of PCs to LysoPCs in pancreatic β-cells.

As stated previously, lipidomics is a relatively new field. For this reason, lipid species can be measured by LC/MS that do not yet have a known identity. It is of interest to note that islets from β-PPARγ2-HFD mice had significantly increased levels of 10 additional unknown lipid species when compared to islets from β-eGFP-HFD control mice. Due to the unknown identity of these species it is difficult to speculate whether or not these species are playing a significant role and impossible to explore any mechanism by which they may be causing changes in β-cell function. In the future, when the identity of more of these lipid species are known, re-analysis of these data may reveal these species are lipotoxic species that increase lipotoxic stress in β-cells of β-PPARγ2-HFD mice and do contribute to the impairments seen in glucose tolerance. For this reason, future reanalysis of these data may prove useful.

As PPARγ2 is a nuclear transcription factor, it was useful to analyze gene expression profiles of isolated islets in order to reaffirm mechanisms for impaired glucose homeostasis arising from changes in lipidomic profiles as well as to identify other potential mechanisms of impaired glucose metabolism in β-PPARγ2-HFD mice. It was previously shown in Dr. Gray's laboratory that overexpression of PPARγ in the β-cell results in changes in plasma insulin levels (Table 3.1). It has also been shown previously that PPARγ can play a role in the production of insulin through binding to promoter regions in PDX1, a protein responsible

for the production of insulin and differentiation of mature β-cells (Gupta et al., 2008). As such, the role of PPARγ on insulin production may provide the simplest explanation for the changes in carbohydrate metabolism seen in β-PPARγ2-HFD mice. For this reason, mRNA expression was measured for the insulin gene as well as PDX1; however, it was found that there was no significant difference in the expression of either of these genes between β-PPARγ2-HFD and β-eGFP-HFD islets (Fig. 3.6), suggesting PPARγ2 overexpression in these β-cells is not inducing changes in insulin production that are responsible for the changes in glucose homeostasis.

Due to the lipogenicity of PPARγ2, genes involved in the formation and recruitment of lipids were analyzed and it was seen that an increase in expression of CD36, a known PPARγ2 target gene (Gupta et al., 2008; Parton et al., 2004), was observed in islets from β-PPARγ2-HFD mice (Fig. 3.6). CD36 is a FFA membrane transport protein; as such, its increased expression would be consistent with the idea that in conditions of elevated FFAs, PPARγ2 would compensate for the increased need for membrane transport of these lipids for entry into the cell for use in lipogenic pathways. However, upon looking at expression of ABCa1, another gene mediating the efflux of cholesterol and phospholipids (Yokoyama, 2006), it was found that there was no significant difference between β-PPARγ2-HFD and β-eGFP-HFD islets. Further evaluation of another lipogenic gene involved in the de novo synthesis of cholesterol and FFAs, SREBP1c (Fernandez-Alvarez et al., 2011), also resulted in no change in expression levels when comparing β-PPARγ2-HFD and β-eGFP-HFD islets (Fig. 3.6), suggesting that perhaps PPARγ2 plays only a minor role in *de novo* lipid synthesis within the islet.

One of the mechanisms of β-cell failure in obesity proposed in the literature is activation of apoptotic pathways as a result of ER stress and oxidative stress (Summers,

2006; van Herpen and Schrauwen-Hinderling, 2008). To assess the role of PPARγ2 overexpression in ER stressor pathways, expression levels of CHOP; a protein that participates in the suppression of metabolic genes in times of ER stress (Chikka et al., 2013), and XBP1s; a protein involved in the maintenance of protein folding during times of ER stress (Iwakoshi et al., 2003), were measured. It was found that there were no changes in the expression levels of CHOP or XBP1s in response to overexpression of PPARγ2. To assess the role of PPARγ2 overexpression in oxidative stress pathways, expression of TXNIP, a protein that interacts with thioredoxin to reduce oxidative stress through redox signalling (Yoshida et al., 2005), was measured and it was also found that there was no difference between the expression in β-PPARγ2-HFD and β-eGFP-HFD islets. In both cases of ER and oxidative stress, it was found that PPARγ2 overexpression in the β-cell had no effect on the expression of these genes (Fig. 3.6), suggesting the role of PPARγ2 on impaired glucose metabolism in these mice may not be due to ER or oxidative stress.

In addition to ER and oxidative stressor pathways, peroxisomal stress on the β-cell must also be considered. My findings that β-PPARγ2-HFD mice exhibit increased expression of AOX are consistent with previously published work that suggest the lack of catalase in pancreatic β-cells results in accumulation of cytotoxic peroxides that can induce apoptosis (Elsner et al., 2011). This finding may suggest that increased β-oxidation specifically through AOX in the peroxisome may induce apoptosis and could account for the decreases observed in β-cell mass in β-PPARγ2-HFD islets (Fig. 3.2a).

Furthermore, β-PPARγ2-HFD mice had significant increases in UCP2 and CPT1, other genes involved in mitochondrial β-oxidation of lipid species (Fig. 3.6). Due to the lipogenic nature of PPARγ2, it was surprising to see an increase in genes involved in β-oxidation; however, these findings are consistent with previous works that would explain an

inhibitory role of PPARγ on insulin secretion and impairments in glucose homeostasis (Ito et al., 2004; Parton et al., 2004). In pancreatic islets it was shown that PPARγ overexpression increased the expression of UCP2 and CPT1 causing a reduction of ATP production, which would decrease the membrane polarization effect, required for mobilization of intracellular calcium stores and the secretion of insulin described in Figure 1.4 (Ito et al., 2004; Parton et al., 2004). This effect is consistent with the changes in plasma insulin levels reported in Table 3.1, and could contribute to the impaired glucose homeostasis observed in β-PPARγ2-HFD mice. Taken together with the findings that β-PPARγ2-HFD mice exhibit increased expression of FFA transport proteins (CD36) and increased expression of genes involved in peroxisomal (AOX) and mitochondrial (CPT1) β-oxidation, it could mean PPARγ2 is responsible for recruitment of lipid species into β-cells for entry into β-oxidative pathways rather than the classic TAG synthesis paradigm seen in white adipose tissue. This theory would be consistent with the lipidomic results reported here where there was no increase in total TAG species as a result of β-cell specific PPARγ2 overexpression (Fig. 3.4).

Finally, genes that have been implicated in signalling of insulin secretion were assessed. In contrast to what would have been expected, it was found that β-PPARγ2-HFD islets showed an increase in expression of GPR40 (Fig. 3.6), a protein that when activated has been shown to increase secretion of insulin from the β-cell as described in Figure 1.4. Due to decreased plasma insulin levels of β-PPARγ2-HFD mice noted in Table 3.1, it would have been expected that a decrease in GPR40 would be observed, however this was not the case.

I have clearly demonstrated here that β-cell specific overexpression of PPARγ2 plays a significant role on the maintenance of plasma glucose levels, and that changes in the β-cell rather than peripheral tissues are responsible for these observed changes. It is difficult to

speculate whether these impairments in glucose homeostasis are a result of changes in islet morphology, such as decreased β-cell mass, or disruptions of insulin secretion brought on by changes in gene expression or lipid profiles. Further exploration is required to determine whether the reductions in LysoPCs observed here is a substantial enough change to impact whole animal glucose homeostasis. In addition, the role of PPARγ2 in the formation of these lipid species is still unknown and requires further attention in order to highlight potential new mechanisms for the role of PPARγ in β-cell lipid metabolism. It is likely that changes in glucose homeostasis observed here are a summation effect of the changes observed in islet morphology and changes in lipid as well as gene expression profiles.

This study has further enforced the idea that PPARγ activation can have varying effects depending on tissue type. As with heart and cardiovascular complications, activation of PPARγ in pancreatic β-cells seems to have a negative impact on β-cell function in obesity. Furthermore, this study outlines the complexity with which lipid metabolism can affect β-cell function in conditions of obesity and further exploration is required to assess the effect of specific lipid profile changes on insulin secretion and signalling in response to secretory cues from peripheral tissues. Tissue-specific activation of PPARγ has become a popular area of research due to the potent anti-diabetic effects of TZD administration. I conclude here that in an obese setting, PPARγ overexpression specifically in pancreatic β-cells impairs whole animal glucose homeostasis, which does not support the use of TZDs to directly improve islet function.

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3.6 APPENDIX

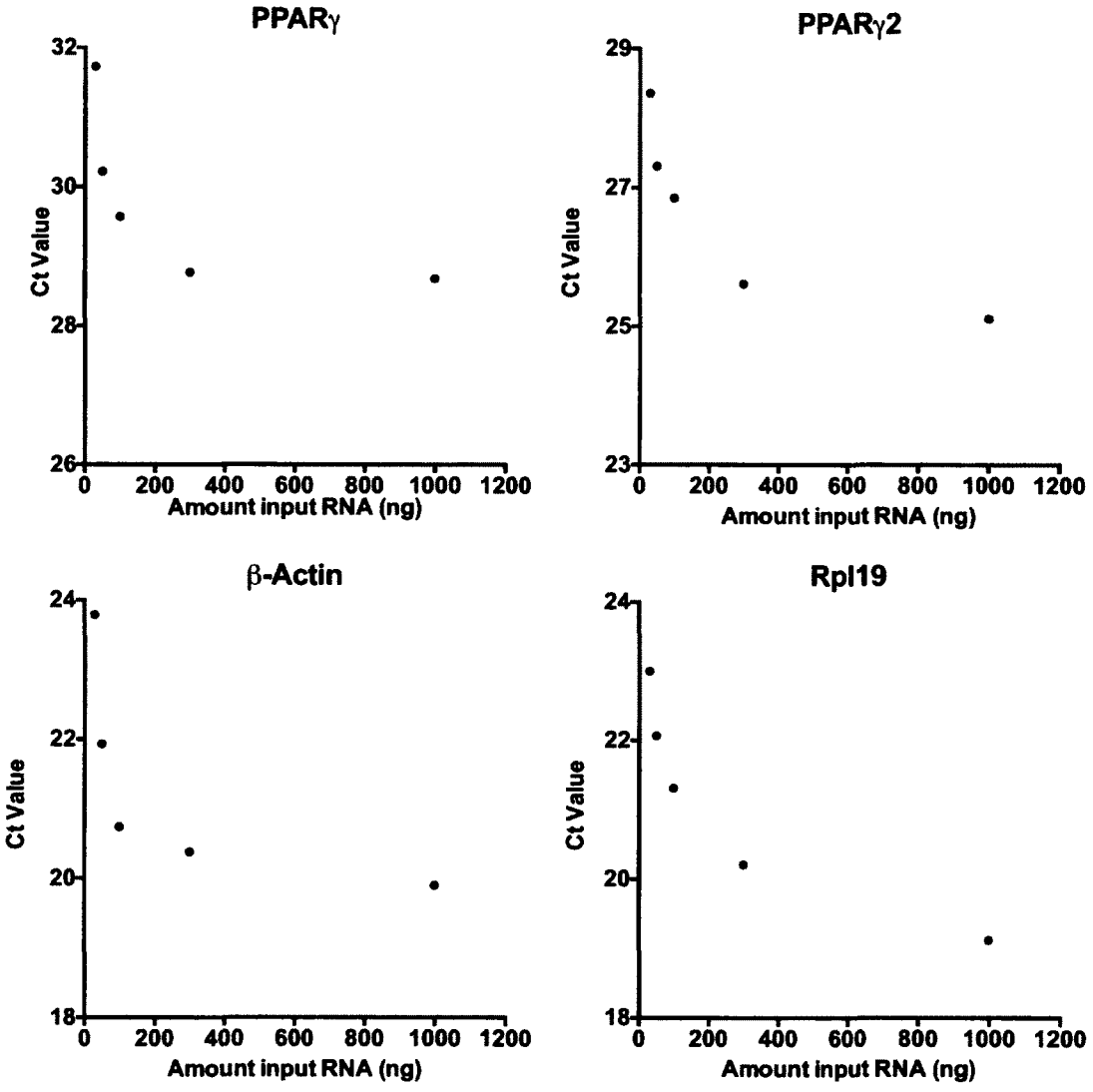

Figure S3.1. Optimization of RNA quantity for use in cDNA synthesis reaction revealed 50ng of input RNA to be the most efficient while maintaining acceptable Ct values.

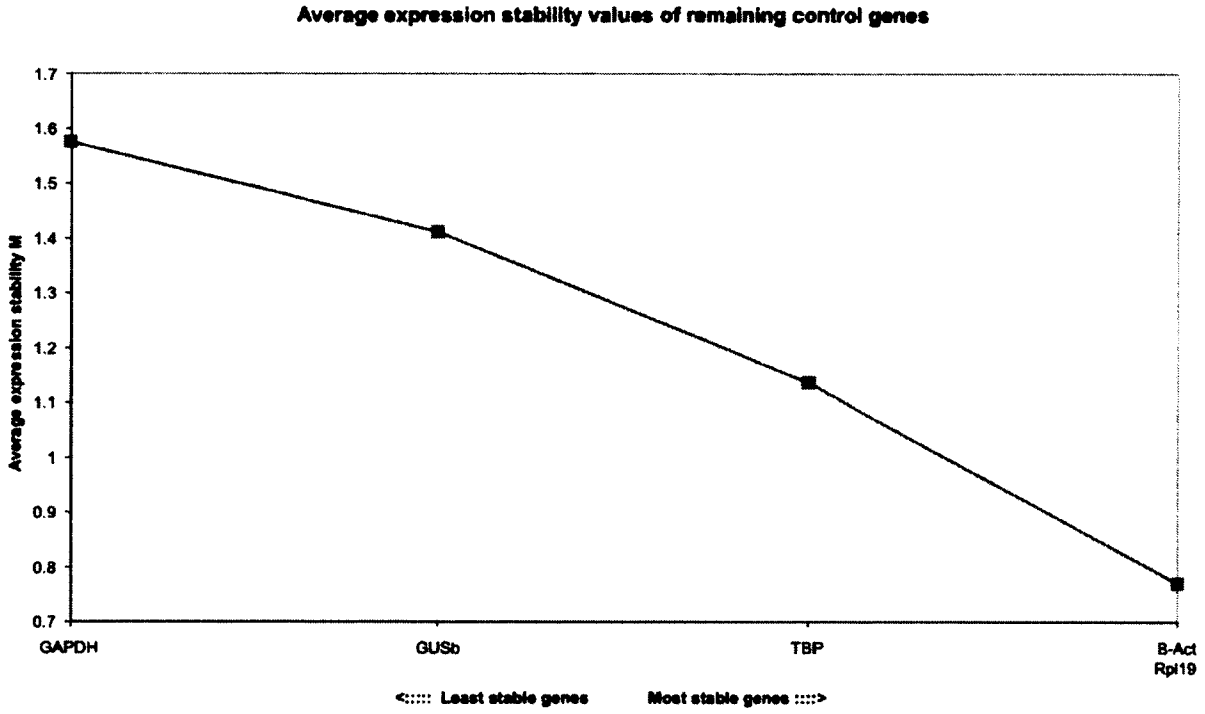

Figure S3.2. Assessment of endogenous control stability using GeNorm algorithm revealed β-actin and Rpl19 to be the most stable endogenous control genes.

CHAPTER 4

Concluding remarks for exploring the role of PPARδ and PPARγ overexpression on pancreatic β-cell function with respect to obesity and T2D

4.1 CONCLUDING REMARKS

Obesity has been established as the primary risk factor for the development of type 2 diabetes (T2D) and complications associated with the metabolic syndrome (Smyth and Heron, 2006). A key hypothesis explaining the link between T2D and obesity is lipotoxicity, where limitations in adipose tissue expandability leading to ectopic deposition of cytotoxic lipid species results in insulin insensitivity and β-cell failure (Ahima, 2006; Chavez and Summers, 2010; Gray and Vidal-Puig, 2007; Kershaw and Flier, 2004). It remains unclear whether elevations in circulating triglycerides, high-density lipoprotein cholesterol, and increased central obesity associated with the metabolic syndrome arise first causing impairments in insulin action and progression towards T2D, or if insulin insensitivity arises first causing dyslipidemia leading to the symptoms associated with the metabolic syndrome described previously (Chapter 1). In an attempt to further elucidate the mechanisms leading to β-cell failure associated with obesity, I have explored the role of peroxisome proliferator activated receptors (PPARs), a family of nuclear transcription factors described as master regulators of lipid metabolism, on β-cell function both *in vitro* and *in vivo.*

Here I outline the effects of PPARδ and PPARγ2 overexpression in a model of obesity in pancreatic β-cells. More specifically, I observed the role of PPARδ overexpression *in vitro* to assess the effect of PPARδ activation on insulin secretion in both lipotoxic and non-lipotoxic conditions. I also report here potential mechanisms specific to pancreatic β-cell failure induced by dyslipidemia associated with β-cell specific PPARγ2 overexpression in an animal model.

By further elucidating the role of PPARs on β-cell function, better treatment strategies can be developed targeting this family of transcription factors. This is an important aspect to consider as systemic administration of PPAR agonists, despite showing marked

improvements in insulin sensitivity and dyslipidemia, has been shown to cause adverse effects, including heart failure and cardiovascular risks, in already at-risk patients (Lago et al., 2007; Nissen and Wolski, 2007).

4.2 SIGNIFICANCE

Here I report the effects of β-cell specific overexpression of PPARδ and PPARγ2 on insulin secretion and carbohydrate metabolism. This study provides a useful addition to the scientific literature related to the effects of obesity on lipid metabolism within the β-cell. I have further elucidated the tissue-specific effects of PPARδ and PPARγ2 overexpression in the β-cell, which will provide insight into the role these transcription factors play in islet function and for development of future treatment strategies aimed at PPAR activation as a tool for improved insulin sensitivity and dyslipidemia in patients with T2D and the metabolic syndrome.

In summary, I have shown that overexpression of PPARδ in MIN6 and INS1 β-cell models yields functional PPARδ protein capable of binding to the peroxisome proliferator response element (PPRE) inducing transcriptional changes (Chapter 2). Furthermore, I have confirmed previous findings that activation of endogenous levels of PPARδ with a synthetic ligand in pancreatic β-cells plays a protective role against palmitate-induced lipotoxicity (Ravnskjaer et al., 2010; Wan et al., 2010). I also report that, under non-lipotoxic conditions, overexpression of PPARδ with subsequent activation by a synthetic ligand causes no significant change in insulin secretion. When observing the effects of PPARδ overexpression and activation in a lipotoxic setting however, I report an impairment on insulin secretion not observed in cells with endogenous levels of PPARδ expression. In examining gene expression profiles, no changes in mitochondrial β-oxidative genes were detected. It is

suggested that additional gene expression studies looking at these pathways be assessed before final conclusions are drawn; however, it may be that additional roles of PPARδ in the β-cell may explain the observed changes in β-cell function. Taken together, these results suggest that treatment strategies involving PPARδ agonists should consider dosing effects and nutrient availability when evaluating the effects of PPARδ on β-cell function and ultimately effects on carbohydrate metabolism.

Previously Dr. Gray's laboratory has demonstrated that mice overexpressing PPARγ1 or PPARγ2 specifically in pancreatic β-cells in obesity exhibit impairments in glucose homeostasis associated with changes in β-cell function (Hogh et al., 2014). Here I report lipidomic and gene expression analysis of islets isolated from obese mice overexpressing PPARγ2 specifically in pancreatic β-cells (Chapter 3). Lipidomic profiling of islets suggests that PPARγ2 may down regulate lipid species previously shown to play a role in insulin secretion. More specifically, I observed decreases in lysophosphatidylcholines, lipid species shown to enhance insulin secretion from β-cells through signalling of G-protein coupled receptors (Overton et al., 2008; Ramanadham et al., 1993; Ramanadham et al., 1999; Soga et al., 2005; Wolf et al., 1991). Furthermore, gene expression analysis of isolated islets revealed that PPARγ2 induced increases in expression of genes involved in the membrane transport of lipids (CD36) as well as mitochondrial (UCP2, CPT1) and peroxisomal (AOX) lipid oxidation. Considering the changes in lipid and gene expression profiles, I suggest that PPARγ2 activation plays an inhibitory role on insulin secretion and may induce cytotoxicity through activation of peroxisomal oxidative pathways leading to the accumulation of cytotoxic peroxide species in the β-cell. These findings will be useful when considering systemic effects of PPAR agonism to assess what role PPARγ activation will have on β-cell function and ultimately whole animal carbohydrate metabolism.

4.3 FUTURE DIRECTIONS

The focus of this thesis has been to examine the role of PPARs, specifically PPARδ and PPARγ2, on β-cell function in a setting of obesity-induced T2D. I have shown here that overexpression and activation of PPARδ in a MIN6 model plays a significant role on the ability to secrete insulin in response to glucose under lipotoxic conditions. The role of PPARγ2 has been further characterized, here I report changes in islet lipid and gene expression profiles associated with impairments in whole animal carbohydrate metabolism of obese mice. These findings have revealed novel insights in which to further explore and characterize the specific role that PPARδ and PPARγ2 play on β-cell function in obesity.

Further studies related to PPARδ in pancreatic β-cells will focus on the effects of PPARδ overexpression specifically in pancreatic β-cells of C57Bl6 mice. A previous study has suggested that β-cell specific knockout of PPARδ causes an increase in insulin secretion due to PPARδ regulation of cellular machinery (Iglesias et al., 2012). Generation of a chow-fed transgenic mouse overexpressing PPARδ will further discern the role of PPARδ on β-cell function and may provide support to the claims of this previous study. Furthermore, overexpression of PPARδ in pancreatic β-cells of mice on a high fat diet will allow for examination into the role of PPARδ in the β-cell under conditions of obesity-induced lipotoxicity. Transgenic mice will be characterized by changes in carbohydrate metabolism as a result of PPARδ overexpression by measuring glucose stimulated insulin secretion, oral glucose tolerance, and insulin sensitivity.

Transgenic mice will be generated using an intraperitoneal injection of a double stranded adeno-associated virus (dsAAV), specifically serotype 8 to target tissues of the foregut (Hogh et al., 2013). Viral vectors will be used to deliver the construct containing the PPARδ gene insert under control of the rat insulin promoter (RIP) to induce overexpression

of PPARδ specifically in pancreatic β-cells. Effectiveness of transfection protocols will be evaluated using immunohistochemistry with PPARδ specific antibodies to visualize fluorescence in pancreatic islet sections to confirm presence of PPARδ protein in addition to measurement of PPARδ mRNA expression using qPCR.

Should any changes in carbohydrate metabolism be observed in PPARδ transgenic mice, islets would be isolated to measure changes in specific lipid species using liquid chromatography in conjunction with mass spectrometry (LC/MS) in addition to changes in gene expression profiles. The aim of these studies will be to examine the *in vivo* effects of PPARδ overexpression on carbohydrate metabolism and to explore possible mechanisms for any observed changes.

Future studies related to the role of PPARγ in pancreatic β-cells will focus on further exploration into the mechanisms related to the impairments in carbohydrate metabolism observed in PPARγ transgenic mice. Here I have explored the changes in gene expression and lipidomic profiles associated in pancreatic islets associated with β-cell specific PPARγ2 overexpression. It will be useful to generate another cohort of mice to examine the effects of PPARγ1 overexpression in β-cells. Through examining lipid and gene expression profiles in PPARγ1 transgenic mice, it may be possible to identify mechanisms common to PPARγ2 transgenic mice in addition to novel mechanisms related to PPARγ1 function. This study will allow for a broader understanding of the role of PPARγ in pancreatic β-cells in an obese setting.

Additional studies related to PPARγ2 expression in the β-cell will focus on mechanisms of β-cell failure that I have eluded to here. Specifically, *in vitro* studies examining the role of PPARγ2 regulation of lysophosphatidylcholine (LysoPC) generation will determine if the decreased LysoPC levels I observed in pancreatic islets are substantial

enough to cause changes in insulin secretion. Furthermore, the idea of cytotoxic accumulation of peroxide species as a result of PPARγ2 overexpression will be assessed to determine whether or not PPARγ2 is shifting lipid metabolism towards preferential peroxidation of long chain fatty acids rather than mitochondrial β-oxidation alone.

The role of PPARs in several tissue types have been well characterized; however, in pancreatic β-cells, their function still remains unclear with many conflicting findings in the literature. The findings that I report here, in addition to the future studies I propose, will aid in elucidating the role of role of PPARs on islet pathophysiology in T2D. Furthermore, I have provided insight into the β-cell specific effects of PPAR agonists and how these effects may or may not contribute to the therapeutic effects of drug treatments in T2D and obesity.

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