CHARACTERIZATION OF PPAR-ALPHA OVEREXPRESSION IN BETA-CELL LIPOTOXICITY AND ITS EFFECTS ON OBESITY-INDUCED TYPE 2 DIABETES

by

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Abstract

Lipotoxicity is implicated as a mechanism for pancreatic β-cell dysfunction in obesity-induced type 2 diabetes (T2D). *In vitro*, peroxisome proliferator-activated receptor alpha (PPARα) protects against lipotoxic β-cell dysfunction preserving insulin secretion. Utilizing an adeno-associated virus (dsAAV8), we induced overexpression of PPARα specifically in pancreatic β-cells of adult, C57Bl6 mice that were fed a high-fat diet for 20 weeks to induce obesity. We show that overexpression of PPARα in pancreatic β-cells, *in vivo,* protects β-cell function in obesity, improving glucose tolerance by preserving insulin secretion compared to obese controls. No change in islet morphology or β-cell mass was observed. Despite metabolic improvements observed in diet-induced obese mice, overexpression of PPARα in pancreatic β-cells of a genetic model of severe obesity (*db/db*) did not improve carbohydrate metabolism. We have developed the first *in vivo* model of β-cell specific PPARα overexpression to elucidate the mechanisms involved in β-cell lipotoxicity in obesity-induced T2D.

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db/db n=6. Mean values with SEM are shown. Statistical significant variation was determined by 2-way ANOVA with Bonferoni Post Tests. *P<0.05, **P<0.01, ***P<0.001.

Figure 4.3 Overexpression of PPARα in β-cells of genetically obese mice (*db/db*) does not affect islet morphology of insulin immuno-positive area. Abbreviations: INS – insulin; GLC – glucagon. Images taken at 20x magnification. Scale bar 50μm.

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DEDICATION

I would like to start by recognizing my family and close friends for their unwavering love and support through the "trials and tribulations" of completing my Masters. This support has allowed me to develop into the procrastinating-comedic-genius I am today.

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CHAPTER 1

A lipotoxic approach to understanding the link between obesity and type 2 diabetes

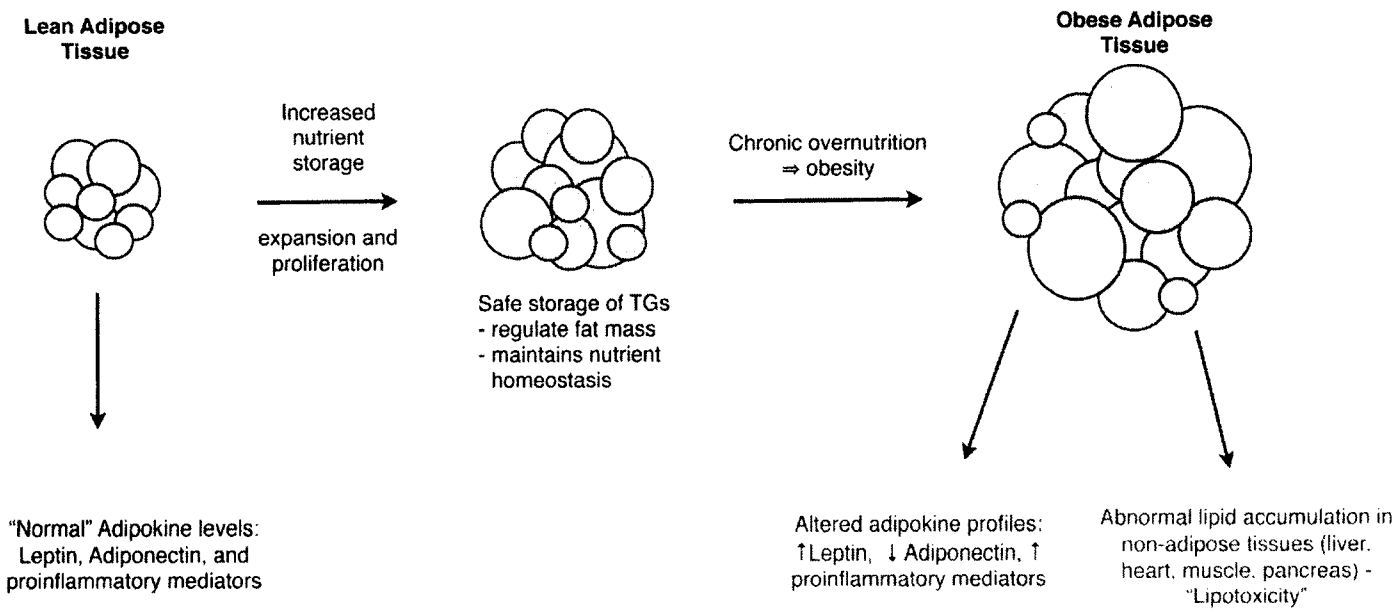
1.1 INTRODUCTION

1.1 General introduction to obesity

Obesity is the number one risk factor for developing type 2 diabetes (T2D) (Smyth and Heron 2006); however, the physiological mechanisms underlying this association is not fully understood. Obesity results from chronic positive energy balance with subsequent increase in triglyceride (TG) storage in adipose tissue (van Herpen and Schrauwen-Hinderling 2008). In addition to T2D, obesity is a risk factor for numerous diseases including and not limited to: insulin resistance, cardiovascular disease, cancer, osteoarthritis, and non-alcoholic fatty liver disease (Kahn et al. 2006; Shoelson et al. 2007; van Herpen and Schrauwen-Hinderling 2008; Kusminski et al. 2009). Recently, rates of obesity, including childhood obesity, have reached epidemic proportions around the world (Deckelbaum and Williams 2001; Smyth and Heron 2006); where it has been estimated that obesity and its comorbidities are responsible for approximately 300,000 deaths annually (Dixon 2010). Therefore it is not a surprise that obesity is associated with a plethora of complications resulting in long term suffering for patients and huge economic burdens to health care systems around the world (Dixon 2010).

Adipose tissues comprise a complex organ composed of adipocytes, vascular tissue, and immune cells that has the ability to expand and proliferate to meet fat storage needs (Gray and Vidal-Puig 2007). In healthy individuals, adipocytes are responsible for the storage of fats as TGs and additionally act as endocrine cells regulating fat mass, nutrient homeostasis and immune response (Spiegelman 1998; Rosen and Spiegelman 2006). As seen in figure 1.1, adipocytes are depots for storing energy in the form of TGs which are

Figure 1.1 The adaptability of adipose tissue and its role as a endocrine organ - modified from (Gray and Vidal-Puig 2007; Shoelson et al. 2007). Healthy adipose tissue is involved in metabolic homeostasis through the production and secretion of adipokines (TNF-α, IL-6, MCP-1, PAI-1, leptin, adiponectin and resistin), as well as the storage of TGs. When the storage capacity of the adipocytes is overloaded, as seen in obesity, lipids spill-over and accumulate in the surrounding non-adipose tissues. This increase in adiposity also alters the adipokine profile.TNF-α -Tumor Necrosis Factor alpha; IL-6 – Interleukin 6; PAI-1 – Plasminogen Activator Inhibitor-1; MCP-1 – Monocyte Chemoattractant Protein-1; TGs - triglycerides


Lean Adipose Tissue
Increased nutrient storage
expansion and proliferation
Safe storage of TGs
- regulate fat mass
- maintains nutrient homeostasis
Chronic overnutrition
⇒ obesity
Obese Adipose Tissue
"Normal" Adipokine levels:
Leptin, Adiponectin, and
proinflammatory mediators
Altered adipokine profiles:
↑Leptin, ↓ Adiponectin, ↑
proinflammatory mediators
Abnormal lipid accumulation in
non-adipose tissues (liver,
heart, muscle, pancreas) -
"Lipotoxicity"

broken down into glycerol and fatty acids (FAs) when metabolic demand is increased and glucose stores are limited (Sethi and Vidal-Puig 2007). Healthy adipocytes have the ability to proliferate and undergo hypertrophy (Fig. 1.1) to accommodate the storage of excess lipids in times of over nutrition (Gray and Vidal-Puig 2007). In the non-obese state, adipocytes are continuously fluctuating between storage and lipolysis of TGs and FFAs to maintain lipid homeostasis (Greenberg et al. 2011). Lipolysis is the finely orchestrated regulation of lipid metabolism, controlled by various lipases including: hormone sensitive lipase (HSL), lipoprotein lipase (LPL) and adipose triglyceride lipase (ATGL) (Arner 2005; Carmen and Victor 2006; Haemmerle et al. 2006); where these lipases catalyze the hydrolysis of ester linkages in TGs and diacylglycerols (DAGs) to produce FFAs and glycerol (Arner 2005; Carmen and Victor 2006). Moreover, the liver is a primary organ involved in lipid metabolism, responsible for the production of cholesterols, TGs and lipoproteins (van Herpen and Schrauwen-Hinderling 2008). A recent study from Haemmerle and colleagues (2006) showed that a decrease in FA availability leads to increased glucose metabolism in adipose tissue and skeletal muscle in ATGL knockout mice (Haemmerle et al. 2006). Furthermore, this study showed that ATGL is a key mediator in TG catabolism in both adipose and non-adipose tissues (Haemmerle et al. 2006).

Increased levels of basal lipolysis has been observed in obesity, and is thought to be due to ineffective actions of insulin (unable to inhibit lipolysis), increased levels of circulating leptin, and tumor necrosis factor-α (Duncan et al. 2007). Therefore, the inability to inhibit lipolysis in both the fed and fasted state greatly contributes to increased circulating levels of TGs and FAs, as well as disruption of TG storage in

adipocytes (Langin et al. 2005; Duncan et al. 2007). In the obese state, when the storage capacity of adipose tissue is challenged, excess lipids "leak" out of the adipocytes into the circulation. This "spillover" of lipids, results in elevated circulating levels of FAs and TGs contributing to abnormal accumulation of lipids in non-adipose tissues such as skeletal muscle, liver, heart and β-cells of the pancreas (Eldor and Raz 2006; van Herpen and Schrauwen-Hinderling 2008), a phenomenon known as lipotoxicity.

1.2 General introduction to lipotoxicity

Lipid accumulation as a result of lipid spillover has been associated with having toxic effects in peripheral, non-adipose tissues; a term referred to as lipotoxicity. It should also be noted that lipotoxicity can also affect individuals who suffer from lipodistrophy (lack of natuarally occurring adipose tissue), as there is no natural storage depot for circulating FAs and TGs, therefore lipids begin to accumulate in non-adipose tissues (Unger 2002).

Increased lipid accumulation in tissues such as liver, skeletal muscle, heart and pancreas have been associated with a plethora of dysfunctional consequences. Lipotoxic effects in skeletal muscle and liver have been associated with low-grade inflammation and insulin resistance; in addition the development of non-alcoholic fatty liver disease is also thought to be a consequence of lipotoxicity in the liver (van Herpen and Schrauwen-Hinderling 2008; Chavez and Summers 2010). Abnormal lipid accumulation in the heart and vasculature has been associated with cardiomyopathy, heart failure ("lipotoxic heart"), inflammation and endothelial dysfunction (contributing to the progression of atherosclerosis) (Unger 2002; van Herpen and Schrauwen-Hinderling 2008; Imrie et al.

2010; Wende and Abel 2010). It is well established that lipid accumulation within the pancreatic β-cells has been associated with insulin resistance, β-cell dysfunction, and β-cell failure (Prentki and Nolan 2006; Summers 2006; van Herpen and Schrauwen-Hinderling 2008; Virtue and Vidal-Puig 2010). Insulin is responsible for activating LPL activity in adipocytes for increased FA storage, and decreases LPL activity in skeletal muscle (Farese et al. 1991; Lewis et al. 2002). In the obese state, LPL activity is inhibited in the adipocytes causing the storage of FAs to be distributed to the circulation and non-adipose tissues (Sadur et al. 1984; Yost et al. 1995; Lewis et al. 2002), thus insulin resistance is a "double-edged sword" further contributing to the vicious lipotoxic cycle. Lipotoxicty and its role in β-cell dysfunction will be discussed later in this review.

There has been great debate in the literature as to whether it is the quantity or quality of lipid species that has the most detrimental toxic effects in non-adipose tissues. Ceramides and sphingolipids have been lipid species of intense study in obesity research as they have been found to be involved in inhibiting glucose uptake in cells as well as inducing oxidative stress (Holland et al. 2007). Ceramides have also been linked to influencing the regulation of various adipokines such as TNF-α (Summers 2006), as well as contributing to apoptosis through increased ROS production and activation of the NF-κB pathways (Kusminski et al. 2009; Chavez and Summers 2010) inducing β-cell dysfunction and insulin resistance, as well as endothelial inflammation and dysfunction. Moreover it has also been shown that ceramides impair the translocation of GLUT4 on cellular membranes in muscle, adipose and liver tissue (JeBailey et al. 2007; Hoehn et al. 2008). Combined, ceramide and sphingolipid species have been implicated as the toxic intermediates involved in lipotoxic accumulation in non-adipose tissues (Chavez et al.

2003; Summers 2006; Wende and Abel 2010), as well as contributing to inflammation through increased ER stress and reactive oxygen species (ROS) (Wende and Abel 2010).

Additionally, adipokines, in association with the lipotoxic effects of lipid accumulation in non-adipose tissues, exacerbates the use of non-oxidative pathways (Kusminski et al. 2009) resulting in cellular dysfunction and apoptosis (Gray et al. 2006; Summers 2006). Thus the combination of lipotoxicity and pro-inflammatory mediators contribute to insulin resistance, oxidative stress, and β-cell dysfunction (Cave et al. 2008).

1.3 Changes in adipokine profiles: role in β-cell dysfunction and insulin resistance

Until recently, it was thought that the primary role of adipose tissue was energy homeostasis and a depot for fat storage (Ahima 2006). It wasn't until the discovery of leptin, an adipocyte derived hormone, that adipose tissue was regarded as an endocrine organ (Zhang et al. 1994; Kershaw and Flier 2004; Ahima 2006; Friedman 2010). Leptin has been identified to play a role in fuel metabolism in peripheral tissues (Suzuki et al. 2007), appetite suppression and reduced fat mass (Schroeder-Gloeckler et al. 2007), FA oxidation in skeletal muscle (Muoio and Lynis Dohm 2002), and glucose metabolism (Huynh et al. 2010). A more in-depth review of leptin will be covered in chapter 4.

The adipose tissue acts as an endocrine organ producing and secreting adipocyte derived hormones (adipokines) that play a role in metabolic regulation (Gray and Vidal-Puig 2007). It has been well established that adipokine production and secretion change in the obese state (Gray and Vidal-Puig 2007) as seen in figure 1.1. The change in adipokine profiles in the obese state is thought to contribute to a low-grade inflammatory

state and insulin resistance (Eldor and Raz 2006; Gray and Vidal-Puig 2007; Lago et al. 2007). Recent studies have shown that adipokines involved in metabolic homeostasis include: tumor necrosis factor-α (TNF-α), interluekin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), plasminogen activator inhibitor-1 (PAI-1), resistin, adiponectin and leptin (Shoelson et al. 2006).

An increase in TNF-α, IL-6, MCP-1, PAI-1, and resistin as seen in obesity promotes a pro-inflammatory state that contributes to developing insulin resistance and also potentiates vascular disease (Esposito et al. 2006; Shoelson et al. 2007). Unlike the other pro-inflammatory adipokines, adiponectin which is reduced in the obese state, has anti-inflammatory, and insulin-sensitizing properties and has been found to be inhibited by TNF-α and IL-6 in obese states (Esposito et al. 2006; Lago et al. 2007). Likewise, leptin acts on the central nervous system and peripheral tissues to reduce fat stores by suppressing appetite, increasing metabolic rate, and activating thermogenesis (Muoio and Lynis Dohm 2002; Ceddia 2005; Unger 2005; Zhao et al. 2006; Gray and Vidal-Puig 2007; Friedman 2010), it also functions in glucose metabolism, cytokine secretion, phagocytosis, and angiogenesis (Otero et al. 2006; Lago et al. 2007). This ultimately contributes to localized disturbances in metabolic pathways contributing to increased macrophage infiltration, insulin resistance, and endothelial damage (Laclaustra et al. 2007; Shoelson et al. 2007). Thus, obesity is a state of heightened inflammation, a process that contributes to the development of obesity-induced insulin resistance; therefore, these inflammatory mediators and the pathways in which they activate could be potential targets for therapeutic interventions or prevention for obesity induced insulin resistance and β-cell dysfunction.

Lipotoxicity and changes in adipokine profiles have been discussed as obesity related hypotheses as contributing factors to insulin resistance and β-cell dysfunction. The remainder of my thesis will focus on the lipotoxic hypothesis and its role in β-cell dysfunction and failure in the progression of obesity-induced T2D.

1.4 The pancreas: insulin secretion and action

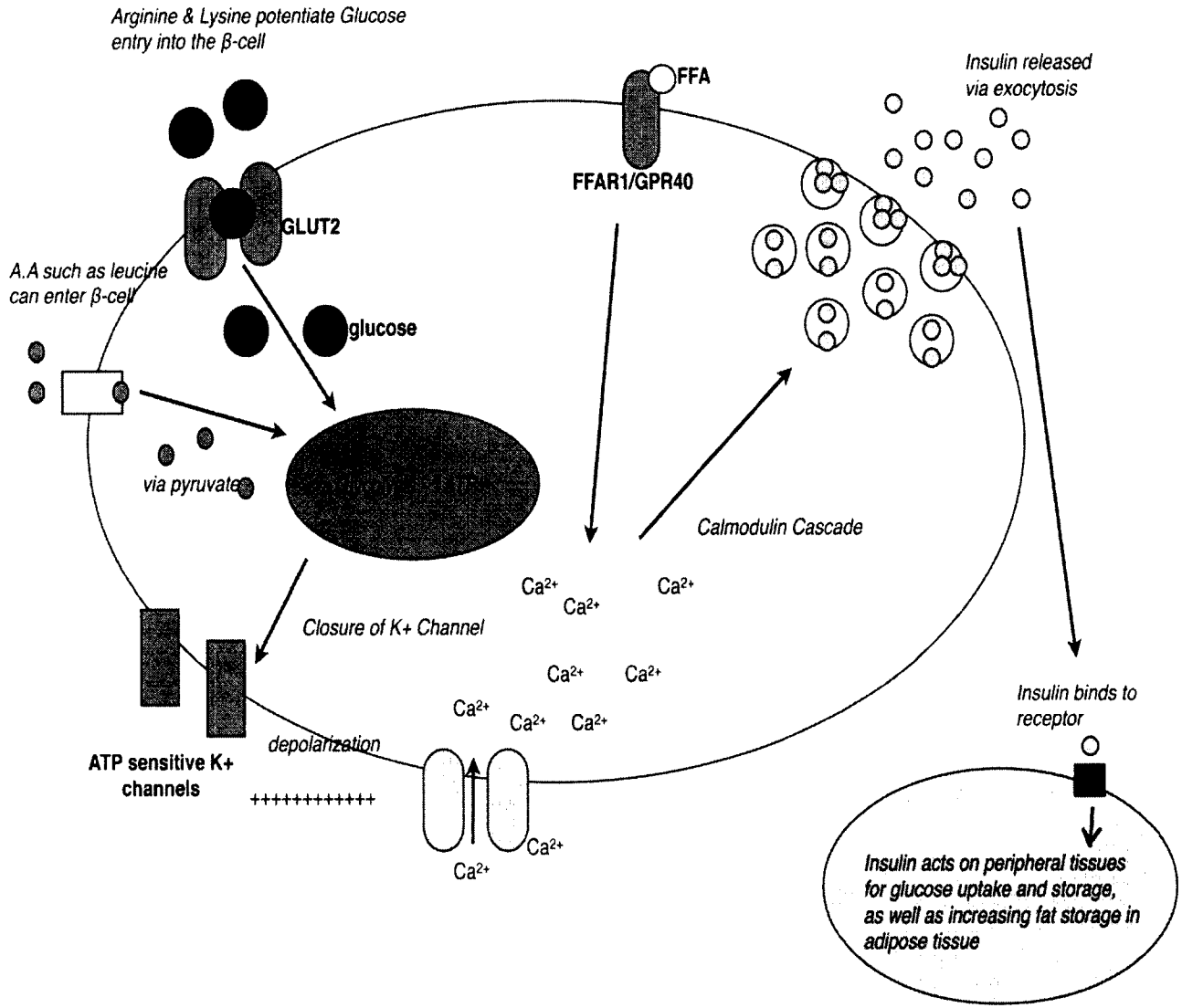
The pancreas functions as both an exocrine and endocrine organ; with the endocrine pancreas responsible for the production and secretion of several hormones that regulate carbohydrate metabolism, including insulin. The endocrine pancreas contains approximately one million islets composed of several cell types (α-cells, β-cells, δ-cells, F-cells, and ε-cells). The pancreatic β-cells produce and secrete insulin promoting glucose uptake and storage within skeletal muscle and adipose tissue and glucose production and storage in liver. In addition to its role in regulating glucose metabolism, insulin also regulates lipid metabolism promoting the storage of fat within adipose tissue (Evans et al. 2004; Wellen and Hotamisligil 2005; Razani et al. 2008), and acting on the brain, specifically the hypothalamus to regulate energy balance by reducing food intake (Choudhury et al. 2005; Kahn et al. 2006).

As shown in figure 1.2, nutrient stimuli such as glucose, amino acids and FFAs, get shuttled into the β-cell through their respected transporters. Once inside the cell, the glucose and amino acids (via pyruvate) undergo glycolysis in the mitochondria increasing intracellular levels of ATP (Las et al. 2006; Nolan et al. 2006). The increase in intracellular ATP causes ATP-sensitive K^+ channels to close, depolarizing the cell and increasing membrane potential; subsequently opening Ca2+ channels and causing an

influx of Ca^{2+} into the cell (Henquin 2000; Nolan et al. 2006). The increase in intracellular Ca^{2+}, activates a calmodulin cascade and release of insulin from secretory granules that undergo exocytosis (Henquin 2000) (Fig 1.2). Additionally, the binding of FFAs to the free fatty acid receptor-1/G-coupled receptor-40 (FFAR1/GPR40) receptors on the β-cell membrane causes an increase in intracellular Ca^{2+} further stimulating the calmodulin cascade and release of insulin (Prentki and Nolan 2006) (Fig 1.2).

Insulin secretion can also be stimulated by the parasympathetic nervous system through the binding of acetylcholine (ACh) to the muscarinic receptor (M2) on the β-cell (Kahn et al. 2006). Binding of ACh can act through either the protein kinase C (PKC) pathway via phospholipase C or through phosphatidylinositol 3-kinase (PI-3K) and subsequent increase in Ca^{2+} to stimulate insulin secretion (Kahn et al. 2006; Nolan and Prentki 2008). Moreover, the insulin secretion from the β-cell is also stimulated hormonally through the binding of incretin hormones such as glucagon-like peptide 1 (GLP-1), glucagon-dependent insulinotropic peptide (GIP), pituitary adenylate cyclase-activating polypeptide (PACAP), and vasoactive intestinal polypeptide (VIP) (Inagaki et al. 1996; Yamamoto et al. 2003; Holst and Gromada 2004; Kieffer 2004). For example, binding of GLP-1 results in an increase in intracellular cyclic adenosine monophosphate (cAMP) causing the stimulation of protein kinase A (PKA) and subsequent closure of K^+ channels and depolarization of the β-cell (Kieffer 2004; Kahn et al. 2006); thus contributing to the influx of Ca^{2+} and increase in intracellular Ca^{2+}.

Figure 1.2 Schematic representation of insulin secretion in the pancreatic β-cell by endogenous stimuli – modified from (Boron and Boulpaep 2005). Nutrient stimuli (such as glucose, amino acids and FFAs) get shuttled into the β-cell through their respective transporters. Once inside the cell, through their respective pathways, the stimuli cause an influx of Ca^{2+} into the cell and release of insulin (via exocytosis of insulin secretory granules). Once secreted insulin binds to insulin receptors and increases glucose uptake and storage as well as increasing fat storage in adipose tissue. Abbreviations: +++ – depolarization; GLUT2 – glucose 2 transporter; aa – amino acids; K^+ channel; Ca^{2+} channels; ATP – adenosine triphosphate; FFAR1/GPR40 – free fatty acid receptor-1/G-coupled receptor-40; FFA – free fatty acid.


Arginine & Lysine potentiate Glucose entry into the β-cell
FFA
FFAR1/GPR40
Insulin released via exocytosis
GLUT2
A.A such as leucine can enter β-cell
glucose
via pyruvate
Calmodulin Cascade
Closure of K+ Channel
Ca2+
Insulin binds to receptor
depolarization
ATP sensitive K+ channels
++++++++++++
Insulin acts on peripheral tissues for glucose uptake and storage, as well as increasing fat storage in adipose tissue

Once secreted, insulin binds to insulin receptors on peripheral tissues such as muscle, liver and fat tissue, and stimulates tyrosine kinase activity and subsequent phosphorylation of insulin receptor substrates 1 (IRS-1) and IRS-2 (Aspinwall et al. 2000). This phosphorylation leads to activation of the phosphatidylinositol 3-kinase (PI3K) pathway and production of downstream intermediates (Summers 2006). One example of a downstream target of this insulin signal transduction cascade is stimulation of the translocation of glucose transporter 4 (GLUT4), expressed in both muscle and adipose tissue, from the cytoplasm to the cell membrane allowing the influx of glucose into the cell for glycolysis or storage (Rose and Richter 2005; Summers 2006) (Fig 1.2). Another molecular action of insulin signaling is its ability to increase the action of lipoprotein lipase (LPL) to promote the storage of FA within the adipocytes (Razani et al. 2008). As highlighted above, insulin secretion and insulin signaling are complex pathways regulated by many stimuli and affecting many molecular targets; therefore disruptions to the signal transduction pathways in a variety of cell types can be detrimental to whole-body carbohydrate metabolism.

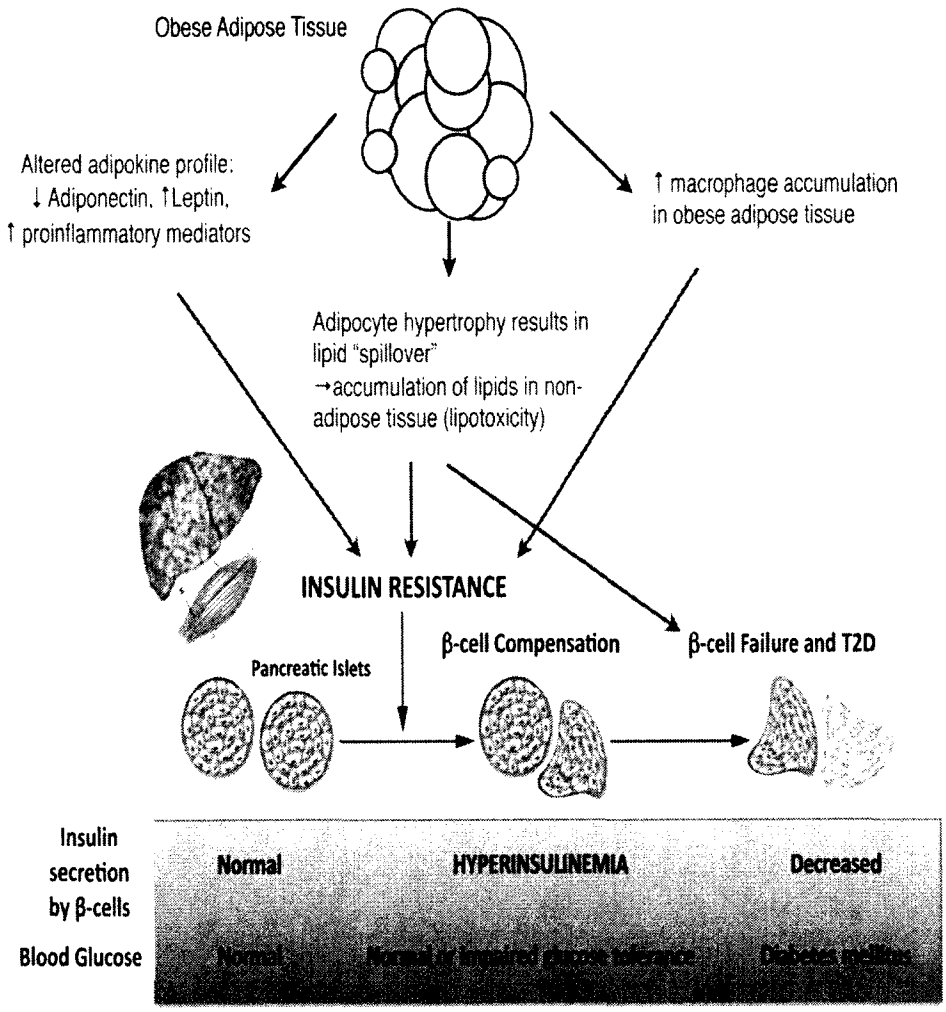
There is great debate in the literature about what comes first in the pathogenesis of T2D: insulin resistance or β-cell dysfunction? Both etiologies are characteristic of obesity-induced T2D and β-cell dysfunction required for the progression to T2D. Insulin resistance is a hallmark of the pre-diabetic state and is thought to be a critical pathophysiological event in the development of obesity-induced diabetes. As discussed above, changes in adipokine secretion, lipid storage and inflammatory profiles in obesity induce peripheral insulin resistance, resulting in compensatory hypersecretion of insulin from pancreatic β-cells to maintain glucose homeostasis (Prentki and Nolan 2006). If β-

cell function cannot maintain compensatory increases in insulin secretion impaired glucose tolerance results and T2D develops (Fig. 1.3) (Gehrmann et al. 2010; Giacca et al. 2011). Hyperglycemia itself can also contribute to the activation of inflammatory pathways involving NF-κB and PKC through increased production of reactive oxygen species (ROS) (Lau et al. 2005) further contributing to β-cell dysfunction. Furthermore, ineffective insulin action effects lipoprotein metabolism due to a decrease in LPL action and decreased suppression of lipolysis resulting in increased small dense low-density lipoprotein (LDL) and decreased high-density lipoprotein (HDL) levels (Razani et al. 2008).

1.5 Pancreatic β-cell dysfunction: the lipotoxic hypothesis

As discussed above, acute exposure of β-cells to free fatty acids (FFAs) is a physiological stimulus for normal insulin secretion and β-cell function (Nolan et al. 2006). However, it is well established that chronic exposure of β-cells to high levels of FFAs is detrimental to β-cell health (Lee et al. 2007; Giacca et al. 2011). For example, Kim and colleagues (2003) showed that reducing circulating lipid in a genetic mouse model of insulin resistance and diabetes (the MKR mouse model - skeletal muscle-specific impairment of IGF-1 receptor) using a hyperlipidemic agent (a fibrate) improved the diabetic state of this animal; suggesting that high circulating is associated with β-cell dysfunction (Kim et al. 2003). Excessive amounts of FFAs within the β-cell have been shown to interrupt glucose-stimulated insulin secretion and increase rates of apoptosis (Bonora 2008; Morgan 2009).

Figure 1.3 Obesity is known to cause insulin resistance and β-cell dysfunction - modified from (Kasuga 2006; Shoelson et al. 2007). Adipose tissue in the obese state contributes to altered adipokine profiles, and lipid accumulation in non-adipose tissues, causing insulin resistance and β-cell dysfunction. In the insulin resistant state, pancreatic islets make more insulin (hyperinsulinemia) to maintain normal glucose homeostasis. The islets "tire" from over-producing insulin and eventually fail resulting in T2D.
Abbreviations: T2D – type 2 diabetes.


Obese Adipose Tissue
Altered adipokine profile:
↓ Adiponectin, ↑Leptin,
↑ proinflammatory mediators
↑ macrophage accumulation
in obese adipose tissue
Adipocyte hypertrophy results in
lipid "spillover"
→accumulation of lipids in non-
adipose tissue (lipotoxicity)
INSULIN RESISTANCE
β-cell Compensation
β-cell Failure and T2D
Pancreatic Islets
Insulin
secretion
by β-cells
Blood Glucose
Normal
HYPERINSULINEMIA
Decreased
Normal
Normal or impaired glucose tolerance
Diabetes mellitus

For instance, increased levels of FAs within the β-cell have been shown to modify the expression of uncoupling protein-2 (UCP2) which is linked to the subsequent decrease in ATP production (van Herpen and Schrauwen-Hinderling 2008), disrupting insulin secretion (Poitout and Robertson 2008).

Mediators of FAs including long-chain acyl-CoAs (LC-CoA) have been implicated in hyperpolarizing the β-cell (reducing the influx of Ca^{2+} into the β-cell) as well as downregulating the PKC pathway, further disrupting the exocytosis of insulin (van Herpen and Schrauwen-Hinderling 2008). Moreover, FFAs have also been linked to increased apoptosis by enhancing ROS production and cellular stress (Prentki and Nolan 2006) further contributing to β-cell dysfunction. Interestingly, studies involving GPR40 knockout mice showed a reduction in hyperglycemia, hyperinsulinemia when on normal chow or high fat diet (Steneberg et al. 2005); thus providing further evidence that FAs shuttled into the β-cell, in excess, can have detrimental effects on β-cell function and whole-body carbohydrate metabolism. More recent studies involving the GPR40 knockout mouse have shown that in response to endogenous stimuli in both the fasted and fed states, insulin secretion is impaired in these mice (Latour et al. 2007; Alquier et al. 2009). Combined, these studies have suggested GPR40 signaling pathways to be responsible for the potentiating effects of FAs on insulin secretion (Latour et al. 2007; Alquier et al. 2009).

There is increasing evidence that the type of lipid accumulating within the β-cell may affect toxicity and β-cell failure (Virtue and Vidal-Puig 2010). *In vitro* studies involving the INS-1E or MIN6 β-cell lines have shown higher rates of apoptosis when treated with the saturated FA palmitate versus the unsaturated FA oleate (Furukawa et al.

1999; Karaskov et al. 2006; Frigerio et al. 2010). For example, the accumulation of diacylglycerols (DAGs) and ceramides in pancreatic β-cells and peripheral tissues have been shown to cause detrimental effects and may be key intermediates in lipotoxic induced β-cell failure and insulin resistance (Chavez and Summers 2010; Virtue and Vidal-Puig 2010). Itani and colleagues (2002) have suggested that DAGs, rather than ceramides, were primary mediators of lipid-induced insulin resistance using lipid-infusion studies in whole animals (Itani et al. 2002). Conversely, Chavez and colleagues (2003) have shown endogenous ceramides were required for inhibitory effects of long-chain saturated fatty acids on insulin signaling (Chavez et al. 2003) and Holland and colleagues (2007) further showed that modulating ceramide levels utilizing myriocin (a potent inhibitor of sphingosine biosynthesis) in obese rodents, ameliorates insulin resistance and subsequent development of diabetes (Holland et al. 2007). Therefore the β-cell is an attractive target for improved therapeutics and new treatment paradigms.

This introductory chapter has highlighted the effects of obesity on peripheral insulin resistance and β-cell dysfunction through the "lipotoxic" accumulation of lipids. To summarize, increases in adiposity causes changes in adipokine profiles and levels of FAs accumulating and being metabolized in non-adipose tissues. These lipotoxic disturbances have a strong association with increased rates of insulin resistance and β-cell dysfunction in obese individuals through the disruption of various signaling pathways involved. The impact of lipotoxic lipid accumulation and metabolism in pancreatic β-cells under conditions of obesity is the focus of my thesis. My work will help elucidate the mechanisms involved in β-cell lipotoxicity and may identify this area as a target for improved therapeutics and treatment paradigms.

As such, manipulating how lipids accumulate within β-cells may be a plausible approach to reducing lipotoxicity and preserving β-cell function. Peroxisome proliferator-activated receptors (PPARs) are a group of nuclear transcription factors involved in lipid metabolism (Chinetti et al. 2000; Lee et al. 2007); where PPARα has been identified as a key regulator of genes involved in β-oxidation, FA uptake, and ω-oxidation (Lee et al. 2007). PPARα will be discussed in detail in chapter 3. Using an adeno-associated virus serotype 8 (dsAAV8; discussed in detail in chapter 2) for targeted overexpression of PPARα in the pancreatic β-cells, my project aims to manipulate lipid metabolism in β-cells of mice on high-fat diet; with the expectation that overexpression of PPARα would affect whole-animal carbohydrate metabolism. Moreover, this would allow us to develop and test the lipotoxic hypothesis in an *in vivo* model of diet-induced β-cell lipotoxicity and T2D. Furthermore, we will be able to look at what subset of PPARα target genes (Acyl-CoA oxidase (AOX), carnitine palmitoyl transferase-1 (CPT1), long chain acyl-coA dehydrogenase (LCAD), and uncoupling protein-2 (UCP2)) are involved in β-oxidative pathways within the β-cells; thus allowing us to elucidate possible mechanisms involved in protecting the β-cell from lipotoxic lipid accumulation in an *in vivo* model.

1.6 Study objectives

Lipid accumulation within the β-cell of the pancreas is a key contributor to inhibition of insulin secretion and β-cell apoptosis in the development of T2D in obesity. As such, manipulating how lipids accumulate within β-cells may be a plausible approach to reducing lipotoxicity and preserving β-cell function. The main objectives of this study are:

1. To establish whether associated-adeno virus serotype 8 (dsAAV8) is an effective and feasible targeted gene delivery tool to generate *in vivo* animal models overexpressing proteins of interest specifically in pancreatic β-cells to study lipotoxcity in obesity induced T2D (Chapter 2).
2. To characterize the *in vivo* effects of PPARα overexpression in pancreatic β-cells on lipotoxicity in a diet-induced obese model of T2D (Chapter 3).
3. To characterize the *in vivo* effects of PPARα overexpression in pancreatic β-cells on lipotoxicity in a genetic model of severe obesity and T2D (*db/db* mouse) (Chapter 4).

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CHAPTER 2

Utilizing dsAAV8 as a Tool to Overexpress Proteins Specifically in the Pancreatic β-Cell *In Vivo*

A version of this chapter has been published as an abstract:

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2.1 INTRODUCTION

Transgenic or knockout mouse models have been used to study various pathways and mechanisms involved in human diseases. Transgenic models are tools utilized to study gene function; specifically they provide *in vivo* models to examine the effects of overexpressing or knocking out genes of interest proving to be a critical link between bedside and bench (Miesfeld 1999). Additionally, the development of the Cre/*loxP* system for animal transgenics has allowed for infinite possibilities for conditional knockouts including developmental stage and tissue-specific gene knockout (Nagy 2000). As outlined in chapter 1, obesity has been implicated in increasing adiposity in non-adipose tissues such as the β-cells of the pancreas contributing to insulin resistance, β-cell dysfunction and development of T2D (Kasuga 2006; Prentki and Nolan 2006; van Herpen and Schrauwen-Hinderling 2008). Thus making the β-cell an ideal target for elucidating the mechanisms involved in β-cell lipotoxicity in obesity-induced T2D. Therefore mouse models for β-cell-specific overexpression or gene knockouts are very relevant to the remainder of this study.

Tissue specific promoters such as the mouse insulin promoter (MIP) and rat insulin promoter (RIP) have been used to develop β-cell specific transgenic mouse models (Gannon et al. 2000; Hara et al. 2003; Dahlhoff et al. 2011) by driving the gene or protein of interest directly to the insulin producing β-cells of the pancreas. It should be noted that the human genome contains a single copy of the insulin gene, whereas two variations are found in the rat (RIP1 and RIP2) (Odagiri et al. 1996; Hay and Docherty 2006). RIP1 and RIP2 vary in size (448bp and 668bp respectively), however they both target the β-cells of the pancreas (Gannon et al. 2000; Hay and Docherty 2006). Hara et

al. (2003) developed transgenic mice expressing enhanced green fluorescent protein (eGFP) in pancreatic β-cells under the direction of the MIP (Hara et al. 2003). In addition, Hara and colleagues (2003) were able to show that under the control of MIP, eGFP was targeted to the β-cells and not the α-cells of the pancreas utilizing FACS to sort isolated islets (Hara et al. 2003), further confirming site-specific expression.

It should be noted that utilizing the RIP to target the β-cells of the pancreas in a RIP-Cre transgenic has been associated with "extrapancreatic" expression in neural tissues of the brain (Gannon et al. 2000; Dahlhoff et al. 2011). Choudhury and colleagues (2005) were able to show expression of eGFP in the hypothalamic and forebrain neurons in addition to the β-cells of the pancreas in their RIPCreZEG transgenic mouse (Choudhury et al. 2005). This neuronal expression utilizing RIP-Cre transgenic mice was further supported by the results obtained by Choi and colleagues (2008) where RIPCre^{+}*Pten*$^{fl/fl}$ mice were found to be PTEN deficient in the insulin secreting neurons in the hypothalamus and not the β-cells of the pancreas (Choi et al. 2008). Moreover, Gannon and colleagues (2000) expressed that caution needs to be exercised when using RIP2-Cre mice, as RIP2 directs expression of the gene of interest during embryogenesis in both the pancreas and brain, as well, the activation of RIP during early development may interfere with the populations of cells becoming β-cells (Gannon et al. 2000). All of this information needs to be taken into consideration when deciding if a transgenic mouse model is the most appropriate tool for β-cell-specific gene expression for *in vivo* overexpression studies.

Novel methods involving the transfer of genes into specific tissues of interest using viral delivery have become more widely accepted and used in place of traditional

transgenic models. One emerging field is the use of adeno-associated viruses as a mode of viral gene delivery. Currently, three classifications of adeno-associated viruses exist: single stranded adeno-associated virus (AAV), recombinant adeno-associated virus (rAAV) and the double-stranded adeno-associated virus (dsAAV) (Wang et al. 2004; Wu et al. 2006); in addition there are several serotypes and hundreds of variations of AAV-based vectors (Wu et al. 2006).

Adeno-associated viruses are small, non-pathogenic, non-replicating, members of the *Parvovirdiae* family, requiring a helper virus for appropriate infection and site-specific integration into tissues of interest (Wang et al. 2004; Inagaki et al. 2006; Wu et al. 2006). AAVs also exhibit low immune response, as well they can be used to transfect both dividing and non-dividing cell types (Gao et al. 2002; Gaddy et al. 2010; Riedel et al. 2010), making them an ideal and efficient tool for viral-mediated gene delivery. Tissue-specific delivery of the AAVs is dependent on the serotype capsid with which it is associated (Inagaki et al. 2006). Serotypes and their tissue specificity include: AAV1 (CNS, skeletal muscle and adipose tissue), AAV2 (kidney), AAV4 (CNS, and photoreceptors), AAV5 (CNS, and photoreceptors), AAV6 (skeletal muscle), AAV7 (skeletal muscle), AAV8 (skeletal muscle, liver, heart and pancreas) and AAV9 (skeletal muscle, liver, lung) (Chao et al. 2000; Gao et al. 2002; Loiler et al. 2003; Wang et al. 2004; Wu et al. 2006). Given AAV8 delivery is directed at tissues in the gut, therefore, site-specific delivery to the β-cells of the pancreas could be enhanced utilizing tissue specific promoters such as MIP or RIP (Wu et al. 2006).

Based on the various tissue types dsAAVs can target as well as their low immune response, dsAAVs are becoming an increasingly attractive tool for gene therapy.

Recently, Gaddy and colleagues (2011) have utilized dsAAV8 driven by MIP to target interleukin-4 (IL-4) as well as combinations of glucagon-like peptide-1 and hepatocyte growth factor/NK1 with IL-4 to the pancreatic β-cells in NOD mice (model of T1D) (Gaddy et al. 2011). Using dsAAV8, they were able to demonstrate that IL-4 as well as the various combinations (GLP-1 + IL-4 and NK1 + IL-4) reverse hyperglycemia in NOD mice through a single ip injection (Gaddy et al. 2011). Moreover, Montane and colleagues (2011) utilized dsAAV8 to target CCL22 (recruits Treg cells) to the β-cells of NOD mice, through an intraductal injection (Montane et al. 2011). Their results suggest that increasing expression of CCL22 in the pancreatic β-cells attenuates the autoimmune destruction of β-cells associated in T1D through the increased recruitment of Tregs (Montane et al. 2011). Combined, these studies show the remarkable potential of utilizing dsAAVs for human gene therapy.

Double-stranded adeno-associated (dsAAV) viral vectors have been used as non-invasive tools for tissue-specific gene delivery to pancreatic β-cells. Extensive *in vivo* studies were conducted administering eGFP with various AAV serotypes under the direction of MIP to C57Bl6 mice via intraperitoneal, intraductal, and intravenous injection (Wang et al. 2006). Wang and colleagues (2006) concluded that viral delivery via interperitoneal injections were as effective as intraductal and intravenous when viral dose was increase to $5x10^{11}$ viral/genomes per mouse (Wang et al. 2006); moreover dsAAV8 was more effective than dsAAV1, dsAAV2, dsAAV5 and dsAAV6 when targeting the pancreatic β-cells (Wang et al. 2006). In addition gene expression in pancreatic islets was observed for four months post infection via intraperitoneal injection (Wang et al. 2006). More recently, Kieffer and colleagues (2011) have conducted long-

term expression studies, showing expression of dsAAV8-MIP-eGFP in the pancreatic β-cells one year post-infection (Kieffer, TJ – personal communication).

Furthermore, under the direction of MIP, Wang and colleagues did not detect eGFP expression in brain tissue of infected mice (Wang et al. 2006), indicating that using dsAAV8-MIP or dsAAV8-RIP to target pancreatic β-cells is an effective method for gene delivery without crossing the blood brain barrier. It should be noted that previous studies have indicated that utilizing dsAAV8 under the direction of MIP or RIP may cross the BBB due to dsAAV8's ability to increase vascular permeability in endothelial cell lining of blood vessels (Inagaki et al. 2006; Wu et al. 2006); further studies investigating the vasculature leakiness of dsAAV8 is required.

More recently, Gaddy and colleagues (2010) have been able to successfully overexpress eGFP within pancreatic β-cells without causing changes in body weight or carbohydrate metabolism utilizing dsAAV8 (Gaddy et al. 2010; Riedel et al. 2010). These studies demonstrate using dsAAV8-RIP-eGFP as a control vector for *in vivo* overexpression studies is an appropriate tool for viral-mediated tissue specific gene delivery. Moreover, the use of dsAAV8 allows for targeted gene delivery at any stage in the rodent's life; essentially providing a model of "inducible transgenics" without the complications associated with conventional transgenic mouse models. Additionally dsAAV8 can be delivered prior to or after a treatment such as high fat diet; as well it can be delivered to various mouse breeds including the "standard" C57Bl6 or various transgenic models including *db/db* or *ob/ob* mouse (to be discussed in chapter four).

The objective of this study is to establish an *in vivo* model and protocol of viral-mediated gene delivery in our lab. This study will serve as control for data collection and

analysis prior to characterizing the *in vivo* effects of dsAAV8-RIP-PPARα overexpression in β-cell lipotoxicity in a diet-induced obese model of T2D.

2.2 MATERIALS AND METHODS

Mice.

All animal studies were approved by the University of Northern British Columbia Animal Care and Use Committee (protocol number 2011-21). For all experiments, 6-week-old, male, C57Bl6 mice were purchased from Charles River Laboratories (Wilmington MA, USA) unless otherwise stated. Mice were maintained on a 12 hr light/dark cycle and received standard rodent chow diet (Rodent LabDiet, 5001, Leduc AB, Canada) *ad libitum* unless otherwise stated. Mice were allowed to acclimatize to the animal facility for 1 week prior to performing any experimental procedures. Body weight, 4hr fasted blood glucose (OneTouch Ultra, Lifescan, Burnaby BC, Canada), oral glucose tolerance tests (OGTT) and insulin tolerance tests (ITT) were performed prior to viral injections to assess baseline carbohydrate metabolism.

Double-stranded adeno-associated virus serotype 8.

dsAAV8 transfer vectors were designed utilizing the rat insulin 1 promoter (RIP) (410 bp) placed upstream of either a sequence encoding eGFP (720bp) or the PPARα (1407bp) open reading frame, followed by the SV40 polyadenylation signal. Complete RIP-eGFP (5328bp) and RIP-PPARα (6015bp) vectors were sent to Children's Hospital of Philadelphia (CHOP) Research Vector Core Services (Philadelphia PA, USA) for AAV8 virus preparation.

Pancreatic β-cell specific expression of eGFP.

At 8 wks of age, C57Bl6 mice were infected with dsAAV8-RIP-eGFP virus by ip injection (n=7; $5x10^{12}$ viral genomes/284 µl/mouse) (β-eGFP-Chow) and control mice (saline) were given an ip injection of saline (n=7; 284µl/mouse) (Gaddy et al. 2010;

Riedel et al. 2010) (Fig. 2.1). Body weights, glucose tolerance and insulin sensitivity were assessed prior to and three weeks post-infection. Three weeks post-infection animals were sacrificed and pancreas was collected and stored in 4% paraformaldehyde for immunohistological analysis.

Insulin tolerance tests.

Mice were fasted for 4hrs and given an ip injection of human synthetic insulin at 0.75U/kg or 1.0U/kg (Novolin Ge, Toronto ON, Canada). Blood was sampled (1-2µl) from the saphenous vein and blood glucose measured (mmol/L) at 10, 20 30, 60, and 120 min post injection, using a handheld glucometer (OneTouch Ultra Lifescan, Burnaby BC, Canada).

Oral glucose tolerance and glucose-stimulated insulin secretion.

Mice were fasted for 16 hrs and given 2g/kg D-glucose by oral gavage. Blood (15-20µl) was sampled at 5, 10 and 180 minutes post glucose gavage from the saphenous vein and blood glucose (mmol/L) measured in 1-2µl blood at 5, 10, 30, 60, 120 and 180 minutes post gavage using a handheld glucometer (OneTouch Ultra Lifescan, Burnaby BC, Canada) (Huynh et al. 2010).

Immunohistological analysis.

Whole mouse pancreas were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned (5µm); n=5 per group (Wax-it Histology Services; Vancouver BC, Canada). All sections were de-paraffinized in xylene, rehydrated in ethanol (100%, 95%, and 70%) and rinsed in 1xPBS as described previously (Riedel et al. 2010). Sections were incubated with polyclonal primary antibody to rabbit anti-eGFP (1:500) (A11122, Invitrogen Molecular Probes, Carlsbad CA, USA), polyclonal guinea pig anti-insulin

(1:1000) (4011-01F, Millipore, Billerica MA, USA) and a monoclonal primary antibody to mouse anti-glucagon (1:1000) (G2654, Sigma, Oakville ON, Canada) overnight. Appropriate secondary whole (heavy and light chain) antibodies conjugated to Alexafluor 488 (A21206 or A21202) or 594 (A11076 or A11032) (1:1000) (Invitrogen Molecular Probes, Carlsbad CA, USA) were used to detect primary antibody immunoreactivity. All samples were visualized using a fluorescent light microscope (Olympus BX61) and images analyzed using Cell Sens Software (Olympus, Markham ON, Canada).

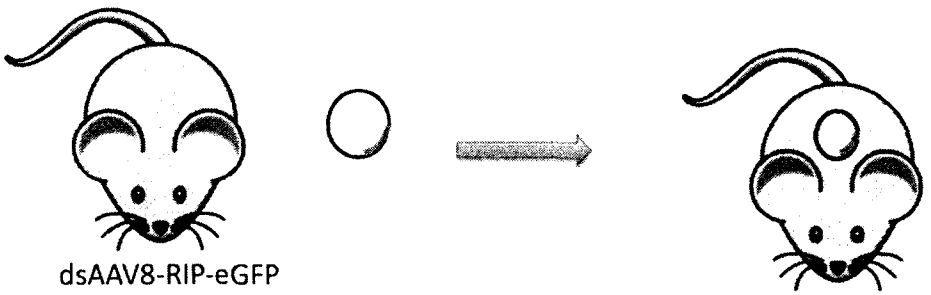
Western blot analysis.

Hypothalamus, liver, intestine, and skeletal muscle samples were collected and flash frozen. Protein was extracted using RIPA buffer (Thermo Scientific, Rockford IL, USA) and quantified using a BCA assay (Thermo Scientific, Rockford IL, USA). Protein (30μg) was separated on a 12% SDS-PAGE gel and transferred to a PVDF membrane (Millipore, Billerica MA, USA). Membranes were blocked with 5% non-fat dry milk in TBS-Tween 20 followed by overnight incubations with a polyclonal primary antibody to rabbit anti-eGFP (1:1000) (A11122, Invitrogen Molecular Probes, Carlsbad CA, USA), a monoclonal primary antibody to mouse anti-β-Actin (A5441, Sigma, Oakville ON, Canada), or a monoclonal primary antibody to mouse anti-α-tubulin (1:3000) (T8203, Sigma, Oakville ON, Canada) were used to normalize protein levels. Secondary horseradish peroxidase-conjugated antibodies (1:5000) (anti-rabbit IgG A4914, anti-mouse IgG, A8924, Sigma, Oakville ON, Canada) were incubated for 1hr and visualized by chemiluminescence (Amersham ECL plus, Baie d'Urfe QC, Canada) using the Carestream Software and Kodak Imager (4000MM Pro) (Kodak, Woodbridge CT, USA).

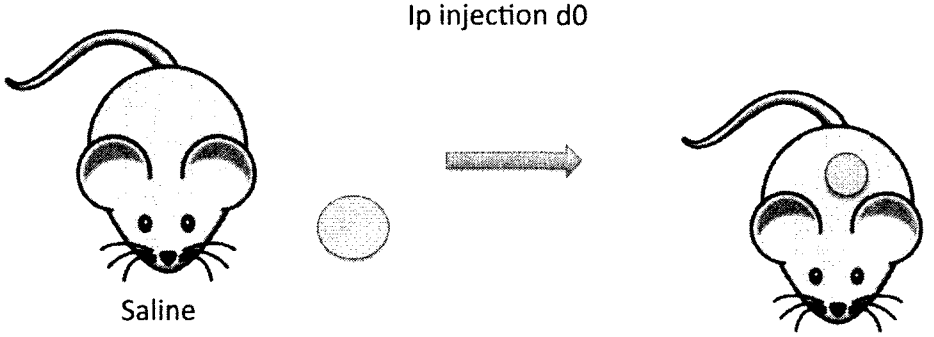
Statistical analysis.

Results are expressed as mean ± standard error of the mean. Analyses were performed using paired student's t-test and 1-way ANOVA (significance between two groups), or 2-way ANOVAs (significance between groups over time) with Bonferoni Post tests using Graphpad Prism 5.0 software (La Jolla CA, USA). Significance was declared if p-values were less than 0.05.

Figure 2.1 *In vivo* experimental approach establishing effects of overexpressing dsAAV8-RIP-eGFP in pancreatic β-cells in mice. Four-month-old male C57Bl6 mice were infected with either dsAAV8-RIP-eGFP (β-eGFP-Control) ($5.0x10^{12}$ viral genomes/mouse) or saline (284µl/mouse). Body weights (weekly), 4hr fasted blood glucose readings (biweekly), oral glucose tolerance (end point), and insulin tolerance (end point) were assessed. Additionally western blot analysis was utilized to determine if eGFP was being expressed in other tissues other than pancreatic β-cells. Abbreviations: dsAAV8 – double stranded adeno-associated virus serotype 8; RIP – rat insulin promotor; eGFP – enhanced green fluorescent protein.



Ip injection d0



Baseline characteristics:	Weekly Monitoring:	Endpoint:
- Body weight	- Body weight	- Body weight
- OGTT	- 4hr FBG (Biweekly)	-OGTT
- ITT		- ITT

2.3 RESULTS

Overexpression of dsAAV8-RIP-eGFP is targeted to β-cells and not the α-cells of the pancreas.

Immunohistochemical analysis of pancreas sections collected from dsAAV8-RIP-eGFP infected mice show eGFP immunofluorescence colocalized with insulin immunofluorescence (Fig. 2.2a) as indicated by the green and red overlay; whereas no colocalization was observed between eGFP and glucagon immunofluorescence (Fig. 2.2b). These results are supported by previous studies from Gaddy et al (2010) and Riedel et al (2010); further confirming that eGFP protein expression using dsAAV8-RIP-eGFP is localized to the β-cell with no expression in the α-cell of the islets or the exocrine tissue of the pancreas. As expected, no eGFP expression was observed in pancreatic sections collected from saline infected mice. Moreover, eGFP was found to be expressed in 60% of the total islet area in eGFP infected islets from an ip injection (Fig 2.2c).

Pancreatic β-cell specific overexpression of eGFP driven by the rat insulin promoter does not cross the blood brain barrier.

Due to the observation that RIP-Cre transgenic mice have been shown to drive recombination of conditional gene knockouts in hypothalamic neurons as well as pancreatic β-cells (Choudhury et al. 2005; Choi et al. 2008), we examined if eGFP expressed from dsAAV8-RIP-eGFP and delivered by dsAAV8 was expressed in hypothalamic neurons of dsAAV-RIP-eGFP infected mice. Western analysis of protein extracts from hypothalamic tissue we detected no expression of eGFP suggesting the adeno-associated virus did not cross the BBB or the RIP promoter was not active in neurons of the hypothalamus in these mice (Fig. 2.3).

Figure 2.2 Immunohistochemical analysis to detect eGFP overexpression in pancreatic β-cells. Overexpression of dsAAV8-RIP-eGFP shows targeted expression of eGFP in β-cells (A) and not α-cells (B) of the pancreas of C57Bl6 mice. (C) Percentage of eGFP immunoreactive area in total islet area. Abbreviations: dsAAV8 – double stranded adeno-associated virus serotype 8; RIP – rat insulin promotor; eGFP – enhanced green fluorescent protein; INS – insulin; GLC – glucagon . Saline-treated n=7, β-eGFP-Control n=7. Images taken at 20x magnification. Scale bar 50μm.

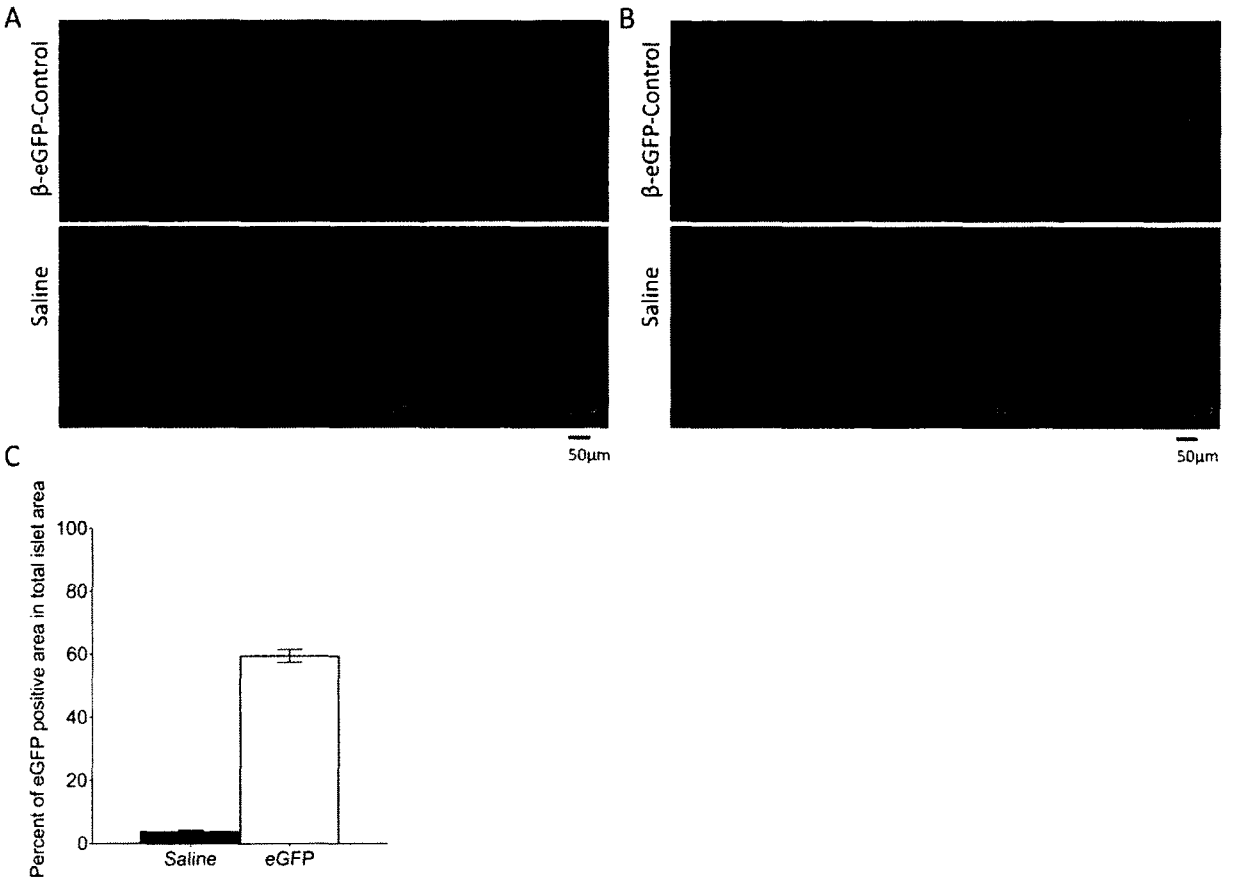
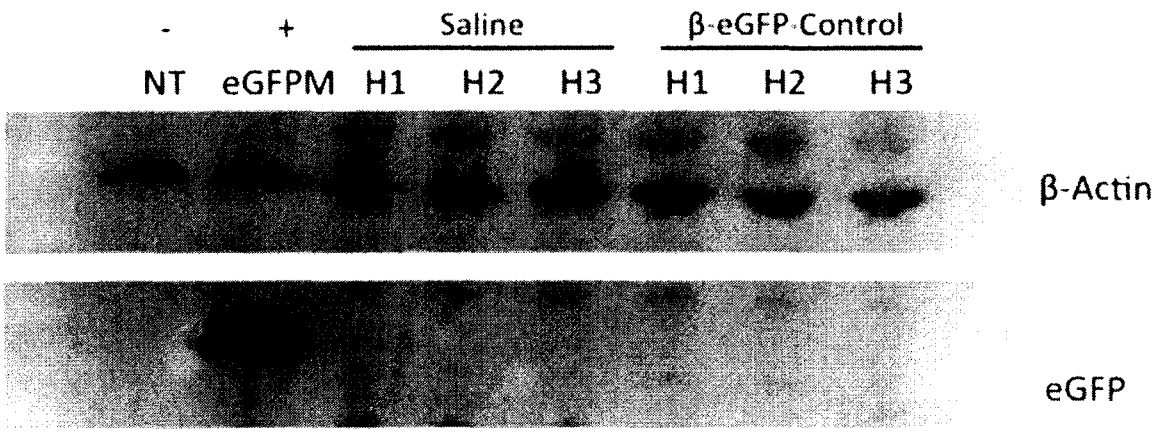

A
β-eGFP-Control
Saline
50μm
B
β-eGFP-Control
Saline
50μm
C
Percent of eGFP positive area in total islet area
100
80
60
40
20
0
Saline
eGFP

Figure 2.3 Western blot analysis of dsAAV8-RIP-eGFP expression in non-pancreatic tissues. Overexpressing dsAAV8-RIP-eGFP in pancreatic β-cells does not cross the BBB showing no expression in the hypothalamus. 30μg of protein per sample was loaded and run (see methods); β-Actin was utilized as a protein control. Abbreviations: dsAAV8 – double stranded adeno-associated virus serotype 8; RIP – rat insulin promotor; eGFP – enhanced green fluorescent protein; NT – non-transfected MIN6 cells; (-) negative control; eGFPM – eGFP transfected MIN6 cells; (+) positive control.


-
+
Saline
β-eGFP Control
NT
eGFPM
H1
H2
H3
H1
H2
H3
β-Actin
eGFP

Overexpression of dsAAV8-RIP-eGFP is not expressed in skeletal muscle or liver.

We examined expression of eGFP in peripheral tissues (liver, intestine and skeletal muscle) of mice that received virus carrying dsAAV8-RIP-eGFP and saline control mice, using western blot analysis. No eGFP expression was observed in skeletal muscle or intestine protein extracts in both dsAAV8-RIP-eGFP expressing mice and saline control mice. Non-specific bands were observed in skeletal muscle and liver protein extracts of both β-eGFP-control and saline mice (Fig. 2.4). It should be noted that the increased brightness observed in liver protein extracts raised concern and required further investigation as dsAAV8 has been reported to target liver and skeletal muscle tissue in addition to the pancreas (Chao et al. 2000; Gao et al. 2002; Wang et al. 2004; Wu et al. 2006); therefore we had to confirm if the non-specific bands were real.

To do this, we decided to perform immunohistochemical analysis using the same antibodies utilized of the western blot. Skeletal muscle and liver tissue samples of both β-eGFP-control and saline mice were sectioned and stained for eGFP using immunohistochemistry. It was observed that eGFP was not expressed in either skeletal muscle or liver tissue (Fig. 2.5a) in both β-eGFP-control and saline mice. Intensity of eGFP observed was identical to the background staining in the no primary (1°) antibody control. As an additional control for immune reactivity, pancreatic sections (Fig. 2.5b) were stained for eGFP and insulin. This allowed us to confirm that under the same conditions and concentrations the antibody to eGFP and blocking solutions utilized for immunohistochemical analysis was able to detect eGFP in pancreatic β-cells as previously shown.

Figure 2.4 Western blot analysis of dsAAV8-RIP-eGFP expression in non-pancreatic tissues. Overexpressing dsAAV8-RIP-eGFP in pancreatic β-cells does not target skeletal muscle or intestine. Non-specific binding of eGFP was observed in liver tissue in both dsAAV8-RIP-eGFP mice and saline controls. 30μg of protein per sample was loaded and run (see methods); β-Actin was utilized as a protein control. Abbreviations: dsAAV8 – double stranded adeno-associated virus serotype 8; RIP – rat insulin promotor; eGFP – enhanced green fluorescent protein; NT – non-transfected MIN6 cells; (-) negative control; eGFPM – eGFP transfected MIN6 cells; (+) positive control; SKM – skeletal muscle.

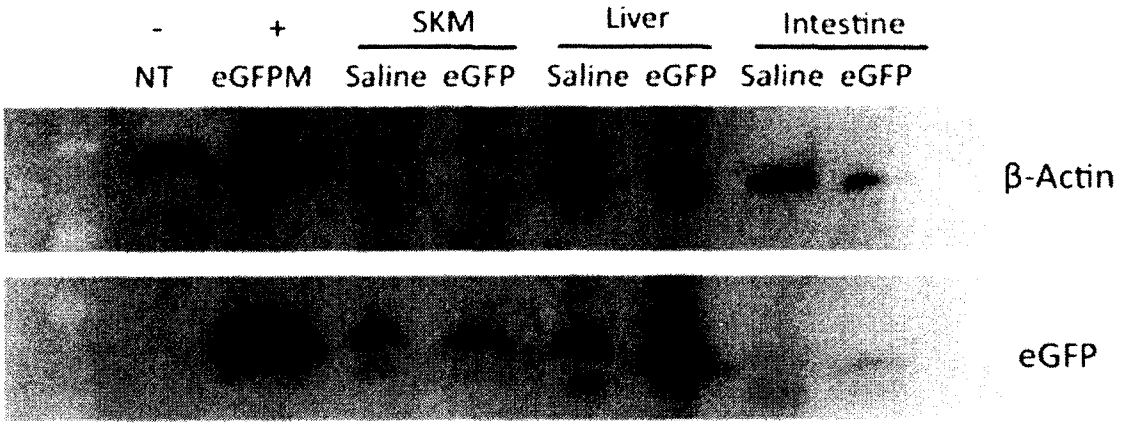
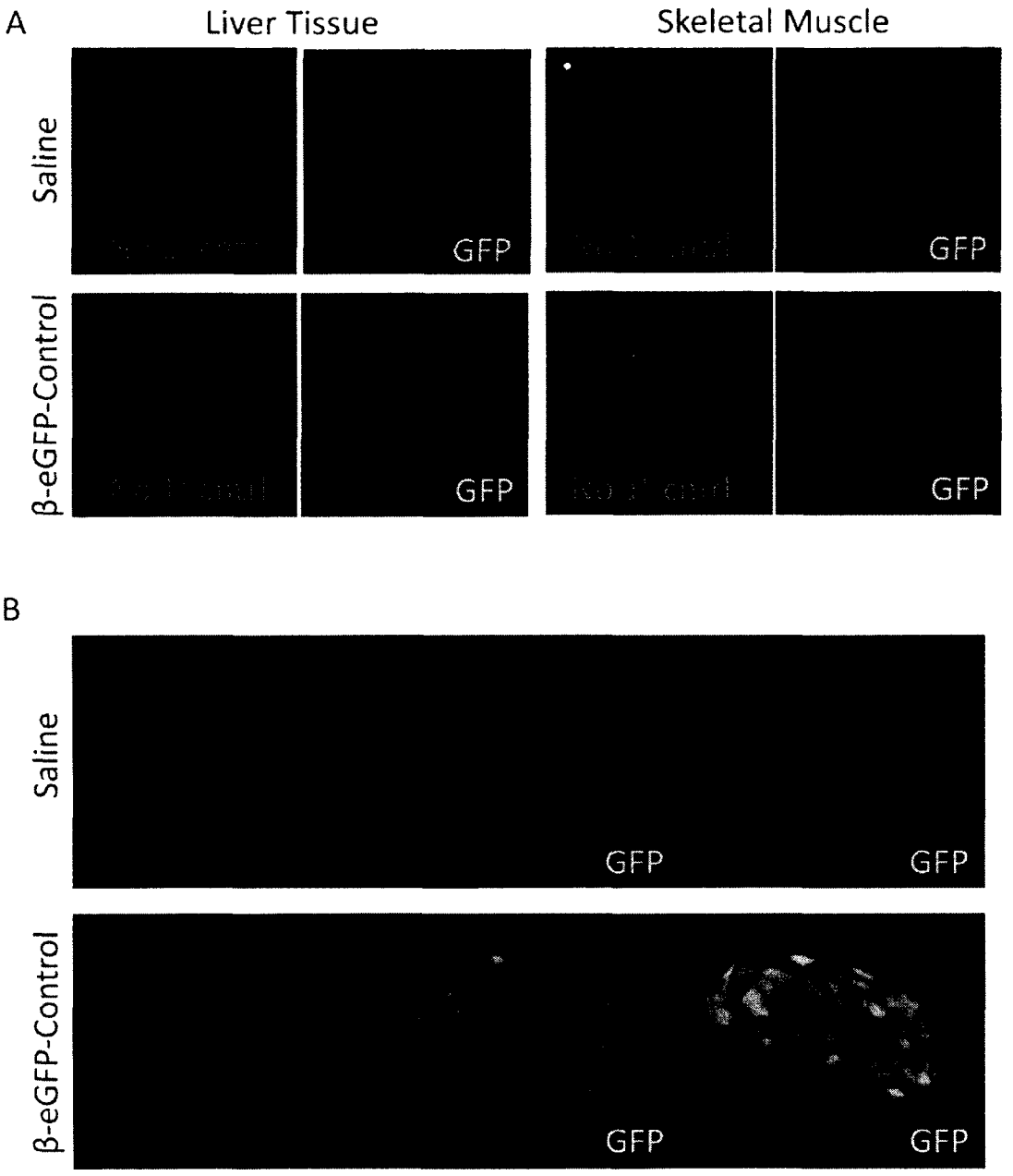

-
+
SKM
Liver
Intestine
NT
eGFPM
Saline
eGFP
Saline
eGFP
Saline
eGFP
β-Actin
eGFP

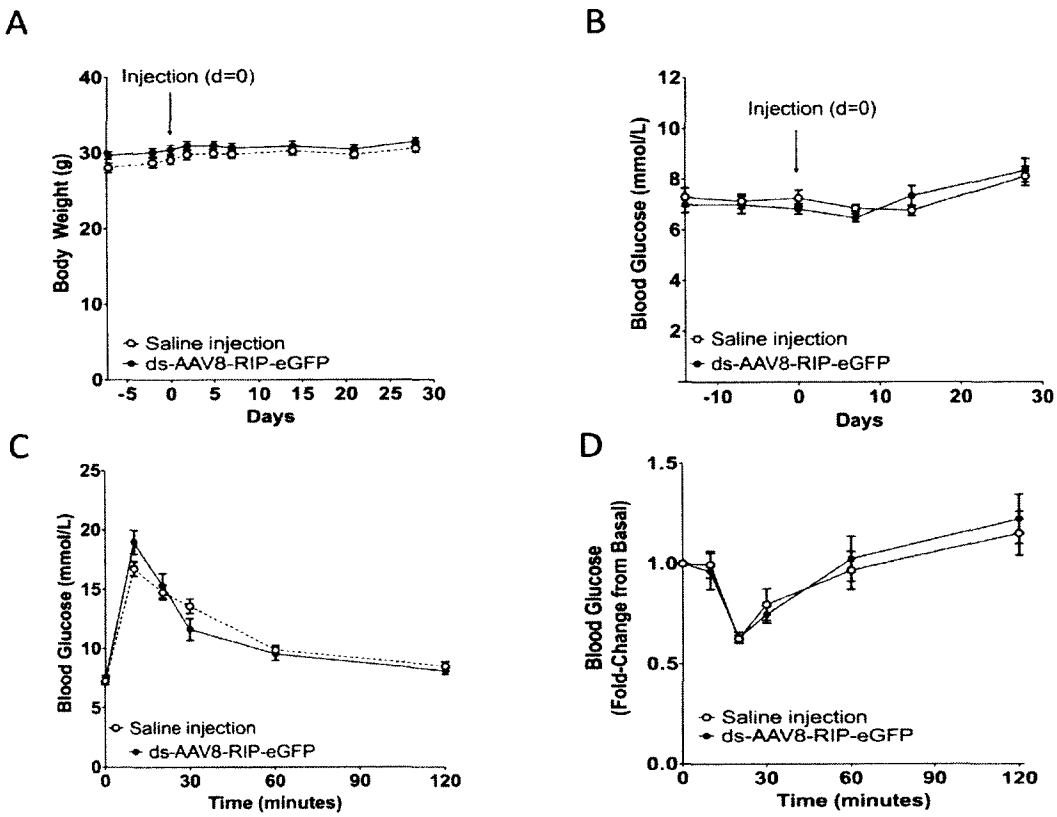
Figure 2.5 Immunohistochemical analysis of dsAAV8-RIP-eGFP overexpression in non-pancreatic tissues. Staining revealed no eGFP expression in liver (A) or skeletal muscle (B) tissues above background staining (secondary antibody AF488 only) in β-eGFP-Control mice when compared to saline controls. Pancreas sections (C) were stained as positive controls to show level of eGFP expression when cells are overexpressing eGFP. Abbreviations: dsAAV8 – double stranded adeno-associated virus serotype 8; RIP – rat insulin promotor; eGFP – enhanced green fluorescent protein; 1° - primary; CNTRL - control. Images taken at 20x magnification. Scale bar 50μm.


A
Liver Tissue
Skeletal Muscle
Saline
β-eGFP-Control
GFP
GFP
GFP
GFP
B
Saline
β-eGFP-Control
GFP
GFP
GFP
GFP

Pancreatic β-cell specific overexpression of eGFP using dsAAV8-RIP in mice does not affect carbohydrate metabolism.

We utilized a RIP driven expression vector delivered by dsAAV8 to generate mice overexpressing eGFP specifically in pancreatic β-cells. We confirmed that control mice expressed eGFP specifically in pancreatic β-cells (dsAAV8-RIP-eGFP) with no effect on body weight, 4 hr fasted blood glucose levels, oral glucose tolerance or insulin sensitivity when compared to control mice injected with saline (Fig. 2.6 a-d). These results, and similar results from others (Gaddy et al. 2010) confirm dsAAV8-RIP-eGFP is an appropriate control virus for *in vivo* overexpression studies using dsAAV8.

Figure 2.6 Body weight and carbohydrate metabolism from 3wk post-infection with dsAAV8-RIP-eGFP (β-eGFP-Control) or saline (284µl/mouse) by ip injection. Overexpression of eGFP in pancreatic β-cells resulted in no change in body weight (A), 4hr FBG (B), glucose tolerance (C, 2g D-glucose/kg), or insulin sensitivity (D, 0.75U insulin/kg) compared to saline-treated mice on regular chow diet. Abbreviations: dsAAV8 – double stranded adeno-associated virus serotype 8; RIP – rat insulin promotor; eGFP – enhanced green fluorescent protein; FBG – fasted blood glucose. Saline-treated n=7, β-eGFP-Control n=7. Mean values +/- standard error of the mean are shown. Statistical significace was tested by 2-way ANOVA (over time) with Bonferoni Post Tests or Student's t-Tests (at specific time points).


A
Injection (d=0)
Body Weight (g)
Saline injection
ds-AAV8-RIP-eGFP
Days
B
Injection (d=0)
Blood Glucose (mmol/L)
Saline injection
ds-AAV8-RIP-eGFP
Days
C
Blood Glucose (mmol/L)
Saline injection
ds-AAV8-RIP-eGFP
Time (minutes)
D
Blood Glucose (Fold-Change from Basal)
Saline injection
ds-AAV8-RIP-eGFP
Time (minutes)

2.4 DISCUSSION

Transgenic mice driven by the RIP start expressing the gene or protein of interest as early as embryonic day 13.5 (Hara et al. 2003); thus, showing overxpression of the gene of interest before birth, from E13.5. In addition, when utilizing RIP-Cre transgenics, there is a possibility that the protein of interest will also be expressed in the hypothalamic neurons of the brain (Choudhury et al. 2005; Choi et al. 2008; Dahlhoff et al. 2011). It has been described previously, that as little as a 10% reduction in body weight can have significant improvements in whole body carbohydrate metabolism (Smyth and Heron 2006). Since the hypothalamus is responsible for appetite regulation (Inui 1999), overexpression of proteins in the hypothalamus by RIP can impact carbohydrate metabolism and β-cell function. Choi and colleagues (2008) speculated that manipulating hypothalamic neuronal activity by overexpressing Cre and increased protein production can regulate whole body growth (Choi et al. 2008) as seen in their RIP-Cre mouse. These characteristics of RIP transgenics were considered when deciding on the most appropriate method for implementing β-cell specific overexpression of PPARα and assessing its effects on β-cell lipotoxicity in a diet-induced obese model of β-cell failure and T2D. If we were to use a RIP-PPARα transgenic mouse overxpressing PPARα, we would induce PPARα expression from E13.5 in the developing pancreas as well as the potential expression in the adult hypothalamus. Thus, we would not be able to easily discern if the physiological results obtained are solely from pancreatic β-cell overexpression of PPARα or additionally related to these cofounding expression patterns. Therefore methods of inducible overexpression seem more favorable when characterizing genes of interest in an *in vivo* model.

Recently, a new gene delivery tool has been developed for targeted delivery of proteins to β-cells of the pancreas via intraperitoneal injection utilizing dsAAV8 vectors (Wang et al. 2006; Gaddy et al. 2010; Riedel et al. 2010) under the direction of the rat-insulin promoter. Here we demonstrate that viral delivery of dsAAV8-RIP-eGFP is specifically targeted to pancreatic β-cells and not α-cells (Fig. 2.2 a, b) or exocrine tissue of pancreas. Overall these results indicate that the delivery of dsAAV8-RIP-eGFP via intraperitoneal injection prove to be an effective, non-invasive method of viral gene transfer for targeted protein delivery to the β-cells of the pancreas.

By overexpressing eGFP in the β-cells of the pancreas, we are forcing the β-cell to increase the production of foreign proteins. This increased protein production could potentially lead to decreased insulin secretion and the development of β-cell dysfunction or β-cell exhaustion. Therefore it was crucial to determine if expressing foreign proteins would affect the overall health of the β-cell. We determined that overexpression of dsAAV8-RIP-eGFP in the pancreatic β-cells did not affect body weight or overall carbohydrate metabolism (Fig. 2.6), further proving to be a useful control vector for future *in vivo* studies involving diabetic models.

Importantly, we have also confirmed that dsAAV8-RIP-eGFP does not cross the BBB (Fig. 2.4) similar to the results obtained by Wang and colleagues (2006), thus avoiding gene expression in hypothalamic neurons that may cause changes in appetite regulation controlled by the hypothalamus (Devaskar et al. 1994; Gannon et al. 2000). eGFP has been shown to be expressed in liver and skeletal muscle (Inagaki et al. 2006; Wu et al. 2006) when using dsAAV8. During western blot analysis non-specific bands were observed in both skeletal muscle and liver protein extracts (Fig. 2.4) from β-eGFP-

control and saline mice. However, after close observation the bands observed were not detected at 27kDa (eGFP positive control) they were found to have a slightly higher molecular weight as seen in the shift on the membrane. It should be noted that protein extracts from liver in β-eGFP-control mice were found to have bands of increased intensity around 27kDa, indicating that eGFP may be expressed in these tissues; thus immunohistochemical analysis was also performed. Moreover, our group concluded that these bands were indeed non-specific based on the observation that chemiluminescence was detected in protein extracts of saline injected mice in both liver and skeletal muscle.

Due to these unexpected results from western blot analysis, our group has confirmed that eGFP is not expressed in skeletal muscle or liver tissue of β-eGFP-control mice, using immunohistochemical analysis of paraffin embedded sections, that no eGFP staining was observed above background fluorescence (Fig 2.5a, b). To show positive staining of eGFP, pancreatic tissue sections were stained and used as a positive control for "real" eGFP staining (Fig. 2.5c). One may argue that there are intensely stained areas of green in the liver section of β-eGFP-control mice, however these intense patches of green were also observed in saline islets (Fig. 2.5c middle panel) indicating that these could be autofluorescing red blood cells, increased background staining, or non-specific staining observed in western results.

Based on our results and those obtained by our colleagues (Gaddy et al. 2010; Riedel et al. 2010), it should be noted that utilizing dsAAV8 for β-cell specific gene delivery is an appropriate vector for developing and characterizing *in vivo* models of β-cell overexpression. In addition we have been able to show effective gene delivery (Fig. 2.2a, b) to the target tissue (β-cells) through a non-invasive interperitoneal injection.

Furthermore, we have shown eGFP expression to be observed in 60% of total islet area of infected islets (Fig 2.2c). Importantly, the use of an ip injection opposed to intraductal injections allows user groups to avoid complications associated with invasive surgery such as recovery time, weight loss and potential death. Having shown the same efficiency of viral delivery as our colleagues (Wang et al. 2006; Riedel et al. 2010), utilizing the interperitoneal injection for our future studies will prove to be critical, as we want to ensure that any changes observed in our model is due solely to the overexpression of PPARs in the β-cells, and not due to weight loss caused by the invasive surgery of performing intraductal injections.

It has been proposed by Wang et al (2006), Gaddy et al (2010) and Riedel et al (2010) that there is the potential of utilizing dsAAV8 as a potential therapeutic for targeted pharmaceuticals or as a method of targeted gene therapy in diabetic individuals (Gaddy et al. 2010; Riedel et al. 2010). Again, the use of dsAAV8 under the control of RIP is also attractive as it specifically targets the pancreatic β-cells, therefore if it were to be used as a method of gene therapy in diabetes treatments, we could potentially avoid systemic complications as seen with some pharmaceuticals. As previously discussed, Gaddy et al. (2011) and Montane et al. (2011) have both utilized dsAAV8 mediated gene delivery to either reverse hyperglycemia or protect against further autoimmune destruction of β-cells in T1D mice (Gaddy et al. 2011; Montane et al. 2011). Moreover, improvements were seen after a single ip or intraductal injection (Gaddy et al. 2011; Montane et al. 2011). These studies have shown that dsAAV8 could be a potential therapeutic for the treatment of T1D, thus making it especially appealing to current patients, as well as parents of T1D kids as an alternative to islet transplantation.

The results obtained in this study were similar to those obtained by Gaddy et al (2010) and Riedel et al (2010); further proving to be an effective tool in *in vivo* diabetic models opposed to traditional models of transgenics. The major findings from this chapter are:

1. dsAAV8 is an effective tool for gene delivery to the pancreatic β-cells.
2. Overexpression of dsAAV8-RIP-eGFP is observed in β-cells and not α-cells of the pancreas as early as three weeks post infection.
3. Overexpression of dsAAV8-RIP-eGFP is not expressed in the hypothalamus of the brain, or peripheral tissues (skeletal muscle, liver and intestine).
4. Overexpression of dsAAV8-RIP-eGFP within the pancreatic β-cells does not affect body weight or carbohydrate metabolism.

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CHAPTER 3

Overexpression of PPARα in the Pancreatic β-cells Improves Carbohydrate Metabolism in a Model of Diet-induced Obesity

A version of this chapter has been submitted as an abstract:
Hogh, K-Lynn N., Uy, Christopher E., Fraser, J., Asadi, A., Baker, Robert K., Riedel, Michael J., Gray, Sarah L. 2011. Overexpression of PPARα in pancreatic β-cells improves glucose tolerance in diet-induced obese mice. *Canadian Journal of Diabetes*. 35(4): 417. Canadian Diabetes Association AGM & Professional Meetings. Toronto ON. Oct 26-29 2011.

A version of this chapter has been submitted:
Hogh, K-Lynn N., Uy, Christopher E., Asadi, A., Baker, Robert K., Riedel, Michael J., Gray, Sarah L. 2012. Overexpression of PPARα in pancreatic β-cells improves glucose tolerance in diet-induced obese mice. *Endocrinology*. Manuscript Submission number: EN-12-1110.

3.1 INTRODUCTION

As previously outlined in chapter one, lipotoxicity is implicated as a mechanism in β-cell dysfunction and development of obesity-induced T2D in (Virtue and Vidal-Puig 2010). Therefore protecting the pancreatic β-cells from increased lipid accumulation may be a plausible approach to reducing lipotoxicity and preserving β-cell function.

The peroxisome proliferator-activated receptors (PPARs) are nuclear transcription factors involved in the regulation of lipid metabolism (Chinetti et al. 2000). PPARα is often expressed in tissues including the liver, skeletal muscle, heart and pancreas (Lee et al. 2003). Activation of PPARα upregulates expression of β-oxidative genes (Tordjman et al. 2002; Lalloyer et al. 2006; Medina-Gomez et al. 2007) with subsequent improvements in glucose homeostasis by decreasing circulating lipids and lipid accumulation in non-adipose tissues such as liver and skeletal muscle, consequently reducing lipotoxicity and increasing insulin sensitivity (Sugden et al. 2003; Lalloyer et al. 2006). Figure 3.1 illustrates the binding of PPARα to RXR where it forms a heterodimer prior to nuclear internalization. Once in the nucleus, the PPARα/RXR heterodimer binds to the PPAR response element (PPRE) where gene transcription remains inactive until the activating ligand (FA or FA derivatives, or PPAR agonist) binds to PPARα/RXR, causing the release of the corepressor complex (CRC) and histone deacetylases (HDACs) and subsequent binding of the coactivator complex (CAC) which recruits histone acetyltransferases (HATs). The PPARα/RXR/CAC complex is then active and the transcription of downstream target genes can occur, including the transcription of genes involved in fatty acid metabolism and β-oxidation (Reddy and Hashimoto 2001; Mandard et al. 2004).

Figure 3.1 – Regulation of gene expression by PPARs (Binding of PPAR/Retinoid Nuclear Receptor Family) modified from (Reddy and Hashimoto 2001). The PPAR/RXR family is involved in controlling gene transcription at the nuclear level. Abbreviations: FA – fatty acid; PPAR – peroxisome proliferator-activated receptor; Pα – PPARα; RXR – retinoid-X receptor; CRC – corepressor complex; HDACs – histone deacetylases; PPRE – PPAR response element; CAC – coActivator complex; HATs – histone acetyltransferases.

Active transport of FA or PPAR agonists into cell
Heterodimer formation
RXR
nuclear localization
FAs or PPAR agonists diffuses through nucleus
HDACs
Nucleus
cytoplasm
Inactive complex (gene repression)
PPRE
Recruits HATs
CAC
FA or PPAR agonist binding
RXR
PPRE
CAC
RXR
PPRE
Active complex (gene transcription)
↑gene transcription

When PPARα is activated, numerous downstream target genes involved in β-oxidation and lipid metabolism are turned on (Mandard et al. 2004). Some of the genes upregulated by PPARα include: Acyl-CoA oxidase (AOX), carnitine palmitoyl transferase-1 (CPT1), long chain acyl-coA dehydrogenase (LCAD), and uncoupling protein-2 (UCP2) (Reddy and Hashimoto 2001; Lee et al. 2003). Expression of these target genes varies depending on tissue selected (Mandard et al. 2004). Increased expression and PPARα activation occurs largely in the liver (Lee et al. 2003), where a decrease in triglyceride synthesis and increased oxidation of fatty acids is observed (Frederiksen et al. 2004). It should be noted that previous studies have reported that prolonged exposure to increased PPARα activity in the liver has been linked to heptacellular carcinoma in rats and mice (Qin et al. 1997; Gonzalez et al. 1998; Reddy and Hashimoto 2001). In contrast, PPARs have been found to be nonmutagenic in nature after increasing metabolic activity (Gonzalez et al. 1998; Reddy and Hashimoto 2001); therefore proving to be a potential tool in tissue-specific gene manipulation in *in vivo* studies.

Studies utilizing PPARα null mice or systemic administration of PPARα agonists have contributed greatly to elucidating the physiological role of PPARα. Loss-of-function studies have shown that animals lacking PPARα are protected against high-fat diet induced obesity and insulin resistance (Guerre-Millo et al. 2001); where PPARα was found to be non-essential for the maintenance of euglycemia, but required to maintain the β-cell adaptive response to hyperglycemia (Guerre-Millo et al. 2001; Bihan et al. 2005; Yessoufou et al. 2009). Moreover, Lalloyer and colleagues (2006) utilized PPARα-null *ob/ob* mice to show that PPARα deficiency in a severely obese model (*ob/ob*) resulted in

increased β-cell dysfunction with reduced islet area and decreased glucose-stimulated insulin secretion (Lalloyer et al. 2006). It should also be noted that PPARα-null mice often suffer from hypoglycemia under prolonged fasting (Guerre-Millo et al. 2001; Lalloyer et al. 2006). This hypoglycemia is believed to be attributed to depleted glycogen stores in PPARα-null mice, (Kersten et al. 1999; Lee et al. 2003; Mandard et al. 2004)

Gain of function studies utilizing systemic administration of PPARα agonists in OLETF rats, prevented FFA-induced β-cell dysfunction (Koh et al. 2003; Lalloyer et al. 2006). PPARα agonists have been shown to improve hepatic insulin sensitivity by enhancing hepatic expression of β-oxidative genes (Bergeron et al. 2006), strongly supporting the notion that PPARα agonists decrease lipid accumulation in peripheral tissues such as liver (Holness et al. 2003; Koh et al. 2003; Bergeron et al. 2006). Additionally, Yajima and colleagues (2003) have shown that administrating a combination of PPARα and PPARγ agonists increased glucose-stimulated insulin secretion by increasing insulin stores in β-cells of *db/db* mice (Yajima et al. 2003). Because the above studies report the effects of systemic PPARα deletion or agonism, the direct effect of PPARα on pancreatic β-cell function cannot conclusively be determined as direct or secondary and thus it remains unclear if the beneficial effects of PPARα on β-cell function are due to reductions in circulating lipid levels via actions on the liver, or direct effects on the β-cell itself.

Studies utilizing both isolated islets and β-cell lines have provided evidence that PPARα activation is able to improve β-cell function directly. Hellemans *et al* (2007) showed that in isolated rat islets, activation of PPARα protected against palmitate-

induced toxicity through increased mitochondrial and peroxisomal β-oxidation (Hellemans et al. 2007). More recently, Frigerio and colleagues (2010) utilized adenoviruses to overexpress PPARα and retinoid X receptor (RXR) in the INS-1E β-cell line (Frigerio et al. 2010). Overexpressing PPARα in this clonal cell line resulted in restored glucose-stimulated insulin secretion in oleate-treated cells by promoting glucose metabolism and fatty acid storage (Frigerio et al. 2010). Frigerio *et al* (2010) proposed that overexpression of PPARα in the INS-1E cells increased fatty acid turnover through consumption via β-oxidation; it was also proposed that the protective effects observed with PPARα overexpression could be due to increased triglyceride synthesis (Frigerio et al. 2010). Further supporting the protective effects of PPARα, Lalloyer *et al* (2007) were able to show that treatment with PPARα agonists under lipotoxic conditions improved insulin secretion in isolated human islets (Lalloyer et al. 2006). These improvements were associated with decreased islet triglyceride content and decreased palmitate-induced apoptosis (Lalloyer et al. 2006). Together, these studies provide evidence that activation of PPARα in isolated islets and β-cell lines may protect against lipotoxic induced β-cell dysfunction through the regulation of lipid metabolism in islets.

Based on the above *in vivo* and *in vitro* studies showing favorable effects of PPARα agonism on β-cell function in the context of lipid exposure and high-fat feeding, we hypothesized that overexpression of PPARα specifically in pancreatic β-cells of obese mice, *in vivo*, would preserve pancreatic β-cell function and delay the onset of obesity-induced diabetes.

3.2 MATERIALS AND METHODS

Cell Culture.

There are several β-cell lines currently utilized to study the *in vitro* effects of lipotoxicity on the β-cell and its role in the pathogenesis of T2D. When available, isolated islets are preferred; however islets are difficult to isolate, and can become expensive to obtain, as whole mice must be sacrificed for the collection of islets. In addition to primary islets, insulinoma cell lines are commercially available for *in vitro* studies looking at β-cell function. Of the β-cell lines, the MIN6 and INS-1E cells are often most utilized for their β-cell characteristics (Xiao et al. 2001; Tordjman et al. 2002; Ravnskjaer et al. 2005; Frigerio et al. 2010). The use of these cell lines will depend on what tests, and data one wants to collect. INS-1E cells have been found to be more easily cultured, however they have less regulation of insulin secretion and higher basal insulin levels without a stimulus (Skelin et al. 2010). Whereas the MIN6 cells, act and respond more like β-cells, in a glucose dependent manner; where insulin is secreted appropriately in response to a glucose stimulus (Skelin et al. 2010).

MIN6 cells (passage 15- 25) were cultured at 37°C and 5% CO_2 in media containing Dulbecco's Modified Eagle's Media (DMEM) (D5671, Sigma, Oakville ON, Canada), 200mM L-glutamine, 50μmol/L β-mercaptoethanol, 10% FBS (vol/vol), and 100 U/ml penicillin-streptomycin). Cells were transfected at 80-90% confluencey as previously described (Dalby et al. 2004) using lipofectamine 2000 (1μl) (Invitrogen, Burlington ON, Canada) and 1.6μg of plasmid (plasmid dsAAV8-RIP-eGFP or plasmid dsAAV8-RIP-PPARα) (Fig. 3.2). Enhanced green fluorescent protein (eGFP) was visualized by fluorescent microscopy to assess transfection efficiency 24hrs post

transfection; experiments that produced 60% or more eGFP expressing cells were considered to be successfully transfected. For experiments requiring palmitate treatment, cells were exposed to the above culture media containing 0μM or 250μM BSA-coupled palmitate (6:1) (Gwiazda et al. 2009) for 48hrs one day post-transfection. 20mM stock palmitic acid was prepared daily as follows: 5.6mg of palmitic acid was dissolved in 1ml of H_2O containing 1μl of NaOH at 70°C; once dissolved 0.5ml of 20mM palmitic acid was added to 1.65ml of 20% FA free BSA, and added to 17.85ml of cell culture media for a final stock concentration of 500μM. Stock palmitate solutions were then diluted to working concentrations of 250μM prior to treating cells.

RNA extraction and cDNA generation.

RNA was extracted (RNeasy kit, Qiagen, Valencia CA, USA) from cells 24 hrs post transfection. Genomic DNA was removed from RNA extracts using 10x TURBO DNAse buffer (5μl) and TURBO DNAse (1μl) and incubated at 37°C for 1 hr as per the manufacturers protcol (TURBO DNAse Kit, Ambion, Burlington ON, Canada). RNA concentration and purity were assessed by spectrophotometry (nanodrop spectrophotometer, ND-1000, Thermo Scientific, Rockford IL, USA) and RNA integrity was assessed by visualizing intact 18s and 28s rRNA bands on a 1.5% agarose gel. Intact RNA (500ng) was reverse transcribed to cDNA using random primers (5μl), dNTPs (1μl), dH_2O (*x*-μl to total volume of 13μl) and incubated at 65°C for 5min, then first strand buffer (4μl), 0.1M DTT (1μl) and superscriptIII (1μl) added to each reaction and incubated in the thermocycler as per the manufacturers protocol (Superscript III Reverse Transcriptase kit, Invitrogen, Burlington ON, Canada).

Quantitative Real-time PCR.

PPARα mRNA expression levels in MIN6 cells and induction of PPARα target gene mRNA expression were assessed using quantitative real-time PCR normalized to the reference genes β-Actin and 18s. Taqman primer and probe sequences for PPARα, carnitine palmitoyl transferase-1 (CPT1), long-chain acyl-CoA dehydrogenase (LCAD), Acyl-CoA oxidase (AOX), uncoupling protein-2 (UCP2), β-actin and 18s are shown in Table 1 (Sigma,) and qPCR master mix contained: iQ Supermix, Taqman forward and reverse primers (5μM), Taqman probes (5μM), RNAse free H_2O, cDNA (1μl) (BioRad Laboratories, Hercules CA, USA; Sigma, Oakville ON, Canada; Ambion, Austin TX, USA). The following reaction conditions were used for each RT-PCR: one cycle of 50°C for 2min, 95°C for 10min followed by 40 cycles of 95°C for 15sec, 60°C for 1min (BioRad iQ5 Multicolor RT-PCR Detection System, BioRad Laboratories, Hercules CA, USA). All qRT-PCR reactions followed the MIQE guidelines (Bustin et al. 2009) where:

1) Each biological replicate (experimental and control) was completed in triplicate
2) RNA extracts were treated with DNAse to remove genomic DNA
3) RNA quality was assessed using slab gel electrophoresis
4) cDNA was prepared immediately after RNA quality assessment
5) Primers and probes were previously designed by groups using qRT-PCR
6) R^2 values were 0.980 or greater, with efficiencies between 90-115%
7) Several reference genes (18S and β-Actin) were used for normalization
8) Each biological replicated was run as a technical triplicate for maximum reproducibility.

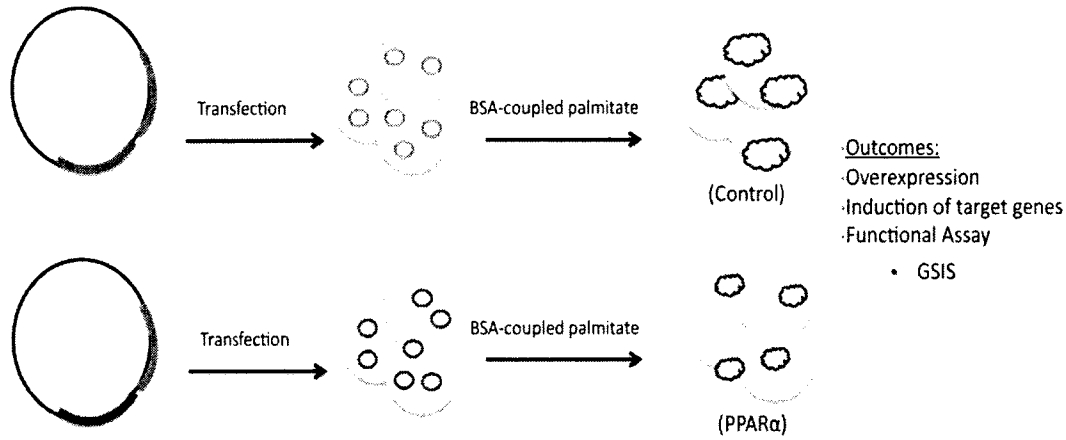
Table 3.1 Taqman primer and probe sequences used to assess mRNA expression levels of PPARα and PPARα target genes using quantitative PCR.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Probe (5'-3')
PPARα	CCTCAGGGTACCACTACGGAGT	GCCGAATAGTTCGCCGAAA	CACGCATGTGAAGGCTGTAAGGGCTT
CPT1	GCGTGCCAGCCACAATTC	TCCATGCGGTAATATGCTTCAT	CCGGTACTTGGATTCTGTGCGGCC
LCAD	GCATGAAACCAAACGTCTGGA	TGTTTTGTAATTCAGATGCCCAGT	TCCGGTTCTGCTTCCATGGCAAAA
AOX	AATTGGCACCTACGCCCAG	AGTGGTTTCCAAGCCTCGAA	CGGAGATGGGCCACGGAACTCA
UCP2	GATCTCATCACTTTCCCTCTGGATA	CCCTTGACTCTCCCCTTGG	CGCCAAGGTCCGGCTGCAGA
β-Actin	GCTCTGGCTCCTAGCACCAT	GCCACCGATCCACACAGAGT	GATCAAGATCATGCTCCTCCTGAGCGC
18S	CGGCTACCACATCCAAGGAA	GTCGGAATTACCGCGGCT	GAGGGCAAGTCTGGTGCCAG

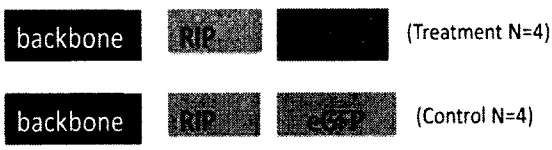
Glucose-stimulated insulin secretion.

MIN6 cells were cultured and transfected with respective plasmids (plasmid dsAAV8-RIP-eGFP and plasmid dsAAV8-RIP-PPARα) and treated with either 0μM or 250μM BSA coupled-palmitate for 48 hrs as described above. Cells were incubated for 1hr in glucose-free KRH Buffer (5M NaCl, 1M KCl, 1M Mg_2SO_4, 1M $NaHCO_3$, 1M $CaCl_2$, 0.5M KH_2PO_4, 1M HEPES, 0.5g BSA) (Sigma, Oakville ON, Canada), before being exposed to basal (2.8mmol/l) and stimulating (20mmol/l) levels of D-glucose for 1 hour each (Frigerio et al. 2010). After each incubation, 1ml of media was collected and insulin levels quantified. 5μl of each sample was run in triplicate with conjugate buffer (75μl) and shaken at 800rpm on a microplate shaker for 2 hrs at room temperature. Samples were then washed with wash buffer and incubated for 15min at 800rpm with TMB substrate. Stop solution was then added to samples and quantified at 450nm on a plate reader (Ultrasensitive insulin ELISA assay, 80-INSMSU-E10, ALPCO, Salem NH, USA). Protein was extracted using 100μl RIPA buffer/well per 24-well plate (10μl HALT buffer and 10μl EDTA per 1ml RIPA) (Thermo Scientific, Rockford IL, USA) and quantified in using a bicinchoninic acid (BCA) assay. 25μl of each sample was run in triplicate with 200μl of working reagent and incubated at 37°C for 30min. Plates were cooled to room temperature prior to quantifying at 560nm on a plate reader (Thermo Scientific, Rockford IL, USA). Secreted insulin levels (ng/ml) were normalized to total cellular protein (mg/ml) per well (ng/ml/mg protein).

Figure 3.2 *In vitro* experimental approach utilized in the expression of PPARα in MIN6 cells (*in vitro*). Abbreviations: PPARα – peroxisome proliferator-activated receptor alpha; MIN6 – mouse insulinoma cell line; BSA – bovine serum albumin; GSIS – glucose-stimulated insulin secretion.



Legend for plasmids:



Mice.

All animal studies were approved by the University of Northern British Columbia Animal Care and Use Committee (protocol number 2011-21). For all experiments, 6-week-old, male, C57Bl6 mice were purchased from Charles River Laboratories (Wilmington MA, USA) unless otherwise stated. Mice were maintained on a 12 hr light/dark cycle and received standard rodent chow diet (Rodent LabDiet, 5001, Leduc AB, Canada) *ad libitum* unless otherwise stated. Mice were allowed to acclimatize to the animal facility for 1 week prior to performing any experimental procedures. Body weight, 4 hr fasted blood glucose (OneTouch Ultra, Lifescan, Burnaby BC, Canada), oral glucose tolerance tests (OGTT) and insulin tolerance tests (ITT) were performed prior to viral injections to assess baseline carbohydrate metabolism.

Double-stranded adeno-associated virus serotype 8.

dsAAV8 transfer vectors were designed utilizing the rat insulin 1 promoter (RIP) (410 bp) placed upstream of either a sequence encoding eGFP (720bp) or the mouse PPARα (1407bp) open reading frame, followed by the SV40 polyadenylation signal. Complete dsAAV8-RIP-eGFP (5328bp) and dsAAV8-RIP-PPARα (6015bp) vectors were sent to Children's Hospital Of Philadelphia (CHOP) Research Vector Core Services (Philadelphia PA, USA) for AAV8 virus preparation

Pancreatic β-cell specific overexpression of PPARα in high fat diet-induced obese mice.

At 4-months old, C57Bl6 mice were infected with dsAAV8-RIP-PPARα virus (β-PPARα-HFD, n=8; $5x10^{12}$ viral genomes/814.3µl/mouse) or dsAAV8-RIP-eGFP virus

(β-eGFP-HFD control group, n=6; $5x10^{12}$ viral genomes/552.5µl/mouse) via ip injection. Body weights were monitored weekly; 4 hr fasted blood glucose was monitored biweekly and glucose tolerance and insulin sensitivity were assessed monthly by OGTT and ITT respectively. Two days post injection mice were switched to a high fat diet containing 45% kcal as fat (D12451, Research Diets, New Brunswick NJ, USA) *ad libitum* throughout the remainder of the experiment (20 weeks) when all animals were sacrificed (Fig. 3.3). Tissues were collected and flash frozen or fixed in 4% paraformaldehyde for 48hrs and then stored in 70% EtOH. Whole pancreas sections were prepared by WAX-IT histological services (Vancouver BC, Canada).

Insulin tolerance tests.

Mice were fasted for 4 hrs and given an ip injection of human synthetic insulin at 0.75U/kg or 1.0U/kg (Novolin Ge, Toronto ON, Canada). Blood was sampled (1-2µl) from the saphenous vein and blood glucose measured (mmol/L) at 10, 20 30, 60, and 120 min post injection using handheld glucometer (OneTouch Ultra, Lifescan, Burnaby BC, Canada).

Oral glucose tolerance and glucose-stimulated insulin secretion.

Mice were fasted for 16 hrs and given 2g/kg D-glucose by oral gavage. Plasma (15-20µl) was sampled for insulin measurement at 5, 10 and 180 minutes post glucose gavage from the saphenous vein and blood glucose (mmol/L) measured in 1-2µl blood at 5, 10, 30, 60, 120 and 180 minutes post gavage using handheld glucometer (OneTouch Ultra, Lifescan, Burnaby BC, Canada) (Huynh et al. 2010).

Overnight fasted and re-fed plasma insulin levels.

Mice were fasted for 16 hrs and blood collected in heparinized capillary tubes (Fisher Scientific, Ottawa ON, Canada) from the saphenous vein. Food was reintroduced for 2 hrs and plasma collected again. Blood glucose was determined using a handheld glucometer (OneTouch Ultra, Lifescan, Burnaby BC, Canada) and plasma insulin levels were assessed by ELISA (Ultrasensitive insulin ELISA assay, 80-INSMSU-E10, ALPCO, Salem NH, USA).

Immunohistological analysis.

Whole mouse pancreas sections fixed in 4% paraformaldehyde and embedded in paraffin were sectioned at 5μm thickness; n=5 per group (Wax-it Histology Services; Vancouver BC, Canada). All sections were de-paraffinized and rehydrated as described previously in chapter 2 (Riedel et al. 2010). Sections were incubated with polyclonal primary antibodies to rabbit anti-eGFP (1:500) (A11122, Invitrogen Molecular Probes, Carlsbad CA, USA), polyclonal guinea pig anti-insulin (1:1000) (4011-01F, Millipore, Billerica MA, USA) and a monoclonal primary antibody to mouse anti-glucagon (1:1000) (G2654, Sigma) overnight. Appropriate secondary whole (heavy and light chain) antibodies conjugated to Alexafluor 488 (A21206 or A21202) or 594 (A11076 or A11032) (1:1000) (Invitrogen Molecular Probes) were used to detect primary antibody immunoreactivity. All samples were visualized using a fluorescent light microscope (Olympus BX61) and images analyzed using Cell Sens Software (Olympus, Markham ON, Canada).

β-cell mass measurements.

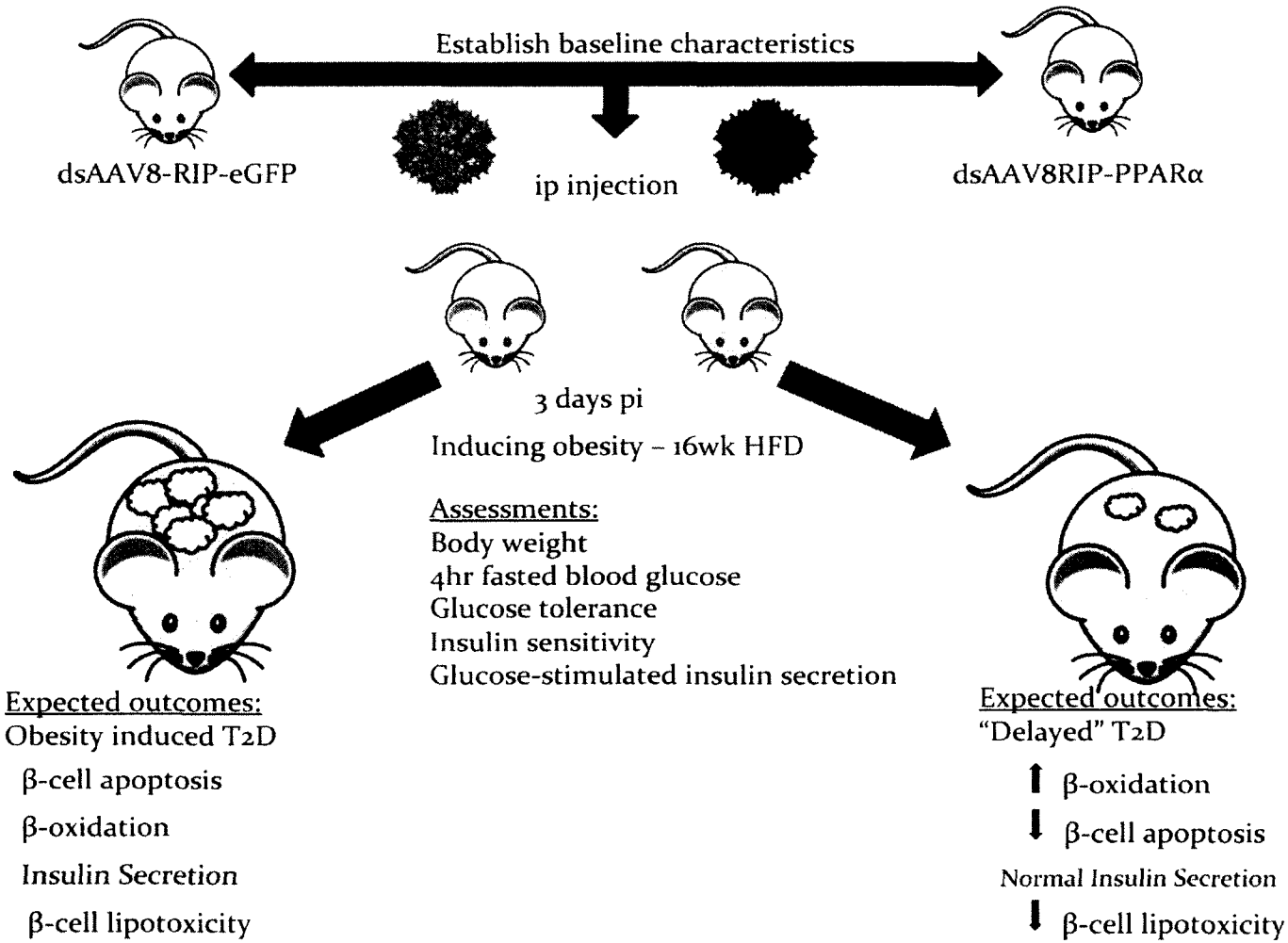
Insulin immunoreactive-positive area was measured in serial sections of the whole pancreas (2 serial sections, 200μm apart from the A. head, B. middle and C. tail of the

pancreas (total 3 sections/mouse, n= 4 per group)). Images were captured using the ImageXpress® Micro Imaging System and analyzed using MetaXpress® Software (Molecular Devices Corporation, Sunnyvale, CA, USA). Total insulin-positive area/total pancreas area was multiplied by weight of the whole pancreas (mg) to determine β-cell mass (Huynh et al. 2010).

Statistical analysis.

Results are expressed as mean ± standard error of the mean. Analyses were performed using paired student's t-test and 1-way ANOVA (significance between two groups), or 2-way ANOVAs (significance between groups over time) with Bonferoni Post tests using Graphpad Prism 5.0 software (La Jolla CA, USA). Significance was declared if p-values were less than 0.05. *P<0.05, **P<0.01, ***P<0.001.

Figure 3.3 *In vivo* experimental approach characterizing the physiological effects of overexpressing PPARα in the pancreatic β-cells of C57Bl6 mice *in vivo*. Four month old male C57Bl6 mice were infected with either dsAAV8-RIP-eGFP (β-eGFP-HFD) or dsAAV8-RIP-PPARα and placed on high-fat diet for 16 weeks. Abbreviations: dsAAV8 – double stranded adeno-associated virus serotype 8; RIP – rat insulin promotor; eGFP – enhanced green fluorescent protein; PPARα - peroxisome proliferator-activated receptor alpha; FBG – fasted blood glucose; HFD – high-fat diet; ip - intraperitoneal.


Establish baseline characteristics
dsAAV8-RIP-eGFP
ip injection
dsAAV8RIP-PPARα
3 days pi
Inducing obesity – 16wk HFD
Assessments:
Body weight
4hr fasted blood glucose
Glucose tolerance
Insulin sensitivity
Glucose-stimulated insulin secretion
Expected outcomes:
Obesity induced T2D
β-cell apoptosis
β-oxidation
Insulin Secretion
β-cell lipotoxicity
Expected outcomes:
"Delayed" T2D
β-oxidation
β-cell apoptosis
Normal Insulin Secretion
β-cell lipotoxicity

3.3 RESULTS

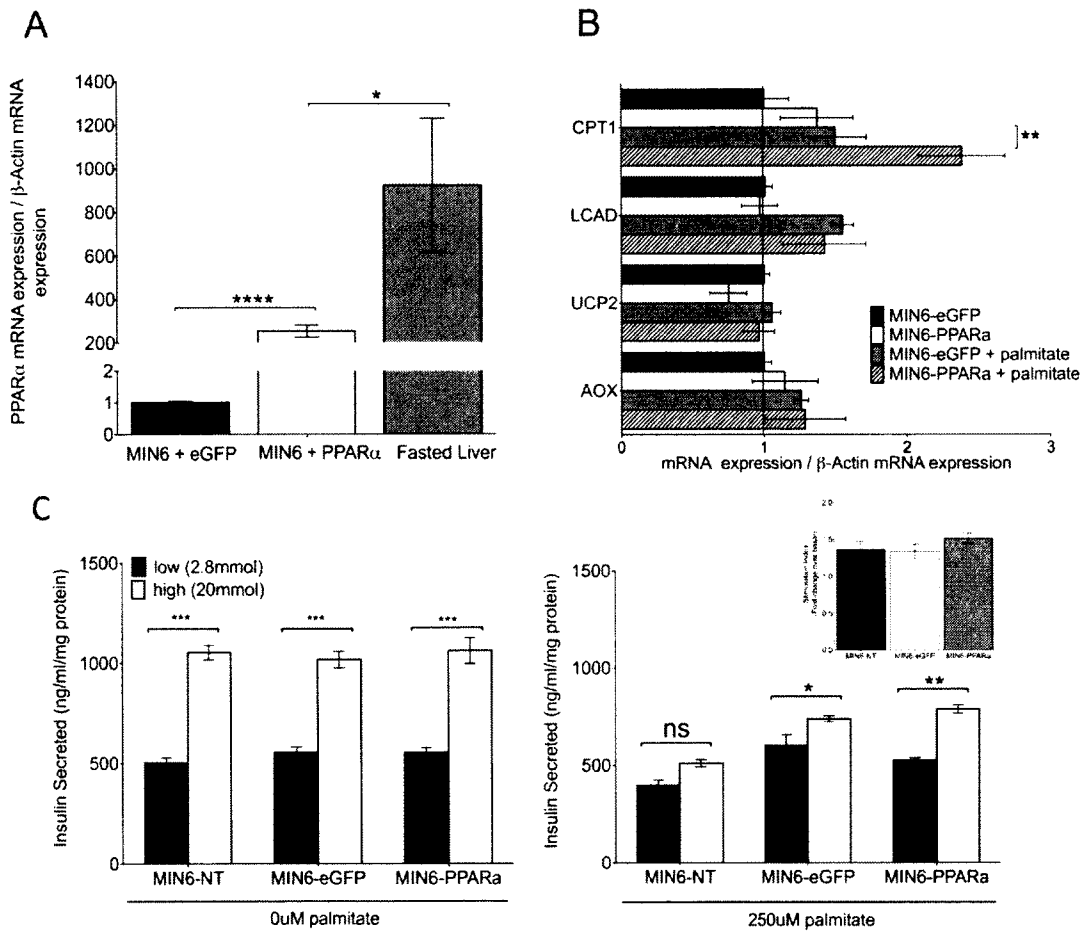
Overexpression of pro-oxidative PPARα in MIN6 cells at a physiological level.

MIN6 cells successfully transfected with dsAAV8-RIP-PPARα plasmid showed a 253 ± 28 fold increase in PPARα mRNA expression compared to control cells overexpressing dsAAV8-RIP-eGFP plasmid (1.0 ± 0.05) (Fig. 3.4a). The level of overexpression in MIN6 cells using plasmid dsAAV8-RIP-PPARα was compared to PPARα mRNA expression levels observed in fasted liver tissue. Comparison allowed us to determine that the levels of PPARα mRNA overexpression achieved in MIN6 cells with the above expression vector were within a physiological range, comparable to levels observed in fasted liver.

Overexpression of pro-oxidative PPARα in MIN6 cells results in upregulation of the PPARα target gene CPT-1.

In cells overexpressing PPARα mRNA and exposed to palmitate (250μM) we detected upregulation of one of the four PPARα target genes measured (CPT1, LCAD, AOX, and UCP2) (Fig. 3.4b). The 2-fold increase in CPT1 expression that we observed has been observed previously in cell models of increased PPARα activity (Xiao et al. 2001; Ravnskjaer et al. 2005; Hellemans et al. 2007; Frigerio et al. 2010). No significant increase in LCAD, AOX or UCP2 was observed in cells overexpressing PPARα with palmitate treatment.

Figure 3.4 Overexpression of PPARα in MIN6 cells. Overexpression of PPARα in MIN6 cells occurs at physiological levels (A) and upregulates expression of CPT1 with palmitate treatment (B). Overexpressing PPARα in MIN6 cells did not change insulin secretion when stimulated with high levels of glucose without palmitate (C). Abbreviations: PPARα – peroxisome proliferator-activated receptor alpha; MIN6 – mouse insulinoma cell line; eGFP – enhanced green fluorescent protein; CPT1 – carnitine palmitoyl transferase-1; LCAD – long-chain acyl-coA dehydrogenase; UCP2 – uncoupling protein-2; AOX – acyl-CoA oxidase . Mean values with standard error of mean are shown. Statistical significance was determined by 2-way ANOVA with Bonferoni Post Tests or Student's T-Tests. *P<0.05, **P<0.01, ***P<0.001. ****P<0.0001


A
PPARα mRNA expression / β-Actin mRNA expression
1400
1200
1000
800
600
400
200
2
1
0
*
****
MIN6 + eGFP
MIN6 + PPARα
Fasted Liver
B
CPT1
LCAD
UCP2
AOX
**
MIN6-eGFP
MIN6-PPARa
MIN6-eGFP + palmitate
MIN6-PPARa + palmitate
0
1
2
3
mRNA expression / β-Actin mRNA expression
C
Insulin Secreted (ng/ml/mg protein)
1500
1000
500
0
low (2.8mmol)
high (20mmol)
***
***
***
MIN6-NT
MIN6-eGFP
MIN6-PPARa
0uM palmitate
Insulin Secreted (ng/ml/mg protein)
1500
1000
500
0
ns
*
**
MIN6-NT
MIN6-eGFP
MIN6-PPARa
250uM palmitate

PPARα overexpression in MIN6 cells protects against palmitate-induced impairment of glucose stimulated insulin secretion (GSIS).

Overexpression of PPARα in MIN6 cells in the absence of palmitate did not alter glucose stimulated insulin secretion (Fig. 3.4c left panel). As expected due to lipotoxic effects of palmitate on β-cell insulin secretion, blunted insulin levels were observed in the non-transfected, eGFP and PPARα MIN6 cells when treated with 250μM of palmitate compared to 0μM palmitate. No significant difference was detected when comparing stimulation indexes of all groups (Fig. 3.4c inset). However, in the presence of palmitate, PPARα overexpression protected against palmitate-induced impairment of glucose-stimulated insulin secretion (Fig. 3.4c right panel). These results are further supported by previous studies utilizing MIN6 and INS1-E β-cell lines (Ravnskjaer et al. 2005; Hellemans et al. 2007; Frigerio et al. 2010).

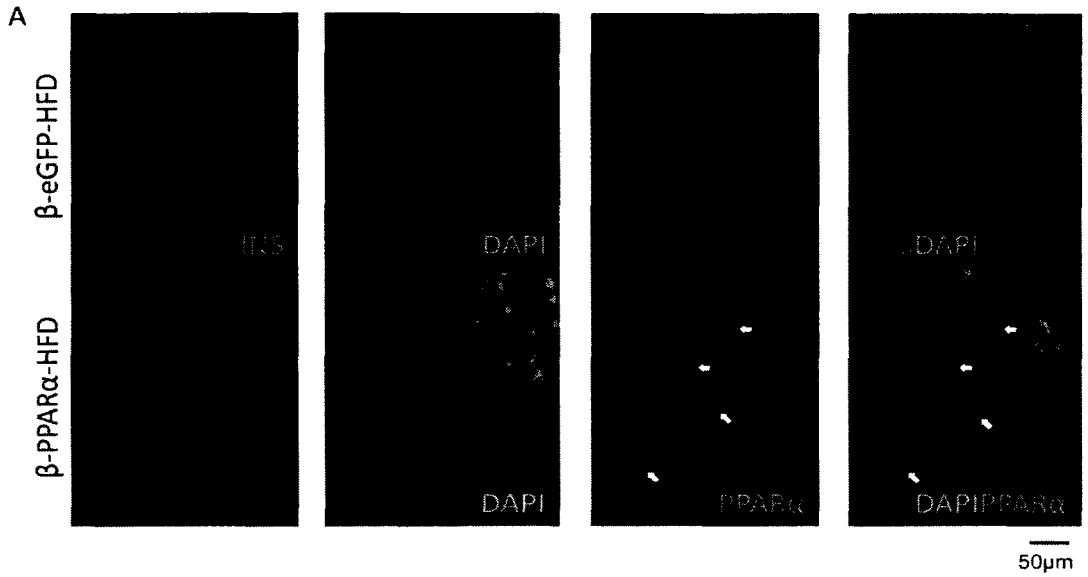
Overexpression of PPARα in pancreatic β-cells protects against obesity-induced glucose intolerance.

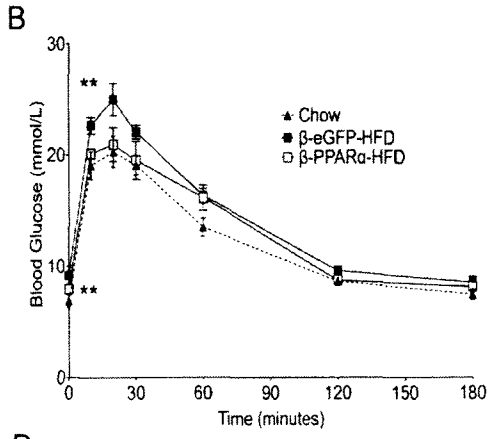
Increased PPARα overexpression was observed in the cytoplasm and nuclei in islets of β-PPARα-HFD mice compared to β-eGFP-HFD controls (Fig. 3a); arrows indicate increased nuclear staining. Fluorescent dyes were reversed (insulin green and PPARα red) in β-eGFP-HFD islets to ensure endogenous levels of PPARα were not mistaken for fluorescence from the presence of our control vector, eGFP. Sixteen weeks post-infection, we observed significantly improved glucose tolerance and lower overnight fasting blood glucose levels in diet-induced obese mice overexpressing PPARα (β-PPARα-HFD) in pancreatic β-cells compared to diet-induced obese control mice (β-

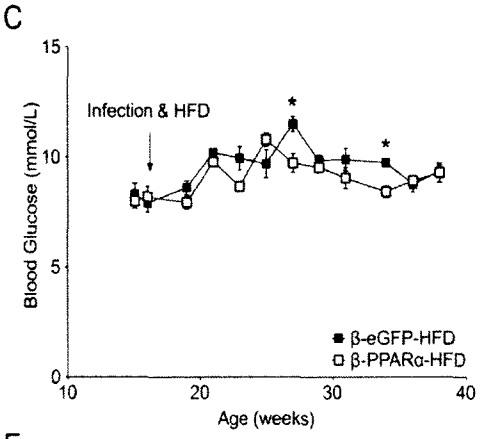
eGFP-HFD) (Fig. 3.5a). In fact, the improvement in glucose tolerance in β-PPARα-HFD mice is equal to that of age-matched, chow-fed control mice; a substantial improvement considering these mice have been on a HFD diet for 16 weeks (Fig. 3.5a).

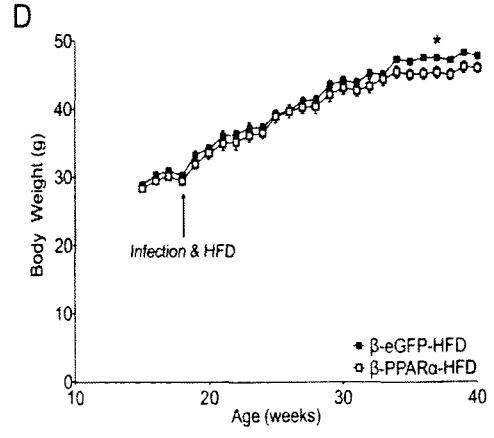
Specifically, blood glucose levels taken 10 minutes post glucose gavage were significantly lower in β-PPARα-HFD (20.13 ± 0.46 mmol/L) mice compared to β-eGFP-HFD controls (22.67 ± 0.75 mmol/L). It should be noted that although not significant over time, significantly lower 4 hr fasted blood glucose levels were observed in β-PPARα-HFD mice at two significantly reduced time points (Fig. 3.5b) compared to β-eGFP-HFD control mice. Improvements in glucose tolerance in obese β-PPARα-HFD mice were not associated with reduction in body weight (Fig. 3.5c) or improvement in whole body insulin sensitivity (Fig. 3.5d) compared to β-eGFP-HFD control mice suggesting improvement in insulin sensitivity did not account for the improvement in glucose tolerance. Moreover, a trend in improving glucose tolerance over time was also observed (Fig. 3.6 a-e).

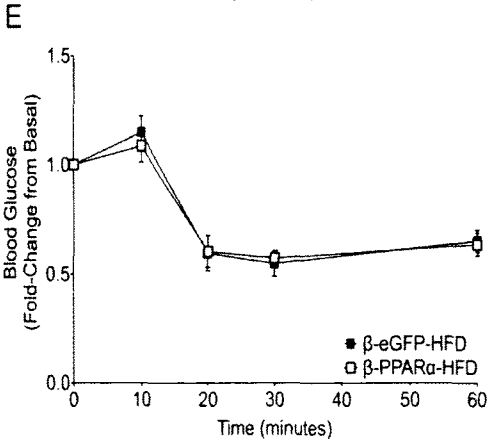
Figure 3.5 (A) PPARα is overexpressed in the cytoplasm and nuclei (arrows) of β-PPARα-HFD compared to β-eGFP-HFD controls. (B) Overexpression of PPARα in pancreatic β-cells of obese mice improved oral glucose tolerance (2g/kg D-glucose) compared to obese controls, with glucose tolerance similar to age-matched, chow mice. (C) Over time, no significant difference between 4 hr fasted blood glucose was observed. (D) PPARα overexpression did not affect body weight. (E), nor was insulin sensitivity affected (1U insulin/kg). Abbreviations: PPARα - peroxisome proliferator-activated receptor alpha; eGFP – enhanced green fluorescent protein; FBG – fasted blood glucose; HFD – high-fat diet. β-eGFP-HFD n=6, β-PPARα-HFP n=8. Mean values with SEM are shown. Statistical significant variation was determined by 2-way ANOVA with Bonferoni Post Tests or Student's T-Tests. *P<0.05, **P<0.01, ***P<0.001.


A
β-eGFP-HFD
β-PPARα-HFD
DAPI
DAPI
DAPI
50μm


B
Blood Glucose (mmol/L)
Time (minutes)
Chow
β-eGFP-HFD
β-PPARα-HFD


C
Blood Glucose (mmol/L)
Infection & HFD
Age (weeks)
β-eGFP-HFD
β-PPARα-HFD


D
Body Weight (g)
Infection & HFD
Age (weeks)
β-eGFP-HFD
β-PPARα-HFD


E
Blood Glucose
(Fold-Change from Basal)
Time (minutes)
β-eGFP-HFD
β-PPARα-HFD

PPARα overexpression in pancreatic β-cells maintains 1st phase insulin secretion in obesity.

Circulating insulin levels in β-PPARα-HFD (2.05 ± 0.19 ng) mice were significantly higher than β-eGFP-HFD controls (1.05 ± 0.20 ng) 5 minutes post glucose gavage (Fig. 3.7a). No significant difference in fasting or refeed plasma insulin levels were observed (Fig. 3.7b) in β-PPARα-HFD mice compared to β-eGFP-HFD control mice (Fig. 3.7b). We did not observe any difference in islet morphology or β-cell mass in the two groups of mice (Fig. 3.7c). Additionally, Figure 3.8 shows no significant difference in body weight or tissue mass (liver, pancreas, gonadal white adipose, or subcutaneous white adipose) from either β-eGFP-HFD or β-PPARα-HFD groups, further suggesting that improvements in whole-body carbohydrate metabolism observed were associated with changes in β-cell function and not body composition.

Figure 3.6 Overexpression of PPARα in pancreatic β-cells shows a trend towards improved glucose tolerance over time (2g D-glucose/kg). Oral glucose tolerance tests over time (A) Baseline, (B) 1-month post-infection, (C) 2-months post-infection, (D) 3-months post-infections (E) 4-months post infection. Abbreviations: PPARα - peroxisome proliferator-activated receptor alpha; eGFP – enhanced green fluorescent protein; FBG – fasted blood glucose; HFD – high-fat diet. β-eGFP-HFD n=6, β-PPARα-HFP n=8. Mean values with SEM are shown. Statistical significant variation was determined by 2-way ANOVA with Bonferoni Post Tests. *P<0.05, **P<0.01, ***P<0.001.

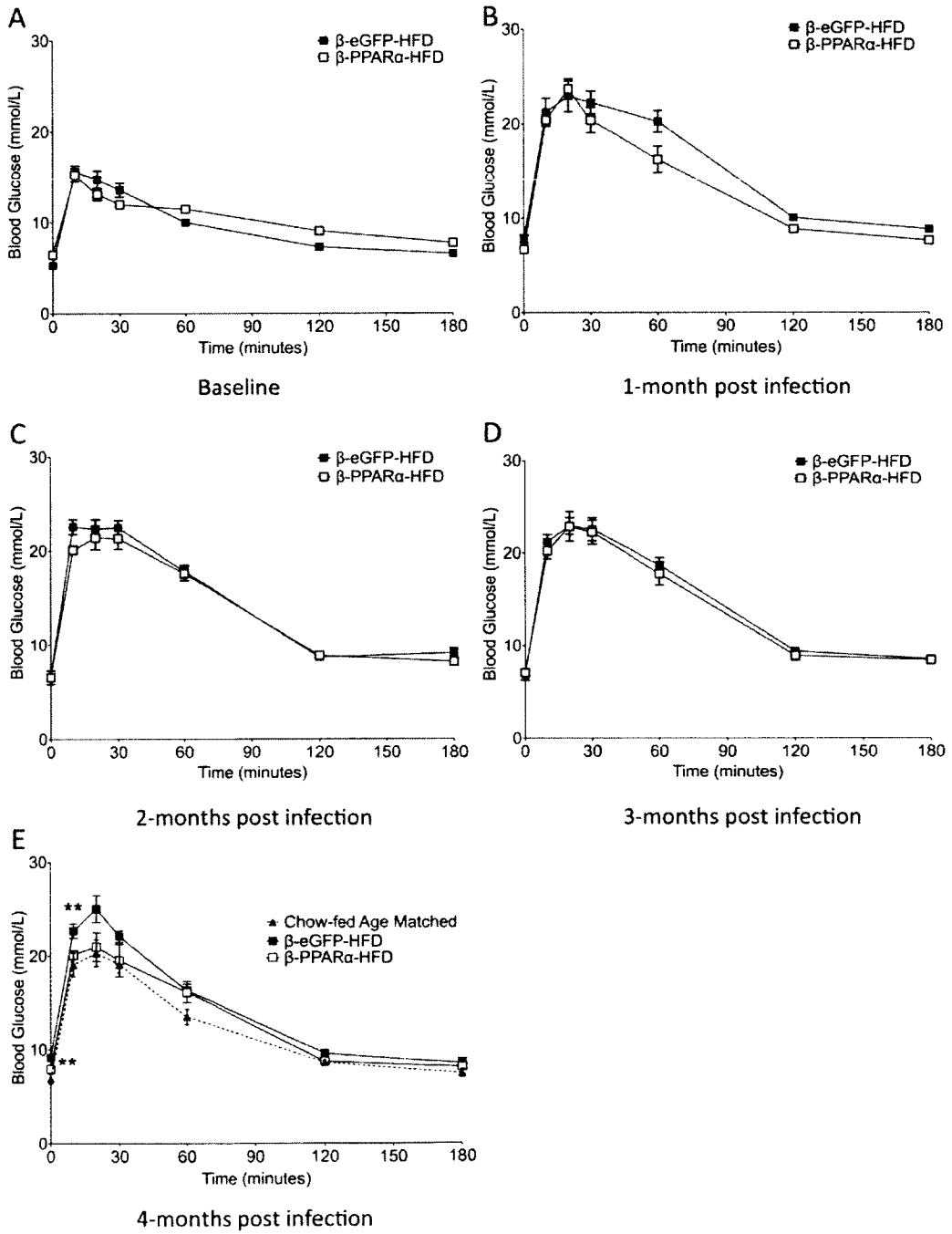

A
β-eGFP-HFD
β-PPARα-HFD
Blood Glucose (mmol/L)
Time (minutes)
Baseline
B
β-eGFP-HFD
β-PPARα-HFD
Blood Glucose (mmol/L)
Time (minutes)
1-month post infection
C
β-eGFP-HFD
β-PPARα-HFD
Blood Glucose (mmol/L)
Time (minutes)
2-months post infection
D
β-eGFP-HFD
β-PPARα-HFD
Blood Glucose (mmol/L)
Time (minutes)
3-months post infection
E
Chow-fed Age Matched
β-eGFP-HFD
β-PPARα-HFD
**
**
Blood Glucose (mmol/L)
Time (minutes)
4-months post infection

Figure 3.7 Overexpression of PPARα in pancreatic β-cells maintains 1st phase insulin secretion in C57Bl6 mice on high-fat diet compared to obese controls. Glucose-stimulated insulin secretion (A) but not overnight and refeed insulin levels (B) was significantly affected. Additionally, overexpression of PPARα in pancreatic β-cells did not change islet morphology or β-cell or α-cell mass (C) when compared to obese controls. Abbreviations: PPARα - peroxisome proliferator-activated receptor alpha; eGFP – enhanced green fluorescent protein. β-eGFP-HFD n=6, β-PPARα-HFP n=8. Mean values with SEM are shown. Statistical significant variation was determined by 2-way ANOVA with Bonferoni Post Tests or Student's T-Tests. *P<0.05, **P<0.01, ***P<0.001. Images taken at 20x magnification. Scale bar 50μm.

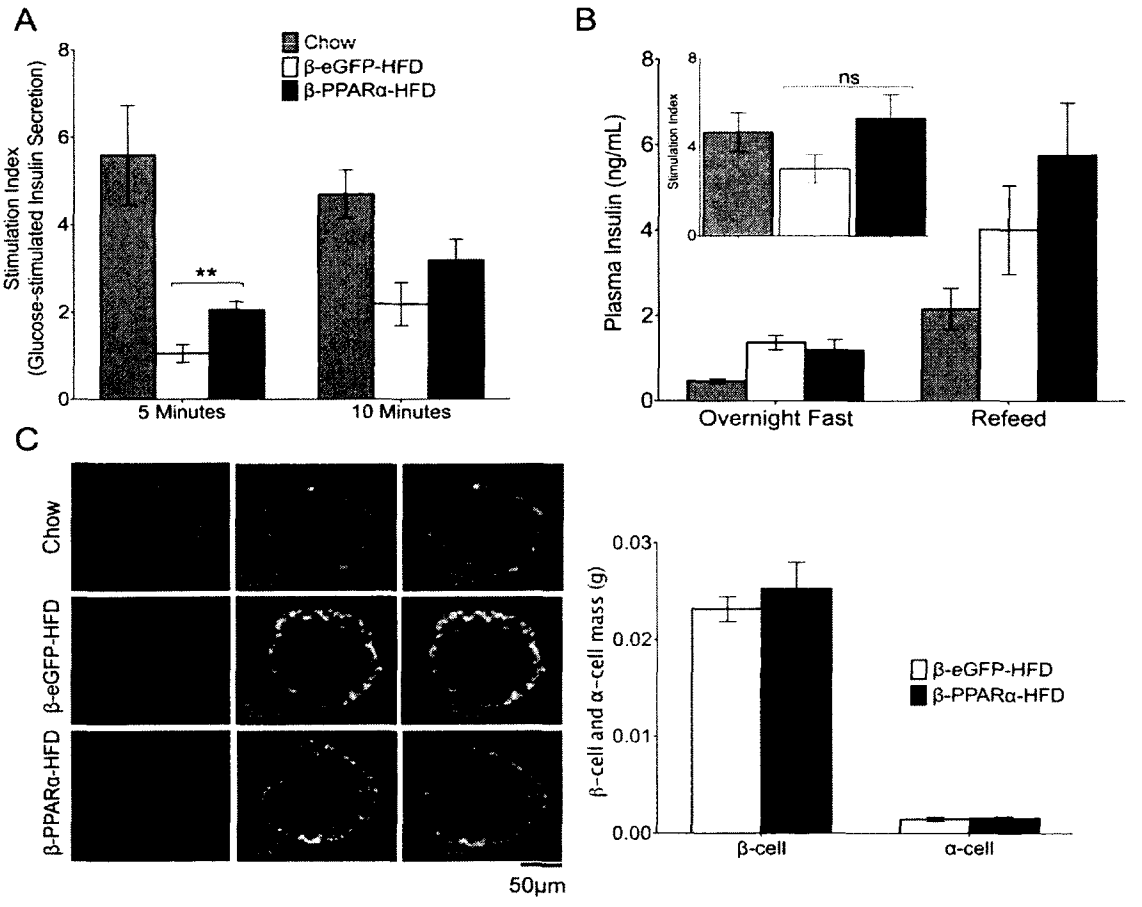
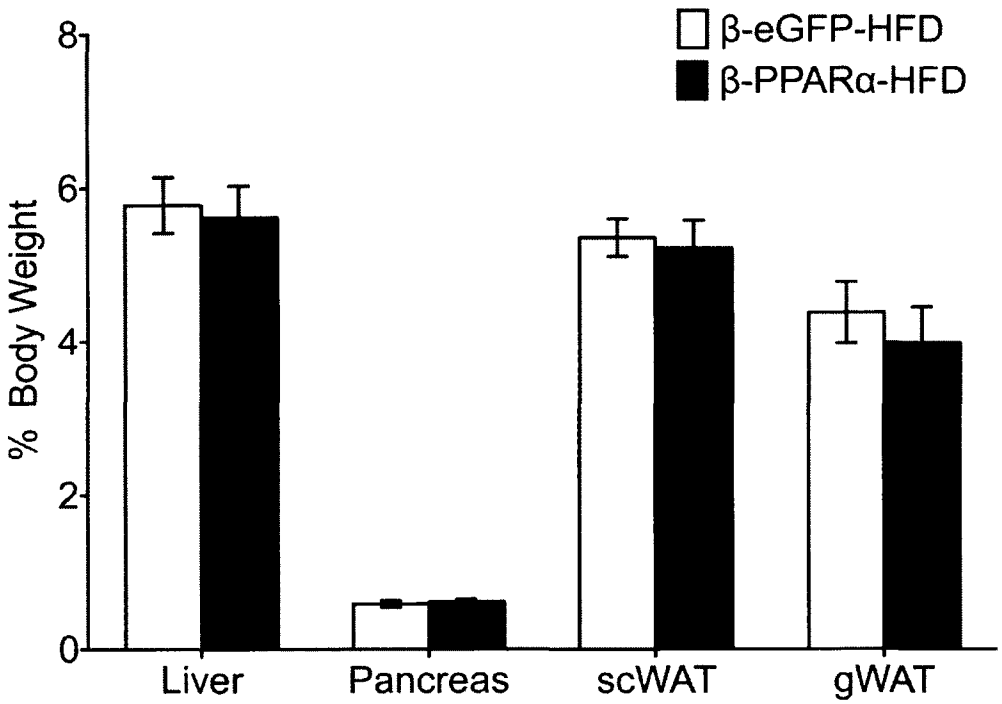

A
Chow
β-eGFP-HFD
β-PPARα-HFD
Stimulation Index
(Glucose-stimulated Insulin Secretion)
8
6
4
2
0
**
5 Minutes
10 Minutes
B
Plasma Insulin (ng/mL)
8
6
4
2
0
Stimulation Index
8
4
0
ns
Overnight Fast
Refeed
C
Chow
β-eGFP-HFD
β-PPARα-HFD
50μm
β-cell and α-cell mass (g)
0.03
0.02
0.01
0.00
β-eGFP-HFD
β-PPARα-HFD
β-cell
α-cell

Figure 3.8 Overexpression of PPARα in pancreatic β-cells does not cause changes in peripheral tissue mass on HFD. Abbreviations: PPARα - peroxisome proliferator-activated receptor alpha; eGFP – enhanced green fluorescent protein; HFD – high fat diet; scWAT – subcutaneous white adipose tissue; gWAT – gonodal white adipose tissue. β-eGFP-HFD n=6, β-PPARα-HFP n=8. Mean values with SEM are shown. Statistical significant variation was determined by student's t-tests.


β-eGFP-HFD
β-PPARα-HFD
% Body Weight
8
6
4
2
0
Liver
Pancreas
scWAT
gWAT

3.4 DISCUSSION

Our group has shown for the first time that PPARα overexpression in pancreatic β-cells, *in vivo*, improves glucose tolerance and maintains 1st phase insulin secretion on HFD in a diet-induced obese model of T2D. Beneficial effects of systemic PPARα agonism on β-cell function and carbohydrate metabolism have been demonstrated (Guerre-Millo et al. 2000; Holness et al. 2003; Bergeron et al. 2006), however the impact of β-cell-specific PPARα overexpression on whole-body carbohydrate metabolism has yet to be explored in an *in vivo* model of obesity-induced T2D. Given the pro-oxidative potential of PPARα, we hypothesized that specific activation of PPARα in pancreatic β-cells of obese mice may prevent lipid-induced β-cell failure. We show *in vivo* overexpression of PPARα specifically in pancreatic β-cells significantly improves whole animal glucose tolerance in diet-induced obese mice to levels equal to that of age-matched, chow-fed, control mice (Fig. 3.5a). This is a substantial improvement considering β-PPARα-HFD mice have been exposed to a HFD for 16 weeks and we would not expect improvements in glucose tolerance to exceed that of the age-matched chow mice. This improvement in glucose tolerance is not due to reductions in body weight or improvements in insulin sensitivity. Instead we show 1st phase insulin secretion (5 minutes post glucose-gavage) is higher in β-PPARα-HFD mice compared to obese control mice. In addition to improvements in glucose tolerance, we observed lower overnight fasted blood glucose levels with a trend of decreased 4 hr fasted blood glucose levels. Despite increased insulin secretion, fasting and fed plasma insulin levels were not significantly higher in β-PPARα-HFD mice compared to control mice. Morphological analysis of pancreatic tissue did not show increased β-cell mass, suggesting β-cell

specific PPARα overexpression improved β-cell function rather than increasing β-cell mass.

Further studies are required to determine the lipidomic profile of any accumulated lipids within the pancreatic β-cells of both our β-eGFP-HFD and β-PPARα-HFD mice. Additionally experiments will need to be conducted to determine the rate of lipid turnover within the pancreatic β-cells, as the majority of *in vivo* work attributes improvements in whole animal carbohydrate metabolism to lipid turnover in the liver. As Lee et al. (2003) have suggested that the increase in liver lipid accumulation and liver mass in PPARα-null mice further supports PPARα's role in lipid regulation, as well the liver being a site of lipid turnover (Lee et al. 2003). The authors also speculated that the hypoglycemia, and elevated FA plasma levels observed in PPARα-null mice were caused by the defective FA oxidation and uptake in the liver (Lee et al. 2003). Furthermore, a study involving fibrate treatment in primates resulted in an increased liver mass associated with an increase in number of peroxisomes, resulting in an increase in β-oxidation (Hoivik et al. 2004). Combined, these studies suggest increased liver mass does affect PPARα's role in lipid metabolism, which subsequently effects whole-body carbohydrate metabolism. Together these studies have indicated that improvements in whole animal carbohydrate metabolism were due to PPARα's role in lipid turnover in the liver. Therefore, it is important that our group rule out the liver as a possible source of carbohydrate improvement. As discussed in chapter 2, our group has ruled out dsAAV8-RIP-eGFP expression in the liver tissue of infected mice; therefore we assume dsAAV8-RIP-PPARα would not be expressed in these tissues. Moreover, as seen in figure 3.8, we have shown no significant difference in liver mass between β-eGFP-HFD and β-PPARα-

HFD mice, thus we suggest improvements observed were not due to changes in liver lipid metabolism. This data supports our observation that improvement in glucose tolerance in β-PPARα-HFD mice was due to the direct action of overexpressing PPARα in the pancreatic β-cells and not due to peroxisome proliferation and increased PPARα activity in the liver.

Utilizing *ob/ob* mice, Lalloyer and colleagues were able to show that PPARα deficiency resulted in impaired GSIS and defective islet function (Lalloyer et al. 2006), suggesting PPARα may play an important role in preserving β-cell function in the obese state. Our results support a direct role for PPARα in improving pancreatic β-cell function, with overexpression of PPARα specifically within the pancreatic β-cells maintaining 1st phase insulin secretion impaired by obesity. Frigerio and colleagues (2010) have performed extensive *in vitro* studies that support this finding. Utilizing the INS1E cell line, they have shown that overexpression of PPARα is able to restore glucose-stimulated insulin secretion in the presence of oleate (Frigerio et al. 2010), while downregulation of PPARα expression exacerbated lipid-induced dysfunction (Frigerio et al. 2010).

As with previous studies, our group has been able to show, *in vitro*, that overexpression of PPARα has protective effects against lipotoxic dysfunction in the presence of palmitate, which may be due to the increased β-oxidative activity from the induction of PPARα target genes. When activated PPARα increases transcription of downstream target genes involved in β-oxidation (Mandard et al. 2004); therefore it would be expected that in the presence of palmitate MIN6 cells overexpressing PPARα would show an induction of target genes. We show in the presence of palmitate, that the

PPARα target gene CPT1 is significantly increased in cells overexpressing PPARα (Ravnskjaer et al. 2005; Hellemans et al. 2007; Frigerio et al. 2010). CPT1 was found to have a two-fold increase (2.4±0.30) compared to eGFP-MIN6 controls (1.0±0.18), similar to results obtained by Frigerio and colleagues (2010). Levels of LCAD, AOX, and UCP2 induction were not found to be significantly increased, therefore we must also consider the fact that these PPARα overexpression studies also overexpressed an adenovirus for RXR in addition to PPARα (Ravnskjaer et al. 2005; Hellemans et al. 2007; Frigerio et al. 2010). Overexpressing RXR in addition to PPARα may allow for increased formation of the RXR-PPAR heterodimer, and subsequent increase in transcription of the downstream β-oxidative genes (Fig. 3.1).

Therefore it is quite possible that the lack of increased β-oxidative gene induction observed is due to an excess of PPARα in the presence of endogenous levels of RXR within the MIN6 cell or the appropriate activating ligand for PPARα is not present in sufficient quantities. However, consistent with the previous studies our group has been able to show the overexpressing PPARα in MIN6 cells protects against lipotoxic impairment of glucose-stimuated insulin secretion (GSIS), showing that *in vitro* PPARα overexpression in the presence of palmitate protects MIN6 cells and enhances glucose-stimulated insulin secretion. These findings are consistent with previous studies that have shown PPARα to protect β-cells from lipid-induced impairment of GSIS in INS-1E cells (Ravnskjaer et al. 2005; Lalloyer et al. 2006; Sun et al. 2008; Frigerio et al. 2010). Frigerio and colleagues (2010) have suggested that the improvement observed with PPARα activation is due to an increase in glucose metabolic pathways as well as safe FA storage in the form of neutral lipids such as TGs (Frigerio et al. 2010). Additionally, this

group was able to show that downregulation of PPARα worsened oleate-induced dysfunction in INS-1E cells (Frigerio et al. 2010); further supporting our results that PPARα overexpression has protective effects against palmitate-induced dysfunction.

The lack of upregulation of PPARα genes may be due to the fact that palmitate may not be the appropriate ligand for PPARα; thus trying *in vitro* experiments with a different FA such as oleate or various ceramides could be used. After reviewing the literature, most studies utilize palmitate as their fatty acid treatment for either their MIN6 or INS-1 cells. Studies have shown that palmitate treatment should be kept to a maximum of 48 hrs before proceeding with the remainder of the desired treatments. Hellemans et al. (2007), Frigerio et al. (2009), and Thorn and Bergsten (2010) have done extensive studies showing that cellular apoptosis increases with palmitate treatments in excess of 48 hrs (Hellemans et al. 2007; Frigerio et al. 2010; Thorn and Bergsten 2010); therefore we kept our palmitate incubations to 48 hrs at a concentration of 250μM. Whereas it was found that oleate was not as toxic to the MIN6 and INS-1 cells in excess of 48hrs (Frigerio *et al* 2009).

It has been documented that the rate of turnover of human pancreatic β-cells is slow (Riedel et al. 2010; Skelin et al. 2010), whereas the turnover time in murine models is approximately 47 days (Wang et al. 2004). Wang and colleagues (2004) have suggested it is very likely that β-cells infected with adeno-associated viruses are lost due to normal maintenance and cellular apoptosis (Wang et al. 2004). Recycling rates of PPARα overexpressing β-cells may explain why significantly lower 4 hr fasted blood glucose was observed during only two weeks (no significance over time) (Fig. 3.5b) and why impaired glucose tolerance was not maintained.

Synthetic PPARα agonists, such as fibrates, are approved for human use to lower elevated lipid profiles in individuals with hypertriglyceridemia; further, their systemic administration has been shown to induce improvements in carbohydrate metabolism (Staels et al. 2008). Our results suggest that PPARα may have direct effects on β-cell function that may be contributing to the improvements in carbohydrate metabolism observed with systemic administration of fibrates. Our model which specifically upregulates PPARα activity in pancreatic β-cells is the first *in vivo* model of β-cell-specific PPARα overexpression and the first model that conclusively shows β-cell specific effects of PPARα agonism can impact whole-animal glucose homeostasis independently of systemic effects on liver and muscle lipid metabolism. Furthermore, our study has shown that targeted delivery of PPARα to the β-cell could serve as a potential site for therapeutic intervention for the preservation of β-cell function, in islet transplantation or in the treatment of obesity-induced T2D. In conclusion, we have demonstrated that targeted delivery of PPARα specifically to pancreatic β-cells *in vivo* preserves β-cell function and protects against glucose intolerance in diet-induced obesity, thus providing a valuable *in vivo* model to elucidate the mechanisms involved in β-cell lipotoxicity in obesity-induced T2D. The major findings from this chapter are:

1. We have developed the 1st *in vivo* model of PPARα overexpression in pancreatic β-cells to further elucidate the mechanisms involved in β-cell lipotoxicity in a diet-induced obese model of T2D.
2. Overexpression of dsAAV8-RIP-PPARα in pancreatic β-cells improves glucose tolerance in a diet-induced obese model of T2D.

3. Overexpression of dsAAV8-RIP-PPARα in the pancreatic β-cells maintains 1st phase insulin secretion impaired by obesity.
4. Overexpression of dsAAV8-RIP-PPARα within the pancreatic β-cells does not affect body weight, insulin sensitivity or weight gain in peripheral tissues.
5. Using AAV8 plasmid, PPARα overexpression in MIN6 occurs at physiological levels and upregulates the β-oxidative target gene CPT1 in the presence of palmitate.
6. Overexpression of PPARα plasmid in MIN6 maintains glucose-stimulated insulin secretion when treated with palmitate.

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APPENDIX

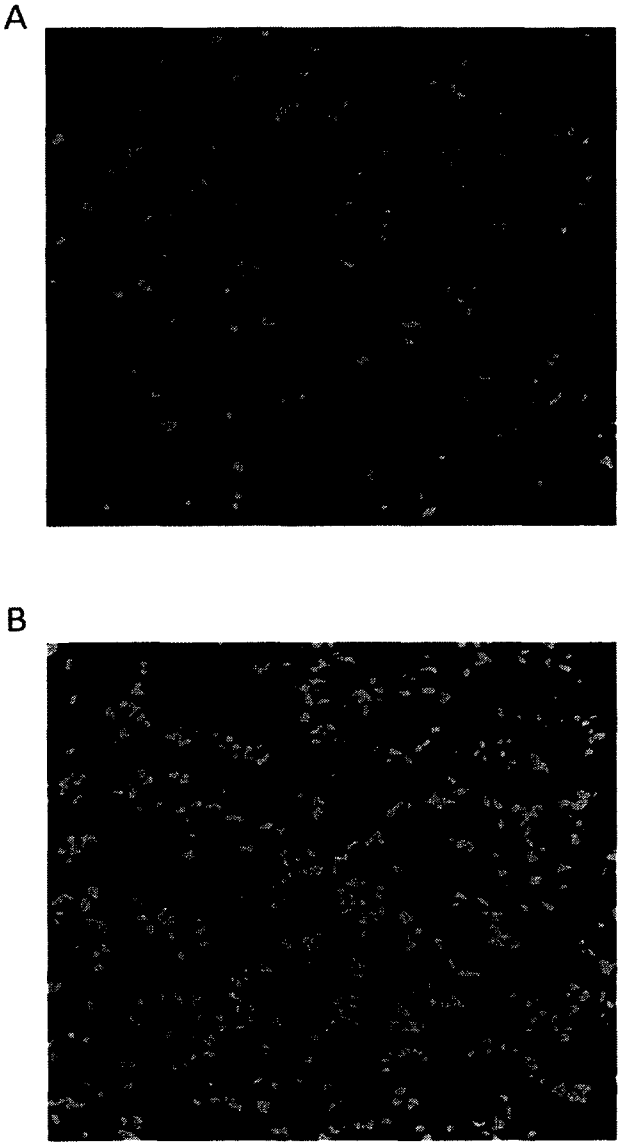


Figure S1. Transfection efficiency of MIN6-eGFP cells. (A) 30% efficiency; 1 day post-transfection. (B) 50-60% efficiency; 2 days post-transfection. Image taken with FITC filter at 10x magnification using confocal microscope (Olympus).

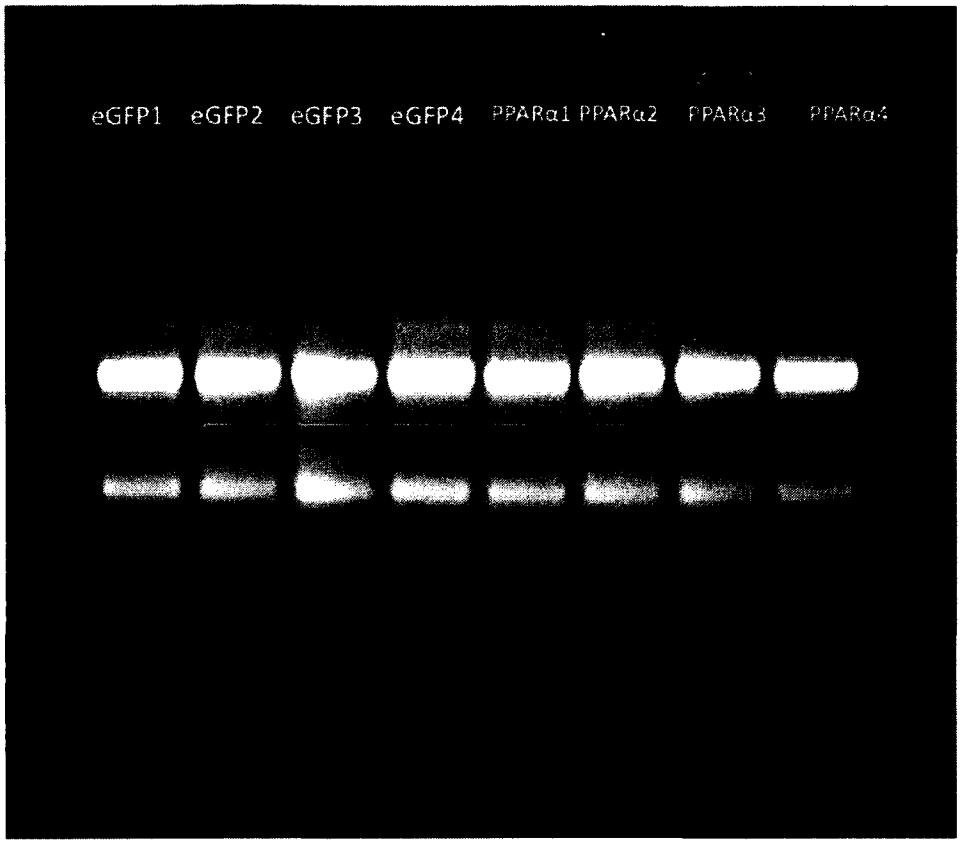


Figure S2. RNA Quality Control Gel for MIN6-eGFP and MIN6-PPARα Transfected Cells. Running Conditions (1.5% Agarose, 1x TAE Running Buffer, 100V, 1 hour, Stained with EtBr). All samples show clean 18S and 28S bands indicating no degradation of RNA collected from the transfected MIN6 cells.

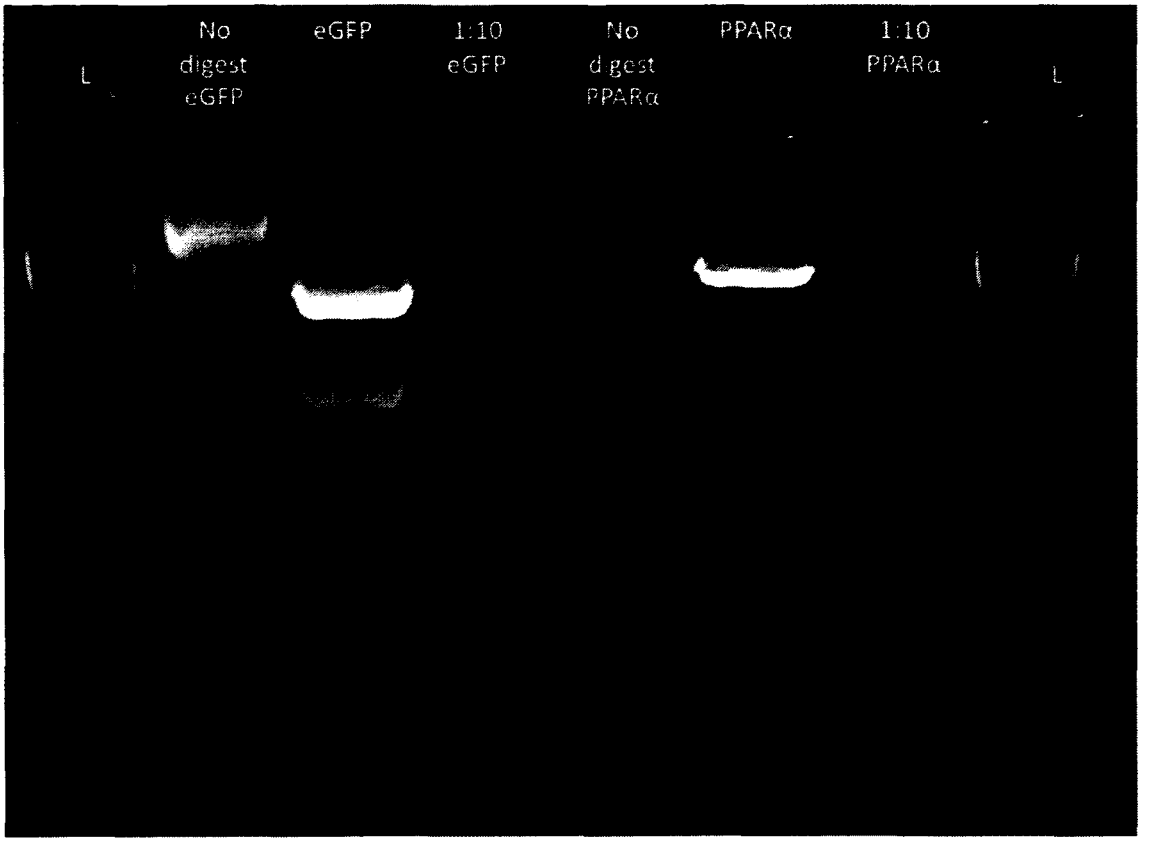


Figure S3. Double Restriction Digests of Purified Plasmid Samples for Viral Construct Preparation. Restriction digests were run using no digest, pure plasmid, and 1:10 dilution of plasmid. Restriction Enzymes for eGFP (MluI and NotI), PPARα (HindIII and MluI). Running Conditions (1.5% Agarose, 0.5x TBE Running Buffer, 100V, 1 hour, Stained with SYBR Safe).

CHAPTER 4

Overexpression of PPARα in Pancreatic β-cells Does Not Improve Glucose Tolerance in a Severely Obese Genetic Model (*db/db* mouse).

A version of this chapter has been submitted:
Hogh, K-Lynn N., Uy, Christopher E., Asadi, A., Baker, Robert K., Riedel, Michael J., Gray, Sarah L. 2012. Overexpression of PPARα in pancreatic β-cells improves glucose tolerance in diet-induced obese mice. *Endocrinology*. Manuscript Submission number: EN-12-1110.

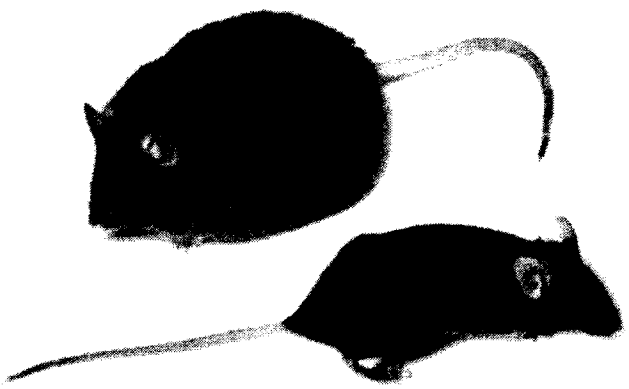
4.1 INTRODUCTION

Animals have been used in diabetes research as early as the 1880's (Rees and Alcolado 2005). The use of animals was best exemplified by Banting and Best in the 1920's, when they used pancreatectomized dogs for the isolation and discovery of insulin (Banting et al. 1922; Bliss 1982; Rosenfeld 2002; Rees and Alcolado 2005). The use of mouse models in research has become increasingly important to elucidate the mechanisms involved in the progression of obesity-induced T2D. In addition to the diet-induced obese model of T2D (C57Bl6 mice on HFD) used in chapter three, there are various obese and non-obese rodent models utilized in diabetes research, including: i) spontaneous/genetically derived (ie. *ob/ob* and *db/db*), ii) diet-induced (ie. C57Bl6), iii) chemically induced (ie. alloxan or streptozotocin), iv) surgically induced (ie. lesion of ventromedial hypothalamus), and v) transgenic/knock-out mice (ie. β_3, GLUT-4, and IRS-1) (Rees and Alcolado 2005; Srinivasan and Ramarao 2007; Shafrir and Ziv 2009). As our lab is interested in elucidating the mechanisms involved in β-cell lipotoxicity and obesity-induced T2D, the severely obese *ob/ob* or *db/db* mouse models (Fig 4.1) are most appropriate for future studies involving the overexpression of PPARα in pancreatic β-cells. Phenotypically the *ob/ob* and *db/db* mice are severely obese, hyperphagic, insulin resistant, hyperinsulinemic and hyperglycemic (Coleman and Hummel 1967; Coleman and Hummel 1973; Srinivasan and Ramarao 2007). Both of these mouse models involve either a mutation in the leptin gene (*ob/ob*) or leptin receptor (*db/db*) (Coleman and Hummel 1967; Coleman and Hummel 1973).

Figure 4.1 Comparing male C57Bl6 littermate controls to *db/db* (A) and *ob/ob* (B) mice. Mice purchased from JAX laboratories at four weeks of age. Images from Jackson Laboratories website: http://jaxmice.jax.org [electronic document] accessed Dec 15 2011.

A

B



The adipocyte-derived hormone leptin acts on the central nervous system and peripheral tissues to reduce fat stores by suppressing appetite, increasing metabolic rate, and activating thermogenesis (Muoio and Lynis Dohm 2002; Ceddia 2005; Unger 2005; Zhao et al. 2006; Gray and Vidal-Puig 2007; Friedman 2010). In a negative feedback loop, leptin acts on the receptors of the hypothalamus to regulate appetite and energy homeostasis (Friedman 2010); where increases in adiposity results in increased leptin levels and vice versa (Schroeder-Gloeckler et al. 2007; Friedman 2010). Leptin has also been implicated to have direct effects on glucose metabolism similar to that of insulin (Huynh et al. 2010). Kieffer and colleagues (1997) utilized patch clamps in *ob/ob* mice to show that leptin reduces insulin secretion through the activation of the K_{ATP} channels in the β-cells of the pancreas (Kieffer et al. 1997). These results were further supported by the work completed by Lee and Romsos (2003), where the authors were able to show that when treated with exogenous leptin, hypersecretion of insulin was normalized in *ob/ob* mice (Lee and Romsos 2003). Further adding to leptin's role in β-cell function, studies involving leptin therapy have shown a reversal in hyperglycemia in diabetic mouse models (Chinookoswong et al. 1999; Hidaka et al. 2002; Wang et al. 2010; Denroche et al. 2011) by lowering blood glucose and plasma insulin without affecting body weight (Kulkarni et al. 1997; Seufert et al. 1999; Gray et al. 2010). Clinically, Farooqi et al. (1999) were able to show that administration of recombinant leptin in humans reduces body weight and decreases food intake, as seen with their nine-year old patient with a frame-shift mutation in the leptin gene (Farooqi et al. 1999). This study further confirmed the importance of leptin in energy balance and appetite regulation (Farooqi et al. 1999).

The discovery of naturally occurring leptin mutations in mice has provided to be a useful tool in diabetes research, as it provides to be a model of severe obesity and diabetes. Leptin deficient (*ob/ob*) and leptin receptor deficient (*db/db*) mice were characterized by George Snell and Doug Coleman and colleagues from the Jackson Laboratories between 1950 and 1970 (Coleman and Hummel 1967; Coleman and Hummel 1973; Friedman 2010). Collectively the *ob/ob* and *db/db* mice show highly elevated plasma insulin levels, severely increased body mass, hyperphagia, no satiety, and hyperglycemia as early as three to four weeks of age (Coleman and Hummel 1967; Coleman and Hummel 1973; Zhang et al. 1994; Kobayashi et al. 2000). In 1994, Zhang and colleagues utilized positional cloning in C57Bl6 and DBA/2JA mice to determine that *ob* gene product was involved in the regulation of body fat deposition (Zhang et al. 1994). More importantly, this study identified the *ob* gene as the leptin gene (Zhang et al. 1994).

The *ob/ob* mouse is characterized by a mutation in the *ob* gene, which results in severe obesity through the disruption of the leptin signaling pathway (Zhang et al. 1994; Farooqi et al. 2002). The *ob/ob* mouse is characterized by an autosomal recessive mutation on chromosome 6 on the *ob* gene (Coleman and Hummel 1973; Srinivasan and Ramarao 2007). In addition to severe obesity, *ob/ob* mice develop extreme hyperglycemia and hyperinsulinemia by nine weeks of age, which results in hypertrophy and degranulation of pancreatic β-cells (Coleman and Hummel 1973; Srinivasan and Ramarao 2007). Interestingly, the *ob/ob* mice have also been observed to have transient hyperglycemia, followed by hyperinsulinemia and euglycemia (Coleman and Hummel 1973).

In contrast to the *ob/ob mouse*, the *db/db* model is characterized by severe obesity, hyperinsulinemia, hyperglycemia, and dyslipidemia as early as three weeks of age (Coleman and Hummel 1967; Kieffer and Habener 2000). The *db/db* mutation is characterized by an autosomal recessive mutation on chromosome 4 of the *db* gene (Coleman and Hummel 1967; Srinivasan and Ramarao 2007; Shafrir and Ziv 2009), resulting in a defective leptin receptor encoded by the *db* gene (Coleman and Hummel 1967; Friedman 2010). It should be noted that degranulation and increased apoptosis can be observed as early as 25 days after birth in *db/db* islets (Coleman and Hummel 1967; Kobayashi et al. 2000). Additionally islets of *db/db* mice have been observed to fail via increased pancreatic necrosis and atrophy (Coleman and Hummel 1967; Kobayashi et al. 2000; Shafrir and Ziv 2009), thus resulting in end-stage β-cell failure. Therefore, as the severity of obesity and development of the diabetic phenotype occurs at an early age in both *ob/ob* and *db/db* mice, these mouse models are ideal for short-term studies elucidating the mechanisms involved in β-cell lipotoxicity, severe obesity and diabetes.

The objective of this study is to utilize the *db/db* model of genetic obesity to replicate and tease apart the observations made in our model of β-cell specific overexpression of PPARα in a diet-induced model of obesity and T2D. We hypothesize that overexpression of PPARα in the pancreatic β-cells of *db/db* mice will delay the progression and severity of β-cell dysfunction in these mice compared to β-eGFP-*db/db* controls. Additionally, this study will allow us to test the effectiveness and potency of PPARα overexpression in a severe model of genetic obesity; thus further characterizing the role of PPARα overexpression in pancreatic β-cells *in vivo*.

4.2 MATERIALS AND METHODS

Mice.

All animal studies were approved by the University of Northern British Columbia Animal Care and Use Committee (protocol number 2011-21). For all experiments, 3-week-old, male, C57Bl6 mice (littermate controls) and *db/db* mice (BKS.Cg-*m* +/+ *Lepr*^{db}/J) were purchased from Jackson Laboratories (Bar Harbor ME, USA). Mice were maintained on a 12 hr light/dark cycle and received standard rodent chow diet (Rodent LabDiet, 5001, Leduc AB, Canada) *ad libitum* unless otherwise stated. Mice were allowed to acclimatize to the animal facility for 1 week prior to performing any experimental procedures. Body weight, 4hr fasted blood glucose (OneTouch Ultra, Lifescan, Burnaby BC, Canada), oral glucose tolerance tests (via OGTT) and insulin sensitivity tests (via ITT) were performed prior to viral injections to assess baseline carbohydrate metabolism as described below and in chapter 3.

Pancreatic β-cell specific overexpression of PPARα in genetically obese mice.

Six-week-old male *db/db* mice were infected with dsAAV8-RIP-PPARα adeno-associated virus (β-PPARα-*db/db*, n=6; $5x10^{12}$ viral genomes/583µl/mouse) or dsAAV8-RIP-eGFP virus (β-eGFP-*db/db* control group, n=7; $5x10^{12}$ viral genomes/574µl/mouse) by ip injection. A group of age-matched, male, wildtype, C57Bl6 mice were given an ip injection of saline to serve as lean controls (n=7; 574µl saline/mouse). Body weight was monitored weekly, and blood glucose levels (OneTouch Ultra, Lifescan Burnaby BC, Canada) and circulating plasma insulin levels (ALPCO) were monitored twice weekly. Insulin sensitivity (ITT) was also assessed. All animals were sacrificed 4 weeks post-infection by CO2 euthanization and cerebral-spinal dislocation. Tissues were collected

by dissection and flash frozen or fixed in 4% paraformaldehyde in 1X PBS for 48 hrs and then stored in 70% EtOH.

Insulin tolerance tests.

Mice were fasted for 4 hrs and given an ip injection of human synthetic insulin at 0.75U/kg or 1.0U/kg (Novolin Ge, Toronto ON, Canada). Blood was sampled (1-2 µl) from the saphenous vein and blood glucose measured (mmol/L) at 10, 20 30, 60, and 120 min post injection (OneTouch Ultra, Lifescan Burnaby BC, Canada).

Glucose-stimulated insulin secretion.

Mice were fasted for 16 hrs and given 2g/kg D-glucose by oral gavage. Blood (15-20 µl) was sampled at 5, 10 and 180 minutes post glucose gavage from the saphenous vein and blood glucose (mmol/L) (OneTouch Ultra, Lifescan, Burnaby BC, Canada) (Huynh et al. 2010).

Overnight fasted and re-fed plasma insulin levels.

Mice were fasted for 16 hrs and blood collected in heparinized capillary tubes (Fisher Scientific, Ottawa ON, Canada) from the saphenous vein. Food was reintroduced for 2 hrs and blood collected again. Blood glucose was determined using a hand held glucometer (OneTouch Ultra, Lifescan, Burnaby BC, Canada) and plasma insulin levels were assessed by ELISA. 5µl of each sample was run in triplicate with conjugate buffer (75µl) and shaken at 800rpm on a microplate shaker for 2 hrs at room temperature. Samples were then washed with wash buffer and incubated for 15 min at 800rpm with TMB substrate. Stop solution was then added to samples and quantified at 450nm on a plate reader (Ultrasensitive insulin ELISA assay, 80-INSMSU-E10, ALPCO, Salem NH, USA).

Immunohistological analysis.

Whole mouse pancreas sections were previously fixed in 4% paraformaldehyde and embedded in paraffin and sectioned at 5μm thickness; n=5 per group (Wax-it Histology Services; Vancouver BC, Canada). All sections were de-paraffinized and rehydrated as described previously in chapters 2 and 3(Riedel et al. 2010). Sections were incubated with polyclonal primary antibodies to rabbit anti-eGFP (1:500) (A11122, Invitrogen Molecular Probes, Carlsbad CA, USA), polyclonal guinea pig anti-insulin (1:1000) (4011-01F, Millipore, Billerica MA, USA) and a monoclonal primary antibody to mouse anti-glucagon (1:1000) (G2654, Sigma) overnight. Appropriate secondary whole (heavy and light chain) antibodies conjugated to Alexafluor 488 (A21206 or A21202) or 594 (A11076 or A11032) (1:1000) (Invitrogen Molecular Probes) were used to detect primary antibody immunoreactivity. All sections were blocked using serum-free blocking solution (DAKO, Burlington ON, Canada) and heat-induced antigen retrieval performed using 6M citrate buffer. All samples were visualized using a fluorescent light microscope (Olympus BX61) and images observed using Cell Sens Software (Olympus, Markham ON, Canada).

Statistical analysis.

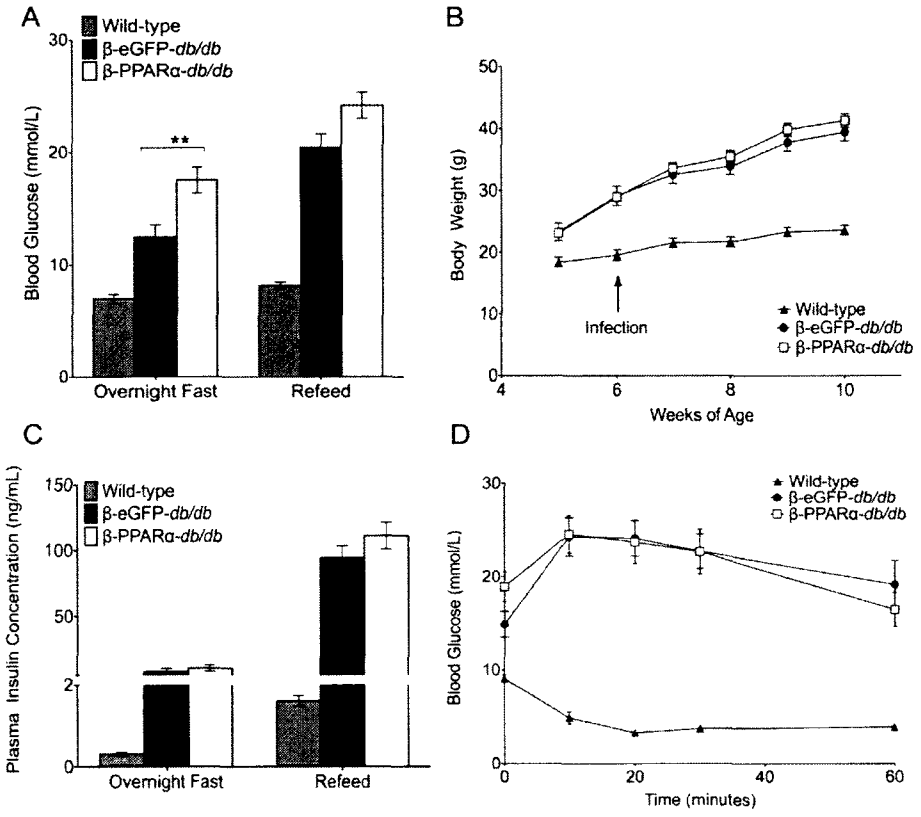
Results are expressed as mean ± standard error of the mean. Analyses were performed using paired student's t-test and 1-way ANOVA (significance between two groups), or 2-way ANOVAs (significance between groups over time) with Bonferoni Post tests using Graphpad Prism 5.0 software (La Jolla CA, USA). Significance was declared if p-values were less than 0.05. *P<0.05, **P<0.01, ***P<0.001.

4.3 RESULTS

Overexpression of PPARα in pancreatic β-cells does not improve carbohydrate metabolism in a model of severe genetic obesity (*db/db*).

β-eGFP-*db/db* control mice and β-PPARα-*db/db* mice had no significant difference for 2 hr fed blood glucose levels (Fig. 4.2a). PPARα overexpression specifically in pancreatic β-cells did not significantly affect body weight (Fig. 4.2b), or plasma (circulating) insulin levels (Fig. 4.2c) when compared to obese controls. As expected, both fasted and fed blood glucose and plasma insulin levels of β-eGFP-*db/db* and β-PPARα-*db/db* mice were significantly higher compared to C57Bl6 littermate controls. Additionally insulin sensitivity did not differ between groups, however as expected, both β-eGFP-*db/db* and β-PPARα-*db/db* are significantly more insulin resistant than the wild type controls (Fig. 4.2d). As expected with the insulin sensitivity (ITT) the wild type controls had a 50% reduction in blood glucose (10 minutes post insulin injection) and by 30 minutes post infection blood glucose levels were on the rise. The complete opposite was observed with the β-eGFP-*db/db* and β-PPARα-*db/db* mice, where a spike in blood glucose levels was observed 10 minutes post-injection; this rise in blood glucose could be attributed to their severe insulin resistance as well as an increase in counter regulatory and stress hormones.

Figure 4.2 Overexpression of PPARα in β-cells of genetically obese mice (*db/db*) did not improve glucose homeostasis. (A) Fast and fed blood glucose levels. (B) body weight, (C) plasma insulin levels, and (D) insulin sensitivity (0.75U/kg insulin for wild-type mice; 1.5U/kg insulin for *db/db* mice) in *db/db* mice. β-eGFP-*db/db* n=7, β-PPARα-*db/db* n=6. *P<0.05.


A
Wild-type
β-eGFP-db/db
β-PPARα-db/db
Blood Glucose (mmol/L)
30
20
10
0
**
Overnight Fast
Refeed
B
Body Weight (g)
50
40
30
20
10
0
Infection
Wild-type
β-eGFP-db/db
β-PPARα-db/db
4
6
8
10
Weeks of Age
C
Plasma Insulin Concentration (ng/mL)
150
100
50
2
0
Wild-type
β-eGFP-db/db
β-PPARα-db/db
Overnight Fast
Refeed
D
Blood Glucose (mmol/L)
30
20
10
0
Wild-type
β-eGFP-db/db
β-PPARα-db/db
0
20
40
60
Time (minutes)

Overexpression of PPARα in pancreatic β-cells does not affect islet morphology in genetically obese mice (*db/db*)

Immunohistochemical analysis of insulin and glucagon in pancreatic β-cells overexpressing PPARα did not reveal any difference in insulin or glucagon positive immunoreactivity when compared to β-eGFP-*db/db* controls (Fig. 4.3). Additionally, islets were stained for eGFP/insulin and eGFP/glucagon immunoreactivity (Fig. 4.4) showing eGFP expression in islets of the *db/db* mice four weeks post infection. It has been suggested that dsAAV8 vectors do not elicit an immune response (Gao et al. 2002; Wang et al. 2006; Gaddy et al. 2010), however further analysis using appropriate apoptotic staining is required to confirm these results in *db/db* mice. Furthermore, total insulin and glucagon positive stained areas were not analyzed as no physiological changes were observed, therefore β-cell and α-cell masses were not calculated.

Figure 4.3 Overexpression of PPARα in β-cells of genetically obese mice (*db/db*) does not affect islet morphology of insulin immuno-positive area. Abbreviations: INS – insulin; GLC – glucagon. Images taken at 20x magnification. Scale bar 50μm.

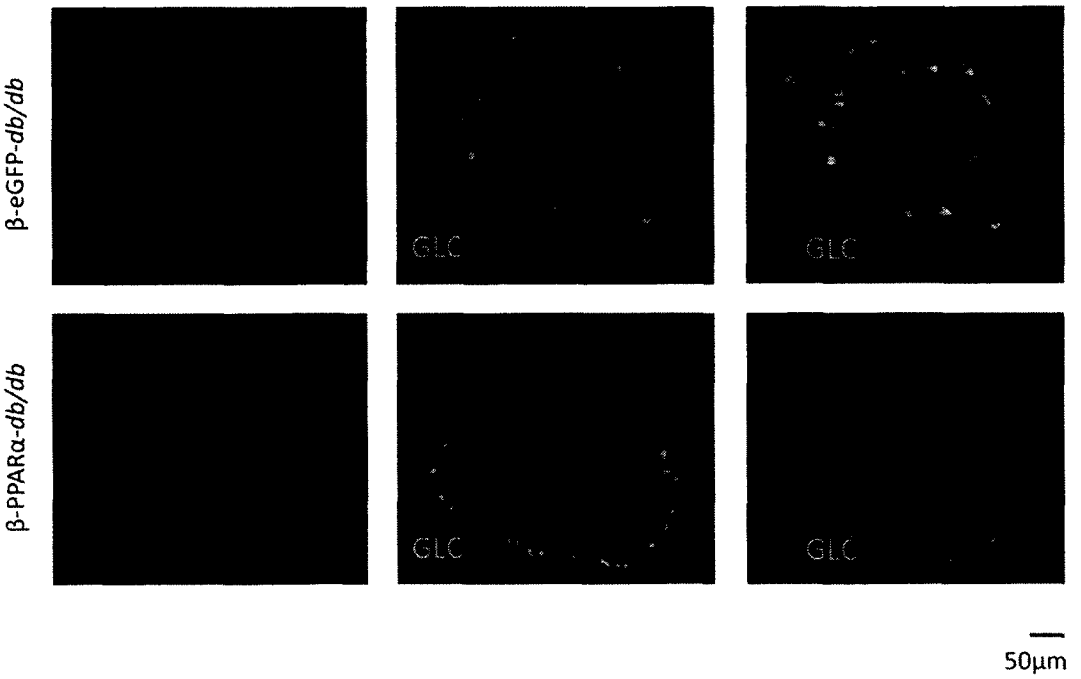
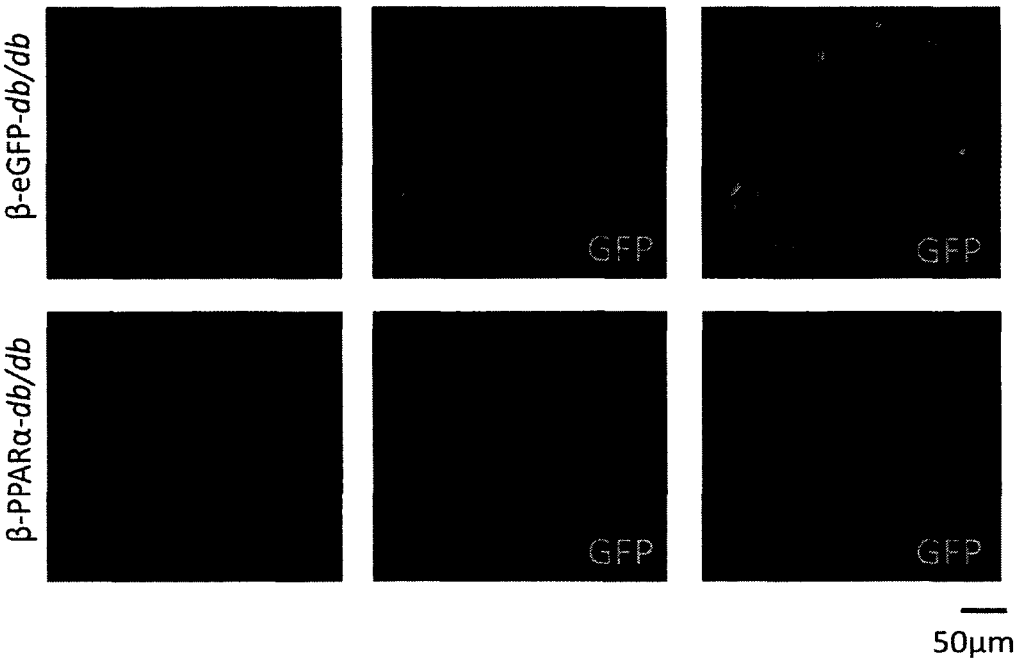
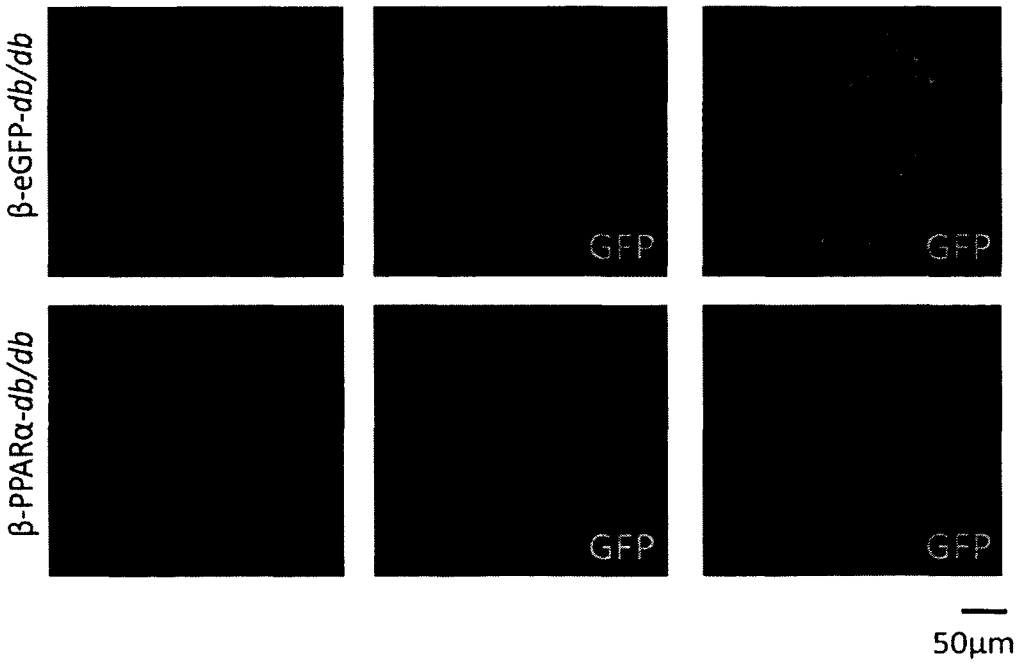

β-eGFP-db/db
β-PPARα-db/db
GLC
GLC
GLC
GLC
50μm

Figure 4.4 Overexpression of dsAAV8-RIP-eGFP in β-cells of genetically obese mice (*db/db*) is targeted to the β-cells and not α-cells of pancreatic islets. Abbreviations: INS – insulin; GLC – glucagon; GFP – green fluorescent protein. Images taken at 20x magnification. Scale bar 50μm.


β-eGFP-db/db
β-PPARα-db/db
GFP
GFP
GFP
GFP
GFP
GFP
50μm


β-eGFP-db/db
β-PPARα-db/db
GFP
GFP
GFP
GFP
GFP
GFP
50μm

4.4 DISCUSSION

Utilizing a genetic model of severe obesity, specifically a mutation with the leptin receptor (*db/db* mice), our group characterized the effects of β-cell specific overexpression of PPARα to ascertain if improvements seen with glucose tolerance in a HFD model of obesity would be observed in a more severe model of genetic obesity. Our group has demonstrated that overxpressing PPARα in the pancreatic β-cells of *db/db* mice has no protective effects against β-cell dysfunction when compared to β-eGFP-*db/db* controls. This study was carried out for a total of four weeks, where mice were infected for four weeks prior to collection. We speculate that the lack of protective effects observed in this model compared to the diet-induced model maybe; i) the *db/db* model of obesity is too severe and the levels of circulating TGs and FAs have overwhelmed the β-cell causing β-cell dysfunction, ii) β-cells of the *db/db* mice are severely impaired and have reached β-cell failure, and iii) four weeks of overexpression is too short to see the beneficial effects of PPARα observed in our diet-induced model.

Gaddy and colleagues (2010) utilized the *db/db* mouse model to determine if expression of GLP-1 via dsAAV8 to the pancreatic β-cells improves the diabetic state (Gaddy et al. 2010). AAV direct targeting of GLP-1 in the β-cells has been shown previously to enhance islet proliferation and delay the progression of diabetes in db/db mice (Gaddy et al. 2010). Additionally, the authors used a dose of $4x10^{11}$vg/mouse for their *db/db* mice and speculated that increasing the viral dose (greater than 10^{12}vg/mouse) would greatly enhance the efficacy of viral transduction compensating for increased body mass of *db/db* mice (Gaddy et al. 2010). Therefore, improvements in glucose tolerance may have been observed in β-PPARα-*db/db* mice had we increased the viral load per

mouse, as opposed to giving the standard $5x10^{12}$ vg/mouse. Additionally it has been suggested that the effectiveness of dsAAV8 virus delivery can be seen as early as four weeks post infection (Wu et al. 2006). We have conclusively shown in chapter 2, that eGFP is expressed in the pancreatic β-cells as early as three weeks post infection when utilizing dsAAV8-RIP-eGFP. Moreover, we have shown eGFP to be expressed in the pancreatic β-cells in *db/db* mice four weeks post infection (Fig. 4.4). Therefore it is possible that PPARα expression is present in the islets of *db/db* mice; however, as the duration of our study lasted four weeks post infection, the physiological effects and beneficial potential of PPARα overexpression may not have been observed. For example we would expect PPARα overexpression to turn on genes involved in β-oxidation, but was the duration long enough to decrease the amount of circulating lipids?

We speculate that had we extended the *db/db* study by four to six weeks, improvements similar to those observed in β-PPARα-HFD mice may have been observed. The severity of the *db/db* phenotype was an indicator to terminate the study four weeks post infection. As shown in figure 4.2 a-c, blood glucose levels, body weights, circulating plasma insulin were significantly higher than age-matched C57Bl6 littermate controls; indicating that the health of the pancreatic β-cells in the *db/db* mice were getting progressively worse. Both β-eGFP-*db/db* and β-PPARα-*db/db* mice showed severe insulin resistance when compared to C57Bl6 littermate controls (Fig. 4.2d); as blood glucose levels reached highs of 24.5mmol/L versus dropping to 4.8mmol/L (C57Bl6) post insulin injection. Moreover, it should be noted that a glucose tolerance test (OGTT) was not conducted on the *db/db* mice due to the severity of their phenotype; additionally we would have expected glucose levels post gavage to be extremely high,

and possibly too high to be detected by a handheld glucometer, given the rise in blood glucose levels observed when mice were challenged with 1.5U/kg of insulin (Fig. 4.2d). It should also be noted that due to a lack of physiological changes observed, further metabolic and immunohistochemical analyses of these islets was not performed.

The whole body improvement in carbohydrate metabolism achieved with pancreatic β-cell specific overexpression of PPARα in a diet-induced model of obesity was not replicated in the severely obese, diabetic *db/db* mouse. The severely impaired carbohydrate metabolism of this genetic model of obesity was not reversed by four weeks of β-cell specific overexpression of PPARα, suggesting that the short duration of PPARα overexpression may not have been sufficient to improve β-cell function or that the metabolic changes induced by PPARα by four weeks of overexpression could not overcome the extreme insulin resistance and lipidemic state induced by the lack of leptin signaling in these severely obese and diabetic mice.

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CHAPTER 5

Developing a model of PPARα overexpression in the pancreatic β-cells *in vivo:* concluding remarks, future directions and significance

5.1 CONCLUDING REMARKS

It has been well established that lipotoxicity is an underlying mechanism involved in β-cell dysfunction and obesity-induced T2D (Kasuga 2006; Prentki and Nolan 2006; Poitout and Robertson 2008). Moreover, obesity, its associated comorbities and T2D have reached epidemic levels in the past few years and are placing an overwhelming economic burden on health care systems worldwide (Dixon 2010). Therefore it is important to elucidate the mechanisms involved in obesity-induced β-cell lipotoxicity to postulate appropriate treatment strategies, and new therapeutic paradigms.

Our group is the first to have developed a new *in vivo* mouse model of β-cell specific overexpression of PPARα in obesity-induced β-cell lipotoxicity. Utilizing dsAAV8 as our tool of gene delivery we have avoided potential complications of utilizing transgenic mouse models (outlined in chapter 2). Furthermore, our study has provided insight into the direct *in vivo* effects of PPARα overexpression in the pancreatic β-cells and its impact in whole-animal carbohydrate metabolism. In addition, our lab has developed a model to study lipotoxic changes in the metabolism directly in the pancreatic β-cells. Having shown improved glucose tolerance and maintained 1^{st} phase insulin secretion, there is the possibility of using dsAAV8 delivered PPARα as a preventable therapeutic for preserving β-cell function in obesity-induced T2D. Moreover, it is likely to utilize PPARα treated isolated islets as a preventable therapeutic for islet transplantation; further protecting the islets from lipid accumulation and increasing the longevity of transplanted islets.

Taken together, our findings have provided new insights and advancements for future studies involving *in vivo* PPARα overexpression in β-cell lipotoxicity. We have

developed an *in vivo* mouse model that can be utilized to elucidate the protective role of PPARα in β-cell dysfunction and lipotoxicity, as well as applying these findings to future treatment and therapeutics in obesity-induced T2D.

5.2 FUTURE DIRECTIONS

As the work in this thesis demonstrates, our lab has developed an *in vivo* model of β-cell specific overexpression of PPARα in the context of obesity-induced T2D to examine the effects of β-cell lipotoxicity. It should be noted that this study has allowed or group to characterize the physiological consequences of β-cell specific overexpression of PPARα *in vivo*; thus we can now move forward with molecular analyses to uncover the mechanisms and specific changes in glucose metabolism in our model of β-cell lipotoxicity. *In vitro*, PPARα overexpression in MIN6 and INS-1E cells has been extensively studied by numerous groups (Ravnskjaer et al. 2005; Hellemans et al. 2007; Frigerio et al. 2010); therefore the following *in vivo* studies will be considered.

There are several avenues that could be explored to further characterize the *in vivo* effects of PPARα overexpression on the pancreatic β-cells in a diet-induced obese mouse model. First and foremost immunofluorescent staining of PPARα in the pancreatic sections needs to be shown, similar to what is observed in mice overexpressing eGFP. This would allow us to correlate the improvements observed in glucose tolerance and maintenance of 1st phase insulin secretion with pancreatic PPARα overexpression on HFD. To do this, antibodies against PPARα must first be optimized utilizing tissue sections that would show positive PPARα staining, such as sections from fasted liver. In addition to optimizing antibodies, the immunohistochemical protocols must also be optimized for heat-induced epitope retrieval and protein blocking. Once optimized, the PPARα antibodies would then be used to stain for PPARα in the β-PPARα-HFD pancreas sections; these sections would be compared against no antibody control pancreas sections stained only with the secondary antibody to determine levels of positive staining over and

above background staining. In addition, dual staining for PPARα + insulin, and PPARα + glucagon would be completed to show that PPARα is being expressed solely in the β-cells and not the α-cells of the pancreas. If PPARα is being overexpressed in the pancreatic β-cells and immunofluorescent staining is not able to detect PPARα above and beyond background fluorescence, colormetric staining of PPARα could be an alternative solution. Colormetric staining could be advantageous for our sections, as it eliminates the autofluorescing from red blood cells that may be present in non-perfused tissue sections.

Once we are able to show PPARα staining in the pancreatic sections, we could then carry out additional studies that would further characterize β-cell specific PPARα overexpression *in vivo*. As work completed in MIN6 cells has been utilized to support our *in vivo* work thus far, utilizing isolated islets from PPARα overexpressing mice would be our next option. A second cohort of mice (ideally n=10 mice per group) would be infected and subjected to HFD as carried out previously by our group. Having a larger cohort of mice would allow our group to collect whole pancreas (n= 1 or 2 per group) with the remainder being sacrificed for isolated islets. The whole pancreases would be sectioned and stained for PPARα to confirm overexpression. The isolated islets would then be utilized for gene expression studies, allowing our group to determine which PPARα target genes involved in β-oxidation are upregulated in our model. From our *in vitro* work, we would identify if acyl-CoA oxidase (AOX), carnitine palmitoyl transferase-1 (CPT1), long chain acyl-coA dehydrogenase (LCAD), and uncoupling protein-2 (UCP2) were upregulated in the pancreatic β-cell. Thus allowing us to correlate improvements in glucose tolerance with increased β-oxidation. These studies would allow us to further elucidate the mechanisms involved in preventing β-cell lipotoxicity.

In addition to gene expression studies, lipidomic profiling in isolated islets will be performed in collaboration with Matej Orešič from the Technical Research Center of Finland, using liquid chromatography-mass spectrometry (LC-MS) lipid profiling (Nygren et al. 2011). This study would allow our group to determine the lipid profiles and lipid levels in β-PPARα-HFD islets compared to islets from β-eGFP-HFD controls. If lipidomic profiling is not a feasible option, our group could also consider isolating islets and determining the levels of triglycerides (TGs) utilizing a standard TG determination kit (Sigma Aldrich, Oakville ON, Canada). This is a crude measure of total TG content, allowing us to determine the levels of lipid accumulation in the β-cells of β-PPARα-HFD and β-eGFP-HFD mice; these results could be correlated with levels of upregulation of β-oxidative genes or other lipid metabolic pathways observed utilizing qRT-PCR. This method would not allow us to determine which lipid species is being accumulated by the β-cells, nor will it allow us to distinguish which lipid species is being utilized as an activating ligand for PPARα *in vivo* as no definitive activating ligand has been identified outside of the liver (Chakravarthy et al. 2009). An alternative option would be to quantify lipid accumulation within pancreatic sections using lipophillic histological staining (either Oil Red O staining or BODIPY 493/503 staining); each of these methods is cost effective and is considered to be a good method for crude quantification of lipids. BODIPY 493/503 is a fluorescent label for lipid droplets (Ohsaki et al. 2010). Spangenburg and colleagues (2011) have demonstrated exceptional staining of lipid droplets using BODIPY 493/503 in skeletal muscle (Spangenburg et al. 2011). Combined immunofluorescent staining for PPARα and BODIPY staining of lipid would allow us to visually quantify lipid accumulation within PPARα overexpressing β-cells.

If feasible, to further understand the mechanisms involved in preserving β-cell function during lipotoxicity, repeating this study with another cohort of mice for taking whole pancreas and isolated islets at various time points would be advantageous. This type of study would allow us to determine levels of PPARα overexpression over time, as well determine the rate at which PPARα overexpressing β-cells are being recycled (Wang et al. 2004). Studying the rate of β-cell recycling could potentially explain why improving trends and improvements in glucose tolerance were only observed at a single time point and significance between β-PPARα-HFD and β-eGFP-HFD mice decreased over time.

In addition to the studies listed above, the use of targeted PPARα delivery using dsAAV8 could potentially severe as a preventative therapeutic. One possibility is to transfect isolated islets for transplantation with PPARα to protect against lipid accumulation and β-cell dysfunction. Isolated islets would be treated with dsAAV8-RIP-PPARα prior to transplanting in either the kidney capsule or hepatic portal vein of streptozotocin (STZ) induced diabetic mice. Mice would be placed on HFD for 20 weeks and monitored as previously described. The PPARα-treated transplanted islets (from kidney capsule or hepatic portal vien) of STZ-treated mice would be isolated and assessed for lipid accumulation. This study would allow us to test the therapeutic possibilities of PPARα as a preventative treatment protecting transplanted islets against lipotoxic dysfunction.

5.3 SIGNIFICANCE

Characterizing the effect of *in vivo* pancreatic β-cell specific overexpression of PPARα in a model of obesity-induced T2D has provided novel insights on obesity-induced β-cell lipotoxicity. More importantly, through the duration of my thesis project, we have developed the first *in vivo* model of β-cell specific overexpression of PPARα in a model of diet-induced β-cell lipotoxicity using the dsAAV8 as our tool for gene delivery. The data collected in this study presents significant information and provides a model of protecting β-cell function in diet-induced obesity. My results are summarized below:

1. We have shown using AAV plasmid, *in vitro,* that PPARα overexpression in MIN6 cells occurs at physiological levels when compared to levels observed in fasted liver. Additionally, PPARα overexpression upregulates the β-oxidative target gene CPT1 in the presence of palmitate. Moreover, MIN6 cells overexpressing PPARα maintains glucose-stimulated insulin secretion when treated with palmitate. Taken together, these results provide *in vitro* support that our construct can be used to overexpress PPARα in a β-cell cell line. Thus allowing us to assess the expected expression levels of PPARα target genes that may be involved in β-oxidation in PPARα overexpressing islets *in vivo*.

2. *In vivo* we have shown that dsAAV8 can be delivered through a non-invasive intraperitoneal injection to overexpress proteins of interest in the β-cells of the pancreas when used with RIP. In addition our group has shown that under the

direction of RIP, eGFP does not cross the BBB and is not expressed in the hypothalamus of infected mice. Thus dsAAV8 is a successful tool for targeting gene delivery to the pancreatic β-cells without expression in peripheral tissues.

3. Overexpression of eGFP under the direction of RIP does not affect body weight or carbohydrate metabolism, therefore can serve as appropriate control vector for *in vivo* studies.

4. Utilizing β-cell specific overexpression of PPARα during diet-induced obesity protects against glucose intolerance. We speculate that these observations may be due to decreasing lipid accumulation in the β-cell, preserving β-cell function. Our group has demonstrated that overexpressing PPARα in pancreatic β-cells maintains 1st phase insulin secretion impaired by obesity without changes in β-cell mass. In addition, PPARα overexpression *in vivo* does not affect body weight gain on HFD or insulin sensitivity when compared to obese controls.

In addition the significance of this study can be addressed as follows:

1. By manipulating the overexpression of PPARα in the β-cell, which makes up approximately 1% of the total weight of the pancreas, we have demonstrated that PPARα agonsim can impact whole-animal glucose homeostasis independently of systemic effects of liver and muscle lipid metabolism. Moreover, we have shown

the physiological phenotype of the first *in vivo* model of β-cell specific PPARα overexpression.

2. We have demonstrated that targeted overexpression PPARα specifically in pancreatic β-cells *in vivo* preserves β-cell function and protects against glucose intolerance in diet-induced obesity. Thus providing an *in vivo* model to elucidate the mechanisms of β-cell lipotoxicity in obesity-induced T2D.

5.4 REFERENCES

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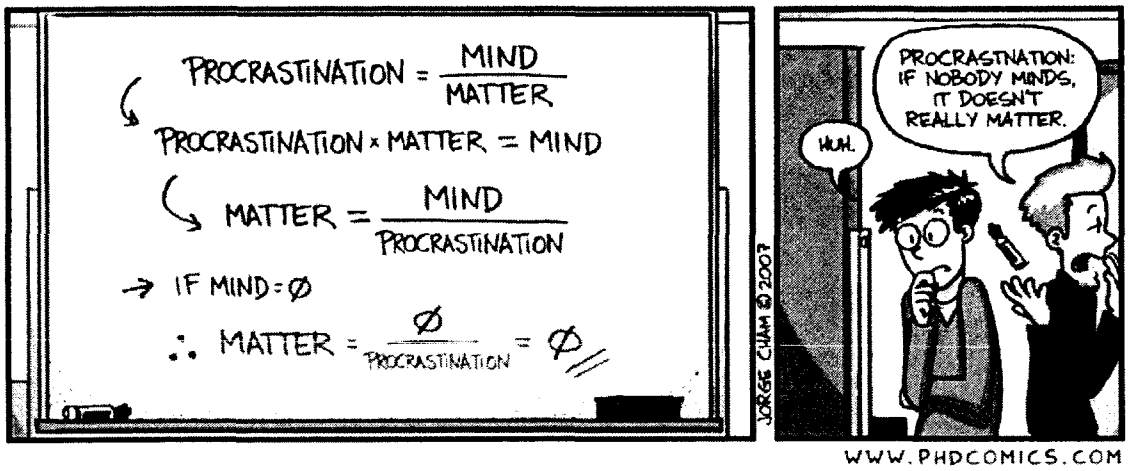
A.



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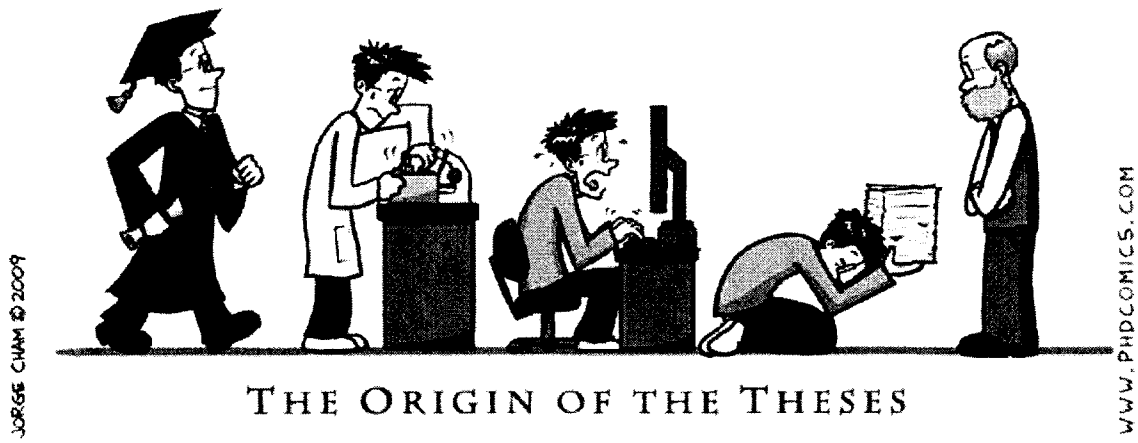

Figure S1 – (A) The mathematical breakdown on how normal levels of procrastination can lead to the development of (B) the Master's thesis. Permission for use of comics granted by PHDcomics.com.