Exploring the Possibility of Changes in SIRT1 Activity in Lipotoxicity-mediated β-cell Dysfunction

by

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Within the β-cell, there is evidence that sirtuin 1 (SIRT1), a key regulator of nutrient metabolism, plays a beneficial role on insulin secretion. Excess circulating fat, as seen in obesity, has been shown to be detrimental to β-cell function ("β-cell lipotoxicity"); an effect that may involve decreased SIRT1 activity. Consequently, SIRT1 activation may have a beneficial role on β-cell function in conditions of nutrient excess. Here we attempted to mitigate lipotoxicity induced β-cell dysfunction *in vivo* using pharmacological and genetic models of SIRT1 activation. Through hyperglycaemic clamps, we found that lipotoxicity resulted in significant β-cell dysfunction as expected in both models. SIRT1 activation, which did not affect β-cell function in the absence of fat, resulted in partial protection from the fat-induced β-cell dysfunction. These results suggest that the mechanisms of lipotoxicity-induced β-cell dysfunction include changes in SIRT1 activity.

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List of Abbreviations

ADP – Adenosine Di-phosphate

AhR – Aryl Hydrocarbon Receptor

AMPK – AMP-activated Protein Kinase

AROS – Active Regulator of Sirt

ATP – Adenosine tri-phosphate

BESTO – Beta-cell Specific SIRT1 Overexpressing

BMI – Body Mass Index

Cdk1 - Cyclin-dependent kinase 1

CHOP - CCAAT-enhancer-binding protein homologous protein

CPT-1 – Carnitine Palmitoyltransferase 1

CR – Calorie Restriction

DAG –Diacylglycerol

DBC-1 – Deleted in Breast Cancer 1

ER Stress – Endoplasmic Reticulum Stress

ETC – Electron Transport Chain

FFA – Free Fatty Acids

FOXO/FOXO1 – Forkhead Box

GIP - Gastric Inhibitory Polypeptide

GLP-1 - Glucagon-like peptide-1

GLUT- Glucose Transporter

GSIS – Glucose Stimulated Insulin Secretion

HFD – High Fat Diet

HOMA-IR - Homeostatic Model Assessment – Insulin Resistance

IKKβ - Inhibitor of nuclear factor kappa-B kinase subunit beta

IL-1β – Interleukin 1 beta

IPGTT – Intraperitoneal Glucose Tolerance Test

IRS – Insulin Receptor Substrate

JNK – c-Jun N-terminal Kinase

K_{ATP} – Potassium ATP

KO - Knockout

LCAD - Long-chain acyl-CoA Dehydrogenase

LC-CoA – Long Chain Acyl-CoA

LDLR – Low Density Lipoprotein Receptor

LKB1 – Liver Kinase B1

LXR – Liver X Receptor

MnSOD – Manganese Superoxide Dismutase

mTORC1 - Mammalian target of Rapamycin Complex 1

NAD – Nicotinamide Adenosine Dinucleotide

NADPH Oxidase - Nicotinamide Adenine Dinucleotide Phosphate Oxidase

NAM – Nicotinamide Adenine Mononucleotide

NAMPT – Nicotinamide Phosphoribosoyltransferase

NF-κB – Nuclear Factor kappa B

NMN – Nicotinamide Mono-nucleotide

PARP - Poly (ADP-ribose) Polymerase

PDE – Phosphodiesterase

PDX-1 – Pancreatic and duodenal homeobox 1

PGC-1α – PPARγ Coactivator 1α

PKC – Protein Kinase C

POMC - Pro-opiomelanocortin

PP – Pancreatic Polypeptide

PPARγ – Peroxisome Proliferator-activated Receptor Gamma

PTP1B – Protein Tyrosine Phosphatase 1B

RER – Rough Endoplasmic reticulum

ROS – Reactive Oxygen Species

RSV - Resveratrol

SD – Standard Diet

Sirt/SIRT1 – Sirtuin/Sirtuin1

STZ - Streptozotocin

TCA Cycle – Tricarboxylic Acid Cycle

TXNIP - Thioredoxin interacting protein

UCP2 – Uncoupling Protein 2

UPR – Unfolded Protein Response

VDCC – Voltage Dependent Calcium Channel

WAT – White Adipose Tissue

WT – Wildtype

ZDF – Zucker Diabetic Fatty Rat

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Chapter 1
Introduction

1.1 Diabetes

Diabetes mellitus, or simply diabetes, is a metabolic disorder that is characterized by elevated levels of blood glucose, or hyperglycemia. Clinically, diabetes can be divided into three main sub-types: Type 1 diabetes, Type 2 diabetes and gestational diabetes. In Type 1 diabetes, the body attacks and destroys its own insulin-producing β-cells, thus leading to a lack of insulin. In Type 2 diabetes, a decrease in insulin sensitivity or insulin resistance along with dysfunctional insulin secretion leads to an impairment in glycemic control and ultimately to diabetes. Finally, gestational diabetes is the development of hyperglycemia during pregnancy and increases the risk that a woman will develop Type 2 diabetes later in life. Ultimately, although the cause of the disease may differ between all three sub-types, the end result is impaired glucose homeostasis.

Of the two main types of diabetes, Type 1 and Type 2, the latter includes approximately 90% of all cases of diabetes. The most common symptoms of diabetes include polyuria (frequent urination), polydipsia (frequent thirst) and polyphagia (increased hunger) but can also include fatigue, weight loss and blurred vision. These symptoms generally apply to both types of diabetes however they may be absent or appear more gradually in Type 2 diabetes. Certain risk factors can also increase susceptibility to developing diabetes. In the case of Type 1 diabetes, family history, race and certain viral infections in childhood are the main risk factors. With Type 2 diabetes, the list is greater and includes race, family history, age, increased Body Mass Index (BMI) and physical inactivity among others. Currently there is no cure for diabetes but it can be managed through diet, exercise and various types of medications, including insulin, which in the case of Type 1 diabetes, is essential due to the lack of endogenous insulin production. As the disease progresses, further complications may develop such as cardiovascular disease, kidney

disease (nephropathy), retinal damage (retinopathy) and nervous system damage (neuropathy). These complications are often reported as the causes of death despite diabetes being the underlying cause of death, resulting in underreporting of diabetes mortality. Ultimately, diabetes is a complex disease that will reduce life expectancy, but if managed correctly the disease progression and its impact on the quality of life can be minimized.

Globally, the impact of diabetes is tremendous and widespread. The World Health Organization estimates that 346 million people currently have diabetes worldwide (WHO, accessed May 2012). In Canada alone, about 9 million people, or nearly one-third of the entire population, have diabetes or pre-diabetes (CDA, accessed May 2012). As shocking as these numbers may be, they are only expected to increase, with some estimates putting the number of people with Type 2 diabetes alone at nearly 400 million by 2025 (1). Traditionally diabetes was a disease mostly associated with North America and its increasing rates of obesity due to the Western lifestyle. However, over the last decade, rates across much of Asia, the Middle East and Africa have been increasing dramatically, with rates of diabetes in the Middle East and North Africa expected to increase by over 80% between 2010 and 2030 (2)– further evidence that diabetes is now truly a global epidemic. As the prevalence of diabetes continues to increase, the economic impact of the disease on healthcare systems will also increase. These costs can be widespread and can include hospital expenses, drug costs and disability due to the disease itself or related complications. In Canada, diabetes already costs the healthcare system billions of dollars annually but that number is expected to balloon to nearly $17 billion by 2020 (CDA accessed May 2012). For publically funded healthcare systems, where scarce resources must be fairly and efficiently distributed, these numbers reflect daunting challenges for future health policy makers.

1.2 **The Islets of Langerhans and Glucose Homeostasis**

The regulation of glucose homeostasis within the body is achieved by the Islets of Langerhans, which constitute the endocrine portion of the pancreas. These structures were discovered in 1869 by the German, Paul Langerhans, and actually make up only 1-2% of the total pancreatic mass. They consist of five known cell types which include β-cells, Alpha cells, Delta cells, PP cells and Epsilon cells (Figure 1.1).

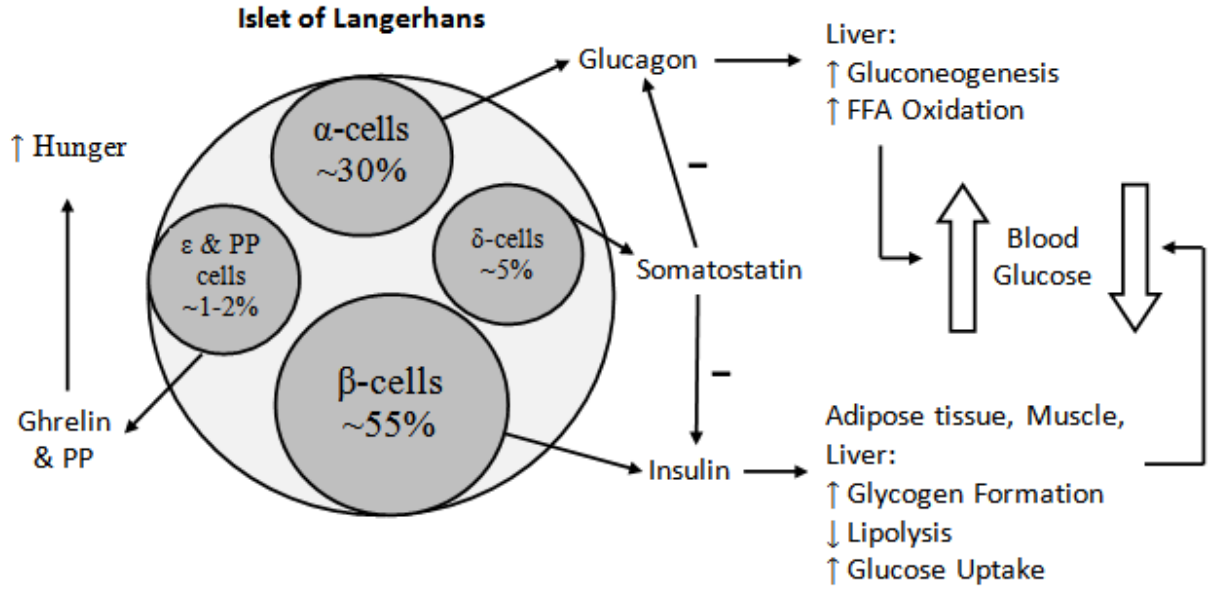

Figure 1.1. The function and actions of the cells and hormones of the Islet of Langerhans. Beta-cells and α-cells make up roughly 80% of all cells followed by δ-cells, then ε-cells and PP cells. Beta-cells release insulin to stimulate body tissues to uptake nutrients and reduce energy production, thus lowering blood glucose. Glucagon is released by α-cells and counters the actions of insulin by increasing energy production and decreasing nutrient storage to increase blood glucose. Delta cells function primarily to release somatostatin, which inhibits the release of both insulin and glucagon. PP cells and ε-cells release pancreatic polypeptide (PP) and ghrelin respectively to influence satiety (ghrelin), but whether PP has a physiological function is not known.

Of these five cell types, the main two that are involved in glucose regulation are the insulin-secreting β-cells and glucagon secreting alpha cells. Insulin is secreted by the β-cells in response to hyperglycemia and its resulting effect is to lower blood glucose by acting on adipose tissue, the liver and muscles to take up excess glucose. Conversely, alpha cells will release glucagon in response to low blood glucose, whereby glucagon will act on the liver to produce glucose and raise plasma glucose levels. Due to these vital roles in glucose homeostasis, it makes sense that alpha cells and β-cells are also the most abundant within the islets, with β-cell and alpha cells making up roughly 50% and 30% of human islets respectively (although these percentages vary within species). In order to ensure tight regulation and quick response, islets are innervated by both parasympathetic and sympathetic nervous fibres and are highly vascularized, receiving 10-15% of all pancreatic blood flow. Hormones released by islets can also act in multiple ways such as in an autocrine or paracrine manner or as mentioned earlier, on distant tissues such as the liver, adipose or muscle. The action of insulin on these tissues is generally to reduce the production of glucose and increase storage of excess nutrients. For example, in the liver, insulin will downregulate gluconeogenesis while in adipose tissue it will enhance uptake and storage of free fatty acids. Glucagon on the other hand, will generally have opposing effects to insulin and encourage breakdown of nutrients and production of glucose.

Since the β-cell is the main target of diabetes and insulin is vital to glucose homeostasis, it is worth exploring how the β-cell functions to produce and release insulin. Insulin itself is a peptide hormone that is produced and secreted by the β-cell. As discussed earlier, the β-cell releases insulin in response to increasing blood glucose levels, hence as glucose levels rise, so will insulin secretion. Insulin is released in a biphasic manner, with an initial, first phase response (within the first 10 minutes) followed by a more sustained, second phase response. The first phase response involves the immediate release of β-cell insulin stores near the cell

membrane whereas the second phase involves the sustained production and subsequent release of insulin. Insulin begins as preproinsulin from the Rough Endoplasmic Reticulum (RER) and then travels to the Golgi apparatus where, either in the Golgi or in released vesicles, proinsulin is cleaved until it reaches the plasma membrane as insulin and c-peptide. Since insulin is co-secreted with c-peptide (both are cleavage products of proinsulin), their levels in the blood should correlate and as such both are often measured when assessing β-cell function and insulin secretion. Insulin release can occur through a number of different mechanisms, although glucose-stimulated secretion is the primary mechanism (Figure 1.2). Glucose-stimulated insulin secretion (GSIS) begins with glucose entering the β-cell through the GLUT2 transporter, although GLUT1 is the most abundant GLUT in human β-cells (3). Once glucose enters the β-cell it will undergo glucose metabolism where the body's primary energy source, adenosine tri-phosphate (ATP), will be generated. An increase in the cellular concentration of ATP will stimulate the closure of ATP-sensitive potassium channels (K_{ATP}). This closure will lead to depolarization of the β-cell membrane, thereby stimulating voltage-dependant calcium channels and causing an influx of calcium ions. This cellular increase in calcium will subsequently cause exocytosis of insulin granules. In addition to glucose, other stimulants of insulin release include certain amino acids, the parasympathetic nervous system (via acetylcholine) and the incretin hormones GIP and GLP-1, which are secreted by intestinal cells in response to food intake.

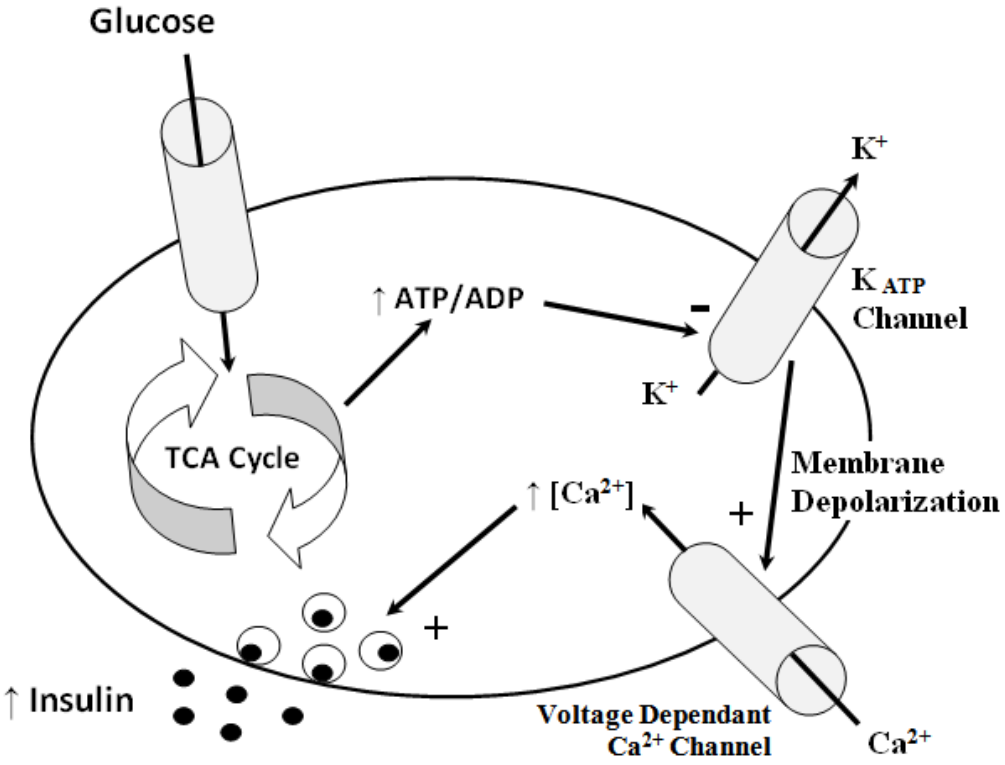

Figure 1.2. Glucose-stimulated insulin secretion (GSIS). Glucose will enter the β-cell through the GLUT2 transporter and will subsequently be metabolized into ATP via glycolysis and the tri-carboxylic acid (TCA) cycle. The rise in cellular ATP levels will inhibit the potassium-ATP (K_{ATP}) channel, thus closing it and depolarizing the cellular membrane. This depolarization will stimulate the voltage-dependant calcium channel (VDCC) to open and increase the cellular calcium concentration. The rise in calcium will then lead to the exocytosis of insulin granules.

1.3 **Pathophysiology of Type 2 Diabetes**

The pathophysiology of type-2 diabetes is complex, multi-factorial and still not completely understood. The number of risk factors that are currently associated with Type 2 diabetes is direct evidence of this complexity. As discussed previously, the defining characteristics of Type 2 diabetes are insulin resistance and impaired insulin secretion. The development of insulin resistance is believed to be one of the initiating factors of the disease. This loss in sensitivity likely develops due to genetic susceptibility and other key risk factors

such as obesity, which will be discussed further in subsequent sections. As this insulin resistance develops, the β-cell will increase secretion of insulin. This compensation is also evident through β-cell hypertrophy and increased proliferation as the β-cells try to meet insulin demands (4). Eventually however, in genetically predisposed individuals, the demand on the β-cells will lead to exhaustion and ultimately β-cell failure. In the later stages of Type 2 diabetes, outright β-cell apoptosis can also take place and outpace the proliferative ability of the β-cells (5). To make matters worse, as the disease progresses, free fatty acids and blood glucose will rise to further exacerbate the β-cell damage. Altogether, the progression of the disease and loss of β-cell mass can lead to a substantial loss of endogenous insulin, thus making the body reliant on external supplies of insulin for glycemic control.

1.3.1 β-cell Lipotoxicity

A key risk factor that plays an important role in the mechanistic development of Type 2 diabetes is obesity. Obesity is clinically defined as having a BMI greater than or equal to 30 kg/m^2. Worldwide the rates of obesity have been increasing dramatically, with nearly a quarter of Canadian adults alone considered obese (PHAC, accessed May 24, 2012). As discussed in section 1.1, rates of Type-2 diabetes have also been steeply rising, which is no surprise as this correlates with increasing rates of obesity. In clinical studies that have assessed this correlation, it has been found that over 85% of patients with type-2 diabetes are obese (6), which is why obesity in considered the main predisposing factor in the development of Type 2 diabetes. Over time, due to expanded and more lipolytically active adipose tissue (7-9), obesity can lead to chronic elevation of circulating free fatty acids (FFA), which can lead to lipotoxicity. The term β-cell lipotoxicity refers to the toxic effects of chronically elevated fatty acids on β-cell function and mass (10). It has been fairly well established through early studies that chronic elevation of

fatty acids leads to impaired glucose-stimulated insulin secretion (GSIS) (11;12). This has been demonstrated *in vitro*, *in vivo* and in human studies (12-14). There is also evidence that elevated FFA can impair insulin gene expression (15-17) and lead to β-cell death via apoptosis (18;19). However, the mechanistic details behind how chronic elevation of FFA leads to β-cell dysfunction remain unclear. Nonetheless, a number of mechanisms have been implicated including reactive oxygen species (ROS) and oxidative stress, protein kinase C (PKC) activation, Endoplasmic Reticulum (ER) stress and inflammation (10;20;21).

1.3.1.1 The Role of Oxidative Stress

Before the mechanisms behind lipotoxicity are discussed, it is worth noting that part of the reason for β-cell dysfunction is due to the inherent characteristics of β-cells themselves. Normally within cells, levels of ROS are controlled by antioxidant enzymes such as superoxide dismutase (22). However, β-cells have very low levels of such enzymes when compared to organs such as the liver (23;24). As a result, β-cells are very susceptible to damage from ROS, especially when levels are increased as with lipotoxicity. In addition, human adult β-cells also have a very low rate of proliferation (25;26). Although this increases following damage and obesity (26), due to the very low basal rate, the overall rate still remains fairly low. This can quickly lead to complete β-cell destruction since increasing ROS levels will eventually cause the cells to die through apoptosis faster than they can replicate or regenerate. Finally, β-cells are further susceptible to lipotoxicity since their ability to store fat, or specifically triacylglycerol, is very limited (27-29). Consequently, in conditions of elevated fatty acids, lipotoxicity can develop very quickly as the cell attempts to deal with the excess fat by redirecting metabolites down less beneficial metabolic pathways (28). Altogether, these characteristics make the β-cell especially vulnerable to lipotoxic conditions and limit its capacity to adapt and survive.

One of the many damaging effects of lipotoxicity is the production of reactive oxygen species (ROS) within β-cells. ROS are highly reactive molecules that contain oxygen and can include radical species such as $OH^{\bullet}$ or non-radical species such as hydrogen peroxide (H_2O_2) (30;31). By being highly reactive, they can induce a variety of harmful effects within the cell such as damaging DNA, inactivating enzymes and altering membranes (32). The condition when the production of ROS exceeds a cell's ability to deal with them through antioxidants, is termed oxidative stress (31). In patients with diabetes, there is a definitive link to ROS since markers of oxidative stress have been shown to be 5 fold higher than in healthy individuals (33). Within the β-cell the major source of ROS is the mitochondria but peroxisomes also produce a significant amount. The major mechanism behind ROS production is β-oxidation of fatty acids in the mitochondria and the resulting increased activity of the electron transport chain (ETC), which results in a greater number of ROS (mainly $O_2^{\bullet}$-) (22;30;31). In the peroxisome, β-oxidation results in the production of H_2O_2 (30). Both organelles oxidize fatty acids but mitochondria preferentially oxidize small to long chain fatty acids whereas peroxisomes prefer very long-chain fatty acids (30;31). Another source of ROS within β-cells that is gaining more interest is the membrane-associated enzyme complex of NADPH oxidase (31;34). Normally this enzyme complex generates superoxide in leukocytes in order to fight off pathogenic organisms. However, components of this complex have been shown to be elevated in the islets of animal models of Type 2 diabetes (31;35) and in cell lines following 24h treatment with the fatty acid palmitate (31;36). As a result, NADPH oxidase may be emerging as another important source of excess ROS production within β-cells.

In the β-cell, ROS play two main roles in the progression to diabetes: impairment of insulin synthesis and secretion, and cell death via apoptosis. Beta-cell death can be explained by the toxic levels of ROS within the cell resulting in mitochondrial damage, which will release

proapoptotic factors such as cytochrome c (37). This will ultimately trigger caspase enzymes to signal cell death via apoptosis once levels of damage reach critical levels (30;37). The effects of ROS on β-cell function are still somewhat unclear. With regard to insulin synthesis, what is known is that ROS can decrease levels and activity of the transcription factors PDX-1 and MafA (38;39). These two molecules are involved in β-cell growth and maturation, and also in insulin gene expression (20). Therefore, a decrease in the levels of these factors will result in decreased insulin gene expression and insulin content within the cell. A decrease in GSIS can occur due to mitochondrial dysfunction through ROS-mediated effects such as disruption of mitochondrial Ca^{2+} transport by directly inhibiting the Na^{+}/Ca^{2+} antiporter, which can subsequently reduce ATP production required for insulin secretion (40). Increases in ROS will also activate the uncoupling protein 2 (UCP2), which is an inner mitochondrial membrane protein involved in ATP production (41). In addition, ROS can activate stress-sensitive pathways such as NF-κB or JNK that disturb the normal insulin secretion mechanism and will be discussed in more detail in subsequent sections (42). Regarding ROS, it should also be noted that high levels of antioxidants reduce GSIS and so it has been suggested that low amounts of ROS can actually increase insulin secretion (32). Related research has also gone into the role of ROS as an important mediator or signal molecule of insulin secretion (22;32). This has changed the perception that ROS are simply harmful by-products and suggest that they may play critical roles in a variety of cellular pathways (22;32). However, the mechanisms behind how ROS may increase insulin secretion remain unclear and so further evidence must emerge to uncover the complete role of ROS within the β-cell.

1.3.1.2 The Role of PKC

Another mechanism through which FFA can contribute to β-cell dysfunction is through the formation of harmful oxidation by-products and the activation of PKC. Under non-pathological conditions, FFA can enter the β-cell and undergo β-oxidation to generate ATP. However, in conditions of excess energy, LC-CoA, diacylglycerol (DAG) and ceramides, which are lipid-derived by-products, can begin to accumulate and interrupt proper β-cell function. One mechanism through which this dysfunction may be mediated is through PKC activation by DAG. PKC proteins exist in numerous isoforms and function to regulate signalling cascades in various tissues through phosphorylation. Upon activation, PKC, and especially the novel isoforms, can phosphorylate serine/threonine residues on the insulin receptor and the insulin receptor substrate (IRS). This phosphorylation can interfere with tyrosine phosphorylation of IRS, which has been demonstrated on human IRS-1 *in vitro*, thus impairing normal insulin signalling (43). Further evidence comes from PKCε whole-body knockout mice that were protected against glucose intolerance when on a high fat diet (HFD) (44). It was also shown that PKCε ablation in diabetic or fat-treated islets enhanced insulin release. The authors attributed their findings to a restoration of GSIS in dysfunctional β-cells and a reduction in hepatic insulin clearance. Interestingly, mice that overexpress another PKC isoform, PKCδ, specifically within β-cells, also demonstrate improved glucose tolerance and serum insulin levels compared to wildtype animals when on a HFD (45). In this case, these findings were attributed to the overexpression of PKCδ resulting in inhibition of nuclear FOXO1 accumulation, decreased apoptosis and prevention of mitochondrial dysfunction. As a result, it is clear that PKC is involved in mediating lipotoxicity, but how exactly remains to be established. As mentioned above, ceramides are another type of lipid-derived product that can contribute to β-cell lipotoxicity. Ceramides form endogenously from fatty acids, in particular saturated fats such as palmitate, and serve as components of cell

membranes and as signalling molecules in processes such as apoptosis. In β-cells, ceramide formation has been shown by numerous studies to play a role in apoptosis-mediated cell death (18;46;47). This was further supported by findings that inhibition of ceramide formation prevents β-cell apoptosis in human and rat islets treated with palmitate or *in vivo* in Zucker Diabetic Fatty (ZDF) rats (48;49). In addition, it has also been observed that ceramides can inhibit insulin gene transcription by altering the normal translocation and expression of PDX-1 and MafA respectively (16;17). However, further details on how ceramides mediate this reduction in insulin gene transcription are currently unknown.

1.3.1.3 The Role of ER Stress and Inflammation

Two other key mechanisms that have been associated with β-cell lipotoxicity are ER stress and inflammation. The β-cell is particularly susceptible to ER stress due to its insulin secretory role, where insulin synthesis makes up over 50% of all protein synthesis (50). As a result, in conditions of excess FFA, the ER is placed under increased demand that can lead to ER stress and even β-cell apoptosis. Many studies have shown that FFA can elevate ER stress markers in islets or β-cell lines (20;51;52) and reduce ER capacity as well (53). Part of this FFA-induced ER stress has been attributed to FFA depletion of ER Ca^{2+} stores, which are essential for proper ER function (51). Under prolonged ER stress, the Unfolded Protein Response (UPR) can become active to alleviate ER stress or initiate apoptosis through markers such as CHOP, which have been shown to be elevated in β-cells exposed to FFA *in vitro* and in the β-cells of patients with Type 2 diabetes (20;50). Closely interrelated with ER stress and oxidative stress as well, are inflammatory pathways which have also been shown to be activated by elevated FFA (42). The two main pathways that are involved are the JNK pathway and the IKKβ/NFκB pathway. JNK, which is a stress-activated kinase, has been linked to β-cell lipotoxicity *in vitro* and to decreased

insulin gene expression (54). JNK can also phosphorylate serine residues on IRS, which as mentioned earlier, can prevent normal tyrosine phosphorylation of IRS (54). In conditions of oxidative stress, it has been shown that FOXO1 can be retained in the nucleus, which results in nuclear exclusion of PDX-1 and the subsequent decrease in insulin gene transcription (55). This effect of oxidative stress on FOXO1 is thought to be at least partially mediated by JNK (55;56). But importantly, the role of JNK is likely more complex since besides oxidative stress, JNK can also be activated by cytokines, PKC, ceramides and even ER stress (21). The majority of these factors can also activate IKKβ, which can subsequently activate the stress-related transcription factor, NFκB. Similar to JNK, IKKβ is also capable of serine phosphorylation of IRS resulting in impaired insulin signalling (57;58). Very recently, it has been shown by our lab that the IKKβ inhibitor salicylate prevents lipotoxicity-mediated β-cell dysfunction *in vitro* and *in vivo* (*Oprescu, A et al,* in preparation). However, some studies have also cited that the IKKβ/NFκB pathway may be beneficial towards GSIS (59-61) and therefore some controversy remains regarding the role of this inflammatory pathway in lipotoxicity.

1.3.2 Glucotoxicity and Glucolipotoxicity

As alluded to earlier, increasing levels of glucose in diabetes can eventually lead to toxicity as well, which is termed glucotoxicity. Acutely glucose and lipids both stimulate insulin secretion in order to regulate physiological levels of nutrients. But just as with lipids, chronic hyperglycemia can lead to glucotoxicity and β-cell damage. Many of the mechanisms of β-cell damage caused by glucotoxicity and lipotoxicity are in fact quite similar. This includes impairment of insulin gene transcription, decreased ATP production and β-cell death, which all revolve around the production of ROS. Glucotoxicity can actually begin very early in Type 2 Diabetes but it generally does not take full effect until the later stages of the disease when more

chronic hyperglycemia takes place. However, experimental evidence has shown that even mild hyperglycemia (around 100mg/dl) can impair insulin secretion (62). Currently there is ample evidence that hyperglycemia can impair β-cell function and insulin secretion both *in vitro* and *in vivo* (63-65). In general, the main mechanisms involved in glucotoxicity induced β-cell dysfunction include oxidative stress, inflammation and ER stress. Clinically, the involvement of oxidative stress in glucotoxicity has been supported by findings that oxidative stress markers such as 8-hydroxydeoxyguanosine, are elevated in patients with Type 2 diabetes compared to healthy patients (33;63). However, many studies exploring glucotoxicity in human and animal models have shown that antioxidants can reduce these markers and prevent or reverse glucose-induced β-cell dysfunction (33;63;64). Many of the mechanisms behind glucotoxicity induced oxidative stress are similar to lipotoxicity. For example, chronic hyperglycemia can increase levels of ROS and damage mitochondrial membranes, leading to the release of proapoptotic factors and ultimately apoptosis. In addition, levels of DAG can also increase through hyperglycemia thus activating PKC (33). Inflammation plays a key role in glucotoxicity as well, most notably through the glucose stimulation of the proinflammatory cytokine IL-1β. This stimulation has been observed in human islets exposed to hyperglycemia and in the β-cells of patients with Type 2 Diabetes (66;67). IL-1β stimulation is also closely linked to NF-κB activation, which can ultimately lead to β-cell apoptosis (66;67). Thioredoxin-interacting protein (TXNIP) has been linked to glucotoxicity mainly via ROS however, it has been shown that TXNIP is required for IL-1β secretion as well (66;68). In addition, TXNIP has also been shown to be elevated by high glucose in islets and INS-1 cells (69). ER stress has also been implicated in glucotoxicity, as supported through evidence *in vitro* (33) and very recently *in vivo* as well (65). Such studies have demonstrated that hyperglycemia will increase markers of ER stress, such as CHOP and GRP78, and activate the UPR, ultimately contributing to β-cell death through

apoptosis. This has also been examined in the islets of patients with Type 2 diabetes and once again ER stress markers were increased along with increased apoptosis and decreased insulin release (70).

Over the last decade, the concept of glucolipotoxicity has emerged, whereby β-cell damage results from a combination of the toxic effects of glucose and FFA. Currently the exact mechanisms remain unclear but a potential mechanism was first proposed by Prentki and Corkey (71) that involved the generation of harmful metabolites through increased FFA esterification. They proposed that in conditions of excess glucose and FFA, the metabolism of glucose will generate citrate and subsequently malonyl-CoA through the Krebs cycle. The increase in malonyl-CoA will then inhibit carnitine-palmitoyl-transferase (CPT-1), which is responsible for the transportation of FFA into the mitochondria. This inhibition will cause long chain fatty-CoA to accumulate within the cytosol and generate diacylglycerol (DAG) and ceramides through esterification. These two molecules have been shown to induce oxidative stress (72;73) but also decrease GSIS and insulin gene transcription (16;20;44;74), as discussed earlier, and thus contribute to β-cell dysfunction. This mechanism is now generally accepted as the manner in which glucolipotoxicity leads to β-cell dysfunction however, it is also believed to be more complex than simply an accumulation of lipid derivatives. For example, cholesterol accumulation, has been suggested since it has previously been shown to induce apoptosis in β-cells and decrease insulin gene expression (20;75). PGC-1α has also been suggested based on recent findings that it inhibits insulin and Beta2/NeuroD transcription levels and reducing PGC-1α expression protected against glucolipotoxicity-induced β-cell dysfunction (76). Overall, the concept of glucolipotoxicity has been gaining momentum through evidence *in vitro*, *in vivo* and from human studies (20) however many details still remain unclear.

1.4 **Sirtuins**

Sirtuins were first discovered at the beginning of the last decade in yeast and were eventually named “silent information regulators” or SIR genes (77). These genes were later discovered in organisms from all kingdoms of life and were found to encode proteins that were primarily NAD-dependant deacetylases (78;79). Initial interest in sirtuins spawned from the finding that they could extend the lifespan of yeast (80;81). This developed into a search for human sirtuins as potential targets for ageing. Over the years, a total of seven sirtuins have been found in mammals and they have been classified as SIRTs 1-7. Although the majority of these mammalian sirtuins share similar core structures and primarily deacetylate (82), their location within the cell and the tissues they are associated with varies greatly.

Table 1.1

Sirtuin	Localization	Functions	Common Substrates	Disease Association
SIRT1	Nucleus, Cytoplasm	Nutrient regulation, neuroprotection, cellular stress responses	FOXO, PGC-1α, NF-κB, p53, LXR, AROS, DBC-1, eNOS, histone H1/4	Metabolic, cardiovascular, neurological, renal, cancer
SIRT2	Nucleus, Cytoplasm	Cell cycle control	FOXO, tubulin, histone H4	Neurological, metabolic, cancer
SIRT3	Mitochondria	ATP production, mitochondrial protein deacetylation	ACS2, GDH, MnSOD	Metabolic
SIRT4	Mitochondria	Amino acid stimulated insulin secretion	GDH, IDE	Metabolic
SIRT5	Mitochondria	Urea cycle regulation	CPS1	Neurological
SIRT6	Nucleus	Chromatin-related functions	Histone H3	Cancer
SIRT7	Nucleus	Transcriptional roles	RNA Pol I, p53	Cardiovascular

Table adapted from (77;83-85)

Table 1.1: General characteristics of the seven mammalian sirtuins including their cellular localization, particular target tissues, primary functions and any known disease associations. Abbreviations/functions: ACS2, acetyl-CoA-synthetase 2; AROS, active regulator of SIRT1 - a direct activator of SIRT1 that together with SIRT1 suppresses p53 activity; CPS1, carbamoyl phosphate synthetase 1 - a mitochondrial ligase protein involved in urea production; DBC-1, deleted in breast cancer 1 - an inhibitor of SIRT1; eNOS, endothelial nitric oxide synthase; FOXO, forkhead box, subgroup O - a family of transcription factors involved in cell growth and differentiation; GDH, glutamate dehydrogenase - a mitochondrial enzyme involved in urea synthesis; IDE, insulin degrading enzyme - a metalloprotease capable of degrading the B chain of insulin; LXR, liver X receptor - a member of a family of transcription factors that are regulators of cholesterol, fatty acids and glucose homeostasis; NF-κB, nuclear factor kappa B; PGC-1α, peroxisome proliferator-activated receptor gamma co-activator 1 alpha; RNA Pol I, RNA polymerase I.

1.4.1 **SIRT1**

Sirtuin 1 or SIRT1 has been the most widely studied of the mammalian sirtuins and is the closest mammalian ortholog to the first sirtuin discovered in yeast. Within the cell, SIRT1 is primarily found in the nucleus (86) but later evidence demonstrated that it could be translocated to the cytosol as well (87;88). To date, SIRT1 has been identified in almost every major body tissue although its role in those tissues varies widely. However, one common theme is that SIRT1 appears to be a key regulator of nutrient metabolism, which is now in fact the general consensus amongst the literature as to the overall role of SIRT1. By virtue of its broad tissue involvement, SIRT1 has also been implicated in various diseases from cancer to diabetes. There have also been a vast number of substrates, activators and inhibitors of SIRT1 identified. Some common examples include the deacetylation targets PGC-1α (89;90) and PPARγ (91) and the transcriptional target UCP2 (92;93), which are all involved in energy or nutrient regulation pathways. In terms of regulation of SIRT1, there are a number of physiological and pharmacological routes that have been uncovered or developed as well and will be discussed further.

1.4.1.1 **The Physiological Role of SIRT1**

Of the various body tissues, the one in which the role of SIRT1 has perhaps been most studied is the liver. Within the liver, SIRT1 deacetylates and activates FOXO1, PGC-1α and LXR. Each of these molecules have differing roles within the liver however, once acted upon by SIRT1, their collective actions are to upregulate gluconeogenesis, fatty acid oxidation and reverse cholesterol transport within the liver (94;95). These responses seem to indicate that SIRT1 negatively influences liver insulin sensitivity. This has been supported by liver-specific knockdown mice that exhibit improved glucose tolerance but enhanced liver steatosis and

inflammation when on a high fat diet (HFD) (85). PGC-1α is also key regulator of mitochondrial biogenesis and so it appears that SIRT1 may increase energy expenditure within the liver. This notion has been supported by experimental evidence which will be examined in subsequent sections. Within white adipose tissue (WAT), SIRT1 primarily acts on PPARγ to increase fatty acid mobilization via direct repression of PPARγ (94). It has also been reported that SIRT1 can increase adiponectin production from WAT by activating FOXO1 and enhancing the FOXO1 and CCAAT/enhancer-binding protein alpha (C/EBPα) interaction (96). This has been supported *in vivo* by one SIRT1 overexpression mouse model that displayed a 30% increase in circulating adiponectin levels (97). However, other models have also found contrary results and therefore some controversy remains with regards to SIRT1 and adiponectin levels. Nonetheless, these are interesting findings since adiponectin levels have been shown to be decreased in patients with diabetes compared to healthy individuals and are inversely proportional to increased adipose tissue (98). As mentioned earlier, SIRT1 can act on PGC-1α to increase mitochondrial biogenesis and this is in fact one of the main roles of SIRT1 within skeletal muscle. Through this mechanism, SIRT1 can increase fatty acid oxidation in muscle tissue (85). In addition, SIRT1 can also repress the expression of PTP1B to increase insulin sensitivity. This was shown through both muscle-specific and whole body PTP1B knockout (KO) mouse models (99;100). These effects of SIRT1 action are contrary to those in the liver (decrease insulin sensitivity) and illustrate the often opposing tissue-specific actions of SIRT1. The last tissue that will be discussed here is the brain. Currently, the role of SIRT1 within the brain is not yet completely understood. SIRT1 is expressed in the hypothalamus, especially in POMC and SF1 neurons, and its expression has been shown to increase following fasting (101;102). Through transgenic mouse models, which will be discussed later, it has been found that SIRT1 in the brain may have a role in energy expenditure and protecting against nutrient excess and obesity (103;104). It has

also been found that SIRT1 plays important roles in regulating neuronal and stem cell differentiation, memory and synaptic plasticity and protection from oxidative stress (105;106). A general consensus is that SIRT1 plays a neuroprotective role through decreasing inflammation and apoptosis (77;107). It is believed that this is accomplished through SIRT1 deacetylation of FOXO to reduce FOXO-mediated apoptosis as well as deacetylation of NF-κB to inhibit its inflammatory pathway.

1.4.1.2 **Regulation of SIRT1**

Since SIRT1 was discovered, a lot has been discovered about its functions and disease implications, but not nearly as much is known about its regulation. However, a few important regulatory pathways are known, including the most obvious one involving its dependence on NAD. Within a cell, the NAD/NADH ratio fluctuates according to nutrient availability and increases during fasting states (77). SIRT1 activity will thus depend on this ratio and peak during periods of maximum NAD availability (108;109). However, competing NAD-dependent molecules such as poly (ADP-ribose) polymerase (PARP) may deplete NAD levels during times of high expression, thus reducing the amount of NAD available to SIRT1 (110). Another method of regulation that can affect SIRT1 activity and protein levels, but has only recently been uncovered is phosphorylation (111). This is one of the most common regulating mechanisms within cells, but only recently have some enzymes that target the serine and threonine phosphorylation sites on SIRT1 been discovered (111). One example is the cell cycle enzyme Cdk 1 (111), which was found to phosphorylate SIRT1 directly and decrease its activity thus affecting progression through the cell cycle. In addition, JNK1 was recently found to phosphorylate SIRT1 directly as well (112). This only occurred when JNK1 was activated under oxidative stress conditions, but upon activation, JNK1 phosphorylation of SIRT1 influenced

cellular localization of SIRT1 and briefly increased SIRT1 activity but eventually resulted in SIRT1 degradation (113), suggesting a regulatory mechanism of SIRT1 during conditions of cellular stress. However, much has yet to be understood and it is likely that other SIRT1 phosphorylating enzymes may exist as well. Aside from phosphorylation, it has also been found that SIRT1 is positively regulated by active regulator of SIRT1 (AROS) and negatively regulated by DBC-1 (deleted in breast cancer-1) (114). AROS is a nuclear protein that directly binds to SIRT1 and increases its deacetylation activity on p53 (115). It is believed that this activation is accomplished by AROS binding to SIRT1 and thus inducing a conformational change (115). However, little else is known about AROS, including how it is regulated. DBC-1 is also a nuclear protein, but it inhibits the activity of SIRT1 by binding to its catalytic site and preventing substrates from binding (116). Very recently, it was shown that DNA damage resulted in phosphorylation of DBC-1, which then bound to SIRT1 and inhibited its activity (117). Conversely, another study found that activation of AMPK resulted in activation of SIRT1 through dissociation of SIRT1-DBC-1 complex (118). But once again, these regulators of SIRT1 have only recently been discovered and so few details about mechanisms or regulation of AROS/DBC-1 are known. The final SIRT1 regulatory mechanism that will be discussed involves calorie restriction (CR) and oxidative stress. The connection between SIRT1 and CR has been extensively studied ever since CR was found to increase lifespan in various other organisms through SIRT1 (110;119;120). This was originally attributed to increases in the NAD/NADH ratio during CR, but more recent data suggests a more complex relationship that involves other mechanisms such as a complicated network with p53 (121;122). Subsequent studies have found that SIRT1 mRNA or protein levels are elevated during CR, potentially explaining the extended lifespan (123-125). Furthermore, the results from studies of elevation or activation of SIRT1 have also mimicked the findings from CR studies (110;120). However, some studies have found

decreased SIRT1 activity during CR in tissues such as the liver as well and so CR may not upregulate SIRT1 activity in all tissues (126). On the opposite end however, oxidative stress, which often results due to nutrient excess, has been linked to reduced SIRT1 protein and mRNA levels. This has been observed with cigarette smoking and the lungs (127), obesity and type 2 diabetes (128;129) and in mice fed a high fat diet (HFD) (130). How these processes mediate the decrease in SIRT1 levels remains fairly unclear, but there is some evidence that oxidative stress can covalently modify the SIRT1 protein making it inactive or mark for proteosomal degradation (127). These are important findings since obesity is a predisposing factor for Type 2 diabetes and decreased SIRT1 levels due to nutrient (fat) excess may help explain the role of SIRT1 in insulin secretion and diabetes. Interestingly, there is also some evidence that oxidative stress can activate SIRT1 to subsequently protect against oxidative damage through activation of FOXO (131;132). This suggests that oxidative stress may activate SIRT1 up to a certain threshold, beyond which oxidative stress decreases SIRT1 activity.

1.4.1.2.a **Resveratrol**

Of all the SIRT1 activators and inhibitors that have been discovered or synthesized over the years, none have attracted more attention than the polyphenol resveratrol. This naturally occurring molecule is found in over 70 plant species, but especially in red wine (or in red grape skin), peanuts and cocoa. On its own, resveratrol is a fairly potent antioxidant that can scavenge ROS within cells through forming stable radicals via resonance structures (133-135). Resveratrol has also been linked to numerous diseases such as cardiovascular disease, cancer, neurological diseases and inflammatory diseases, with new associations constantly being discovered (136). For example, resveratrol may benefit atherosclerosis through its ability to inhibit platelet aggregation, whereas its anticancer properties have been attributed to inducing mitochondria-

mediated apoptosis of cancer cells (136-138). Due to its structural similarity to estrogen, resveratrol has also been cited as a phytoestrogen and may mimic the effects of estrogen to help combat conditions such as osteoporosis (138). The phenolic structure of resveratrol has also linked it to aryl hydrocarbon receptors (AhR), which are transcription regulators that mediate the toxic effects of chemical dioxins. Resveratrol was found to antagonize AhR, which implicated resveratrol in preventing dioxin toxicity (139). As a result of these disease associations, tremendous focus has gone into trying to develop resveratrol-based drugs. However, due to its broad effects and very low bioavailability of the parent compound (resveratrol is readily metabolized in the intestine), effective drug interventions have remained elusive until further information about the effects and mechanism of action of resveratrol are uncovered (136;138).

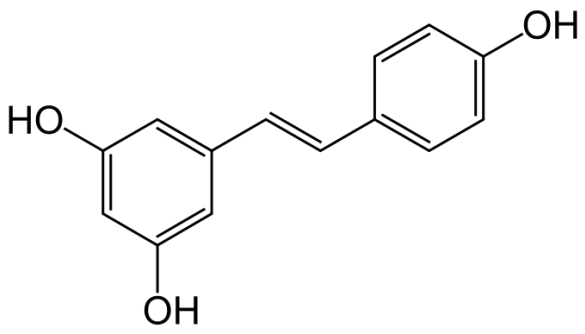

Figure 1.3. The chemical structure of resveratrol (RSV). Resveratrol is a polyphenol compound that is naturally found in a wide variety of plants. The two phenol rings and multiple hydroxyl groups make it an effective ROS scavenger and antioxidant. Resveratrol exists as either a cis or trans isomer with the trans isomer (shown) being the more biologically active version.

Although resveratrol was discovered in the late 1930s, it was not until the mid 2000s that it gained interest after it was found to extend the lifespan of yeast (140). As research expanded, its beneficial effects were believed to occur through SIRT1. This sparked interest to see whether it could do the same in higher animals and ultimately humans. This knowledge was then applied

to disease models and was thought to explain concepts such as the French Paradox, i.e. the observation that French people have very low rates of heart disease despite diets rich in saturated fats (believed to be due to high red wine consumption). Currently resveratrol is believed to be an activator of SIRT1 however, whether it is a direct or indirect activator remains controversial. Initial findings *in vitro* suggested that it was a direct activator (141;142), but subsequent studies found that this finding may be an artifact of the substrate used in the activation assay (143;144). More recent studies have suggested that resveratrol actually activates SIRT1 indirectly via AMPK mediated increases in cellular NAD (145-147). These studies have also suggested that resveratrol may in fact be an activator of AMPK and that activation of SIRT1 is a downstream effect of AMPK activation. How resveratrol may activate AMPK is unclear but it may be through inhibition of phosphodiesterases (PDEs) or ATPase, which through the latter can increase the AMP/ATP ratio to activate AMPK (146;148). However, these results are conflicting since both AMPK and SIRT1 KO mice were shown to be resistant to many of the beneficial metabolic effects of resveratrol, indicating some sort of dependence on one another to mediate the effects of resveratrol (145;147). Alternatively, it is also possible that SIRT1 may be upstream of AMPK, which has been supported by evidence that SIRT1 overexpression increases AMPK activity, possibly through deacetylation of the upstream AMPK kinase LKB1 (97;149). As a result, it is quite clear that the relationship between SIRT1, AMPK and resveratrol is very complicated and will require further study to uncover mechanistic details. Nonetheless, regardless of how resveratrol may activate SIRT1, it is now quite apparent that the beneficial effects of resveratrol likely occur through SIRT1.

1.5 **Sirtuins and the β-cell**

In the β-cell, the best described effect of SIRT1 is to decrease transcription of the uncoupling protein 2 (UCP2) (92;93). UCP2 is a member of a group of uncoupling proteins that is found on the inner mitochondrial membrane and primarily functions to dissipate the proton gradient created by the electron transport chain (41). By doing so UCP2 decreases the efficiency of oxidative phosphorylation and reduces the amount of ATP produced (41). In addition, while dissipating the proton gradient it also reduces the amount of ROS produced by the electron transport chain (22;150).

In addition to acting on UCP2, SIRT1 also deacetylates FOXO proteins within the β-cell. FOXO proteins are transcription factors that are involved in a variety of cellular processes related to cellular stress, with FOXO1 being the most common in β-cells (151). One way that FOXO1 reduces cellular stress is by upregulating antioxidants such as Manganese Superoxide Dismutase (MnSOD) (152). However, whether SIRT1 mediates this upregulation via FOXO1 has not been completely established. SIRT1 activation of FOXO1 can also cause nuclear exclusion of PDX-1 (17;153). PDX-1 is a transcriptional factor that plays a crucial role in insulin gene expression (154). Therefore, release of PDX-1 from the nucleus decreases insulin gene transcription. This has been supported by studies have examined decreased levels of PDX-1 and have found insulin secretion and β-cell development to be

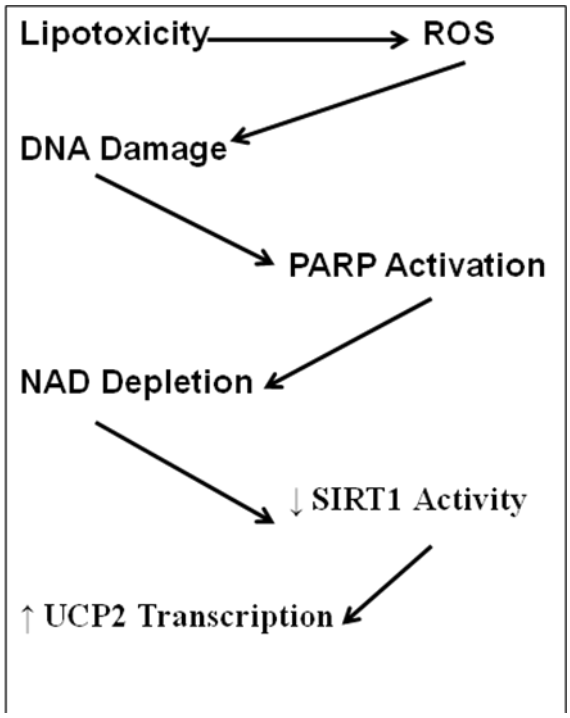

Figure 1.4. A flow chart outlining a possible connection between lipotoxicity, SIRT1 and UCP2 in the β-cell.

defective (see review (154)). However, one study has also found that FOXO1 may form a complex with SIRT1 that activates expression of the transcription factors MafA and NeuroD (153). Together with PDX-1, MafA and NeuroD work to regulate β-cell maturation and insulin gene transcription.

Under lipotoxic conditions, UCP2 transcription may be increased due to decreased SIRT1 activity. This decrease in SIRT1 could result indirectly through lipotoxicity since as discussed earlier, lipotoxic conditions result in an increase in ROS within the β-cell. This increase in ROS can result in cell damage but especially damage to DNA. To counter this damage, cells activate repair enzymes including poly (ADP-ribose) polymerase (PARP) (110). What is key about PARP enzymes is that they are also NAD-dependent in that they use NAD to repair DNA (110). As a result, during lipotoxic conditions, PARP activity may increase to repair DNA damage thus depleting NAD within the cell (155). This depletion may decrease SIRT1 activity due to lack of NAD availability (155) and so UCP2 activity increases resulting in a decrease in ATP and ROS production (156). Since ATP is crucial to the release of insulin, a decrease in ATP also results in a decrease in insulin secretion (156). Individual parts of this relationship, which is summarized in Figure 1.4, have been demonstrated in β-cells but not in its entirety. For example, the connection between lipotoxicity and ROS generation in the β-cell has been well studied (21;157). The connection between PARP activation, decreased NAD and decreased SIRT1 activity has also been well studied, including using whole-body PARP knockout mice that showed increased SIRT1 activity (158;159). However, this relationship has not been demonstrated within the β-cell. Very recently, it was shown *in vitro* and *ex vivo* that incubation with palmitate or intravenous infusion of Intralipid reduced SIRT1 mRNA and protein levels in rat islets and β-cell lines (160). Nonetheless, neither PARP nor UCP2 were discussed and limited mechanistic details were provided. There is however, evidence supporting the relationship between SIRT1 and

UCP2 in the β-cell. In studies that have either knocked out UCP2 or decreased its expression, GSIS has been shown to increase due to an increase in ATP levels within β-cells (93;150;161;162). Similarly, increased levels of UCP2 have decreased insulin secretion and ATP production (156;163). It has also been shown that increasing or decreasing SIRT1 levels decreases or increases UCP2 levels respectively (92;93). Evidence further supports the fact that UCP2 is elevated during oxidative stress and that there is a link between UCP2 expression and lipotoxicity (151;164;165). Finally, there is also evidence that elevated SIRT1 or decreased UCP2 protects against decreased insulin secretion after a high fat diet (161;166;167). However, currently the physiological role of UCP2 remains unclear due to conflicting findings. For example, studies overexpressing UCP2 have also found no changes in GSIS or ATP within the β-cell (168;169). As a result, although these studies are slowly beginning to piece together the connection between SIRT1, UCP2 and lipotoxicity, the relationship appears more complex than originally thought.

1.5.1 **SIRT 3**

Very recently, another mammalian sirtuin, SIRT3, has been gaining some interest with regards to metabolic syndrome and glucose homeostasis. As touched upon earlier, SIRT3 is an important mitochondrial sirtuin that regulates fatty acid oxidation during fasting and ATP production through deacetylation of mitochondrial metabolic enzymes such as long chain acyl-CoA dehydrogenase (LCAD) (170). It is also upregulated by the mitochondrial regulator, PGC-1α, which is also a substrate of SIRT1. Currently the relationship between SIRT1 and SIRT3 has not been explored, but it is possible that SIRT1 may upregulate SIRT3 indirectly via PGC-1α. However, little else is know about the regulation of SIRT3. The role of SIRT3 in metabolism has been examined quite extensively by Hirschey et al using SIRT3 KO mice and HFD (171). They

found that chronic HFD feeding in normal mice resulted in decreased SIRT3 mRNA but interestingly, acute HFD feeding actually increased levels of SIRT3 mRNA. This association becomes more intriguing when considering that there is also evidence that CR increases SIRT3 expression (172). Furthermore, SIRT3 KO mice were found to have impaired glucose tolerance on a HFD at 3 and 12 months of age, and also at 12 months of age on a standard diet (SD). These mice also displayed hyperlipidemia, increased inflammation and hepatic steatosis and decreased fatty acid oxidation. Altogether this evidence makes the relationship between SIRT1, SIRT3 and NAD much more interesting and complex in terms of understanding nutrient regulation and metabolism.

1.5.2 Models of Altered SIRT1 Expression

In order to further explore the role of SIRT1 *in vivo*, a number of animal models have been developed where SIRT1 has been overexpressed or knocked-down/knocked-out in the whole body or in specific tissues. The first transgenic animal developed was a whole body SIRT1 KO mouse (173). Unfortunately, these animals died postnatally due to developmental defects, but on an outbred background some survived to adulthood. This lack of viability did however uncover the importance of SIRT1 to embryological development (174). The outbred animals have been characterized by a number of studies (93;124;126;175). Morphologically, the islets of the KO animals did not differ from wildtype (WT) animals in terms of islet area, absolute size or staining for major hormones, however KO animals did have significantly lower plasma insulin levels in both fed and fasted states (93). Correspondingly, GSIS experiments also revealed significantly lower insulin secretion from KO islets. Surprisingly, blood glucose levels in KO animals were lower than WT controls, indicating increased insulin sensitivity, the origin of which was not confirmed. One final important finding from this study was that KO animals

had increased levels of UCP2 within the whole pancreas. Furthermore, Li et al found that SIRT1 null mice had a blunted response to LXR agonists suggesting that SIRT1 plays a role in mediating LXR activity (175). In addition to SIRT1 null mice, there have also been heterozygous (+/-) SIRT1 mice developed that are phenotypically normal (176). These mice display decreased energy expenditure and increased fat mass and susceptibility to fatty liver. The latter may be mediated by an increase in PPARγ and NF-κB activity, which would lead to increased inflammation. These mice have not been characterized with regards to glucose metabolism and the β-cells. To avoid the developmental defects of KO animals, two groups have recently developed new models of SIRT1 KO mice. The first model was created by the group that originally developed the SIRT1 KO mouse model, but this time they introduced a point mutation in the SIRT1 gene that eliminates its catalytic activity but maintains normal SIRT1 protein levels (177). These mice still had significant developmental defects, although the phenotype was much milder than the original SIRT1 KO mice. This indicates that SIRT1 protein has effects independent of SIRT1 activity, but further study is needed to better characterize these mice. Another group has also developed a tamoxifen induced SIRT1 KO model that demonstrated no phenotypic differences from WT animals (147). This model was mainly examined in the context of resveratrol and will be discussed further.

To further explore the effects of lack of SIRT1, a number of tissue specific knockout mice have been developed, focusing mainly on the brain and liver. Within the brain there have been two models developed, one whole brain KO model on a C57BL/6 background (178) and one POMC neuron SIRT1 KO on a mixed C57BL6/129Sv/FVB background (103). The POMC KO is an interesting model since POMC neurons are involved in appetite and glucose homeostasis. In these KO mice, it was found that on a high calorie (HC) diet, females gained significant fat mass and had decreased energy expenditure along with elevated leptin levels

(leptin resistance). Remarkably however, these mice had all normal parameters of glucose and lipid homeostasis, suggesting that SIRT1 is required for defence against diet-induced obesity. On the other hand, whole brain KO mice (178) were actually smaller than their WT counterparts due to reduced pituitary growth hormone (GH). Furthermore, older KO mice displayed impaired glucose tolerance (that was prevented on a calorie restricted diet), which was absent in young mice. The authors suggested that this impairment likely arose from a disruption in the brain-liver neuronal circuit that failed to downregulate gluconeogenic genes in response to glucose stimulation. Another target organ for tissue-specific knockouts has been the liver, where once again two different models have been developed, both on a C57BL/6 background (126;179). Both of these animal models display no visible phenotypes or altered metabolic parameters on normal diets. However, when placed on a HFD, the Purushotham liver KO (LKO) model gained weight and developed hepatic steatosis, which was in complete contrast to the Chen LKO model that had lower weight, less liver and WAT fat accumulation, lower insulin and glucose levels and improved glucose tolerance compared to WT animals. The Purushotham LKO model also displayed altered PPARα signalling, reduced fatty acid oxidation and increased inflammation and ER stress on a HFD.

At the other end of the spectrum, there have also been a number of SIRT1 overexpressing models developed, including four whole body overexpressing mouse models (97;166;180;181). Three of the four studies developed models on C57BL/6 backgrounds and with moderate overexpression (2-4 fold) of SIRT1 protein, except for Bordone et al who used a mixed C57BL/6 and 129/Sv background and did not quantify their level of SIRT1 overexpression. For the most part, findings from these studies were in agreement, although some differences exist, likely due to the differing methods of generating the transgenic animals. The most consistent finding that was reported was improved glucose tolerance when assessed through a glucose tolerance test.

Interestingly, the transgenic mice developed by Banks et al only reported this finding when the mice were on a HFD, whereas mice on a standard diet (SD) did not show improved glucose tolerance. Similarly, Bordone et al also reported lower fed plasma insulin levels in their transgenic mice but this was only found by Banks et al in transgenic mice on a HFD and was not assessed in the other two studies. Fasting plasma glucose was reported to be lower by both Banks and Bordone, but once again Banks et al only observed this in transgenic mice on a HFD. With regard to body weight, Bordone et al found lower body weights in transgenic mice whereas Banks and Pfulger did not find any significant difference in body weight, regardless of the diet.

Apart from whole body overexpression models, there have also been a few tissue-specific SIRT1 overexpression models developed. Recently there was a model developed by Wu et al that overexpresses SIRT1 by 1.5-2.5 fold in the striatum and hippocampus of the brain (182). Surprisingly, they found was that these mice had increased fat mass and body weight, and impaired glucose tolerance, which they attributed to a significant decrease in muscle GLUT4 mRNA levels. These mice also displayed decreased energy expenditure which was evident through decreased body temperature and decreased mRNA levels of many mitochondrial genes in muscle tissue. Another interesting study was done Li et al where they overexpressed SIRT1 2-3 fold in the livers of low density lipoprotein receptor (LDLR) KO mice and ob/ob mice using adenovirus (183). They found that overexpression of SIRT1 in the LDLR KO mice significantly improved glucose tolerance and lowered plasma glucose and insulin levels, likely attributable to a ~60% decrease in the mRNA of gluconeogenic genes. Nearly identical results were found in the ob/ob mice overexpressing SIRT1, with improved glucose tolerance and HOMA-IR scores and lower plasma glucose and insulin levels. In both these models, there was also reduction in serine IRS-1 phosphorylation, which is a key regulator of insulin sensitivity. Finally, SIRT1 prevented obesity-induced expression of UPR markers such as CHOP. They also found that

SIRT1 overexpression reduced insulin resistance by suppressing mTORC1 activity through reduced serine phosphorylation, which resulted in reduced serine phosphorylation of IRS-1.

One particular study that is worth discussing further was done by Moynihan et al in 2005 (92). This study examined glucose tolerance and insulin secretion in pancreatic beta-cell-specific SIRT1 overexpressing (BESTO) transgenic mice. They found that both were improved in BESTO islets with no morphological differences between control and BESTO islets. UCP2 protein levels were also decreased in the BESTO islets and ATP levels were increased. Finally, the study also examined the BESTO mice at 8 months (initial results were determined at 3 months) to see if they still maintained enhanced β-cell function and in fact they did. However, in a follow up study (167) the group looked at old BESTO mice aged 18-24 months and found different results. They found that although SIRT1 was still overexpressed, the improved glucose tolerance and GSIS, the repression of UCP2 and the increased ATP levels were all lost in the aged mice. Interestingly, they did find that improved glucose tolerance and GSIS was still maintained under a high fat diet. They attributed the loss of these effects to the age-related decrease in NAD levels within the cell. In a follow-up study, Yoshino et al (184) indeed demonstrated that NAD levels and protein levels of Nicotinamide phosphoribosyltransferase (NAMPT), which mediates biosynthesis of NAD, are decreased in older mice. However, supplementation of nicotinamide mononucleotide (NMN), a NAD precursor, restored impaired glucose tolerance in these mice after just one IP dose of NMN. They also explored NAD biosynthesis during HFD feeding and found that NAMPT-mediated NAD synthesis was compromised by HFD in WT mice. NMN supplementation restored glucose tolerance and insulin sensitivity in HFD mice.

1.5.3 Pharmacological Models of SIRT1 Activation & Inhibition

Despite benefits to both increasing and decreasing SIRT1 levels depending on the tissue, the majority of research has focused on activation of SIRT1 because of the potential to increase lifespan. In fact, there have been over 3500 SIRT1 activating compounds developed (83). However, as discussed earlier, resveratrol was one of the first SIRT1 activators found and remains the most commonly cited natural activator.

Over the last few years, research on resveratrol has exploded with dozens of studies employing it at various doses both *in vitro* and *in vivo*. A common study protocol that has been used involves mice on a high fat or high calorie diet (HFD/HC) followed by some form of resveratrol administration. This general protocol has now been used by numerous groups and generally the results seem to agree (134;145-147;185-188). Mice on a HFD with resveratrol show improved glucose tolerance, decreased oxidative stress and improved glucose and lipid metabolism compared to mice on a HFD alone (146;187;188). It has also been shown that such a diet protects against obesity and produces mice with a greater aerobic capacity and survival (134;145;146;185). These beneficial effects have been attributed to activation of AMPK and PGC-1α by resveratrol, resulting in increased fatty acid oxidation and higher mitochondrial numbers in muscle. Furthermore, with regard to glucose homeostasis, resveratrol has been found to enhance GSIS and cellular ATP levels (147;189) and improve insulin sensitivity not only in mice and rats but also in humans (145;190;191). However one group has also produced contrary findings by demonstrating that resveratrol suppresses insulin secretion *in vitro* (192). In addition, two recent studies found that resveratrol supplementation to either non-obese women or obese men resulted in no beneficial metabolic effects in either group (193;194). Dramatic results have come from models of diabetes using streptozotocin (STZ) in rats where resveratrol was shown to have significant antidiabetic potential by enhancing insulin secretion, greatly improving

metabolic parameters and essentially preventing STZ-induced diabetes and β-cell dysfunction (195-198). One contributing factor may be the inherent antioxidant properties of resveratrol (133;135), however this has typically not been examined in the context of such studies. Interestingly, it has also been found that mice on a standard diet supplemented with resveratrol do not show increased survival as do mice on a HFD (199). This has supported the notion that resveratrol does not mimic all aspects of CR (199-201). This also suggests that the benefits of resveratrol may be dependent upon additional factors.

Although resveratrol is currently the best natural activator of SIRT1, a more potent synthetic activator has also been developed known as SRT1720. This molecule was initially described by Milne et al in 2007 using high-throughput screening and was found to activate SIRT1 at a rate nearly four-fold that of resveratrol (202). Based on these findings, the group tested SRT1720 *in vivo* and found results similar to those using resveratrol (202). A more recent study further supported initial findings by showing that SRT1720 extended lifespan in HFD mice and also increased insulin sensitivity and reduced liver steatosis and inflammation without any toxicity (203). However, other studies have also found that the effects of SRT1720 did not resemble resveratrol and so the benefits of this molecule are still somewhat unclear (97;143;200). In addition, it is worth noting that SRT1720 and other synthetic activators share the same problem as resveratrol in that they have been shown to have other SIRT1 independent effects and may not activate SIRT1 directly as first proposed (see review (204)). Despite uncertainties, there are currently dozens of clinical trials involving resveratrol and other synthetic SIRT1 activators taking place (83;136;204). These trials will determine how beneficial these molecules are at treating various metabolic conditions and whether they are safe for therapeutic use.

Although focus has been on developing SIRT1 activators, many SIRT1 inhibitors have also been developed and discovered. Inhibitors have often been used to verify if experimental

effects occur through SIRT1 (such as with resveratrol), but more clinically, inhibiting SIRT1 has been proposed in treating diseases such as HIV and cancer, where in the latter SIRT1 expression has been observed to be increased (205). The main natural inhibitor of SIRT1, and all sirtuins in fact, is nicotinamide (NAM), which is an intermediate involved in generating NAD and a by-product of the deacetylation reaction. Through high-throughput screening methods, many synthetic inhibitors have been identified including Suramin, Cambinol and EX-527 among many others (see review (206)). Interestingly, many of these SIRT1 inhibitors can also inhibit SIRT2 and even SIRT3 and SIRT5 to a lesser degree. However, as with SIRT1 activators, the majority of these inhibitors still require extensive study before any therapeutic interventions can be considered.

1.6 Rationale and Significance

Over the last decade it has become quite evident that sirtuins and SIRT1 are key players in the regulation of nutrients and metabolism. At the same time, our lab and others have demonstrated that lipotoxicity, which can result from nutrient excess, plays a crucial role in mediating β-cell dysfunction. However, there has been limited research connecting these two areas of study.

Two key studies have examined the importance of SIRT1 within the β-cell and demonstrated that SIRT1 overexpression or knock-out mouse models display increased or decreased insulin secretion respectively (92;93). The latter SIRT1 knockout study attributed the decrease in insulin secretion to upregulation of UCP2, which is a transcriptional target that is repressed by SIRT1. Subsequent studies further demonstrated that a lack of NAD availability, which is essential to sirtuin function, may be responsible for decreased SIRT1 activity (184). It is well established that oxidative stress is a mediator of lipotoxicity, but a couple of studies linked

this notion to SIRT1 by demonstrating that oxidative stress induced DNA damage could activate NAD-dependent PARP enzymes, thus reducing NAD availability for SIRT1 and ultimately SIRT1 activity (158;159).

Currently, the role of decreased activity of sirtuins (SIRT1) as a possible mechanism initiated by β-cell lipotoxicity *in vivo* is not known. However, based on the evidence from the literature presented above, we believe that a connection can be drawn whereby oxidative stress generated by high fat infusion can deplete cellular NAD via activation of PARP enzymes, thus decreasing SIRT1 activity due to a lack of NAD availability. This would subsequently lead to upregulation of UCP2 and decrease the availability of ATP for β-cell function. As a result, in this study I sought to examine whether increasing SIRT1 activity could mitigate lipotoxicity-induced β-cell dysfunction *in vivo* and if so, how these effects were being mediated.

1.7 **Hypothesis**

We hypothesized that either pharmacological or genetic activation of SIRT1 would prevent fat-induced β-cell dysfunction *in vivo*.

Chapter 2
Materials & Methods

2.1 Procedures

2.1.1 Experimental Animal Model and Surgical Procedures

2.1.1.1 Animals

Rats: normal female Wistar rats (Charles River, Quebec, Canada) aged 10-12 weeks and weighing 250-300g were used. **Mice:** male Beta-cell Specific Sirt1 Overexpressing (BESTO) mice (Dr. S. Imai, St. Louis, Missouri, USA) and wild type (WT) littermates aged 12-16 weeks and weighing 25-35g were used. All animals were housed in the University of Toronto's Department of Comparative Medicine. They were exposed to a 12 h light/dark cycle. The animals were fed a Teklad Global diet containing 21% protein with 64% carbohydrate and 14% fat, Harland Teklad Global Diets, Madison, WI.

2.1.1.2.a Rat Surgery

All procedures were in accordance with the Canadian Council of Animal Care Standards and were approved by the Animal Care Committee of the University of Toronto. After a week of adaptation to the facility, rats were anaesthetized with ketamine:xylazine:acepromazine (87:1.7:0.4 $mg.ml^{-1}$, 1 $ul.g^{-1}$ of body weight) or with isofluorane, and indwelling catheters were inserted into the right internal jugular vein for infusions and the left carotid artery for sampling. Polyethylene catheters (PE- 50; Cay Adams, Boston, MA), each extended with a segment of silastic tubing (length of 3 cm, internal diameter of 0.02 inches; Dow Corning, Midland, MI), were used. The venous catheter was extended to the level of the right atrium, and the arterial catheter was advanced to the level of the aortic arch. Both catheters were tunnelled subcutaneously and exteriorized. The catheters were filled with 4% heparinized saline to maintain patency and were closed at the end with a metal pin. The rats were allowed a minimum

3-4 days period of post-surgery recovery before experiments, after which they were connected to the infusion apparatus. The infusion lines ran inside a wire tether that was fitted to the subcutaneous implant. Each rat was placed in a circular cage, and the infusion lines were protected by a tether and run through a swivel, which was suspended on top of the cage to give complete freedom of movement to the rat. Infusions were started through the jugular vein, whereas a slow infusion of heparinized saline was used to keep the carotid artery patent for sampling. The rats had ad libitum access to tap water and standard rodent chow.

2.1.1.2.b Mouse Surgery

All procedures were in accordance with the Canadian Council of Animal Care Standards and were approved by the Animal Care Committee of the University of Toronto. After reaching 12-16 weeks of age, male BESTO mice or wildtype littermate controls were anaesthetized with isofluorane and the left jugular vein was cannulated using a two-part catheter consisting of polyethylene tubing (PE-10; Cay Adams, Boston, MA) and silastic tubing (length of 1.5 cm, Dow Corning, Midland, MI). The free catheter end was exteriorized at the back of the neck through a subcutaneous tunnel created using a 16G needle, filled with heparinized saline (40 units/ml) and closed by creating a knot at the free end. Mice were allowed a minimum of 3-4 days post-surgery recovery before infusions. The infusion line ran inside a wire tether that was fitted to a polyethylene harness. Each mouse was placed in a harness and the infusion line was run through the tether and attached to a swivel suspended above the mouse cage. The mice had complete freedom of movement and had ad libitum access to standard rodent chow and tap water.

2.1.1.3 Mouse Islet Isolation

Pancreatic islets were isolated from male BESTO and WT mice as previously described (207). Briefly, mice were anesthetized with ketamine:xylazine:acepromazine (87:1.7:0.4 $mg.ml^{-1}$, $1\mu l.g^{-1}$ of body weight). The visceral contents were exposed and mice were exanguinated through an incision in the abdominal aorta. The pancreatic duct was perfused with 3 ml of collagenase V (0.8 mg/ml; Sigma, St. Louis) in RPMI-1640 containing 2.8mmol/l glucose, 10 mmol/l Hepes, 1% Penicillin. The pancreas was then excised and digested for 20 minutes at 37 °C. Islets were hand-picked from acinar tissue debris, and transferred into Krebs Ringer buffer containing 10mmol/l HEPES (KRBH) and 2.8 mmol/l glucose.

2.1.2 Infusion Period and Preparation of Infused Solutions

2.1.2.1 Rat Infusion Period and Solutions

Rats were infused for 48 h with either: 1) Saline (SAL), 2) Oleate (OLE; 1.4 μmol/min), 3) resveratrol (RSV; 0.025mg/kg.min) or 4) oleate + resveratrol (OLE + RSV). The artery line was also infused with heparinized saline (0.2-0.4% at 5 μl/min) for 44 h or until the onset of the clamp to maintain artery line patency. During the 48 h infusions the rats had free access to food and water. Blood samples for FFA, glucose, and insulin were taken at 18, 24, and 44 hours after the onset of infusion. Two-step hyperglycemic clamp studies were performed in overnight fasted, conscious rats at the end of the 48 h infusion period.

We have established an *in vivo* model of β-cell lipotoxicity in rats (14), for which we have used a 48 h i.v. infusion of oleate, a monounsaturated fatty acid, bound to bovine serum albumin (BSA). The BSA is used to prevent the detergent action of the fatty acid, and in this way, the oleate infusion can be given to animals through a central i.v. line (208). We found a two fold elevation of plasma FFA obtained with 48 h oleate infusion, which did not induce

significant insulin resistance, decreased GSIS during 2 step hyperglycemic clamps in rats (14). The fat emulsions were freshly prepared (209) as in our previous studies (210) and protected from light. The resveratrol (RSV; Sigma, St. Louis) was prepared as in previous studies in our lab (211). Briefly, resveratrol was dissolved in 20% cyclodextrin (dissolved in saline; Sigma, St. Louis) and the pH was adjusted to 7.4. The solution was protected from light.

2.1.2.2 Mouse Infusion Period and Solutions

Mice were infused for 48 h with either 1) Saline (SAL, 0.5 μl/min) or 2) Oleate (OLE, 0.4 μmol/min). During the 48 h infusions the mice had free access to food and water. Samples for FFA, glucose, and insulin were taken at 0 and 46 hours after the onset of infusion via tail vein. One-step hyperglycemic clamp studies were performed in conscious mice fasted for 4 hours at the end of the 48 h infusion period.

The oleate was prepared as described in 2.1.2.1 except that a 2:1 dilution was used instead of a 3:1 dilution as with the rats.

2.1.3 Hyperglycemic Clamp Protocol

2.1.3.1 Two-Step Hyperglycemic Clamp in Rats

After the 48 h infusion period, basal samples were taken over 20 min (basal period). The two-step hyperglycemic clamp was performed in conscious rats fasted overnight. GSIS was evaluated from the plasma insulin and C-peptide response to the rise in plasma glucose. Both C-peptide and insulin were measured because they are co-secreted but cleared by different mechanisms (kidney and liver). Therefore, a change in both indicates a change in secretion. Furthermore, insulin clearance may be decreased by FFA, whereas there is no effect of FFA on C-peptide clearance (212). At –20 min, the continuous arterial infusion of heparinized saline was stopped in all rats, since the same total amount of heparinized saline was used to dilute the

erythrocytes that were re-infused into the rats after plasma separation from blood samples. The venous infusion saline/treatment was continued throughout the experiment. The glucose infusion was given through the jugular catheter. Both the glucose and the saline/treatment infusion lines were connected to the jugular line through a Y shaped connector. Using an infusion of 37.5% glucose, at time = 0 min, glucose level was gradually raised and then maintained at 13 $mmol \cdot l^{-1}$ (upper physiological glucose level for rats) for 120 min. The gradual rise in glucose avoids any oleate bolus from the dead space of the infusion line which may cause heart arrhythmia and sudden death of the rat. At time = 120 min the glucose level was again raised and then maintained at 22 $mmol \cdot l^{-1}$ (maximum stimulatory levels) for another 120 min. Plasma glucose was 'clamped' at either 13 $mmol \cdot l^{-1}$ or 22 $mmol \cdot l^{-1}$ by a variable glucose infusion adjusted according to frequent (every 5-10 min) glycemic readings obtained on a Beckman 2 or Analox GM9 glucose analyzer. Samples for insulin, C-peptide and FFA were taken at regular intervals. The sample volume was minimized to avoid anemia. A total of 2.5ml of blood was withdrawn from the rats. After removal of plasma from centrifuged whole blood samples, erythrocytes were suspended in heparinized saline (4U/ml) and re-infused into the rats. FFA levels were measured by colorimetric kits (Wako and Boehringer Chemicals, resp.), rat insulin and C-peptide by RIA kits (Linco Research Inc, MO). In each experiment, an index of insulin sensitivity was obtained from the hyperglycemic clamp data. At the end of the experiment, the rats were deeply anaesthetized and the pancreas was excised for histochemical analysis. The pancreas was fixed immediately in 10% formalin overnight, and then transferred to 70% ethanol. Samples were processed and embedded in paraffin within 7 days of collection.

2.1.3.2 One-Step Hyperglycemic Clamp in Mice

After the 48 h infusion period, basal samples were taken over 20 min (basal period). Thereafter, the hyperglycemic clamp was performed to evaluate GSIS from the plasma insulin and C-peptide response to the rise in plasma glucose. The venous infusion of saline/treatment was continued throughout the experiment. The glucose infusion was given through the jugular catheter. Both the glucose and the saline/treatment infusion lines were connected to the jugular line through a Y shaped connector. Using an infusion of 37.5% glucose, at time = 0 min, glucose level was gradually raised and then maintained at 22 $mmol \cdot l^{-1}$ (maximum stimulatory levels) for 120 min. Plasma glucose was 'clamped' at 22 $mmol \cdot l^{-1}$ by a variable glucose infusion adjusted according to frequent (every 10 min) glycemic readings obtained on a Hemocue glucose analyzer (HemoCue, Lake Forest, CA) from tail vein blood samples. Samples for insulin, C-peptide and FFA were taken during the last 20 minutes of the hyperglycemic clamp. The sample volume was minimized to avoid anemia. A total of 0.3 ml of blood was withdrawn from the mice. FFA levels were measured by colorimetric kits (Wako and Boehringer Chemicals, resp.), mouse insulin and C-peptide by RIA kits (Linco Research Inc, MO). In each experiment, an index of insulin sensitivity and a Disposition Index (see 2.3.2) was obtained during the last 30 minutes of the hyperglycemic clamp data. At the end of the experiment, the mice were deeply anaesthetized and the pancreas was excised for histochemical analysis. The pancreas was fixed immediately in 10% formalin overnight, and then transferred to 70% ethanol. Samples were processed and embedded in paraffin within 7 days of collection.

2.2 Laboratory Methods

2.2.1 Plasma Glucose

In rats, plasma glucose concentrations were measured by the glucose oxidase method using a Beckman Glucose Analyzer II (Beckman, Fullerton, CA) or Analox GM9 Analyzer (Analox Instruments, London, UK). A 10µl sample of plasma containing D-glucose is pipetted into a solution containing oxygen and glucose oxidase. The glucose reacts with oxygen in the following reaction catalyzed by glucose oxidase:

$$\textbf{D-glucose} + O_2 + H_2O \text{ ----------> } \textbf{Gluconic Acid} + H_2O_2$$

In the reaction, oxygen is used at the same rate as glucose to form gluconic acid. A polarographic oxygen sensor is used to detect oxygen consumption, which is directly proportional to the glucose concentration in the sample. Results are obtainable within 30 seconds following sample addition. Plasma samples were reanalyzed until repeated measurements were within a difference of 3 $mg.dL^{-1}$. The analyzer was calibrated before use and frequently during the experiment with the 150/50 glucose/urea nitrogen standard (Beckman Instruments Inc., Nguabo, Puerto Rico, USA) or 144/50 standard (Analox Instruments, London, UK) that accompanied each kit.

In mice, the glucose concentration in whole blood was measured using the Hemocue Glucose 201^+ System (HemoCue, Lake Forest, CA). Approximately 5µl of blood is required per sample. Glucose is analyzed first through hemolysis of erythrocytes and conversion of α-D-glucose to β-D-glucose, which is then oxidized by glucose dehydrogenase (GDH) in the presence of NAD^+ to form NADH. Thus the amount of NADH produced is proportional to amount of glucose in the sample. Using diaphorase as a catalyst, a tetrazolium salt is formed to a coloured formazan in the presence of NADH. This coloured formazan is then quantified photometrically at 660nm and 840nm.

2.2.2 Plasma Insulin Assay

Radioimmunoassay (RIA) kit specific for rat insulin from Linco Research Inc. (St. Charles, MO, USA) were used to determine plasma and islet insulin concentrations. This kit has 100% reactivity to rat insulin I and II. Cross-reactivity to rat proinsulin has not been tested. Insulin in the sample competes with a fixed amount of ^{125}I-labelled insulin for the binding sites on the specific antibodies. A standard curve was generated using insulin standards at 0, 3, 10, 30, 100, 240 μU/ml in duplicate. ^{125}I-labelled and rat insulin antibody were mixed with plasma sample. The tubes were then vortexed and incubated overnight at 4^{o}C. Precipitating reagent was added to all tubes followed by vortexing and incubating for 30 minutes at 4^{o}C. The tubes were then spun at 3000g for 30 minutes. The supernatant was aspirated and the radioactivity in the pellet was counted for 4 minutes in a gamma counter (Beckman Instruments, Fullerton Ca, USA). The counts (B) for each of the standards and unknowns were expressed as a percentage of the mean counts of the "0 = standard" (Bo):

% activity bound = B (Standard or Sample)/Bo x 100%

The % activity bound for each standard was plotted against the known concentration in order to construct the standard curve. The unknown sample concentration was determined by interpolation of the standard curve. The coefficient of interassay variation determined on reference plasma was less than 10%.

2.2.3 Plasma C-Peptide Assay

Rat C-peptide RIA kit was used to determine plasma C-peptide levels. The kit uses an antibody specific for rat C-peptide (Linco Research, Inc, St. Charles, MO, USA), with no crossreactivity to rat insulin I and II. Cross-reactivity to rat proinsulin has not been tested. The principle is the same as insulin RIA as described above. The procedures are the same as insulin

RIA with the exception of one extra day. In the first day, only rat C-peptide antibody was added followed by an overnight incubation at 4^{o}C. In the second day, ^{125}I-rat C-peptide was added followed by vortexing and overnight incubation at 4^{o}C. In the last day, precipitating reagent was added followed by vortexing and incubation for 20 minutes at 4^{o}C. Then, the tubes were centrifuged at 3000rpm for 30 minutes. The supernatant was then aspirated and the radioactivity in the pellet was counted for 1 minute in a gamma counter. The % activity bound was calculated in the same manner as insulin RIA. The % activity bound for each standard was plotted against the known concentration to obtain standard curve. The unknown concentrations of the samples were determined by interpolation of the standard curve. The coefficient of interassay variation determined on reference plasma was less than 10%.

2.2.4 Plasma Free Fatty Acid Assay

Plasma levels of FFA were analyzed using a colorimetric kit under enzymatic reaction from Wako Industrials (Neuss, Germany). The method relies upon the acylation of coenzyme A by the fatty acids in the presence of added acyl-CoA synthetase (ACS). The acyl-CoA produced is oxidized by adding acyl-CoA oxidase (ACOD), which generates H_2O_2. H_2O_2, in the presence of peroxidase (POD) permits the oxidative condensation of 3-methyl-N-ethyl-N-(B-hydroxyethyl)-aniline (MEHA) with 4-aminophenazone to form the final reaction product, which is a purple coloured adduct. This can be measured colorimetrically at 550 nm. The results are correct to within 1.1%. The reactions of this assay are listed below:

ACS

FFA + ATP + CoA ------> Acyl-CoA + AMP + PPi

ACOD

Acyl-CoA + O_2 -----> 2,3-trans-Enoly-CoA + H_2O_2

POD

2 H_2O_2 + 4-aminoantipyrine + MEHA -----> Final Reaction Product + 3 H_2O

2.2.5 Western Blotting

Pancreatic islets were isolated as described in 2.1.1.3. Approximately 100-150 isolated mouse islets were hand-picked and washed with PBS prior to lysis directly in RIPA buffer containing protease inhibitor cocktail (Roche Diagnostics, Laval, QC). The cell lysates were then spun at 12,000rpm and the supernatant was loaded onto 4-15% SDS-PAGE gradient gel (Biorad, Hercules, CA) and transferred onto PVDF membrane using the Turbo Blotter Transfer System (Biorad, Hercules, CA). The membrane was probed with anti-SIRT1 antibody (Thermo Scientific) at a 1:500 dilution and imaged using a Kodak imager 4000pro and Carestream Imaging Software (Carestream, Rochester, NY). Images were then quantified using Image J software.

2.2.6 RT-PCR and real-time PCR

Pancreatic islets were isolated as described in 2.1.1.3. Approximately 50-100 isolated mouse islets were hand-picked and washed with PBS. The total RNA was extracted from isolated mouse islets using an RNeasy Kit (Qiagen) and converted to cDNA using SuperScript II reverse transcriptase. The real-time PCR was performed as previously described (213). RT-PCR was performed using platinum Taq DNA polymerase on the Dual Block DNA Engine Thermal

Cycler (MJ Research, Inc., MA, USA). The software used for real-time PCR primers is Primer Express (Applied Biosystems) and for RT-PCR primers is Primer3.

2.3 Calculations

2.3.1 Insulin Sensitivity Index

The insulin sensitivity index (M/I) was calculated at individual time points during the last 30 minutes of each step of the hyperglycemic clamp according to the following formula:

$$\mathbf{M/I=GINF/Insulin}$$

where **GINF** is the rate of glucose infusion, Insulin is the insulin concentration, and Glucose is the plasma glucose level at individual time points during the last 30 minutes of each step of the hyperglycemic clamp. This equation assumes that the change in glucose uptake and production induced by a change in insulin concentration is proportional to the ambient insulin concentration. M/I is reported in units of decilitre per kilogram per minute per microunit per millilitre. Unfortunately, there are limitations to using this method to assess insulin sensitivity at elevated insulin levels. It has been reported that the relationship between circulating insulin levels and insulin action is not linear at high insulin concentrations (214).

2.3.2 Disposition Index

In vivo, there is a hyperbolic relationship between insulin sensitivity and insulin resistance. To account for changes in sensitivity, insulin secretion *in vivo* should be assessed by taking into account β-cell compensation for insulin resistance. The disposition index (DI), which was used as an index of insulin secretion corrected for the ambient degree of insulin resistance, was calculated at each step of the hyperglycemic clamp according to the following formula:

$$\mathbf{DI = M/I * C\text{-}peptide}$$

where M/I is calculated as described above during the last 30 minutes, and C-peptide is the C-peptide concentration at individual time points during the last 30 minutes of each step of the hyperglycemic clamp.

2.3.3 Insulin Clearance

The C-peptide to insulin ratio was used as an index of insulin clearance. The C-peptide level was divided by the insulin level at each time point in the last 40 minutes of each step of the hyperglycemic clamp, and the average ratio was calculated.

2.4 Statistical Analysis

Data are presented as means +/- SE. One way non parametric analysis of variance (ANOVA) for repeated measurements followed by Tukey's t test was used to compare differences between treatments. Calculations were performed using SAS (Cary, NC).

Chapter 3
Results

3.1 Studies in Rats

3.1.1 Plasma Free Fatty Acid (FFA) Levels

Basal plasma FFA levels did not differ between groups prior to the start of the 48 h infusion period (Table 3.1). Following the 48 h infusions, plasma FFA levels of both the OLE and OLE+RSV groups were significantly elevated ($p<0.01$) compared to the SAL control and RSV alone groups (Table 3.1). During the clamp period, plasma FFA levels in all groups decreased compared to basal (pre-clamp) levels (Figure 3.1). This decrease is expected and due to the effect of hyperglycemia and hyperinsulinemia and subsequent tissue response to decrease lipolysis and uptake plasma FFA. Throughout the clamp, plasma FFA levels in the OLE infused group remained significantly higher than both the SAL and RSV alone groups. The FFA levels in the OLE+RSV group remained significantly different from the SAL and RSV alone groups for part of the clamp but this significance was lost towards the end of the clamp period. There was no significant difference in FFA levels between the SAL and RSV alone groups during the clamp period.

Table 3.1

Group	Time (h)	FFA (µmol/l ± SEM)	Significance (vs SAL)
SAL	0	634 ± 83	-
	48	660 ± 62	-
OLE	0	663 ± 101	NS
	48	1026 ± 97	$p < 0.01$
OLE+RSV	0	633 ± 39	NS
	48	995 ± 63	$p < 0.01$
RSV Alone	0	615 ± 92	NS
	48	711 ± 56	NS

Table 3.1. Plasma FFA levels during the 48 h infusion period. Rats were infused with: 1) Saline (SAL, n=6), 2) Oleate alone (OLE, n=7) at 1.4µmol/min, 3) Oleate + Resveratrol (RSV, 0.025mg/kg.min, n=6) and 4) RSV alone (0.025mg/kg.min, n=8). Data are means ± SEM.

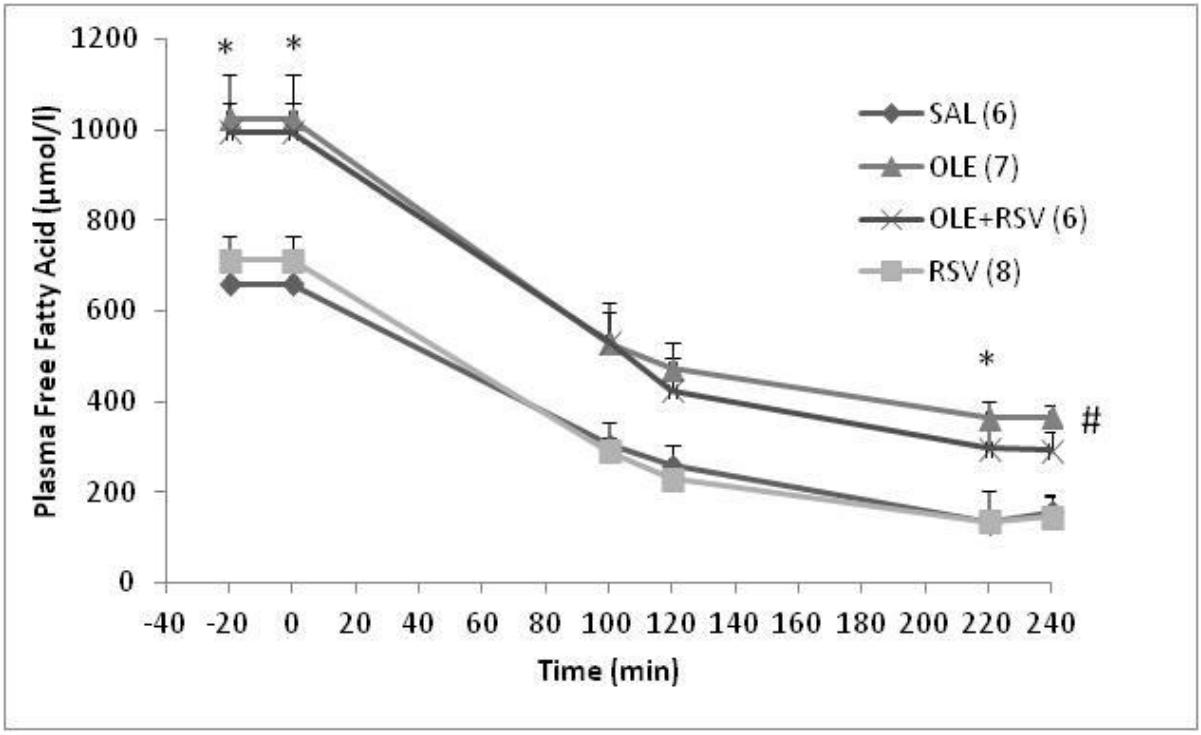

Figure 3.1. Plasma FFA levels during the two-step hyperglycemic clamp. Rats were infused with: 1) Saline (SAL, n=6), 2) Oleate alone (OLE, n=7) at 1.4µmol/min, 3) Oleate + Resveratrol (RSV, 0.025mg/kg.min, n=6) and 4) RSV alone (0.025mg/kg.min, n=8). Data are means ± SEM. # $p < 0.05$ at all points for OLE group vs. both SAL and RSV alone. * $p < 0.05$ for OLE+RSV group vs. both SAL and RSV alone.

3.1.2 Two-step Hyperglycemic Clamps

After infusion of treatment for 44 h, a two-step hyperglycemic clamp was performed to assess β-cell function, while continuing treatment infusions until completion of the clamp. During this time, blood glucose was taken every 10 minutes and plasma samples were taken for insulin, c-peptide and FFA measurements (see 2.1.3.1 for full details).

Basal plasma glucose levels prior to the start of the clamp did not significantly differ between groups (Figure 3.2). At time 0 min, a 37.5% glucose infusion was started and plasma glucose was gradually raised to 13mM until 120 min and then to 22mM until 240 min. There were no significant differences in plasma glucose levels between groups during the clamp (Figure 3.2).

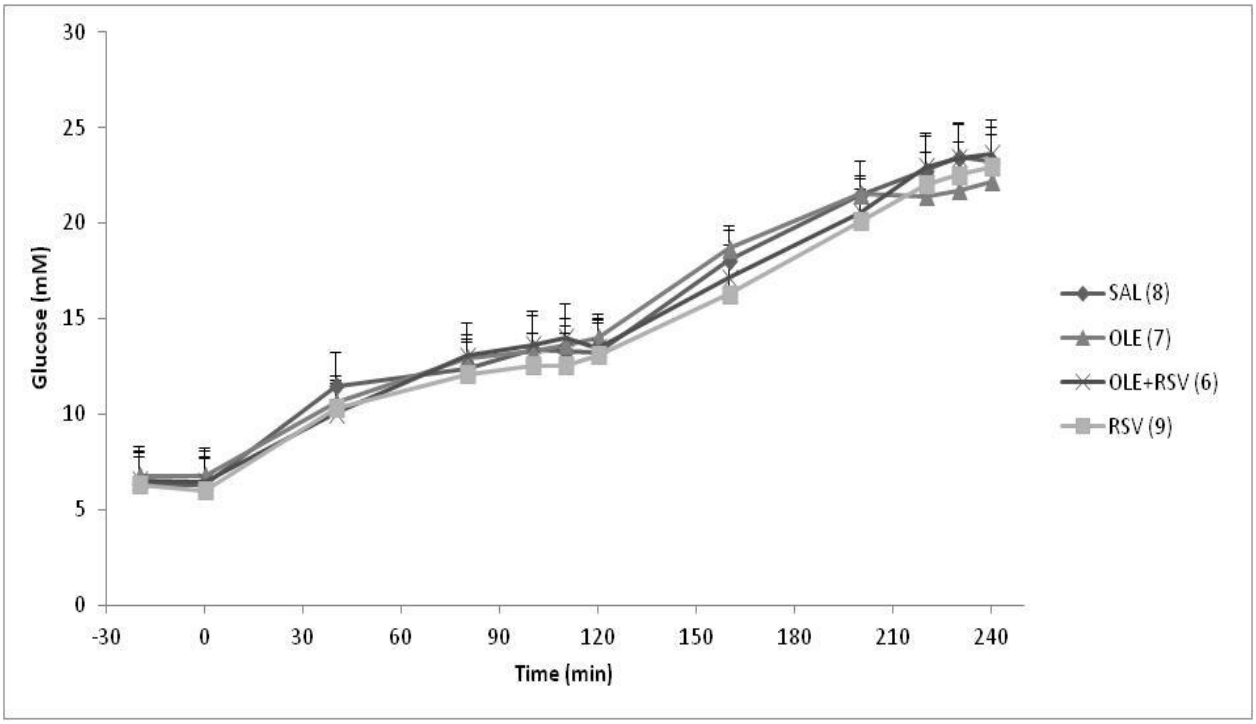

Figure 3.2. Plasma glucose levels during the two-step hyperglycemic clamp. Rats were infused with: 1) Saline (SAL, n=8), 2) Oleate alone (OLE, n=7) at 1.4μmol/min, 3) Oleate + Resveratrol (RSV, 0.025mg/kg.min, n=6) and 4) RSV alone (0.025mg/kg.min, n=9). Data are means ± SEM.

The glucose infusion rate (Ginf), which reflects the amount of glucose infused to reach and maintain a steady state of glucose, was significantly lower during both stages of the clamp in OLE infused rats compared to both the SAL controls and RSV alone groups (Figure 3.3). The group infused with OLE+RSV had a partially restored Ginf as evident through an intermediate Ginf compared to the SAL and OLE groups. There were no significant differences between SAL and RSV alone.

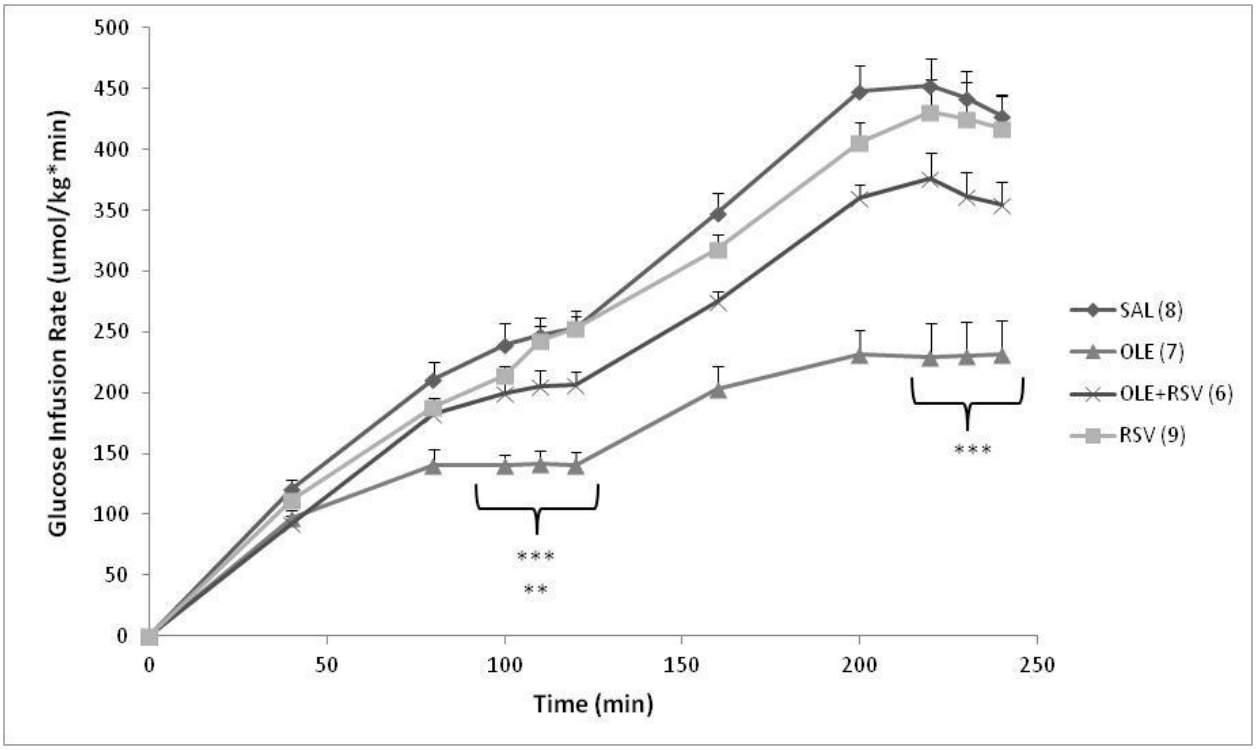

Figure 3.3. Glucose infusion rate (Ginf) during the two-step hyperglycemic clamp. Rats were infused with: 1) Saline (SAL, n=8), 2) Oleate alone (OLE, n=7) at 1.4µmol/min, 3) Oleate + Resveratrol (RSV, 0.025mg/kg.min, n=6) and 4) RSV alone (0.025mg/kg.min, n=9). Significance is indicated during the last 30 minutes of each clamp stage. Data are means ± SEM. *** $p<0.001$ vs SAL, ** $p<0.01$ vs RSV alone

Basal plasma insulin and c-peptide levels were similar in all groups prior to the start of the clamp (-20 min; Figure 3.4A and 3.4B). Upon starting the clamp, the rising glucose levels caused an increase in both plasma insulin and c-peptide levels as expected, reflecting increased secretion (due to agreement in both insulin and c-peptide response). Similar to the Ginf, both insulin and c-peptide levels were significantly lower in the OLE group at both stages compared to the SAL control. There were no significant differences between the SAL, RSV alone or OLE+RSV groups.

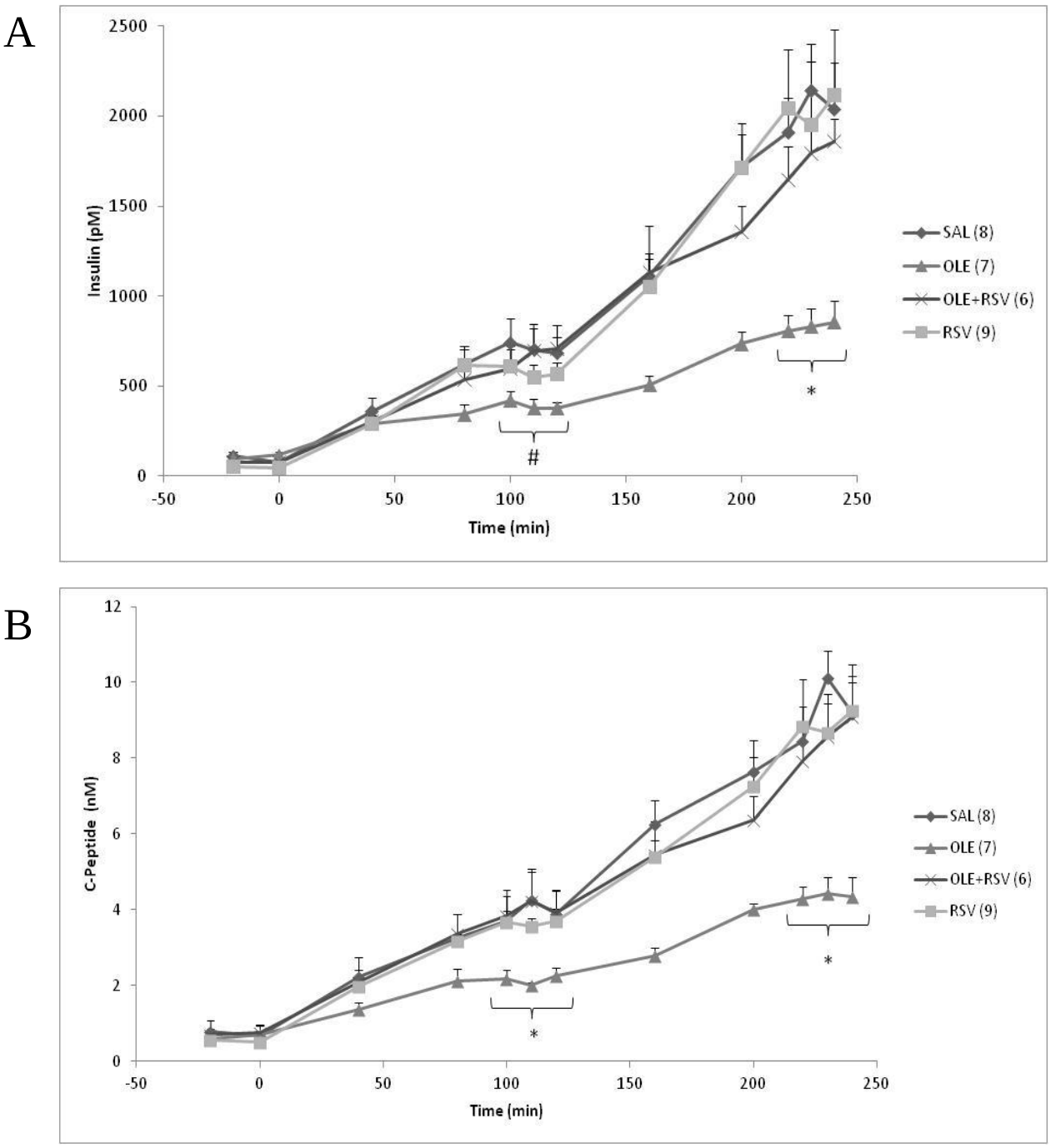

Figure 3.4. Plasma insulin and c-peptide during the two-step hyperglycemic clamp. Rats were infused with: 1) Saline (SAL, n=8), 2) Oleate alone (OLE, n=7) at 1.4µmol/min, 3) Oleate + Resveratrol (RSV, 0.025mg/kg.min, n=6) and 4) RSV alone (0.025mg/kg.min, n=9). Significance is indicated during the last 30 minutes of each clamp stage. Data are means ± SEM. * $p<0.05$ vs both SAL and RSV alone, # $p<0.05$ vs SAL

The sensitivity index (M/I index, see section 2.3.1 for details) was not significantly different between any groups at either 13mM or 22mM (Figure 3.5A). The disposition index (DI), which more accurately reflects β-cell function in cases of insulin resistance (see 2.3.2), was significantly lower in the OLE group compared to both SAL and RSV alone at both 13mM and 22mM (Figure 3.5B). Since there were no changes in the sensitivity index, the decreased DI corresponds with the Ginf and insulin or c-peptide levels and reflects a decrease in β-cell function in the OLE group compared to SAL. The OLE+RSV group had a partially restored DI that was not significantly different versus any other group. There were no significant differences between SAL and RSV alone.

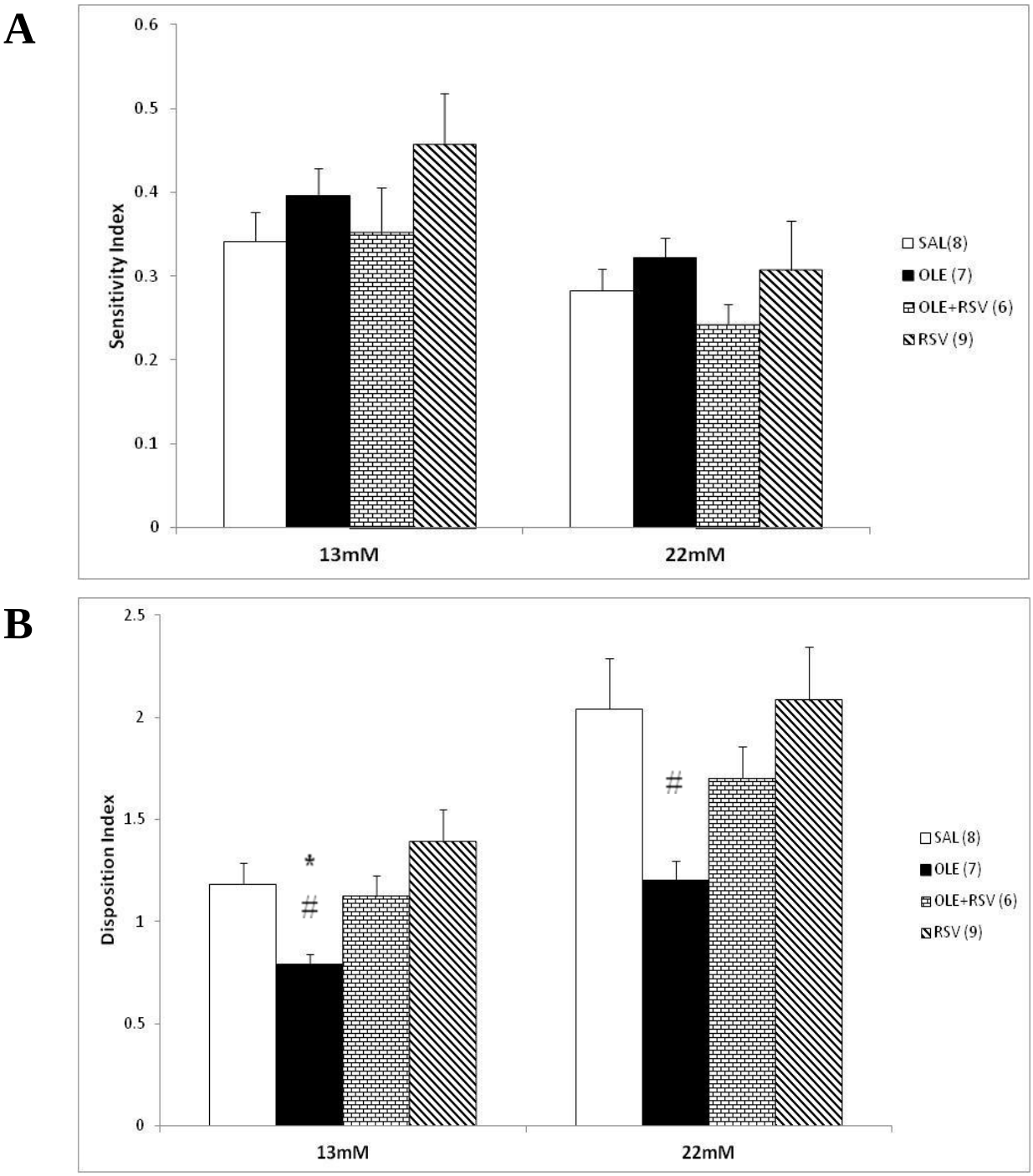

Figure 3.5. Sensitivity and Disposition Indices during the two-stage hyperglycemic clamp. Rats were infused with: 1) Saline (SAL, n=8), 2) Oleate alone (OLE, n=7) at 1.4μmol/min, 3) Oleate + Resveratrol (RSV, 0.025mg/kg.min, n=6) and 4) RSV alone (0.025mg/kg.min, n=9). (**A**) Sensitivity Index. (**B**) Disposition Index. Data are means ± SEM. # $p<0.05$ vs. SAL and RSV alone, * $p<0.01$ vs. SAL

Due to the different mechanisms by which insulin and c-peptide are cleared from the blood, a Clearance Index was calculated to examine any significant changes in clearance. This was calculated by dividing the plasma c-peptide values by their corresponding insulin values at each point. The averages during each stage are shown in Figure 3.6. There were no significant differences between groups during either stage of the clamp.

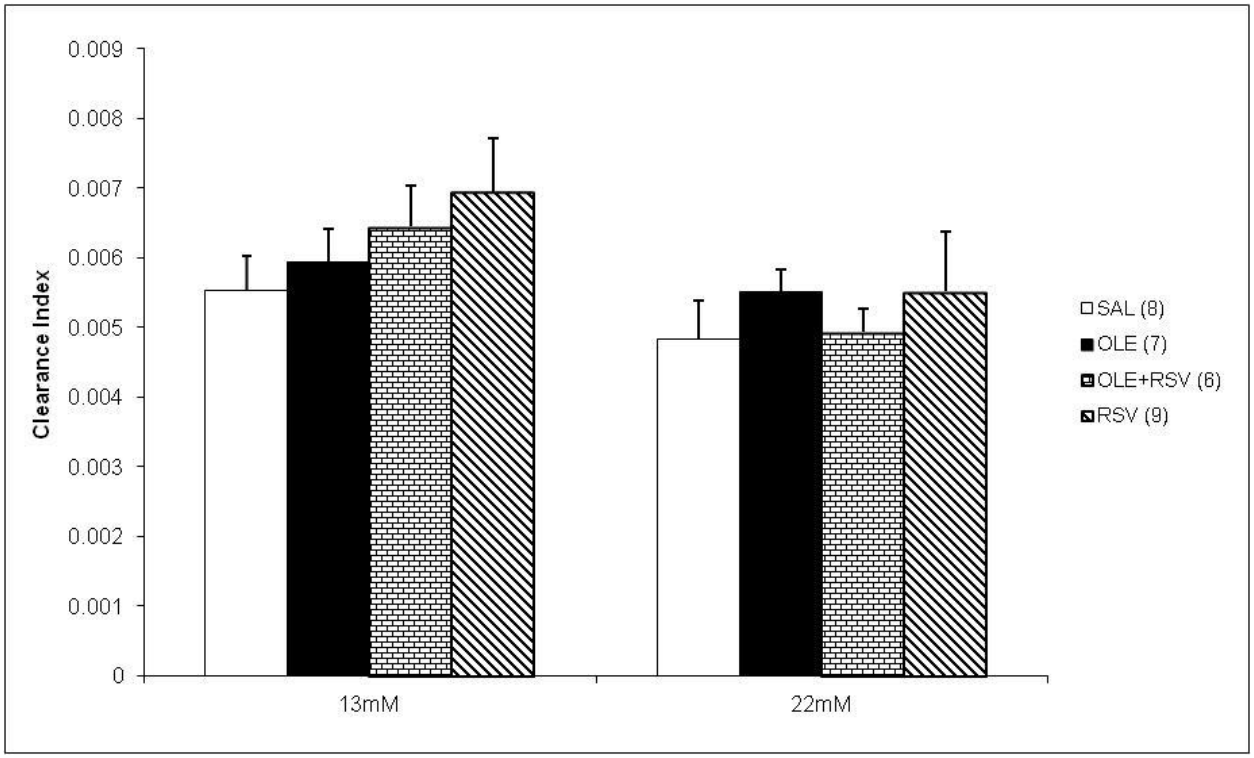

Figure 3.6. Clearance Index during the two-stage hyperglycemic clamp. Rats were infused with: 1) Saline (SAL, n=8), 2) Oleate alone (OLE, n=7) at 1.4µmol/min, 3) Oleate + Resveratrol (RSV, 0.025mg/kg.min, n=6) and 4) RSV alone (0.025mg/kg.min, n=9). Data are means ± SEM.

3.2 Studies in Mice

3.2.1 Genotype and Overexpression Data

Mice genotypes were verified through tail clips. Tail samples were submitted to the Centre for Applied Genomics at The Hospital for Sick Children for genotyping. Bands were compared against a positive control (CON) and bright bands were identified as transgenic mice (Tg) and mice with an absent band were identified as wildtype (WT) littermates (Figure 3.6).

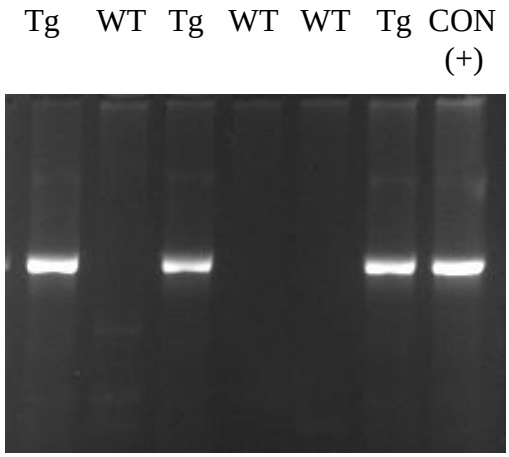

Figure 3.7. Representative Mouse Genotype Blot. Genotypes were assessed at weaning through tail DNA. BESTO primer sequences were obtained from (92).

SIRT1 overexpression levels were verified in transgenic BESTO mice compared to their WT littermates through qPCR and western blot (Figure 3.7A and 3.7B). qPCR data revealed that SIRT1 mRNA was overexpressed about 16 fold in transgenic mice compared to their WT littermates (Figure 3.7A). Western blot data showed much higher SIRT1 protein levels in transgenic mice compared to WT littermates (Figure 3.7B). Quantification of the data from 3 individual experiments revealed that SIRT1 protein was overexpressed by about 15 fold in BESTO mice versus WT littermates (data not shown).

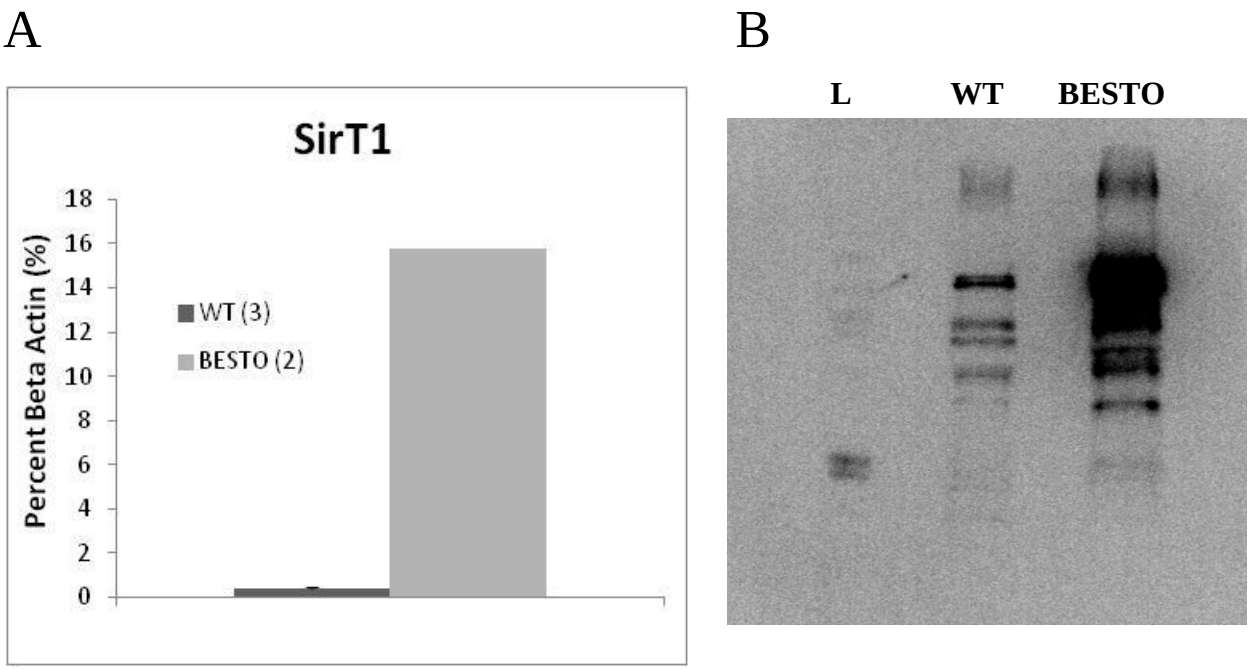

Figure 3.8. SIRT1 overexpression levels. (A) SIRT1 mRNA. (B) SIRT1 protein. SIRT1 mRNA was assessed through qPCR and BESTO mice revealed approximately 16 fold higher levels compared to WT mice. Protein levels were assessed through western blot and revealed approximately 15 fold higher protein levels compared to WT mice. A representative blot is shown in (B), 'L' indicates the protein ladder band.

3.2.2 Plasma Free Fatty Acid (FFA) Levels

Basal plasma FFA levels did not differ between groups prior to the start of the 48 h infusion period (Table 3.2). Following the 48 h infusions, plasma FFA levels of both the WTOLE and TGOLE groups were significantly elevated ($p<0.05$) compared to the WTSAL and TGSAL groups (Table 3.2). During the clamp period, plasma FFA levels in all groups decreased compared to basal (pre-clamp) levels (Figure 3.2). Throughout the clamp, plasma FFA levels in the WTOLE and TGOLE infused groups remained significantly higher than both the WTSAL and TGSAL groups. There was no significant difference in FFA levels between the WTSAL and TGSAL groups or the WTOLE and TGOLE groups during either the basal or clamp periods.

Table 3.2

Group	Time (h)	FFA (µmol/l ± SEM)	Significance (vs. WTSAL/ TGSAL)
WT SAL	0	1011 ± 160	-
	48	1354 ± 169	-
WT OLE	0	1281 ± 110	NS
	48	2222 ± 167	$p<0.05/p<0.01$
TG OLE	0	1299 ± 174	NS
	48	2229 ± 155	$p<0.01/p<0.001$
TG SAL	0	1161 ± 178	NS
	48	1319 ± 80	NS

Table 3.2. Plasma FFA levels during the 48 h infusion period. BESTO mice or wildtype (WT) littermates were treated for 48 h with either 1) saline (WT SAL, n=6; TG SAL, n=7) or 2) oleate (WT OLE, n=8; TG OLE, 0.4 µmol/min, n=8). Data are means ± SE.

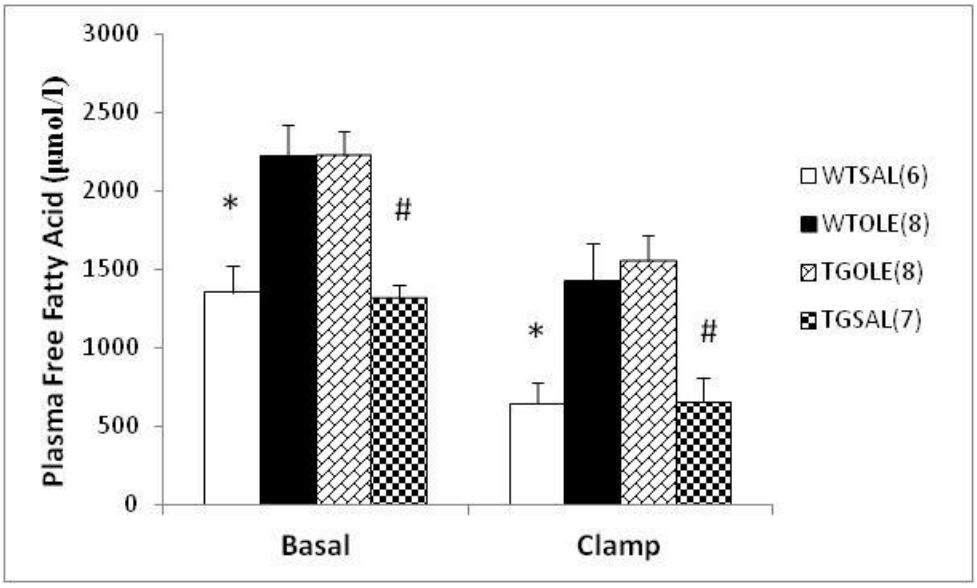

Figure 3.9. Plasma FFA levels prior to and during the one-step hyperglycemic clamp. BESTO mice or wildtype (WT) littermates were treated for 48 h with either 1) saline (WT SAL, TG SAL) or 2) oleate (WT OLE, TG OLE, 0.4 µmol/min). Data are means ± SEM. *$p<0.05$ vs. WT SAL, #$p<0.01$ vs. TG SAL.

3.2.3 One-step Hyperglycemic Clamps

After infusion of treatment for 46 h, a one-step hyperglycemic clamp was performed to assess β-cell function, while continuing treatment infusions until completion of the clamp. During this time, blood glucose was taken every 10 minutes and plasma samples were taken for insulin, c-peptide and FFA measurements at 0, 100 and 120 minutes (see 2.1.3.2 for full details).

Basal plasma glucose levels prior to the start of the clamp were slightly lower in both oleate infused groups compared to both saline control groups (Figure 3.9). There were no differences between the two saline groups or between the two oleate groups. At time 0 min, a 37.5% glucose infusion was started and plasma glucose was gradually raised to 22mM until 120 min. There were no significant differences in plasma glucose levels between groups during the clamp (Figure 3.9).

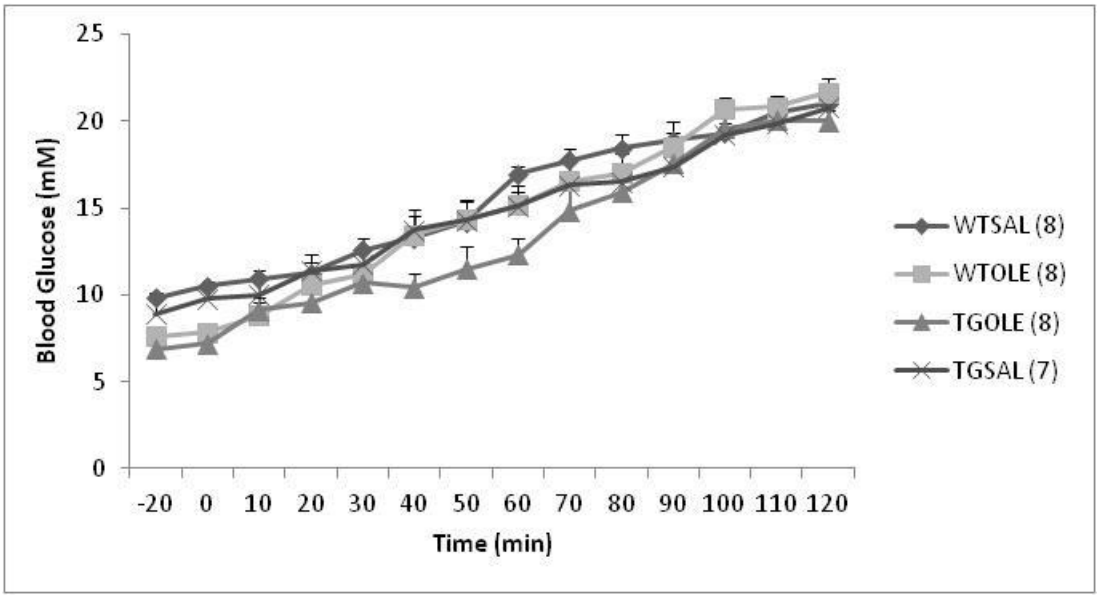

Figure 3.10. Rise in plasma glucose levels during the one-step hyperglycemic clamp. BESTO mice or wildtype (WT) littermates were treated for 48 h with either 1) saline (WT SAL, TG SAL) or 2) oleate (WT OLE, TG OLE, 0.4 μmol/min). Clamp glucose levels were determined by taking the average glucose during the last 30 minutes of the clamp. Data are means ± SEM.

The glucose infusion rate (Ginf) was significantly lower in the WT OLE group compared to all other groups (Figure 3.10). The TG OLE group was partially protected from the oleate induced decrease in Ginf. The TG OLE group was not significantly different from either the WT SAL or TG SAL groups. There was no significant difference between the WT SAL and TG SAL groups.

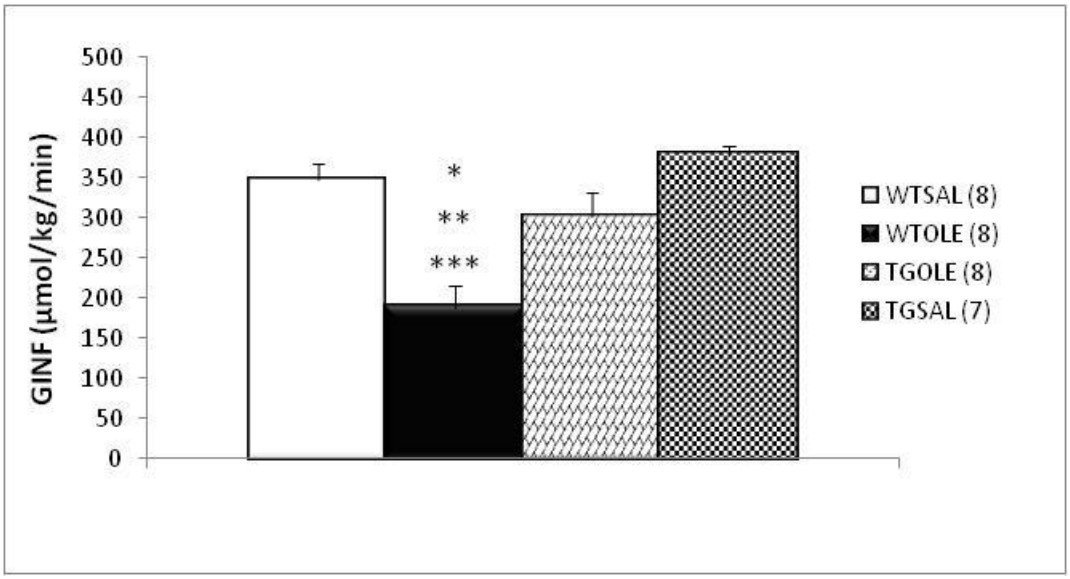

Figure 3.11. Glucose infusion rate (Ginf) during the one-step hyperglycemic clamp. BESTO mice or wildtype (WT) littermates were treated for 48 h with either 1) saline (WT SAL, TG SAL) or 2) oleate (WT OLE, TG OLE, 0.4 µmol/min). The Ginf rate was determined by taking the average rate during the last 30 minutes of the clamp. Data are means ± SEM. *p<0.05 vs. all, **p<0.01 vs. WT SAL, ***p<0.001 vs. TG SAL.

Basal plasma insulin and c-peptide levels were similar in all groups prior to the start of the clamp (Figure 3.11A-B). During the clamp, both plasma insulin and c-peptide levels were elevated compared to basal levels across all groups. Compared to both SAL treated groups, the OLE treated groups had significantly higher insulin and c-peptide levels. This elevation is due to

insulin resistance induced by oleate treatment. There were no significant differences between the two SAL treated groups or the two OLE treated groups in either insulin or c-peptide.

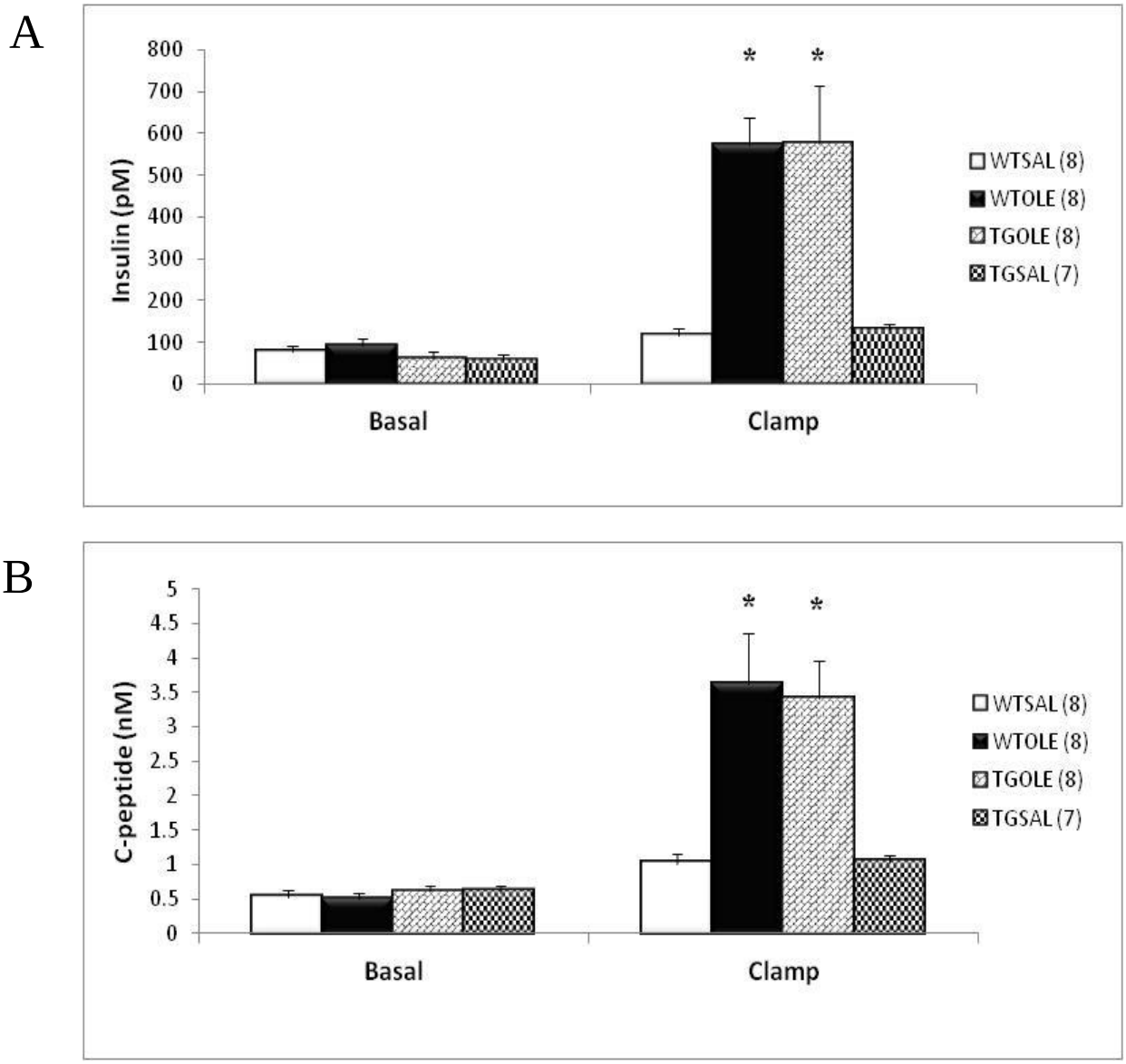

Figure 3.12. Plasma insulin and c-peptide levels during the one-step hyperglycemic clamp. BESTO mice or wildtype (WT) littermates were treated for 48 h with either 1) saline (WT SAL, TG SAL) or 2) oleate (WT OLE, TG OLE, 0.4 μmol/min). (**A**) Plasma insulin. (**B**) Plasma c-peptide. Clamp insulin and c-peptide levels were determined by taking the average of the 100 and 120 minute plasma samples. Data are means ± SEM. *$p<0.001$ vs. both WT SAL and TG SAL.

The sensitivity index (M/I index, see section 2.3.2 for details) was significantly lower in both OLE treated groups compared to the SAL treated groups (Figure 3.12A). This is in line with the elevated insulin and c-peptide levels in both OLE treated groups and reflects the decreased sensitivity caused by oleate treatment. The TG OLE group had slightly improved sensitivity compared to the WT OLE group however this difference was not significant. There was no significant difference in sensitivity between the WT SAL and TG SAL groups. The disposition index (DI) was significantly lower in the WT OLE group compared to both the WT SAL and TG SAL groups (Figure 3.12B). In this case, the DI more accurately reflects β-cell function compared to insulin/c-peptide levels since it takes into account the insulin resistance caused by the oleate treatment. The TG OLE group was partially protected from the oleate induced β-cell dysfunction as evident through an intermediate DI compared to both saline groups. There was no significant difference between the TG OLE group and any other group. There was also no significant difference between the two saline groups.

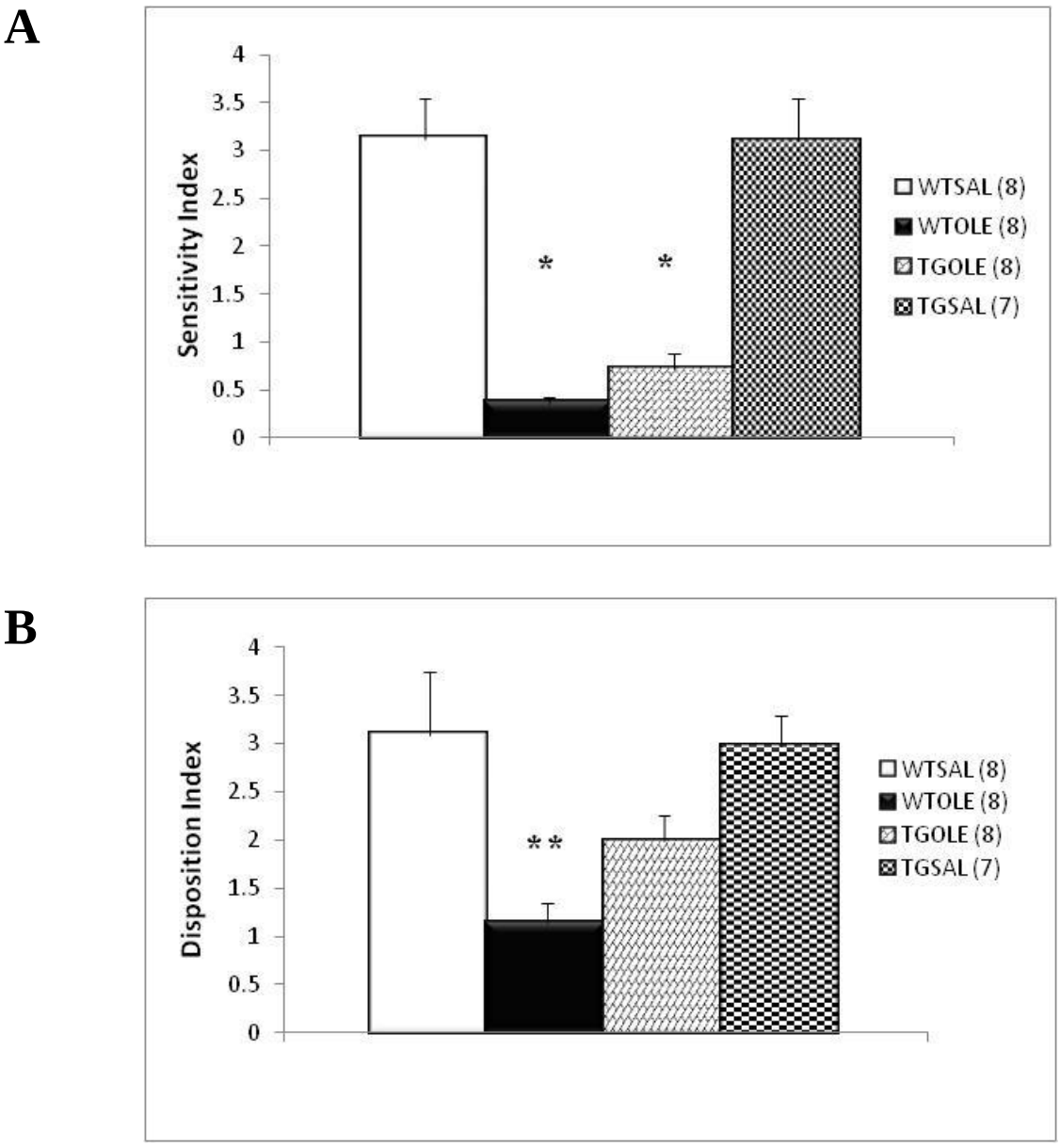

Figure 3.13. Sensitivity and Disposition Indices during the one-step hyperglycemic clamp. BESTO mice or wildtype (WT) littermates were treated for 48 h with either 1) saline (WT SAL, TG SAL) or 2) oleate (WT OLE, TG OLE, 0.4 μmol/min). (**A**) Sensitivity index. (**B**) Disposition index. Both indices were calculated by taking the average of the 100 and 120 minute values. Data are means ± SE. **p<0.01, *p<0.001 vs. both WT SAL and TG SAL.

Due to the different mechanisms by which insulin and c-peptide are cleared from the blood, a Clearance Index was calculated to examine any significant changes in clearance. This was calculated by dividing the plasma c-peptide values by their corresponding insulin values at each point. The averages during each stage are shown in Figure 3.13. There were no significant differences between groups during the clamp.

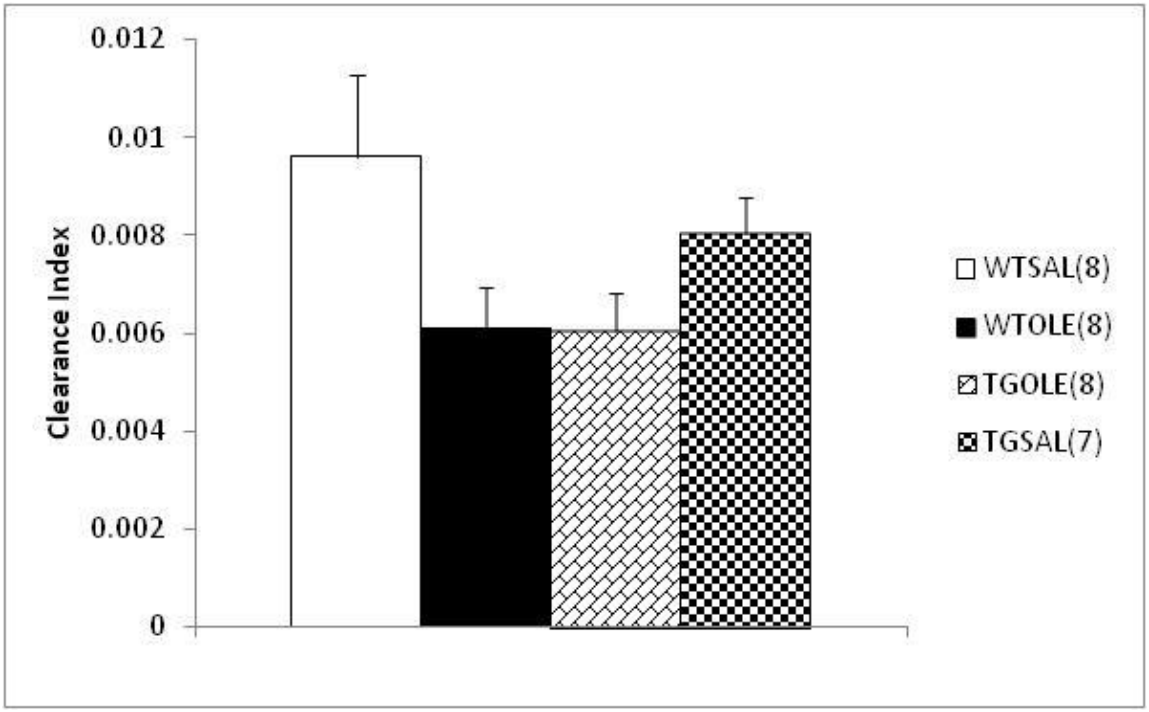

Figure 3.14. Clearance index during the one-step hyperglycemic clamp. BESTO mice or wildtype (WT) littermates were treated for 48 h with either 1) saline (WT SAL, TG SAL) or 2) oleate (WT OLE, TG OLE, 0.4 μmol/min). The index was calculated by taking the average of the 100 and 120 minute values. Data are means ± SE.

Chapter 4
Discussion

In the decade or so since research on sirtuins really emerged, enormous strides have been made in understanding the roles of these molecules, but much remains to be uncovered. Of all the mammalian sirtuins SIRT1 has received the most attention, spawning from its ability to extend the lifespan of yeast. It has since been found to have key metabolic roles in various tissues, with new information literally emerging weekly. SIRT1 has also been implicated in numerous diseases, but determining its exact role in pathogenesis or even successfully targeting it generally remains elusive. Here we have tried to further understand the role of SIRT1 in pancreatic β-cells by observing how enhancing its activity affects β-cell function in conditions of lipotoxicity-mediated β-cell dysfunction.

4.1 Studies in Rats

Within the literature, there is ample evidence that resveratrol and SIRT1 can be beneficial towards improving β-cell function and more generally metabolic parameters. Early studies that simply supplemented resveratrol through diet to animals on high fat or high calorie diets saw dramatic improvements in insulin sensitivity and glucose tolerance along with reduced oxidative stress among many other benefits (134;145;185). Although controversy remains as to whether resveratrol activates SIRT1 directly or indirectly (141-144), resveratrol is still generally considered an activator of SIRT1. More specific to β-cells, resveratrol has been shown in other studies to enhance GSIS and increase cellular ATP levels as well (147;189). There is also evidence that SIRT1 mRNA and protein levels can be decreased in conditions of oxidative stress, including Type 2 diabetes and obesity (128-130). Having previously established a model of lipotoxicity-mediated β-cell dysfunction, here we attempted to explore whether resveratrol could

prevent this β-cell dysfunction in rats, which would suggest that SIRT1 activity may be decreased in conditions of lipotoxicity.

In line with previously published work (157), we observed through hyperglycemic clamp that rats infused with oleate for 48 h had significant β-cell dysfunction. This was seen through decreased levels of plasma insulin and c-peptide and also a decreased glucose infusion rate (Ginf) and Disposition Index (DI) in rats treated with oleate compared to saline controls. Importantly, we observed no changes in sensitivity due to oleate infusion. Although FFA can induce insulin resistance, the lack of this finding was not unexpected since our previous studies using the same oleate infusion protocol and animal model also did not observe any significant changes in sensitivity (14). The absence of any changes in sensitivity may be due to relatively moderate elevation of monounsaturated FFA, the female sex of the rats and the use of hyperglycaemic clamp rather than the gold-standard hyperinsulinemic-euglycemic clamp. *In vivo*, insulin secretion should be assessed in the context of insulin sensitivity since the β-cell will compensate for insulin resistance by increasing insulin secretion. As a result, the Disposition Index (DI) is calculated to more accurately assess β-cell function by taking into account any changes in sensitivity (see 2.3.2). In this case, since there was no change in insulin sensitivity, the DI corresponds with the insulin and c-peptide results. In rats that were co-infused with both oleate and resveratrol we saw a partial prevention of the oleate induced β-cell dysfunction. In addition, there were no differences between saline controls and resveratrol alone. Our results suggest that resveratrol prevents the oleate-induced β-cell dysfunction.

The attention on resveratrol prompted many groups to use it to try and ameliorate the detrimental effects of a HFD. In general, our findings are in line with such studies that have demonstrated improved glucose tolerance, improved levels of plasma glucose and insulin and enhanced GSIS (134;185;188). Unfortunately, most of these HFD studies assess only glucose

tolerance without accounting for changes in insulin sensitivity and so the effect of resveratrol specifically on the β-cell is difficult to assess in these studies. However, two studies have shown that resveratrol can improve insulin secretion *in vivo*/*ex vivo* and *in vitro* after a HFD or high fat culture respectively (160;188). The study by Zhang et al (188) fed mice a HFD with or without resveratrol and attributed the beneficial effects of resveratrol to increased SIRT1 mRNA and protein levels in the HFD plus resveratrol group, compared to HFD alone, where SIRT1 mRNA and protein levels were decreased. In general, most of these studies supplementing resveratrol to a HFD attribute the actions of resveratrol on either SIRT1 or AMPK, but the mechanisms remain largely unclear due to conflicting evidence. Some groups suggest that resveratrol activates SIRT1 (134;189), whereas others suggest it activates AMPK (146;185), while both AMPK KO mice and SIRT1 KO mice are resistant to the beneficial effects of resveratrol (145;147). This mechanistic controversy is something which we have yet to examine in our model.

In interpreting these results, it is important to consider differences between our intravenous (i.v.) fat model and the HFD model. First, compared to dietary fat, our i.v. infusion model avoids the incretin effect and GI involvement that can release incretin hormones such as GLP-1 to stimulate insulin secretion. By bypassing the GI system, our model minimizes this effect and can therefore be considered more β-cell specific compared to a HFD. Furthermore, HFD models are typically more chronic compared to our 48h infusion. As a result, we may not observe some effects seen with chronic HFD treatment such as protein or mRNA changes over time. Another difference is that our infusion model roughly doubles plasma FFA levels (see 3.1.1), which may differ from HFD where lower plasma FFA levels are observed. Furthermore, in comparing HFD models to our model, expanded adipose tissue may play a larger role in HFD models through the release of cytokines and other factors.

Very recently, a study by Wu et al demonstrated decreased SIRT1 expression primarily *in vitro* through palmitate-induced β-cell dysfunction (160). They found that rat islets cultured with palmitate had reduced SIRT1 protein and mRNA levels but adding resveratrol to the culture prevented this reduction. In addition, they showed that acetylation of the SIRT1 target p53 was increased in INS-1 cells cultured in palmitate, indicating decreased SIRT1 deacetylation activity. They also showed that 24h infusion of Intralipid in rats reduced SIRT1 mRNA and protein levels, but they did not assess prevention of this decrease using resveratrol. Although this study focused on *in vitro* data, we would expect similar findings *in vivo*, especially with regards to SIRT1 expression levels and the effect of resveratrol on preventing β-cell dysfunction. An important distinction between our study and this one however, is our use of resveratrol *in vivo*, which raises the possibility of brain involvement. Recently, the hypothalamus has been implicated by many studies as having in important role in GSIS and in regulating β-cell function. One study by Osundiji et al (215) is particularly interesting since they showed that hypothalamic glucose sensing plays an important role in GSIS by infusing glucose specifically into the hypothalamic brain region and subsequently observing increased GSIS. They also showed that glucose infusion along with pharmacological inhibition of glucokinase within the hypothalamus could reduce the subsequent GSIS response. Other studies have further demonstrated that hypothalamic inflammation, which can occur in obesity, and reduction in insulin receptors within the hypothalamus can also lead to impaired GSIS (216;217). SIRT1 has been shown to be expressed and play a crucial role in both POMC and SF1 hypothalamic neurons, which help regulate appetite, body weight and glucose homeostasis. In mouse models where SIRT1 has been knocked out in POMC and SF1 neurons, both models show increased susceptibility to diet-induced obesity (103;104). However, administration of resveratrol directly to hypothalamic neurons or to the rodent brain resulted in improved insulin sensitivity and glucose homeostasis,

effects which were reversed by inhibiting or impairing SIRT1 (218;219). Interestingly, it has also been found that UCP2 mediates obesity-induced loss of glucose sensing in POMC neurons (220). These findings suggest that the hypothalamus and neuronal SIRT1 may be at least partially involved in mediating our observed effects. Unfortunately, the role of SIRT1 in the brain and hypothalamic regulation of GSIS remains poorly understood and so determining how the brain may be implicated in our model may be difficult.

Due to the controversy regarding resveratrol and SIRT1 activation, it is possible that the action of resveratrol on other molecules or the properties of resveratrol itself may be mediating some of the observed effects. One such aspect that we have not yet examined is the effect of resveratrol on oxidative stress. A number of studies have used STZ models and shown that resveratrol reduces oxidative stress and STZ-mediated β-cell destruction (195-198). Considering our model mediates β-cell dysfunction through oxidative stress it would be beneficial to examine the effect of resveratrol and determine if it does reduce oxidative stress within the β-cell. Furthermore, since resveratrol can scavenge free radicals and upregulate antioxidant enzymes via SIRT1-mediated activation of FOXO1 (152), the possibility arises that oxidative stress may be reduced by resveratrol through both SIRT1 dependent and independent mechanisms (138;195;196). Since it has been suggested that resveratrol may actually activate SIRT1 via AMPK-mediated increase in NAD (146), it is possible that SIRT3, which like all sirtuins also depends on NAD, may be activated as well. Furthermore, SIRT1 can influence SIRT3 through deacetylation and activation of PGC-1α, which positively regulates SIRT3 expression (172). Recent studies demonstrated that loss of SIRT3 negatively influenced glucose and lipid homeostasis while HFD feeding decreased levels of SIRT3 mRNA in mice (171). In addition, SIRT3 can also reduce ROS through activation of the antioxidant MnSOD through direct deacetylation (221). This raises the possibility that resveratrol may be mediating our observed

effects through SIRT3 rather than SIRT1, or possibly through both sirtuins. Furthermore, resveratrol has been shown to both activate PARP enzymes in apoptotic HepG2 cells and inhibit PARP enzymes in STZ treated rat islets (196;222). This creates an interesting competitive situation since both PARP and SIRT1 are NAD-dependent, so depending on whether resveratrol activates or inhibits PARP, SIRT1 activity may be decreased or increased respectively. In addition, PARP-2 has been shown to be a direct negative regulator of the SIRT1 promoter (159), which may also result in resveratrol indirectly affecting SIRT1 activity. The structural similarity between resveratrol and estrogen raises the interesting possibility that some of the effects of resveratrol on the β-cells occur through estrogen receptors. Although this association has not been explored within the literature, the role of estrogen on the β-cells has been investigated. Through the 3 β-cell estrogen receptors, estradiols have been shown to increase insulin production, GSIS and β-cell survival, although the exact mechanisms remain unclear (223). Further support comes from the findings that female animal models of Type 2 diabetes typically display less severe phenotypes compared to male counterparts (223). Even in humans, menopausal women have a higher prevalence of diabetes than premenopausal women, a risk that has been shown to be reduced through estrogen replacement therapy (ERT) (224). In addition to estrogen, resveratrol may also be mediating effects in the β-cell through aryl hydrocarbon receptors (AhR), which are cytosolic transcription factors that can mediate the toxic health effects of chemical toxins such as dioxins. Within the β-cell, it has been shown that AhR receptors can mediate dioxin toxicity and impair insulin secretion, although the exact mechanisms remain unclear (225;226). More recently, AhR receptors have also been linked to obesity and fat metabolism (227). Due to the phenolic structure of resveratrol, it was linked to AhR through evidence that it could bind to and antagonize AhR and inhibit the toxic effects of dioxins (139;228). Furthermore, it appears that there is also crosstalk between AhR and estrogen

receptors where for example, activated AhR can inhibit estrogen receptor activity (229;230). Altogether, this raises the possibility that AhR may be implicated in our model through resveratrol however, this connection between resveratrol, AhR and possibly estrogen receptors has currently not been examined in the β-cell.

Although SIRT1 has been explored extensively with regards to metabolism in various tissues, including the β-cell, its precise role in nutrient regulation remains unclear. In this study we investigated the role of SIRT1 in lipotoxicity-mediated β-cell dysfunction using the SIRT1 activator resveratrol. Our *in vivo* studies revealed that resveratrol could prevent fat-induced β-cell dysfunction suggesting that SIRT1 plays an integral role in maintaining β-cell function. However, mechanistic details remain to be examined to verify whether these effects are SIRT1-mediated. Nonetheless, a recent study has demonstrated that SIRT1 expression can be reduced in islets by fat (160), supporting our functional data. This also suggests that resveratrol may be affecting SIRT1 in our model through two mechanisms: 1) resveratrol may directly increase SIRT1 activity/expression, which is decreased by FFA-generated ROS, or 2) resveratrol may reduce FFA-generated ROS, thus preventing the ROS-mediated decrease of SIRT1. In order to further implicate SIRT1 in the prevention of lipotoxicity we have also performed these studies in mice overexpressing SIRT1 in the β-cells.

4.2 Studies in Mice

To complement our studies in rats, we examined β-cell function in transgenic BESTO mice, where SIRT1 was specifically overexpressed in the β-cells. Using qPCR and western blotting, we verified the SIRT1 overexpression levels within the islets of our cohort of BESTO mice and they revealed very high SIRT1 levels that were comparable to the original cohort of BESTO mice (see 3.2.1). Our 48 h high fat infusions and hyperglycaemic clamps revealed that

BESTO mice were partially protected against the fat-induced β-cell dysfunction. These mice had similar plasma insulin and c-peptide levels to WT mice in both the SAL and OLE groups, but the BESTO OLE group revealed slightly higher, but not significant, insulin sensitivity compared to the WT OLE group. Both WT groups were similar to our previous studies with the WT OLE group demonstrating significant β-cell dysfunction as expected. Altogether, these findings suggest that SIRT1 overexpression in the β-cells is beneficial in protecting against lipotoxicity-induced β-cell dysfunction.

In the original paper describing and characterizing the BESTO mice (92), there were a number of observations that were in line with our studies. We chose to study mice 3-4 months of age as was done by Moynihan et al. In line with what was originally observed, we too did not observe any differences between WT and BESTO mice in terms of baseline metabolic characteristics or body weight. In a subsequent study (167), BESTO mice were placed on a HFD and exhibited improved glucose tolerance and enhanced GSIS. These findings are perhaps most closely relatable to our findings and support the notion that BESTO mice are more resistant to β-cell dysfunction due to nutrient excess. However, as discussed earlier, there are important differences between our model and HFD models that should be considered when interpreting these results. An interesting finding that was contrary between our study and their studies was with regards to enhanced insulin secretion in untreated BESTO mice. Moynihan et al originally reported that BESTO mice and their islets displayed enhanced insulin secretion after an IPGTT or secretion studies respectively. In our hyperglycaemic clamp studies we did not observe any enhanced β-cell function in BESTO mice compared to their WT controls. There are however a few possible explanations for these contrary findings. Firstly, since we assessed β-cell function through a hyperglycaemic clamp over a two hour period, we would more likely be assessing second phase insulin secretion. Moynihan et al used an IPGTT and observed enhanced secretion

at 30 minutes and so they likely examined first phase insulin secretion. They confirmed this finding through pancreatic in-situ studies as well. This would suggest that BESTO mice do display enhanced insulin secretion but only in terms of first phase secretion. Another possibility is that our BESTO cohort may have slightly lower levels of SIRT1 overexpression compared to the original cohort, although it may be debatable if a few fold lower expression would make a difference since the original overexpression levels were so high. Nonetheless, our novel findings in a selective model of β-cell lipotoxicity support the original observations made by Moynihan et al and suggest that SIRT1 overexpression is beneficial towards protecting β-cells from dysfunction due to nutrient excess.

Compared to other models of altered SIRT1 expression, our findings in BESTO mice generally seem to correspond. Our main finding that BESTO mice were partially protected from fat-induced β-cell dysfunction follows other KO and overexpression studies that showed reduced or improved glucose tolerance respectively (97;166;178;180;181). We also observed a non-significant improvement in insulin sensitivity in our BESTO OLE group, which has also been observed in other SIRT1 overexpression models and surprisingly in SIRT1 KO mice as well (93;97;180;183). In most of these studies, the authors attributed the increased sensitivity to involvement from other tissues, but how specifically, was not addressed. One possibility is overexpression of SIRT1 within the hypothalamus, which was not checked or reported by all studies (BESTO mice did not show any SIRT1 overexpression in the brain), but may be involved in altered sensitivity. In the whole body SIRT1 KO mice used by Bordone et al (93), it was unclear why there was an increase in sensitivity but it likely involved reduced insulin secretion involving increased UCP2 expression as well as involvement from other tissues. Nevertheless, the increase in UCP2 corresponds with Moynihan et al, who reported reduced UCP2 in BESTO mice, which correlates with the role of SIRT1 in repressing UCP2 transcription. Whether UCP2

expression is altered in our BESTO cohort or after fat infusion is something we have yet to assess.

Compared to other overexpression models, BESTO mice have rather high levels of SIRT1 overexpression (up to 18 fold), which can be considered a limitation of the model. Despite this however, our studies revealed only a partial prevention of β-cell dysfunction. One possible explanation for this finding is that although there is overexpression of SIRT1 protein, their activity is limited by NAD availability, which is likely lower during conditions of nutrient excess. Yoshino et al (184) recently addressed this notion by demonstrating that NAMPT-mediated NAD synthesis is compromised by HFD in mice. However, supplementation of NMN, a NAD precursor, can restore glucose tolerance and insulin sensitivity among many other effects. This suggests that even normal levels of SIRT1 may be enough to ameliorate the negative effects of nutrient excess, provided that there are sufficient levels of NAD available. Considering that PARP enzymes may be highly active in conditions of nutrient excess due to increased cellular stress and damage, it would make sense that they would be depleting NAD thus reducing SIRT1 activity regardless of its protein expression levels. This would also be an interesting notion to test in our model and see whether NMN infusion would completely prevent the fat-induced β-cell dysfunction in BESTO or even WT mice. Resveratrol may be another option as well since it has been suggested that it activates SIRT1 through increasing NAD levels.

Since the discovery of SIRT1, numerous whole body and tissue specific animal models have been developed to better understand its physiological role. In this study, we attempted to better understand the role of SIRT1 in β-cell lipotoxicity using the BESTO transgenic mouse model. Our *in vivo* results indicated that BESTO mice maintained better β-cell function after 48h fat infusion compared to their WT counterparts. These findings supported previous data on BESTO mice and implicate SIRT1 in β-cell lipotoxicity.

4.3 General Discussion & Future Directions

The goal of this thesis was to explore the role of SIRT1 in lipotoxicity-induced β-cell dysfunction using two different models of SIRT1 activation. In our studies in rats we used the SIRT1 activator resveratrol to try to ameliorate fat-induced β-cell dysfunction as assessed through hyperglycaemic clamp. We found that resveratrol partially prevented oleate-induced β-cell dysfunction in rats that were co-infused with both oleate and resveratrol. To further implicate SIRT1, in our second study we attempted to see if mice that overexpressed SIRT1 specifically within the β-cells were also protected from fat-induced β-cell dysfunction. Once again we discovered that these transgenic mice displayed partial prevention of β-cell dysfunction. Altogether, these two studies demonstrated that activation of SIRT1 can at least partially prevent lipotoxicity-induced β-cell dysfunction *in vivo*.

Since early studies discovered that SIRT1 could extend the lifespan of lower organisms, research into the physiological and pathological role of SIRT1 has been unrelenting. Through animal models and using various activators and inhibitors of SIRT1, it has emerged as an important regulator of nutrient metabolism in nearly every major tissue. Type 2 diabetes has long been known to be associated with obesity-related nutrient excess that ultimately contributes to and exacerbates β-cell dysfunction. Research specifically on the role of SIRT1 within the β-cell has been fairly limited, although two influential studies demonstrated that SIRT1 was vital to insulin secretion and proper β-cell function (92;93). Using our well established model of lipotoxicity-induced β-cell dysfunction, we have shown at a functional level *in vivo* that decreased activity of SIRT1 initiated by lipotoxicity may be a mechanism involved in β-cell dysfunction. These findings correspond with early work on SIRT1 in the β-cell and support the notion that SIRT1 activation may be of therapeutic interest to improve β-cell dysfunction in Type 2 diabetes. However, before that can be achieved, the precise mechanisms behind the actions of

SIRT1 in the β-cell need to be uncovered. Initially UCP2 was implicated in the SIRT1 mediated decrease in insulin secretion (92;93), although the role of UCP2 within the β-cell still remains unclear (168;169). FOXO1 is another important target of SIRT1 that has been shown to upregulate antioxidants to prevent ROS damage (152), but it may also reduce insulin gene expression through nuclear exclusion of PDX-1 (55). In addition, SIRT1 activity may be reduced by PARP-mediated depletion of NAD or PARP repression of the SIRT1 promoter (158;159). Reduced SIRT1 activity may then decrease deacetylation of PGC-1α and NFκB, which could subsequently reduce SIRT3 expression (172) and increase inflammation (231) within the β-cell respectively. Nonetheless, despite unanswered questions and uncertain mechanisms, this study and others, have demonstrated that SIRT1 plays an important role in the β-cell and in preventing β-cell dysfunction.

Though our results indicate that SIRT1 can prevent β-cell dysfunction *in vivo*, these findings must be considered along with the limitations of our studies. The most obvious involves the use of resveratrol as a SIRT1 activator. Although initially resveratrol was shown to directly activate SIRT1 (141;142), subsequent studies refuted this data (143;144) and so whether or not resveratrol activates SIRT1 remains unclear. However, the general consensus is that it does activate SIRT1 but perhaps indirectly through other molecules such as AMPK (146;147). Resveratrol itself, having many non-specific effects, poses another problem since it raises the possibility that SIRT1 independent effects may account for some of our results (133;135;138). This possibility is compounded when considering that other tissues aside from the β-cell may also be involved. In our transgenic BESTO mouse model the primary limitation is the high level of SIRT1 overexpression (up to 18x). This has been argued to exaggerate the beneficial results of SIRT1 overexpression that may not be apparent at lower levels of overexpression. However, subsequent whole body overexpression models displayed more moderate levels of SIRT1

overexpression but the results did not differ greatly and generally seemed to agree with findings using the BESTO model (97;166;180;181). Ideally, a β-cell specific mouse model with a more moderate overexpression of SIRT1 would best address this issue. More generally, there are also limitations with our lipotoxicity model. Due to the acute nature of the model (48 h), a disadvantage is that chronic effects on the β-cell are not observed. This differs from human Type 2 diabetes pathogenesis where development takes many years and therefore involves gradual cellular changes. Thus a more prolonged high fat model, such as a HFD, may be a better option, although this has previously been done with BESTO mice. However, with diet models, weight gain or the incretin effect can serve as confounding factors that limit the specificity of the model. Another drawback is that these studies cannot be replicated in humans since oleate infusion poses significant health risks. This somewhat limits the implications of our findings since they cannot be directly translated to humans, although Intralipid is another fat preparation that can be safely infused in humans to test these findings (232).

For both sets of studies, although we provide valuable *in vivo* evidence outlining the role of SIRT1 in the β-cell, future studies should focus on uncovering mechanistic details. In rats, since we use a non-specific activator of SIRT1 with inherent effects on its own, the priority should be to examine SIRT1 mRNA or protein levels to determine if our oleate infusion results in lower expression of SIRT1 and whether co-infusion of oleate with resveratrol prevents this decrease. However, due to the dependence of SIRT1 on NAD availability, it may be more useful to examine SIRT1 activity instead, since this would more accurately address whether SIRT1 is actively mediating our observed effects. This can be assessed indirectly through examining the acetylation status of downstream SIRT1 substrates such as p53 or PGC-1α, as was done by Wu et al. Alternatively, NAD levels within islets can be measured to assess SIRT1 activity as well as supplement SIRT1 expression data. Due to the controversy involving resveratrol, SIRT1 and

AMPK, it would also be beneficial to further examine markers of AMPK activity and AMPK protein levels as well. However, within the β-cell, AMPK acts contrary to SIRT1 by inhibiting insulin release (233), which would contrast with our findings if AMPK levels were increased. ROS are another important aspect of our lipotoxicity model and an important target for the scavenging ability of resveratrol as well. In order to determine how significant the role of ROS reduction by resveratrol is in our model, measuring cellular ROS levels using fluorescent imaging techniques or examining FOXO1 acetylation levels (or even antioxidant gene mRNA) should be done to help clarify these mechanisms. In addition, examining SIRT3 mRNA and PARP activity or DNA damage would also help clarify the possible role of these molecules in our model and their relationship with SIRT1. Once basic mechanistic details have been uncovered, we will need to repeat these studies *ex vivo* or *in vitro* to corroborate our *in vivo* data and eliminate other tissue effects.

In mice, since the β-cells specifically overexpress SIRT1, our data provides solid evidence that the observed effects are due to SIRT1. However, as with the rats, mechanistic details remain unclear and so future studies should once again focus on assessing how the results are being mediated by SIRT1. This can most easily be accomplished by assessing the mRNA or protein expression levels of SIRT1 targets such as UCP2, which has been shown by others to be altered within the β-cell with changes in SIRT1 expression. Previously, Yoshino et al demonstrated that the NAD precursor NMN could prevent fat induced β-cell dysfunction (184). Considering our only partial prevention of β-cell dysfunction in BESTO mice infused with oleate, it raises the possibility that a lack of NAD limits the activity of SIRT1 in BESTO mice, despite the high overexpression. Therefore, co-infusing BESTO and/or WT mice with either NMN or resveratrol (which may act by increasing NAD levels) would certainly help clarify this issue. SIRT1 can also influence ROS levels by repressing UCP2 transcription or upregulating

antioxidants via FOXO1. As a result, it may be of interest to assess ROS levels within BESTO and WT mice before and after oleate infusion, since BESTO mice may be more resistant to lipotoxicity due to lower baseline levels of ROS.

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