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The effect of simvastatin and pitavastatin on insulin secretion from clonal pancreatic ß-cells (INS-1)

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Thesis

THE EFFECT OF SIMVASTATIN AND PITAVASTATIN ON INSULIN SECRETION FROM CLONAL PANCREATIC ß-CELLS (INS-1)

by

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THE EFFECT OF SIMVASTATIN AND PITAVASTATIN ON INSULIN SECRETION FROM CLONAL PANCREATIC ß-CELLS (INS-1)

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ABSTRACT

Objective. The 10th leading cause of death in the United States is heart disease. Most of the deaths by heart disease has a correlation with an occlusion of the coronary arteries. While diabetes mellitus is currently the 7th leading cause of death, which is a chronic condition that affects more than 37 million people in America. The global epidemic of obesity largely explains the dramatic increase in the incidence and prevalence of type 2 diabetes (T2D) over the past 25 years. Statins are well known drugs to decrease LDL for individuals who suffer from hypercholesterinemia; however, there is also an increased risk of developing diabetes mellitus. An estimation of 10-20 per 10,000 patients per year demonstrated an excess risk of T2D with the long-term use of statin. Here we examine the effects of simvastatin and pitavastatin on pancreatic ß-cell function to determine whether altered insulin secretion may contribute to an increased risk of T2D.

Methods. The experiments were performed using clonal pancreatic ß-cells (INS-1). The cells were grown in 4 mM glucose in RPMI media. Cells were grown for three days before adding the different types of statins: simvastatin and pitavastatin for one day. Then the cells were used to perform the glucose-induced insulin secretion (GSIS) experiment. Insulin secretion and insulin content were assay using a fluorescence-based immunoassay. The study was calculated using Microsoft Excel. Standard variance and standard error were used to assess the difference sets of data.

Results. INS-1 cells responded to acute glucose stimulation after chronic culture in both low (4 mM) and high (11 mM) glucose. Secretion from cells cultured at 4 mM glucose was higher than cells cultured at 11 mM glucose at all glucose concentrations tested, characteristic of the effects of glucolipotoxicity (GLT). Insulin content in cells cultured at high glucose was decreased 8.6-fold compared to cells cultured at the more physiological low glucose condition. When normalized to basal secretion cells cultured at high glucose exhibited basal hypersecretion and increased GSIS compared to those in low glucose.

Simvastatin (100 nM, 24 hrs) increased basal insulin secretion to a greater extent than Pitavastatin. The effects of pitavastatin on basal insulin secretion were less consistent than seen with simvastatin. Simvastatin was also shown to inhibit GSIS from cells cultured at 4 mM glucose, while pitavastatin increased GSIS.

Conclusion. Both pitavastatin and simvastatin alter insulin secretion from pancreatic ß-cells. The effect of simvastatin to both increase basal and decrease GSIS, characteristic of GLT suggests pitavastatin may be the statin of choice to reduce the risk of statin-induced T2D.

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LIST OF ABBREVIATIONS

Ac-CoA ……...…. Acetyl-CoA

ADP …….…….. Adenosine Diphosphate

apoB …… Apolipoprotein B

α-GP ……. α-glycerophosphate

ADP …….…….. Adenosine Diphosphate

ATP …….….. Adenosine Triphosphate

BME…….ß-mercaptoethanol

BSA…….Bovine Serum Albumin

Ca2+ ……... Calcium

CoQ10 ……..… Coenzyme Q10

CPT1 ……. Carnitine palmitoyltransferase 1

CYP …….. cytochrome

DAG …….. diacylglycerol

DMSO …….. Dimethyl Sulfoxide

ETC …….…….. electron transport chain

eNOS …… endothelial NOS

FA ……... Fatty Acid

FBS……...…… Fetal Bovine Serum

FRET ……..… Fluorescence Resonance Energy Transfer

FH ……...…….Familial Hypercholesterolemia

FPG ……...…. Fasting Plasma Glucose

GLT …….. Glucolipotoxicity

GLUT2 ……. Glucose Transporter 2

GLUT4 ……. Glucose Transporter 4

GPR40 ……. G-protein-coupled receptor 40

GSIS..... Glucose-stimulated insulin secretion

HDL ……...…. High Density Lipoprotein

HEPES ……... N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

HI .....Hyperinsulinemia

HMG-CoA …….. D3-hydroxy-3-methylglutaryl coenzyme A

HTFR …….…. Homogenous Time-Resolved Fluorescence

INS-1 …….…… Insulinoma Cells

K+ ……...…. Potassium

K_{ATP} ……..… ATP-sensitive potassium channel

KREBS…….……Krebs-Ringer Bicarbonate Buffer

LCA-CoA …….…… Long chain acyl-CoA

LC-CoA ……..….. Long chain-CoA

LDL ……. Low Density Lipoprotein

LDLR ……..… Low Density Lipoprotein Receptors

LPA ……. Lysophosphatidic Acid

MOG ……..… Mono-oleoyl-glycerol

OGTT …… Oral Glucose Tolerance Test

PFK ……..…….. Phosphofructokinase

PIP2 ……. Phosphatidylinositol 4,5-bisphosphate

PLC ……... Phospholipase C

ROS ……. Reactive Oxygen Species

RPMI ……...…… Roswell Park Memorial Institute

SREBPS ……...……. Sterol Regulatory Element Binding Proteins

TG ……...… Triglycerides

T1D ……..... Type 1 Diabetes

T2D ..... Type 2 diabetes

Vm ……...…… Membrane Potential

INTRODUCTION

Coronary Heart Disease

Cholesterol is an important molecule for the proper functioning of human organs; however, the overproduction of cholesterol leads to coronary heart disease. Carl Müller made the first genetic connection between cholesterol and heart attack (Goldstein & Brown, 2015). He examined families who had an inherited trait of high LDL-cholesterol levels and premature heart attacks, which later became known as familial hypercholesterolemia (FH). A recent study conducted by the CDC showed that the 10th leading cause of death in the United States is heart disease (Heron, 2018). Most deaths by heart disease has a correlation with an occlusion of the coronary arteries.

Low density lipoprotein (LDL) is a causative agent of coronary heart disease. Each particle of LDL contains cholesteryl ester in a hydrophobic core enclosed in a polar phospholipid case with an apolipoprotein B (apoB) layer that has a molecular with of 500 kDa shown in figure 1. Low-density lipoprotein receptors (LDLRs) are on the outer surface of the cells, where they collect circulating LDLs in the bloodstream and move them into the cell. If there was an increase in the amount of low-density lipoprotein receptors it would increase the removal of LDL. However, some mutations reduce the low-density lipoprotein receptors (LDLRs), which increases LDL and causes premature heart attacks because the body would take a longer time to remove the LDL (Goldstein & Brown, 2015). This buildup of LDL could cause atherosclerosis plaques to form causing cardiovascular disease. The risk factors for the increase of LDL include obesity, high blood pressure, and diabetes.

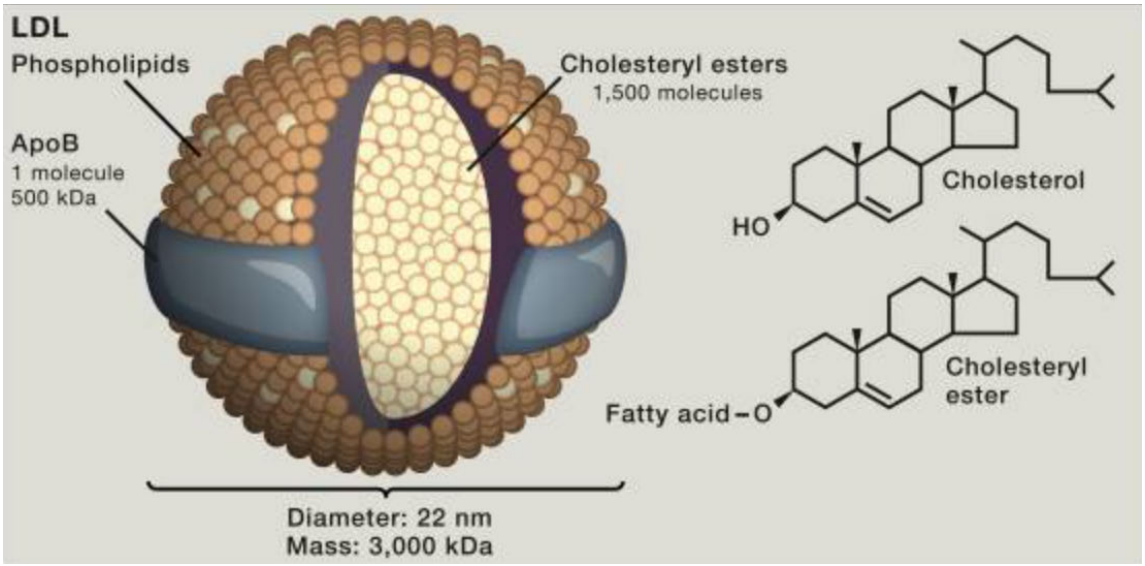

Figure 1: Low Density Lipoprotein, Cholesterol Carrier. From Goldstein, J. L., & Brown, M. S. (2015). Cell, 161(1), 161–172.

Atherosclerosis is a disease of the arteries due to plaque buildup in the inner wall. It decreases blood flow, which could ultimately lead to coronary heart disease and stroke. Major risk factors, such as hypercholesterolemia and high blood pressure, are closely linked with the degree of fibrous plaques (Lewis et al., 2020). In the coronary arteries, fatty streaks gradually become fibrous plaques through the continuation of lipid accumulation and chronic inflammation. Additionally, LDL cholesterol is positively correlated, while high density lipoprotein (HDL) cholesterol is negatively correlatedly with atherosclerosis. HDL cholesterol is also known as good cholesterol because of its ability to absorb cholesterol in the blood and carry it back to the liver. Hence the liver can flush the cholesterol from the body. When a person has high levels of HDL cholesterol it could significantly lower the risk for cardiovascular disease (Kauvery Hospital, 2016).

Figure 2 demonstrates the difference between LDL and HDL cholesterol. Diets focused on regulating cholesterol and fats are major factors that contribute to decreasing the risk of atherosclerosis.

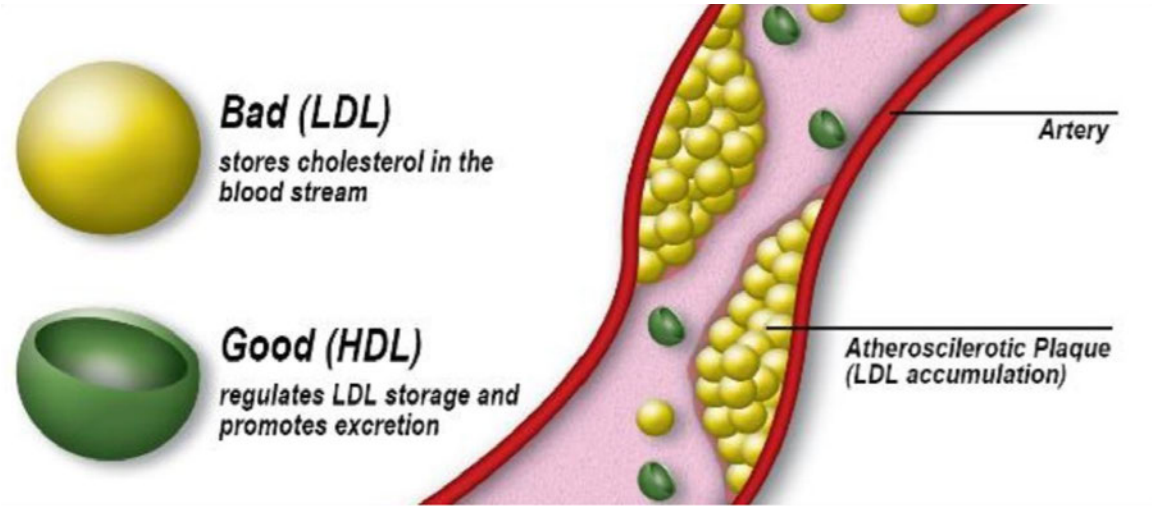

Figure 2: The difference between LDL and HDL cholesterol. From Kauvery Hospital. (2016). Kauvery Hospital.

Statin

Statins are drugs used to lower LDL-cholesterol. The biochemist, Akira Endo, discovered statin in 1987. However, the first approved statin for human consumption was by Merck's Mevacor (Goldstein & Brown, 2015). Mevacor's findings showed that statins reduce the chance of heart attacks and decrease mortality. Statin works on hepatocytes to reverse and competitively block HMG-CoA reductase. HMG-CoA is an enzyme that converts HMG-CoA to mevalonate, which is used for cholesterol synthesis seen in Figure 3. Statins alter the conformation of the enzyme once it binds to the active

site. This alteration prevents the functioning of HMG-CoA reductase, which allows the LDLR to replenish and reduce the LDL plasma levels because the receptors would be able to remove LDL at a higher rate. (Stancu & Sima, 2001). However, this is a reversible process if statins are discontinued. Statins lead to the decrease of esterified cholesterol into macrophages, decrease atherosclerotic plaques, and decrease inflammation.

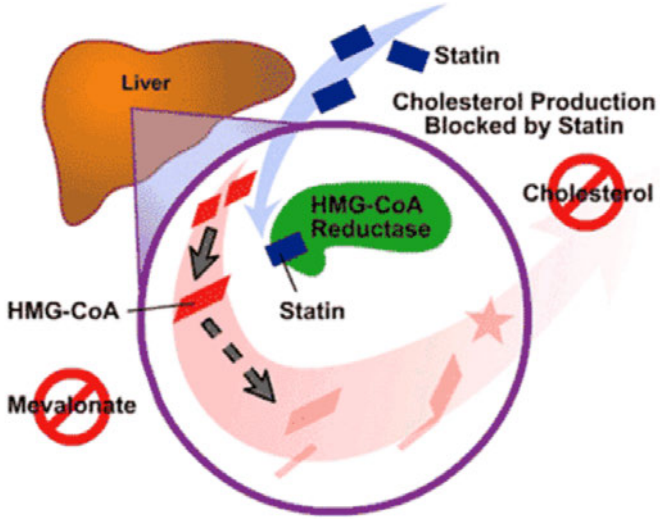

Figure 3: Statin Mechanism in Reducing Cholesterol. From Davidson, M., & Jacobson, T. (2001). Medscape.

Statin's ability to inhibit HMG-CoA reductase also has a role in activating protease. This process slices the sterol regulatory element binding proteins (SREBPS) from the endoplasmic reticulum. The activation of the SREBPs increase the gene expression of the LDL receptors as there is a decrease in cholesterol in hepatocytic cells. There are parallels in the reduction of LDL cholesterol and triglyceride. Statins inhibit the synthesis of apoB, which reduces the synthesis of triglyceride rich lipoproteins and

increases the receptor of apoB (Stancu & Sima, 2001). It was discovered that statins prevent the prenylation of p21 Rac protein, which lessens the production of superoxide anion in endothelial cells. Thus, inhibiting LDL oxidation by retaining the endogenous antioxidant system.

Hypercholesterolemia decreases the amount of available endothelial cells to make nitric oxide (NO). Statins increase endothelial function, which helps to reduce cholesterol. This process is partly independent of the decrease in lipid levels (Stancu & Sima, 2001). Statins significantly decrease the risk of coronary disease by being an effective hypolipidemic drug. The drug is tolerated well; however, there are adverse effects on the liver and muscles. Additionally, the effect that statins have on pancreatic ß-cells is still being studied as there is an increased risk of insulin resistance with the prolonged use of statins.

Since the early 2000s, there has been a variety of different types of statins, such as simvastatin, lovastatin, pitavastatin, and fluvastatin. These statins primarily have the effect of reducing LDL cholesterol non-linearly when the patients consistently take a daily dose of the drug. Also, it has an effect of reducing triglycerides that correlates with the decrease of LDL cholesterol.

This thesis focuses on two statins: simvastatin and pitavastatin. In table 1, it demonstrates the different classification, derivation, liver metabolism, specific activity, and chemical properties of the different statins.

Statins	Trade Name	Chemical Property	Potency	Classification
Simvastatin	Zocor, Simcor	Lipophilic	Moderate Potency	- HMG-CoA Reductase Inhibitor - Decreases LDL cholesterol
Pitavastatin	Livalo, Zypitamag	Lipophilic	Low Potency	- HMG-CoA Reductase Inhibitor - Significantly increase HDL cholesterol

Table 1: Types of statins used in this study

Simvastatin

Simvastatin is one of the earliest types of statins that are derived from fungal fermentations. It reduces LDL in patients with homozygous hypercholesterolemia, where the LDL is usually not functioning properly. Simvastatin is administered as an inactive form that is converted to an active form in the body. Common side effects associated with simvastatin include headaches and gastrointestinal complaints (Mauro & MacDonald, 1991). Statins have a moderate effect on the increase of HDL and barely any influence on the concentration of lipoproteins. Simvastatin is known to induce the transcription of endothelial NOS (eNOS) gene in endothelial cells. eNOS is an enzyme that regulates cellular function and maintains endothelial homeostasis (Heiss et al., 2015). Simvastatin also restores the antioxidant potential in patients with hyperlipemia. This is due to the

reduction in the amount of reactive oxygen species the cells produce or a decrease in LDL binding to the surface of phospholipids (Stancu & Sima, 2001).

Pitavastatin

Pitavastatin is derived synthetically. It is administered as an active compound similar to simvastatin. Pitavastatin has a 2-fold higher HMG-CoA inhibitor rate than simvastatin (Alagona, 2010). Most lipophilic compounds are metabolized in the liver by cytochromes (CYP), specifically CYP450. Pitavastatin is mostly lipophilic; however, it is only minimally metabolized by CYP. It is efficient in decreasing LDL cholesterol, increasing HDL cholesterol, and lowering triglycerides.

Effect of Statins on Diabetes

Statins are well known drugs to decrease LDL for individuals who suffer from hypercholesterinemia; however, there is also an increased risk of developing diabetes mellitus. An estimation of 10-20 per 10,000 patients per year demonstrated an excess risk of T2D with the long term use of statin (Pinal-Fernandez et al., 2018).

There are three mechanisms behind the onset of statin-induced diabetes. First, statins affect insulin secretion through direct, indirect, or combined effects on the pancreatic ß cells' calcium channels. Second, statin decreases products from the mevalonate pathway, such as Coenzyme Q10, pyrophosphate, and dolichol. This lessens intracellular signaling. Third, statins interfere with the glucose transporter 4 (GLUT4) and its presence leads to hyperglycemia and hyperinsulinemia (Brault et al., 2014).

Additionally, it was recently discovered that during a population-based study statins cause roughly a 25% decrease in insulin sensitivity and a 12% decrease in insulin secretion over long term use (Cederberg et al., 2015). Statins affect insulin signal transduction pathways by inhibiting phosphorylation and decreasing GTPase. Also, it decreases leptin, which leads to the inhibition of pancreatic ß cells proliferation and insulin secretion.

Statins have a side effect of downregulating the production of coenzyme Q10 (CoQ10), which protects against oxidative stress (Urbano et al., 2017). Mevalonate is a precursor of CoQ10, so the affects that statin has on inhibiting the mevalonate pathway also decreases the amount of CoQ10. Clinical trials have shown that patients with hypercholesterolemic patients have reduced CoQ10 levels. Reduction in CoQ10 increases oxidative stress leading to the deterioration of mitochondrial respiratory function and inhibition of insulin secretion from pancreatic ß cells (Urbano et al., 2017).

Diabetes

Diabetes mellitus is a chronic condition that affects more than 37 million people in America. It is currently the 7th leading cause of death (CDC, 2022b). The two major forms of diabetes are type 1 and type 2. Type 1 Diabetes (T1D) is the result of an autoimmune disease, which causes the body to stop producing insulin. Roughly, 5-10% of people are diagnosed with T1D and require a daily dose of insulin to survive (CDC, 2022b).

Type 2 diabetes (T2D) is diagnosed when blood sugar levels remain persistently elevated due to insulin resistance or the inability to produce enough insulin. If the body remains in this state, it could cause serious health problems such as obesity, loss of vision, kidney disease, and cardiovascular disease (CDC, 2022b). Most patients with T2D are overweight or obese. The global epidemic of obesity largely explains the dramatic increase in the incidence and prevalence of T2D over the past 25 years (CDC's Division of Diabetes Translation, 2017). Figure 4 depicts the prevalence of T2D and obesity in the U.S.

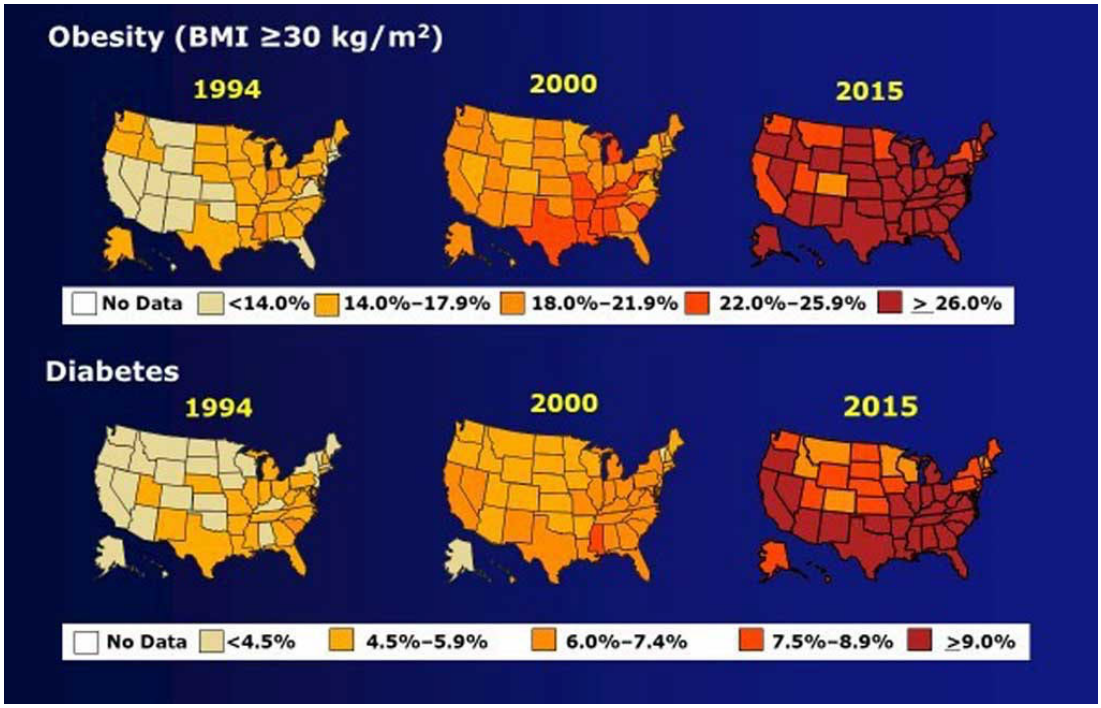

Figure 4 Age-Adjusted Prevalence of Obesity and Diagnosed Diabetes Among US Adults. From the CDC. (2017). CDC Map Obesity and Diabetes.

Proper functioning of pancreatic ß cells is important for controlling the homeostasis of blood glucose levels. During fasting blood glucose, the normal physiological range is between 4.4 to 6.1 mmol/L. The diagnostic criteria for T2D are A1C 6.5% or higher, fasting plasma glucose (FPG) of 126 mg/dl or higher, or an oral glucose tolerance test (OGTT) of 200 mg/dl or higher demonstrated in figure 5 (ADA, n.d.).

In the United States, more than 1 out of 3 adults have prediabetes and more than 80% are unaware that they have it (CDC, 2022a). Patients with prediabetes are not diabetic; however, they have blood glucose that is higher than normal. These patients are more susceptible to developing diabetes if they do not focus on diet and exercise. Patients who become diabetic usually are diagnosed with prediabetes first, before the full onset of T2D. Prediabetes correlates with impaired glucose tolerance.

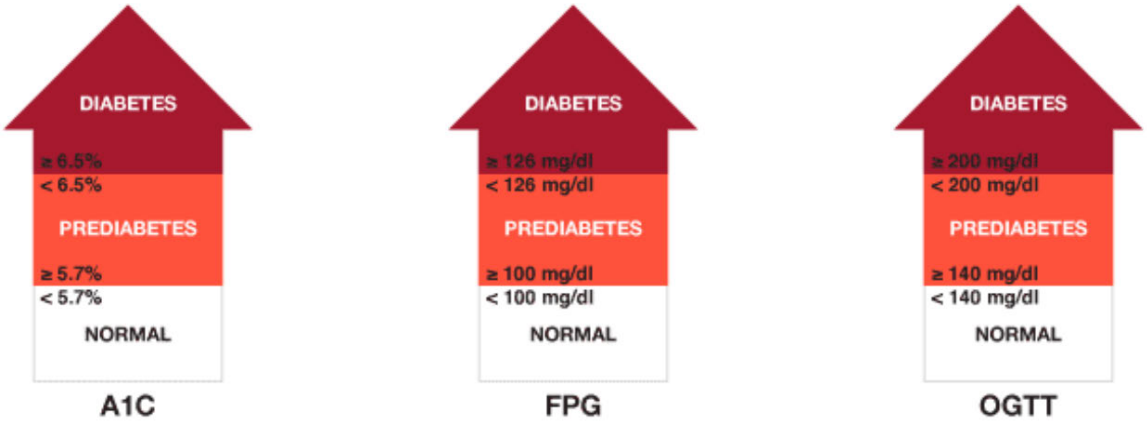

Figure 5: Three ways to diagnose diabetes. From ADA. (n.d.). American Diabetes Association.

Mechanisms of ß-Cell Insulin Secretion

Triggering of Insulin Secretion

Pancreatic ß-cells are located in the islets of Langerhans within the pancreas. They play a role in producing insulin and regulating blood glucose levels (Da Silva Xavier, 2018). Normal glucose-stimulated insulin secretion requires glucose to be metabolized in the cytosol through glycolytic generation of pyruvate and further in the mitochondria through the TCA cycle and electron transport chain producing ATP. Glucose enters ß-cells by Glut2 receptors, which are non-insulin dependent. This leads to phosphorylation of glucose by glucokinase (GK). GK is known as the glucose sensor of the ß-cell as it has a high K_m for glucose allowing response to a range of physiological glucose concentrations. Pyruvate is converted to acetyl-CoA by pyruvate dehydrogenase (PDH) and enters the citric acid cycle leading to the production of NADH. NADH provides reducing equivalents to the electron transport chain (ETC). This leads to ATP production and oxygen consumption. The increase in the cytosolic ATP/ADP ratio induces the closing of ATP-sensitive potassium (K_{ATP}) channels. This closure leads to depolarization of the plasma membrane and opening of voltage-dependent calcium channels. Calcium comes into the cell, which increases the concentration of calcium as it goes down its concentration gradient. Lastly, the influx of calcium triggers the secretion of insulin (Skelin Klemen et al., 2017). Oscillations in insulin secretion are generated by oscillations in the ATP/ADP ratio produced by the glycolytic enzyme Phosphofructokinase (PFK). PFK is inhibited by ATP and activated by AMP and ADP.

PFK is also activated by its product, fructose 1-6, bisphosphate (F1,6 BP) (Skelin Klemen et al., 2017). Figure 6 illustrates the process of insulin secretion.

Amplification of Insulin Secretion

Lipid signaling has been proposed to amplify GSIS. Glucose is metabolized through glycolysis and the citric acid cycle. Citrate produced in this cycle can be transported out of the mitochondria and converted in the cytosol to malonyl-CoA by the actions of citrate lyase (CL) and acetyl-CoA carboxylase (ACC). Malonyl-CoA inhibits carnitine palmitoyltransferase 1 (CP1), which is required for transporting long chain CoA (LC-CoA) into the mitochondria to be oxidized. With the inhibition of CPT-1, LC-CoA is proposed to increase and combine with α-glycerophosphate (α-GP) to form complex lipids The effect of glucose to inhibit fatty acid (FA) oxidation and increase insulin secretion through LC-CoA and its incorporation into lipids such as lysophosphatidic acid (LPA) and diacylglycerol (DAG) has been proposed as the LC-CoA hypothesis (Rustenbeck et al., 2021).

Long chain acetyl-CoA (LCA-CoA) has multiple effects. LC-CoA has been shown to both positively and negatively affect PKC isoforms (Yaney et al., 2000) and directly stimulate insulin exocytosis (Erion et al., 2015). The level of LC-CoA and lipid products have been proposed to oscillate along with glucose metabolism. When cells are exposed to high glucose, they do not require FA as a fuel. In fact, fat oxidation is reduced and LCA-CoA would putatively increase. When glucose concentration is low FFA oxidation will increase along with lipid signals. It is proposed that similar oscillations in

lipid metabolism occur as a result of oscillations in PFK activity during glycolysis. Gulzhan Narmuratova demonstrated oscillations in ACC phosphorylation/activity were out of phase with oscillations in insulin secretion (Narmuratova, 2022). Low ACC phosphorylation representing high ACC activity and increased malonyl-CoA would be coincident with high insulin secretion. In that state, FA oxidation would be inhibited and LC-CoA signaling and incorporation into lipids increased to amplify secretion.

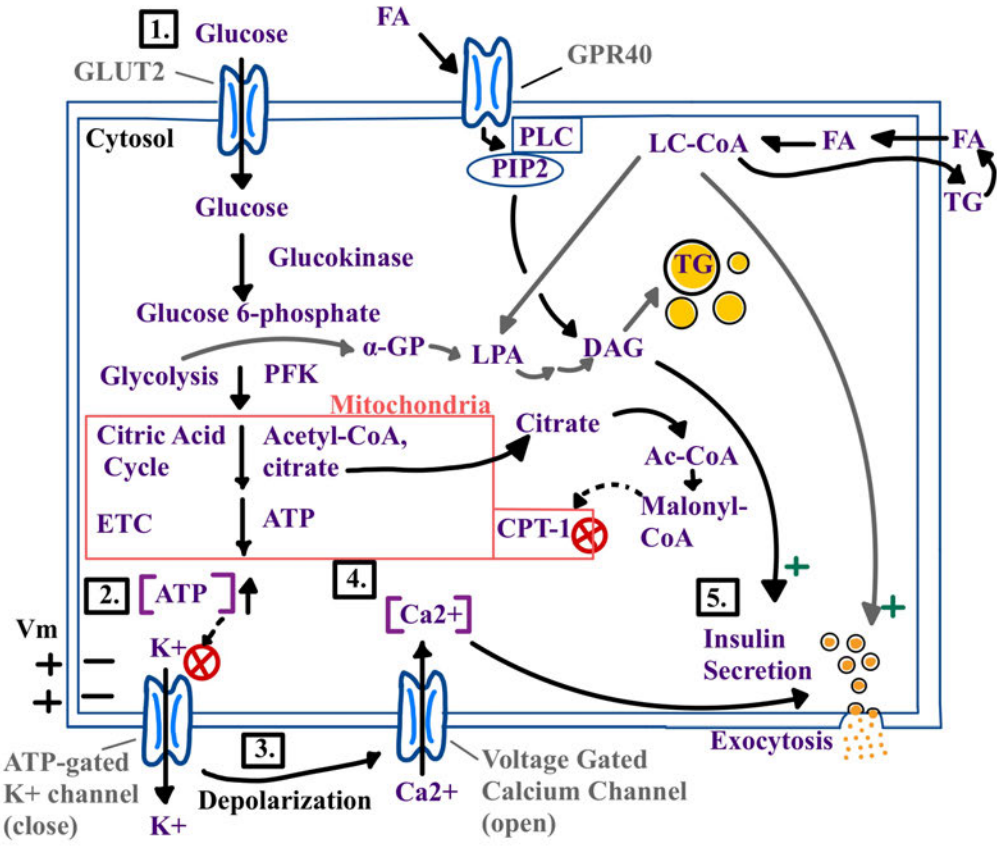

Figure 6: Metabolic regulation of insulin secretion. Abbreviations used are: PFK; phosphofructokinase, ETC; electron transport chain, ATP; adenosine triphosphate, V_m; membrane potential, K+; potassium, Ca2+; calcium, CPT-1; Carnitine palmitoyltransferase 1, α-GP; α-glycerophosphate, LPA; lysophosphatidic acid, DAG;

diacylglycerol, FA; fatty acid, GLUT2; glucose transporter 2, PLC; phospholipase C, PIP2; Phosphatidylinositol 4,5-bisphosphate, LC-CoA; Long chain-CoA, TG; Triglycerides, Ac-CoA; Acetyl-CoA, and GPR40; G-protein-coupled receptor 40

Ca^{2+} influx also elicits the activation of phospholipase C. Phospholipases C activation provides diacylglycerols and IP3. IP3 mobilizes calcium from endoplasmic reticulum; therefore, increases the concentration of cytoplasmic calcium (You et al., 2021). Diacylglycerol binds to munc13, which could be bound to syntaxin. Syntaxin as well as synaptobrevin and SNAP-25 are part of the SNARE protein complex, which docks secretory granules awaiting calcium influx that drives membrane fusion (Chen et al., 1999) and insulin release.

Glucolipotoxicity

Insulin secretion is normally stimulated by an increase in glucose and FA. However, a chronic excess nutrient state (high glucose and FA), results in glucolipotoxicity (GLT) characterized by increased basal and decreased GSIS. GLT is accompanied by increased lipid accumulation and decreased insulin content. This theory is used to explain the pancreatic ß-cell dysfunction in T2D. There is a reduced ß-cell function when the cells are in a GLT environment. This reduction GSIS is theorized to be because of either two reasons. The first reason could be that the pathway leading towards insulin secretion is interrupted. The second reason could be that the storage of insulin is exhausted (Erion et al., 2015). In GLT state, excess nutrients directly increase basal

insulin secretion, decrease GSIS and cause a left-shift in the glucose-sensitivity of the ß-cell. Excess nutrients result in insulin hypersecretion at low glucose, which is independent of insulin resistance seen in figure 7.

Insulin is kept in a very narrow range, so interruption and abnormality to this range could lead to the onset of diabetes. Previous studied showed that if membrane depolarization and calcium influx are prevented, depletion of insulin content can be avoided in cultured islets exposed to chronic excess nutrients (Erion et al., 2015). This showed that insulin hypersecretion contributes to decreasing insulin content under glucolipotoxicity conditions. Insulin hypersecretion was also confirmed as a characteristic of GLT in cells with normal insulin content.

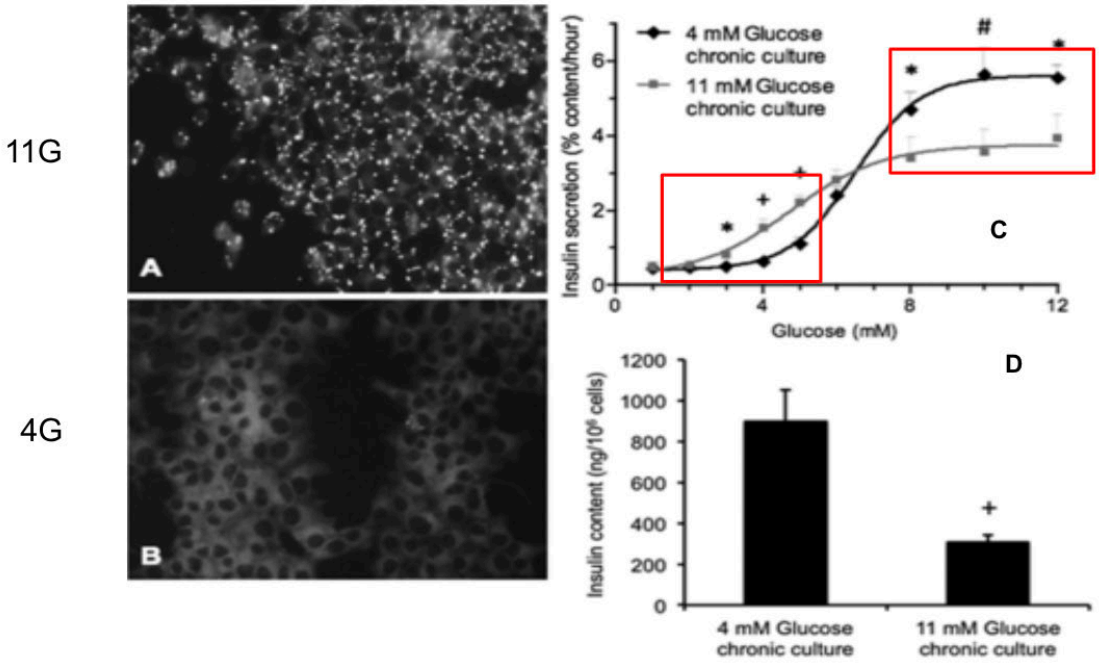

Figure 7: Excess nutrients increase lipid stores, left-shifts the dose-response curve of GSIS, and decreases insulin content in INS-1 cells. Lipid stores presented for 11G cells

(A) and 4G cells (B). Stimulated insulin secretion shown in 4G and 11G cells (C). Total insulin content assessed in 4G and 11G (D). From Erion, K. A., Berdan, C. A., Burritt, N. E., Corkey, B. E., & Deeney, J. T. (2015). Journal of Biological Chemistry, 290(26), 16191–16201.

Hyperinsulinemia

Hyperinsulinemia (HI) is elevated plasma insulin at basal glucose. The exact mechanism is not well understood. However, prolonged HI leads to underlying health problems, such as obesity, T2D, and insulin resistance. The generally accepted view is that HI results from insulin resistance, although chronic excess nutrients have been shown to stimulate basal insulin secretion. Therefore, an alternative hypothesis is HI may be the cause rather than the consequence of the progression of insulin resistance and T2D. The resulting insulin hypersecretion from the ß-cell will also lead to hyperinsulinemia; therefore, it could cause the onset of T2D.

Studies Leading to the Present Aim

The first thesis from this lab's Kowa funded study demonstrated the effect of chronic (3 days) statin exposure to inhibit insulin secretion from clonal pancreatic ß-cells (Datu Tasik, 2019). In this study it was shown that both simvastatin and pitavastatin inhibited GSIS. Simvastatin was more effective than pitavastatin in this regard as determined by the dose response of the statin. Simvastatin also significantly decreased insulin content in the same experiments. When secretion was normalized to basal insulin release the effect of simvastatin to inhibit secretion was only seen at the highest statin

concentration. This suggested that the effect of statin to inhibit secretion was primarily due to loss of content.

Loss of content is a characteristic of cells cultured in excess nutrients (glucose and FA) leading to GLT. There are a number of contributors to this loss of content. Excess nutrients leads to increased lipid accumulation, which increases basal insulin secretion. Thus, insulin hypersecretion from INS-1 cells cultured at high (11 mM) glucose depletes the cell of its insulin content. Under these conditions it has been shown that the insulin synthetic pathway does not compensate for this loss and the net result is a decreased content. Thus, we were struck with the interesting possibility that statin treatment did not inhibit but rather stimulated GSIS.

A subsequent study showed that statins also inhibited secretion and reduced insulin content in INS-1 cells cultured at 4 mM glucose. If this was also due to hypersecretion it would suggest that statins increased glucose-sensitivity of the cells allowing for greater stimulation at lower glucose concentrations (Sao, 2020).

To test this intracellular Ca^{2+} oscillations were monitored in single cells cultured in 4 mM glucose on glass bottom dishes and mounted on a wide field fluorescence microscope. INS-1 cells were loaded with fura and Ca^{2+} oscillations were measured in control and statin treated cells at 1,3 and 12 mM glucose. It was determined that pretreatment with simvastatin stimulated Ca^{2+} oscillations at 3 mM glucose while no oscillations occurred at this glucose concentration in the control. The effect of pitavastatin to stimulate Ca^{2+} influx at low glucose was significantly less (Sao, 2020).

The exact mechanism of why statins increase the risk of T2D is still not fully understood. The experiments described in the section included only chronic statin treatment. In this thesis we set out to determine whether basal insulin hypersecretion could be observed from clonal pancreatic ß-cells after shorter statin exposure.

AIMS

1. Investigate the effects of simvastatin and pitavastatin on basal insulin release and GSIS from clonal pancreatic β-cells (INS-1) cultured in 4 mM glucose.

METHODS

Cell Culture

The experiments were performed using clonal pancreatic β-cells (INS-1). The cells were grown in 4 mM glucose in Roswell Park Memorial Institute (RPMI) media. The composition of the RPMI consisted of 50 μg/ml streptomycin, 10 mM of N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 1 mM pyruvic acid, 50 IU/ml penicillin, 10% fetal bovine serum (FBS), 50 μM β-mercaptoethanol (BME), and 2mM glutamine. The cells were split on a weekly basis. The experiments were conducted in a 24 well plate, where the statins: simvastatin and pitavastatin were eventually distributed throughout the plate. The 24 well plate contained roughly 400,000-500,000 cells/wells. The cell number in the 24 well plate was based on the doubling time which was 72 hours in 4 mM glucose media.

Cells were grown for three days before adding the different types of statins: simvastatin and pitavastatin for one day. Then the cells were used to perform the glucose-induced insulin secretion (GSIS) experiment (Figure 8).

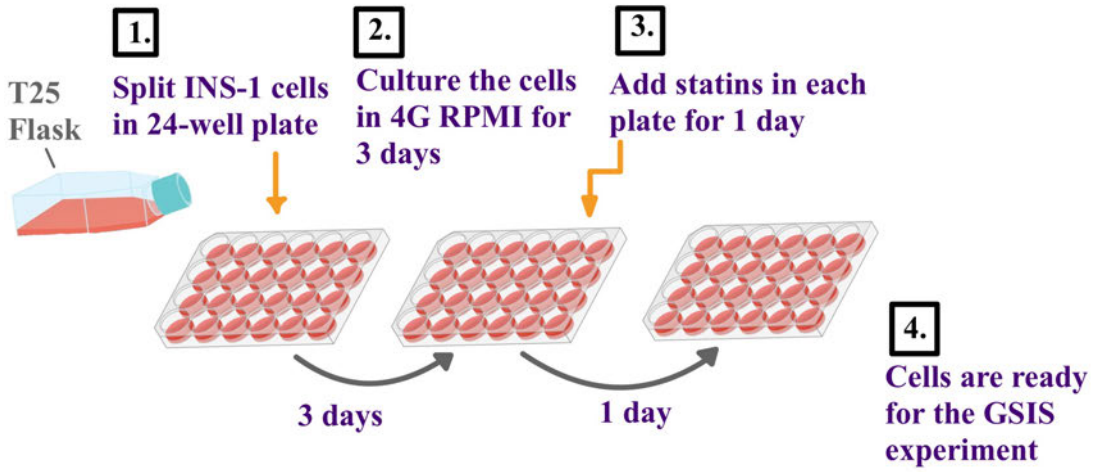

Figure 8: Timeline of the statin treated INS-1 cells. Adapted from Datu Tasik, G. M. (2019). OpenBU.

Cell Count

The cells were counted before the start of the GSIS experiment. The cells for each statin conditions: simvastatin and pitavastatin were counted for as well as the control, dimethyl sulfoxide (DMSO). Within the 24 well plate 0.4 ml of 0.05% trypsin-EDTA were added into the wells of the last row in the plate. The trypsin was kept inside the wells for 4 minutes to decreases the cell's ability to form adhesive bonds by reducing the integrins on the cell membrane (Brown et al., 2007). After the cells were examined for trypsinization, then they were placed inside three different 2.0 ml Eppendorf tube where all three conditions: simvastatin, pitavastatin, and control were counted. The cells were stored in Eppendorf tubes that also contained 80µl of 0.05% trypsin.

The Eppendorf tubes were placed inside of an ice bath to prevent trypsinization from continuing while counting the cells. A hemocytometer was used to measure the cell

number and the total cell count was based on the milliliter per well. The remainder of the cells inside the Eppendorf tubes were centrifuged at 4000 rpm for 4 minutes so that the cells were separated from the solution. The 0.05% trypsin-EDTA was aspirated from each of the Eppendorf tubes without removing the cells. Then 150µl 1% BSA and 150µl of PBS, 0.1% triton x-100 and 25 mM NaOH were added to the tubes. The tubes were vortex three times to ensure proper mixing. Afterward, the tubes were stored in the freezer for later use.

Insulin Secretion

Insulin secretion was measured with the GSIS experiment for both simvastatin and pitavastatin for one day. GSIS started with the cells being incubated inside a laboratory incubator for two hours in 1 mM glucose (1G) RPMI. Each of the 24 wells contained 0.5 ml of 1 mM glucose of RPMI.

Once the incubation period of two hours is completed then the 1mM glucose was removed from the plate and 0.5ml of Krebs-Ringer Bicarbonate Buffer (KREBS) was added into each well for 30 minutes in a water bath at 37ºC. KREBS contained 10 ml 5x KREBS, 38 ml deionized water, 1 ml $CaCl_2$, 1 M glucose, and 0.025 grams Bovine Serum Albumin (BSA) at a pH of 7.4. After the 30 minutes incubation period the KREBS was removed from the well. Two test solution of 4G and 12G KREBS were added to the cells and each well contained 0.5 ml of the solution. Then the plate was placed back into the water bath at 37ºC for 1 hour. Once the incubation period was finished then the plate

was placed on an ice bath and the insulin samples are collected for further analysis. This is shown in figure 9.

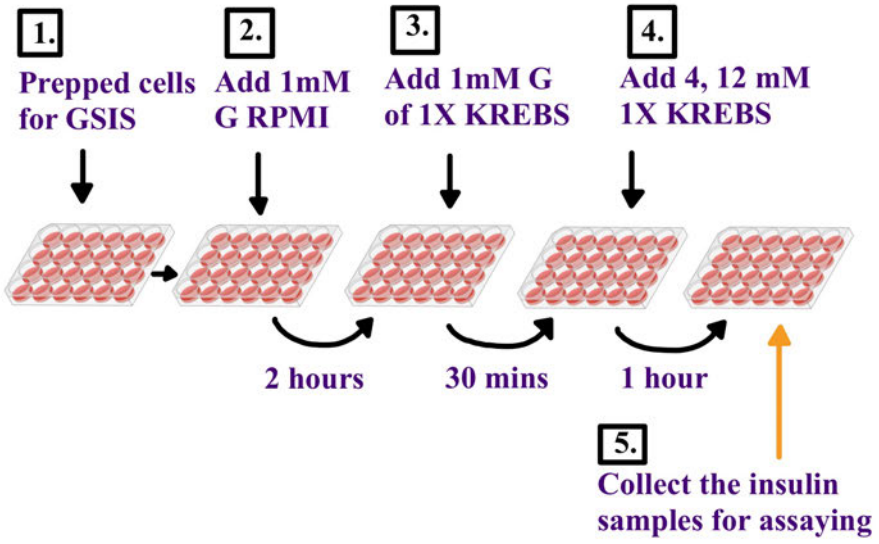

Figure 9: Steps of Glucose-Stimulated Insulin Secretion. Adapted from Datu Tasik, G. M. (2019). OpenBU.

Insulin Secretion Assay

Once the insulin samples were prepared then the samples for each of the statins and the control are transferred to a 96 well plate. The samples were diluted at 1:1 in 1% BSA and then it was vortex three times to ensure proper mixing of the sample. After the samples were further diluted based on the test assay previously performed. The test assay involves using two dilutions for each glucose condition (4G and 12G) in order to determine the best dilution factors for the experiment and 0.5% BSA is used for these dilutions. For 4G, a 1:2 and 1:4 dilution was used and for 12G 1:4 and 1:8 dilution was used.

The insulin secretion assay was performed with a fluorescence resonance energy transfer (FRET) based HTFR assay and the Team Infinite M 1000 Pro spectrophotometer was used to measure fluorescence. The insulin samples and content were stored in the freezer at a temperature of -25ºC for later use. Once the dilutions were determined then the samples were transferred to a microplate and centrifuged for 1 minute. After centrifuging the plate, the antibodies were added in each well of the microplate and centrifuged. This ensure that the samples were bounded to the insulin for the FRET signals to be properly measured. The microplate was sealed and kept overnight to ensure that the sample and antibodies had settle before analyzing the insulin content.

Following the insulin secretion assay, on the next day insulin secretion was analyzed. The results are calculated using Microsoft Excel, where the insulin standard curve was used to assist in the analysis of the sample. Each standard curve was specific to each experiment to ensure a greater accuracy in the experiment. The dilution factor, volume of the cell solution, and the cell number per well were also used to assess the data.

Insulin Content Measurement

Insulin content was assayed following the GSIS analysis. The cells previously stored in the Eppendorf tubes with PBS, 0.1% triton x-100, and 25 mM NaOH were further used to measure total insulin content. Each condition (simvastatin, pitavastatin, and DMSO) was diluted 1:128 for the insulin content of 4G with 0.5% BSA. Then those samples were transferred on a microplate to analyze the data.

Statin Preparation

Simvastatin

Simvastatin is an antihyperlipidemic drug that reduces 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. It contains a lactone derivative and hydrophobic ring (Figure 10). Originally, simvastatin is not in its active derivative, so it needs to be converted to its active state by dissolving it in 4 mg of ethanol and adding 150 µl of 0.1 N NaOH to the solution. After, the solution is incubated for two hours and the pH is at 7.0. Once the simvastatin is activated, then it is dissolved in ethanol until it reaches 20 mM and diluted in DMSO until it gets to 200 µM and aliquoted. The simvastatin stock and aliquots were then stored away in the freezer.

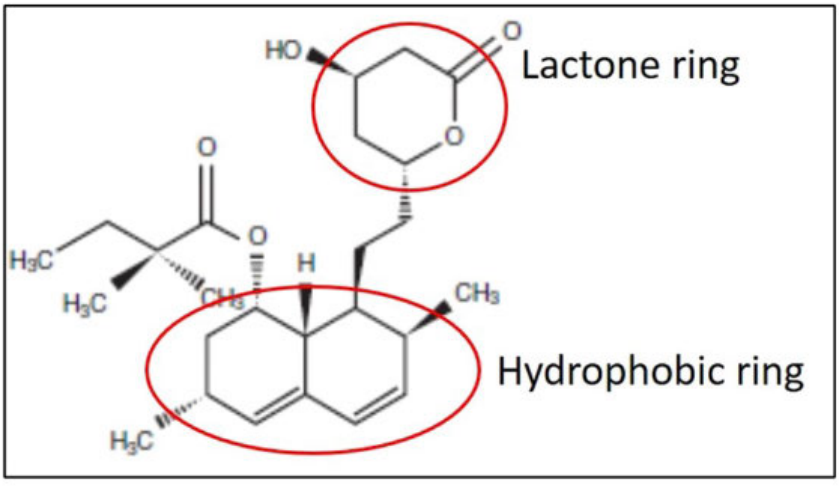

Figure 10: Simvastatin structure. From Sariisik, E. (2019). Biochimica et Biophysica Acta (BBA) - Biomembranes, 1861(4), 810–818.

Pitavastatin

Pitavastatin is usually represented as a calcium salt. It is a lipophilic statin that contains heterocyclic compounds (Figure 11). It has the ability to form a bridge between two statins, which is double the amount of simvastatin. Pitavastatin is dissolved in DMSO until it reaches 10 mM. Then it was diluted with DMSO until it reaches 200 μM and aliquoted. The statin was stored and put away similarly to simvastatin.

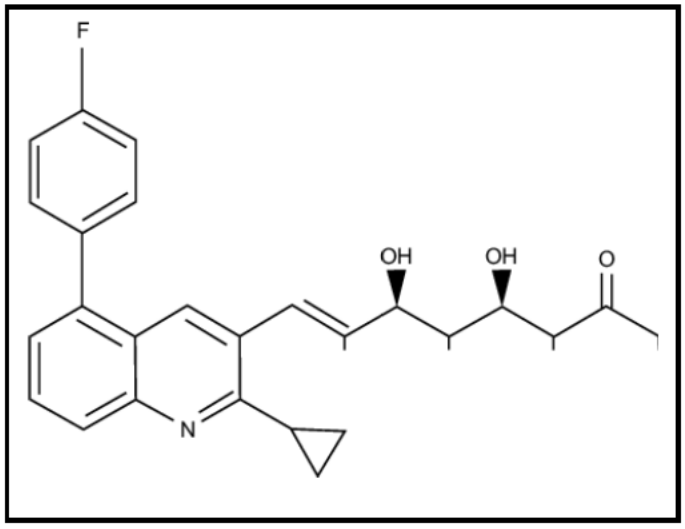

Figure 11: Pitavastatin structure. From Alagona, P. (2010). Core Evidence, 91.

The cells were fed the night before the GSIS experiment with statins. The statin stocks were removed from the freezer, defrosted, and vortex. Three test tubes for each condition: control, simvastatin, and pitavastatin are gathered to feed the cells. First, each of the three test tubes was filled with 5ml of 4G RPMI each labeled with its respective statins and control. For the control, 5μM of DMSO stock is added to the test tube. The simvastatin test tube has 5μM of the simvastatin stock and the pitavastatin has 2.5 μM DMSO and 2.5 μM pitavastatin stock. Pitavastatin is double the amount of simvastatin

because of its ability to bridge together, so when creating the solution, it needed to be halved to have equal amounts of pitavastatin and simvastatin.

Statistical Analysis

The study was calculated using Microsoft Excel. Standard variance and standard error were used to assess the difference sets of data. Additionally, insulin secretion and insulin content were calculated using Excel as well.

RESULTS

Glucose-Stimulated Insulin Secretion (GSIS) from INS-1 Cells Cultured in 4 mM and 11 mM Glucose

To demonstrate the effects of excess nutrients on ß-cell function we examined insulin secretion from clonal pancreatic ß-cells (INS-1) cultured in low (4 mM) and high (11 mM) glucose. Figure 12 shows the results of insulin secretion from cells cultured at conditions mimicking both normal blood glucose levels (4 mM G, red bars) and a hyperglycemic blood glucose level (11 mM G, blue bars). INS-1 cells were cultured in 24 well plates in 11 mM glucose for 1-2 days before changing the media to 4 mM and 11 mM glucose for three days. GSIS was then tested at 1, 4, and 12 mM glucose for 1 hr.

Basal insulin secretion (1 mM G) was 15 ng insulin/10^6 cells and 10 ng insulin/10^6 cells from cells cultured in 4 mM G and 11mM G respectively. Increasing the glucose in the test incubation to 4 mM glucose did not increase insulin release in either 4 or 11 mM glucose. Once the glucose was increased to 12 mM, insulin secretion at 4 mM G rose to about 50 ng/10^6 showing a 3.33-fold increase, while cell culture at 11 mM G increased 2-fold to 20 ng insulin/10^6 cells. The results demonstrated that the cells were responsive to acute glucose stimulation after chronic culture in both low and high glucose. Secretion from cells cultured at 4 mM G was higher than cells cultured at 11 mM G at all glucose concentrations tested, characteristic of the effects of GLT.

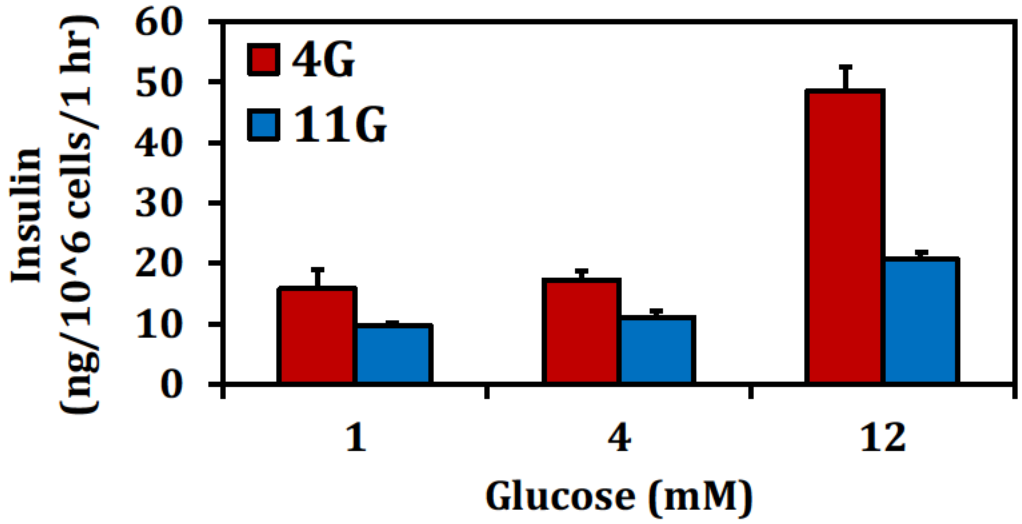

Figure 12: Cells cultured in 11 mM glucose exhibited a decrease in both basal and GSIS compared to cells cultured in 4 mM glucose. INS-1 cells were cultured in 4G and 11G to stimulate GSIS. The cells were cultured in 4G for 72 hours and in 11G for 48 hours. The cells cultured in 4 mM glucose had a higher insulin secretion than cells cultured in 11 mM glucose.

Insulin Content

We then measured whether the GLT conditions changed the insulin content of the cells when cultured in different glucose concentrations. Figure 13 shows the insulin content extracted from INS-1 cells cultured at low (4 mM, red) and high (11 mM, blue) glucose. Insulin content was 995 ng/10^6 cells and 116 ng/10^6 cells for INS-1 cells cultured at 4 mM G and 11 mM G respectively. This represented an 8.6-fold decrease in

content when cells were switched from low to high glucose and is characteristic of cells cultured in GLT conditions.

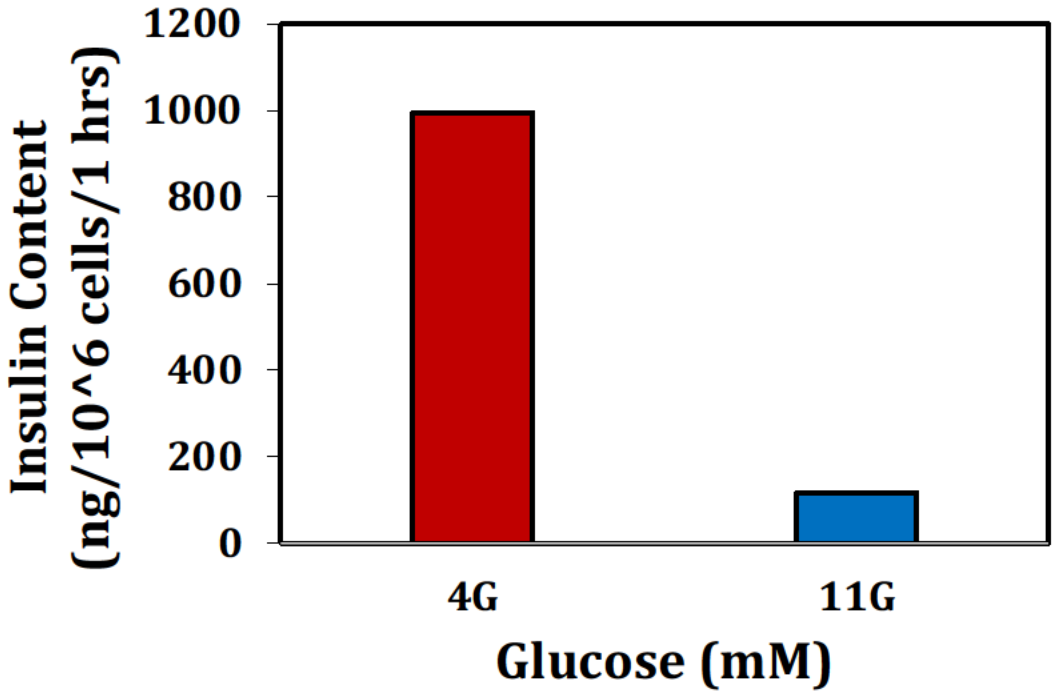

Figure 13: Insulin content was decreased in cells cultured in 11 mM glucose compared to cells cultured in 4 mM glucose. The insulin content was obtained from INS-1 cells from both 4 mM glucose and 11 mM glucose, where 11G decreased by 8.6-fold when compared to the 4G.

GSIS Corrected for Insulin Content

Insulin secretion from INS-1 cells depicted in Figure 13 was corrected for cellular insulin content. Secretion from cells cultured at both 4 mM and 11 mM glucose was calculated as a percentage of the total insulin contained in the cell under those conditions.

After normalizing to basal insulin release, the relative secretion of cells cultured at 4 mM and 11 mM glucose was inverted. The low secretion from cells cultured in 11 mM glucose had a greater percentage of the cell insulin content compared to cells cultured at 4 mM glucose (Figure 14). The relative changes due to acute glucose stimulation were not affected. Increased basal secretion in 11 mM G cells compared to 4 mM cultured cells is consistent with GLT effects of high glucose culture.

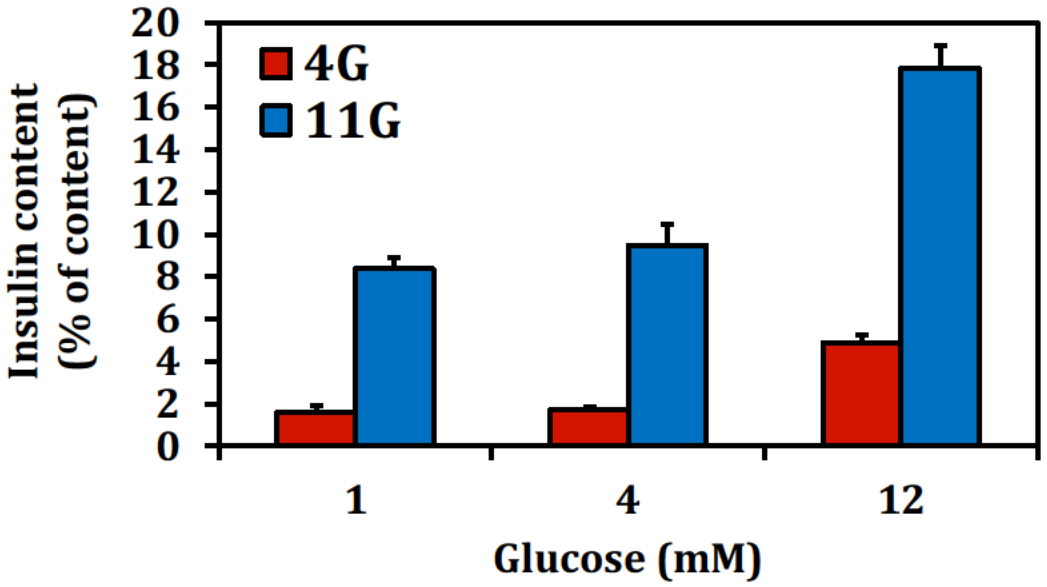

Figure 14: Cells cultured in 11 mM glucose exhibited an increase at basal and GSIS compared to cells cultured in 4 mM glucose when corrected for insulin content. INS-1 cells were corrected for insulin content. Where the data was calculated as a percentage of the total insulin. The cells cultured in 11 mM glucose had a higher insulin secretion than cells in 4 mM glucose representing a shift between the 4G and 11G insulin secretion levels.

Insulin Secretion Normalized to Basal Release

In the previous analysis secretion at 12 mM glucose was not inhibited as expected with GLT. For this reason, secretion (from Figure 12) was reanalyzed and normalized to basal insulin release.

In figure 15, the insulin release from INS-1 cells was normalized to basal insulin secretion from cells cultured in 4 mM G. To do this all secretion from cells cultured at 11 mM G was divided by 0.6 to increase secretion to match the basal secretion for the two conditions. Thus, secretion induced by acute incubation of 1 mM glucose was about the same percentage. Secretion stayed the same in 11G when glucose was increased to 4 mM in the test incubation. There was an increase in the cells in 4G once the glucose was increased to 12 mM glucose. When examining the acute 12 mM glucose condition in this way the results were more characteristic of GLT.

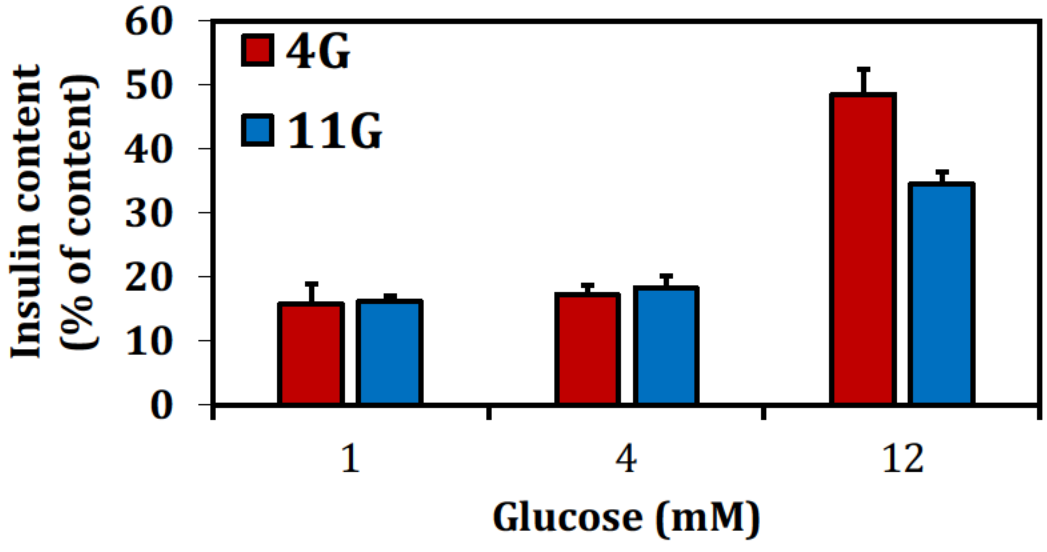

Figure 15: Cells cultured in 11 mM glucose exhibited a decrease in GSIS compared to cells cultured in 4 mM glucose when normalized to basal insulin release. Cells

cultured in 4 mM glucose were lower than 11 mM glucose at the basal end. At the high end, the 4G cells were higher. This is characteristics of GLT.

Statins Effect on INS-1 cells

We then examined the effect of statin on insulin secretion from cells chronically cultured at 4 mM glucose for 2-3 days. Since secretion was not changed when acute glucose exposure was increased from 1 to 4 mM glucose, we used 4 mM glucose as a basal glucose concentration. We then tested the effects of 18-24 hrs exposure of 100 nM simvastatin and pitavastatin on 4 mM (basal, red bars) and 12 mM (stimulated, blue bars) GSIS.

Insulin secretion from control (DMSO) cells increased by 25% from 40 ng insulin/10^6 cells to 50 ng insulin/10^6 cells when acutely incubated in 4 mM and 12 mM glucose respectively. When cells were treated with simvastatin the basal level (4G) was increased to 64 ng insulin/10^6 cells compared to 40 ng insulin/10^6 cells (Figure 16), a 60% increase. Pitavastatin did not significantly increase basal release (only to 42 ng insulin/10^6 cells) increasing secretion by only 5%. The results showed that simvastatin increased basal insulin secretion compared to the control and pitavastatin. In this experiment, the control cells barely showed any insulin secretory response between the 4 mM and 12 mM glucose. This was also true for cells treated with simvastatin, while pitavastatin treated cells exhibited about a 1.7-fold increase in GSIS.

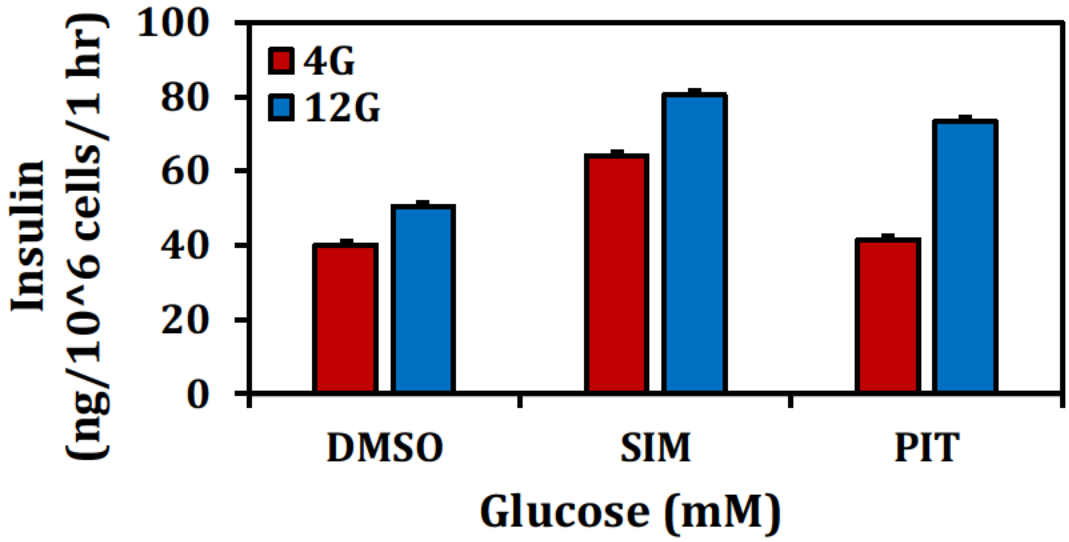

Figure 16: Simvastatin increased basal insulin secretion in cells cultured in 4 mM glucose compared to pitavastatin. Cells were incubated in 4G RPMI for 3 days and then statins (100 nM) were added for 1 day. The simvastatin was higher basally than the control and pitavastatin. The control showed a reduced insulin secretion than the statins.

Due to the poor secretion that was observed in Figure 16, culture in 4 mM G RPMI was shortened to 2 days. In this experiment, GSIS was improved. Secretion from control cells (DMSO) went from 32 to 66 ng insulin/10^6 cells. Simvastatin increased basal secretion (red bars) to 45 ng insulin/10^6 cells or about 40% while the increase with pitavastatin was to 41 ng insulin/10^6 cells. This is seen in figure 17. The effect of statins on GSIS was markedly different in this experiment. Simvastatin completely inhibited GSIS (blue bars) while pitavastatin increased GSIS by 20%.

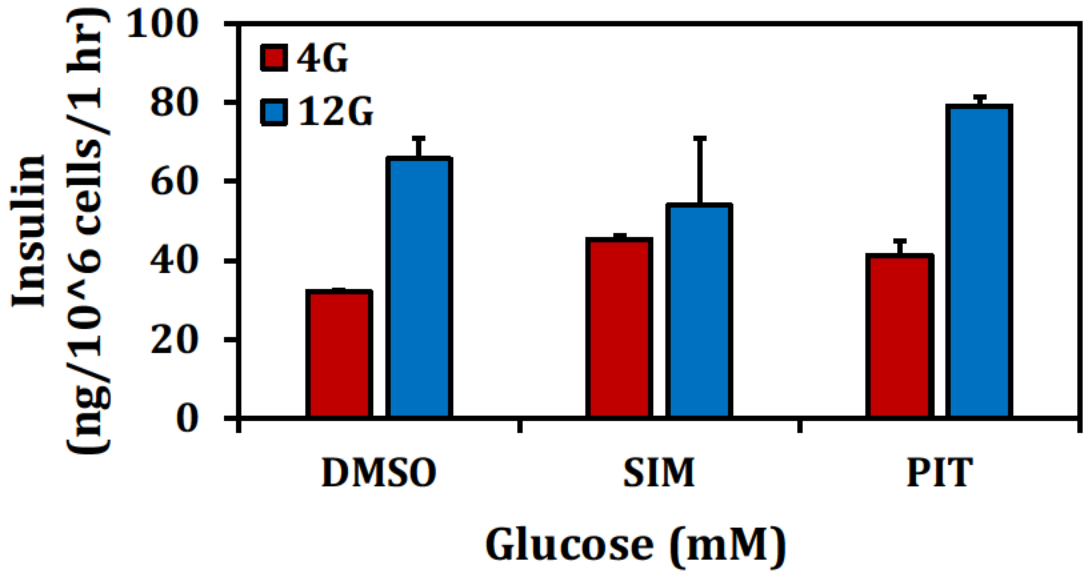

Figure 17: Simvastatin increased basal insulin secretion and decreased GSIS in cells cultured in 4 mM glucose compared to pitavastatin. Cells were incubated in 4G RPMI for 2 days and then statins were added for 1 day. The simvastatin was higher basally than the control and pitavastatin. Pitavastatin showed higher insulin secretion between 4G and 12G.

DISCUSSION

The goal of the thesis was to investigate the effects statins have on insulin secretion under physiological normal blood glucose levels (4 mM glucose). This investigation stems from the increased incidence and prevalence of T2D and its association with cardiovascular disease. Recent studies have shown a link between the use of statins and an increased risk of T2D; however, further investigation was needed to know the exact mechanism and its effects on pancreatic ß cells. In this study, we compared the effects of simvastatin and pitavastatin on insulin secretion from clonal pancreatic ß-cells (INS-1) cultured at low physiological 4 mM glucose. The preliminary data showed that simvastatin consistently increased basal insulin, while also inhibiting GSIS. Pitavastatin increased basal secretion to a lesser degree than simvastatin and did not inhibit GSIS. The results suggest that pitavastatin may be less deleterious to ß-cell function than simvastatin and thus be less likely to lead to T2D.

Initial experiments were designed to replicate previous results demonstrating chronic culture in excess nutrients increase basal and decreases GSIS. Figure 12 demonstrates that cells culture at low glucose secreted much more insulin at both basal and glucose stimulated conditions. This fit with an 8-fold increase in insulin content in cells cultured at low glucose but did not fully exemplify the characteristics of GLT. After correcting for insulin content (Figure 14), cells cultured at high glucose exhibited increased basal insulin secretion and an uncharacteristic increase in GSIS. There may be a number of contributors to this finding. For one, it is noted that the level of insulin content drops to a very low level in the cells cultured at 11 mM glucose yet secretion

from these cells is not reduced to the same extent. In fact, secretion from cells cultured at 11 mM glucose was only reduced by about 30-40% at basal glucose and about 60-70% at high glucose compared to secretion from cells cultured at 4 mM glucose. Thus, it seems that the reduced content does not accurately reflect the insulin available for release by the cell. It could be that the rate of new insulin synthesis, which would be secreted preferentially over insulin from a deep reserve pool, is high enough to support much of the secretory needs of the cell. The reserve pool is a large pool of insulin that will not normally be secreted, but rather is destined for degradation. Reduction in this pool due to insulin hypersecretion could lead to a dramatic decrease in content that might not have too much effect on insulin release. Normalizing to such a low content would artificially increase the relative insulin secretion between cells cultured at 4 and 11 mM glucose. It should be noted that when loss of insulin content was prevented in islets exposed to chronic excess nutrients, basal insulin secretion was still increased (Erion et al., 2015). This confirmed that the basal insulin hypersecretion under these conditions was not simply an artifact of changes in content in the intact islet.

Due to the significant decrease in insulin content from cells cultured at 11 mM compared to 4 mM glucose, seen in figure 13, we chose to also normalize the data to basal insulin secretion. In this case, the cells were normalized by 60%. This was based on the premise that the low content would still allow for normal secretion at 1 mM glucose. GSIS (at 12 mM glucose) from cells cultured in 11 mM glucose was decreased by about 30% compared to cells cultured at the low glucose (4 mM) condition (figure 15). Normalizing to basal insulin secretion thus unveiled an impaired GSIS, which is a

characteristic of GLT. We believe the answer to correcting for content is somewhere in between these two methods of normalization. Additional experiments are warranted to determine a threshold level of insulin content that allows for proper normalization of secretion from cells cultured at high 11 mM glucose.

In a previous study conducted by Saadeh, it showed that the addition of fatty acid cultured in 11mM glucose cells caused an increase in basal without affecting stimulated insulin release. Once the fatty acid continues to increase higher, then the glucose stimulated condition began to decrease, which represents GLT. Additionally, it was concluded that mono-oleoyl-glycerol (MOG) stimulated basal secretion without increasing intracellular calcium (Saadeh et al., 2012). Intracellular lipids also amplify insulin secretion through a series of mechanisms. This amplification assists in achieving the results of GLT. Another factor to consider is reactive oxygen species (ROS). It plays a role in basal insulin secretion as well. An increase in the production of ROS showed an increase in insulin secretion basally (Saadeh et al., 2012). This is due to an increase in the mitochondrial redox state, which is independent of the GSIS (Saadeh et al., 2012). The lack of lipids or ROS could have been part of the reason insulin secretion was not left-shifted in cells cultured in high glucose. Glucose hypersensitivity is a characteristic of GLT. The size of this left shift (increased secretion from 1 mM to 4 mM glucose) has been associated with lipid content in the cells (Erion et al., 2015). Lipid content in cultured cells is, in part determined by the lipid in the fetal bovine serum (FBS) component of the media. The level of the lipid in the FBS in our system will contribute to GLT in the cultured cells.

In addition to affecting the left-shift in insulin secretion, the lipid content of the media also affects GSIS from cells cultured in 4 mM glucose. Results indicate shorter incubation at 4 mM glucose leads to increased GSIS.

It is known that GSIS requires a level of intracellular lipid to support cell functions. Islets isolated from fasted rats do not secrete insulin in response to increased glucose until fatty acid is added to the incubation media. This suggests that fatty acid is permissive for GSIS. This is supported by studies that show orlistat, a lipase inhibitor also inhibits GSIS until fatty acid is added to test incubations (Kujawska-Łuczak et al., 2017). Whether this effect is due to fatty acid or a complex lipid is not clear and needs further exploration.

We hypothesize that low 4 mM glucose culture conditions cause INS-1 cells to switch from glucose to fat oxidation. It may be that longer incubations in 4 mM glucose media because the lipids in cells are reduced through increased oxidation to a level below the threshold to support GSIS.

When analyzing the INS-1 cells in statins, during the first experiment, represented in figure 16, the cells were cultured for 3 days in 4 mM glucose RPMI. While in the second experiment represented in figure 17, the cells were cultured for 2 days in 4 mM glucose RPMI. Results indicate shorter incubation at 4 mM glucose leads to increased GSIS. It would be interesting to add fatty acid to our high glucose secretion incubations after longer culture in 4 mM glucose to determine if this would recover normal GSIS. This could be an advantage to our studies as the membrane lipids would be low when

looking at basal insulin secretion as proposed to be the case when cells are cultured for 3 days in low glucose (Figure 16).

The time that the cells were cultured in 4G RPMI was decreased in the second statin experiment to prevent poor secretion at 12G, which is an essential component of the insulin secretion process. For instance, during the first experiment, the control barely gave an insulin secretory response; however, with a shorter cultured time in 4G RPMI, the control increased in its insulin secretory response. Shorter culture in low glucose helped to avoid lowering lipid levels below its threshold; thus, supporting GSIS.

In the first experiment with statins seen in figure 16, simvastatin increased basally, while pitavastatin did not. This triggers early insulin secretion and an increase risk of hyperinsulinemia. Pitavastatin and simvastatin both increase GSIS; however, pitavastatin showed improved GSIS at a higher rate compared to simvastatin. In the second statin experiment seen in figure 17, pitavastatin also showed an increase in insulin secretory response compared to simvastatin. The basal level of both statins was elevated, but simvastatin was higher basally.

When cholesterol levels are drastically lowered it stimulates the hydrolysis of PIP_2. The reduction of PIP_2 increases the ATP sensitivity of the K_{ATP} channels in pancreatic ß cells (Skelin Klemen et al., 2017). This leads to the depolarization of the cell membrane and the opening of the calcium voltage gated channels. The calcium triggers phospholipase C (PLC) and furthers hydrolyzes PIP_2 and closes the K_{ATP} channels. Hence causing the release of insulin (Fu et al., 2013). A previous study performed by Keanu Sao in our lab showed that statins increase calcium oscillations at basal levels;

therefore, triggering the early release of insulin secretion. Without statins, the calcium oscillation barely showed at the basal level (Sao, 2020). The overnight incubation of simvastatin in INS-1 cells cultured in 4G RPMI showed that GSIS is impaired as the cells secreted more insulin. This is important because if there is more insulin being secreted then it could cause hyperinsulinemia. However, further studies need to be done to study the underlying mechanisms of GSIS mediated by statins through intracellular calcium oscillations.

In summary, the insulin secretion in simvastatin increased basally compared to the control and pitavastatin. Also, pitavastatin had a higher increase in insulin secretion compared to simvastatin. The data supports that patients who are on a statin should be cautious of the increased risk of T2D; therefore, both experiments on statins suggest that pitavastatin may be the statin of choice to reduce the risk of statin-induced T2D.

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