2019

Differential effects of statins on the pancreatic beta cell

https://hdl.handle.net/2144/36260

BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

DIFFERENTIAL EFFECTS OF STATINS ON THE PANCREATIC BETA CELL

by

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B.Sc., University of New England, 2016

Submitted in partial fulfillment of the
requirements for the degree of
Master of Science
2019

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DEDICATION

I would like to dedicate this work to my loving family and friends.

ACKNOWLEDGMENTS

I’d like to acknowledge Dr. James Hamilton and Dr. Jude Deeney for their continued support and wonderful mentorship. This has been possible only with their guidance.

DIFFERENTIAL EFFECTS OF STATINS ON THE PANCREATIC BETA-CELL

JACOB BODDE

ABSTRACT

Statins are widely used in the treatment of atherosclerosis and hypercholesterolemia, both of which are comorbidities of obesity. However, the effects statins have on insulin homeostasis are relatively unknown and may increase one's risk for type-II diabetes mellitus. INS-1 pancreatic β-cells, were cultured in 11 mM glucose with 25, 50, 100, 200 nM statin or without. Specifically, this study observed the effects that pitavastatin, simvastatin, lovastatin, and pravastatin have on insulin secretion, insulin content, and ROS levels. GSIS was measured after statin and non-statin exposed cells were incubated in 12 mM glucose KRB. Insulin content was measured after trypsinization and subsequent lysing of cells. Both were analyzed via FRET based HTRF assay. ROS levels within cells were measured following statin exposure during a 2-hour period of 12 mM glucose oxidation after DCF was added. Analysis was done using a Tecan™ fluorescent microplate reader. Pitavastatin, simvastatin, and lovastatin decrease glucose stimulated insulin secretion and insulin content as compared to control. All concentrations of pitavastatin reduced insulin secretion proportionally to insulin content, suggesting it does so through impairment of insulin synthesis or storage. Simvastatin reduced insulin secretion and content in a dose dependent manner, however when secretion was adjusted for % content, data showed that high doses of simvastatin reduced insulin content in a greater fashion than insulin secretion, suggesting both secretory mechanisms and storage/synthesis were impaired. Lovastatin reduced insulin secretion by a greater amount than its reduction of insulin content, suggesting that it impaired insulin secretion via secretory mechanism impairment. Pravastatin did not have an effect on either insulin secretion or insulin content at any concentration. Cells were also tested to determine if pitavastatin, simvastatin, or lovastatin induced a change in ROS levels within the cell. None of the three statins tested caused a statistically significant change in ROS levels at all concentrations. These results suggest that pitavastatin, simvastatin, and lovastatin may impair insulin secretion in patients with high blood glucose. As such, clinical guidelines for statin therapy use in those who are at risk, or suffer from, diabetes may need to be reevaluated.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACS	Acute coronary syndrome
BMI	Body Mass Index
CETP	Cholesteryl ester transfer protein
CVD	Cardiovascular disease
FFA	Free fatty acids
GSIS	Glucose stimulated insulin secretion
HDL	High-density lipoprotein
IDL	Intermediate-density lipoprotein
KRB	Krebs-Ringer bicarbonate buffer
LCAT	Lecithin cholesterol acyltransferase
LDL	Low-density lipoprotein
LDLr	Low-density lipoprotein receptor
LPL	Lipoprotein lipase
NAFLD	Non-alcoholic fatty liver disease
ROS	Reactive oxygen species
SREBPs	Sterol regulatory element binding proteins
TAG	Triglyceride
T2DM	Type-II diabetes mellitus
VLDL	Very low-density lipoprotein

INTRODUCTION

OBESITY: A CENTRAL CAUSE AND LINK TO TYPE 2 DIABETES; THE ROOT OF THE PROBLEM

Approximately one third of all adults in the United States are overweight, a statistic that is shared globally [Bastien, et al., 2014]. The percentage of obese adults however differs, as nearly 10% of all adults and 26% of US adults are obese [Bastien, et al., 2014]. Obesity is defined as a body mass index (BMI) equal to or greater than 30.0 [Centers for Disease Control and Prevention, 2017]. The prevalence rate of obesity among infants, 2-to-19 year old children, and even adults has not changed [Ogden, et al., 2014]. The obesity problem is considered an epidemic, with a very incomplete understanding of the causes, both physiological and social/psychological. Researchers and medical professionals have sought to ascertain the risk factors that contribute to obesity as well as the outstanding comorbidities that continue to persist.

Obesity can be caused genetically by a defect in leptin, a hormone produced primarily by white-adipose tissue [Zhang, et al., 1994]. Leptin acts on the hypothalamus to reduce food intake [Weigle, et al., 1995], and acts peripherally (such as on the liver and pancreas) to increase fatty acid oxidation and reduce lipid synthesis [Shimabukuro, et al., 1997]. However, typical obesity, not caused by a congenital defect, is associated with a high circulating concentration of leptin due to a form of resistance to the hormone [Frederich, et al., 1995]. There are many other genetic factors that predispose individuals to obesity as well, such as dysfunction within both the leptin-melanocortin pathway [Krude et al., 1998] and the ghrelin hormone receptor [Baessler, et al., 2005].

However, it is estimated that genetic causes only account for approximately 5% of the global obesity cases [Bouchard, 2010]. It stands to reason that environmental factors influence weight gain more than genetics, as proposed and studied by many researchers. High-fat diets can cause weight gain and have been known to do so for a while [Lichtenstein, et al., 1998; Teodoro, et al., 2014], but high-carbohydrate diets have similarly been found to cause weight gain [Crescenzo, et al., 2015]. Additionally, the *quality* of fat within an individual's diet can affect weight gain, with diets rich in saturated fats being more detrimental than unsaturated fats. The latter being found to even diminish the negative impact that saturated fats can have on an individual's weight gain and blood lipid profile [Crescenzo, et al. 2015]. More recent studies are illuminating beneficial effects of smaller amounts of polyunsaturated fatty acids such as omega 3 that are stored in membranes (phospholipids) and in fat (triglycerides). In humans, they have not been shown to aid weight loss, however they can attenuate future weight gain and counter metabolic changes from obesity [Albracht-Schulte, et al., 2018].

COMORBIDITIES OF OBESITY: AN OVERVIEW

Obesity carries with it a milieu of dangerous medical comorbidities. As such, it is pertinent to dive into the identity of a few of these comorbidities and how they originate from obesity. An apparent comorbidity of obesity is joint pain caused by arthritis, specifically in the knees [Stürmer, et al., 2000]. This seems to be an issue that is greatly associated with a person's BMI during their 20's and is unique to the knee joint [Gelber, et al., 1999]. The cause of osteoarthritis in an obese person's knees appears to be mechanical in nature [Stürmer, et al., 2000].

Several types of cancers also seem to be more prevalent in those who are obese. Obesity prolonged throughout adulthood increases the risk for kidney, colorectal, liver, pancreatic, stomach, breast (post-menopausal), endometrial, and other forms of cancer [World Cancer Research Foundation, 2018]. One mechanism in which obesity influences the development is through the over secretion of leptin [Somasundar, et al., 2003]. Which has been shown to increase the growth of prostate, esophagus, and breast cancers [Somasundar, et al., 2003]. Additionally, leptin has been shown to have detrimental effect on colorectal cancer, enhancing cell invasion [Jaffe and Schwartz, 2008]. Obesity can also increase one's risk for cancer through the proinflammatory cytokines that adipocytes release [O'Rourke, 2009]. It has been studied that the serum levels of such pro-inflammatory cytokines as TNF-α, IL-6, and even C- reactive protein are elevated in the obese [O'Rourke, 2009]. The increased pro-inflammatory molecules are linked to many different cancers [Coussens and

Werb, 2002]. This is an area of emerging research interest since other diseases are linked to systemic inflammation [Taylor, et al., 2018].

The inflammation promoting cytokines that adipose tissue secretes are also linked to non-alcoholic fatty liver disease (NAFLD) and type-II diabetes mellitus (T2DM) [O'Rourke, 2009]. These systemic inflammatory states are further exacerbated by visceral adipose tissue's proximity to the liver's portal vein, allowing for drainage of inflammation promoting cytokines and abnormally high level of fatty acids directly to the Liver [O'Rourke, 2009]. The increased fatty acid synthesis within the liver and uptake from the plasma, while in an obese state, causes steatosis as their rates dwarf those of fatty acid export and oxidation [Fabbrini, et al., 2010]. NAFLD continues to be an important public health concern in part to its high prevalence, it's risk towards developing liver failure, as well as its ability to influence other disease states, such as T2DM and dyslipidemia [Marchesini, et al., 2003].

Another prevalent heath problem linked to obesity is insulin resistance, which can similarly, and often in conjunction with NAFLD, lead to cardiovascular disease as well as T2DM and dyslipidemia [Ormazabel, et al., 2018]. The increased adiposity contributes to systemic inflammation which causes insulin resistance through release of pro-inflammatory cytokines, ultimately decreasing the effectiveness of insulin's action on both adipocytes and hepatocytes [Halberg, et al., 2009]. The pro-inflammatory molecule TNF-α is elevated in adipose tissue and acts through p55 to inhibit IRS-1, a key component in the insulin signal transduction pathway [Peraldi, et al., 1996]. Insulin resistance is further induced by TNF-α through its effect on PPARγ, a nuclear hormone receptor that drives fat storage and lipid synthesis, This occurs by different mechanisms [Ye, 2008]. The first being at the transcriptional level, wherein TNF-α inhibits the expression of C/EBPδ [Zhang, et al., 1996]. C/EBPδ binds directly to DNA in order to activate the gene promoter for PPARγ [Kudo,et al., 2004]. Thus, TNF-α indirectly reduces PPARγ. The second model of PPARγ inhibition comes from TNF-α's action on HADC3, a nuclear corepressor of PPARγ [Gao, et al. 2006]. TNF-α degrades IkBα, a cytoplasmic binding protein for HADC3, causing HDAC3 nuclear translocation and further elevation of HDAC3 levels within the nucleus, which leads to a lack of PPARγ expression [Gao, et al., 2006]. TNF-α is one of the many ways in which obesity perpetuates insulin resistance, which goes so far as stimulating Kupffer cell disruption of insulin signaling within the liver [Ye, 2013].

Hyperinsulinemia, a constant high level of insulin in the plasma, is considered to be caused by insulin resistance, but may however reciprocally act to induce insulin resistance. Normal insulin secretion requires plasma free fatty acids (FFA) [Stein, et al. 1996] Insulin secretion and is normally a part of a negative feedback loop. The plasma level of insulin is determined by the rate of clearance of insulin as well as the rate of production [Ye, 2007]. Because of the abnormally high concentrations of serum free fatty acids in obesity, the rate of insulin production is tipped into a pathological range that promotes hyperinsulinemia [Stein, et al. 1996; O'Rourke, 2009; Milburn, et al., 1995].

Studies also suggest that both pancreatic β-cell function and cell number is enhanced in obesity, which is attributed to fatty acid and glucose stimulation related to severe weight gain [Corkey, 2011]. Additionally, leptin inhibits insulin production in β-cells [Zhao, et al., 1998]. However, leptin resistance associated with obesity [Fredrich, et al., 1995] contributes to an over production of insulin. Hyperinsulinemia may also occur when there is deficient clearance of insulin, normally performed via the insulin receptor and insulin degrading enzymes in primarily the kidney and liver [Polidori, et al., 2016]. When both of these pathways were disabled in knockout mice, insulin resistance and T2DM occurred [Dodson, et al., 2000; Farris, et al., 2003]. It is important to note that a reduction in intra-abdominal adiposity reduces resolves the insulin resistance [Katzel, et al., 1997]. Ultimately, both overproduction of insulin and deficient insulin clearance contribute towards obesity's ability to induce insulin resistance, an important facet of dyslipidemia.

LIPID METABOLISM AND HOMEOSTASIS

While the links between obesity and the aforementioned disease states are clear and noteworthy, obesity is also linked to dyslipidemia. This is perhaps more deleterious as both are significant risk factors for atherosclerosis and thus cardiovascular disease (CVD) [Feingold and Grunfeld, 2000]. For this issue, it is important to understand key mechanisms of lipid transport and metabolism.

Normal lipid homeostasis is a complex pathway that involves several lipoproteins, which are emulsion particles that encapsulate a large core of neutral lipids. Apo B containing lipoproteins are the primary carriers of triglycerides within the blood and serve to transport them to the peripheral parts of the body [Bays et al., 2013]. These are mainly (1) chylomicrons and chylomicron remnants (Apo B_{48}), which house dietary lipids and are constructed in the enterocyte, and (2) very-low density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), and (3) low-density lipoprotein (LDL) (all of which contain Apo B_{100}) [Bays, et al., 2013].

Exogenous lipids are absorbed and packaged into chylomicrons, which circulate through the blood after exiting the lymph system [Feingold and Grunfeld, 2000]. These chylomicrons release packaged TAG via hydrolysis from lipoprotein lipase (LPL), and as it does so the chylomicron grows smaller, enriches in cholesterol esters, becomes a chylomicron remnant and acquires Apo E. Several apolipoproteins on the surface of the chylomicron are transferred to HDL and other lipoproteins [Feingold and Grunfeld, 2000]. The liver then clears these chylomicron remnants via binding of the hepatic LDL receptor (LDLr) to Apo E, and the cholesterol within the remnant is utilized in the future to form VLDL [Feingold and Grunfeld, 2000].

Cholesterol and endogenous triglycerides are transferred to Apo B100 to create the VLDL particle if triglyceride supply is not limited [Tiwari and Siddiqi, 2012]. VLDL circulates to the peripheral tissues and functions similarly to chylomicrons, wherein triglycerides within the particle are hydrolyzed by LPL, forcing it to decrease in size, leave a higher concentration of cholesterol esters, and become its remnant, IDL (which can also be removed by the liver via the ApoE-LDLr interaction) [Dallinga-Thie, et al., 2010]. The new IDL particle's remaining triglycerides are depleted by hepatic lipase and its exchangeable apolipoproteins are transferred, causing it to form LDL. LDL primarily consists of cholesterol esters and apo B100, with its function to deliver cholesterol to cells [Zhang, et al., 2012]

The liver removes approximately 70% of plasma LDL via the LDL receptor, with the remaining 30% attributed to extra-hepatic removal [Feingold and Grunfeld, 2000]. Ultimately, the number of LDL receptors is what determines the plasma LDL levels, and the expression of the LDL receptor is regulated by cellular cholesterol content [Zhang, et al., 2012; Brown, et al., 2018]. As the levels of cholesterol within a cell decrease, the sterol regulatory element binding proteins (SREBPs) become active, which are transcription factors that induce the transcription of the LDL receptor and HMG CoA reductase, the rate limiting enzyme in the synthesis of cholesterol [Brown, et al., 2018]. The opposite occurs when cholesterol levels are too high within the cell [Brown, et al., 2018].

Peripheral cells receive cholesterol from lipoproteins and can synthesize it on their own, but do not have the capability of catabolizing cholesterol [Feingold and Grunfeld, 2000]. Cholesterol removal involves efflux of cholesterol, via the ABCA1 transporter, from hepatic and extrahepatic tissues to the structural protein Apo A-I, which is known as a nascent high-density lipoprotein (HDL) particle [Rosenson, et al., 2012]. Cholesterol that has effluxed from a cell to HDL remains localized as free cholesterol within the surface of the nascent particle. In order for the HDL particle to become mature, lecithin:cholesterol acyltransferase (LCAT) removes an ester group from a fatty acid within a phospholipid and transfers it to the free cholesterol, creating a cholesterol ester [Ossoli, et al., 2016]. LCAT is an associated enzyme of HDL and is activated by Apo A-I [Ossoli, et al., 2016]. As the cholesterol esters increase, a core is created within the center of the HDL particle, causing it to grow [Feingold and Grunfeld, 2000]. Cholesteryl ester transfer protein (CETP) exchanges the cholesterol within HDL for triglycerides from lipoproteins containing Apo B [Mabuchi, et al., 2014]. HDL then becomes filled with triglycerides and can be metabolized by hepatic lipases [Feingold and Grunfeld, 2000]. The cholesterol carried by the HDL particle is primarily taken up by the liver, a process which is mediated by the SR-BI [Trigatti, 2017].

OBESITY AS A LINK TO DYSLIPIDEMIA

Dyslipidemia itself is defined by a lipid profile of elevated VLDL, LDL, Apo B, serum triglycerides, and a decreased level of HDL [Feingold and Grunfeld, 2000; Miller, 2009]. There are many aspects that link obesity to dyslipidemia, such as adiposity location and metabolic function.

Elevated VLDL, a key component of dyslipidemia, is caused by the increased synthesis of triglycerides from the liver [Donnelly, et al., 2005]. A fatty liver in conjunction with a greater visceral and total body adiposity is the source of a larger access to FFAs for triglyceride synthesis [O'Rourke, 2009], pushing it towards pathological levels [Donnelly, et al., 2005]. A failure to attenuate triglyceride lipolysis, due to insulin resistance, also contributes to increased fatty acid delivery to the liver, resulting in the elevated VLDL profile [Donnelly, et al., 2009; Yu and Ginsberg, 2005].

Additionally, obesity causes a reduction in mRNA expression of lipoprotein lipase in adipose tissue. This directly reduces the activity of LPL, and thus the lipolysis of triglyceride-rich lipoproteins [Clemente-Postigo, et al., 2011]. This leads to an elevation in FFA levels, causing LPL displacement from the endothelium to VLDL and IDL, and further TAG depletion from these lipoproteins [Peterson, et al., 1990; Klop, et al., 2013]. The increased TAG displacement contributes to greater exchange of TAG from these remnants to HDL for cholesterol-esters via CETP, resulting in elevated LDL [Capell, et al., 1996; Klop, et al., 2013]. In hypertriglyceridemia however, the cholesterol-ester content of LDL decreases, and TAG content increases due to CETP activity. Further alterations occur via hepatic lipase hydrolysis of the TAG content within the LDL particle, creating small dense LDL, a pro-atherogenic lipoprotein [Tchernof, et al., 1996; Deighan, et al., 2001].

Obesity’s role in reducing levels of HDL in obese persons is particularly detrimental as HDL has been known to be atheroprotective [Poti, et al., 2014]. Specifically, the subforms of HDL_2 and apolipoprotein A-I [von Eckardstein, et al., 1994]. HDL_2 is less dense and contains more cholesterol on a per particle basis than HDL_3. Apo A-I, the major protein component of HDL, is in high concentration within the HDL_2 subfraction [Rashid and Genest, 2007]. Apo A-I has been shown to prevent the self-aggregation and oxidation of LDL particles within the arterial wall [Khoo, et al., 1990].

A reduction in HDL_2 population, which directly lowers apo A-I levels, can also indirectly occur in obesity due to obesity’s deleterious effect on metabolism. Hypertriglyceridemia, following an increase in hormone sensitive lipase activity due to obesity-related insulin resistance, causes an increase VLDL particles [Ginsberg, 2000]. Higher VLDL numbers result in an increase in TAG transfer to HDL, creating more triglyceride-rich HDL_2 particles, the desired substrate for hepatic lipase, allowing for greater hepatic uptake of HDL_2 [Rashid and Genest, 2007]. This issue is three-fold, as both HPL and CETP activity are greater in obese individuals, with the HPL activity being further increased in insulin resistant states [Arai, et al., 1994; Baynes, et al., 1991].

ATHEROSCLEROSIS: A MAJOR HEALTH ISSUE

As previously mentioned, obesity is a major risk for atherosclerosis, the underlying cause of CVD. Dyslipidemia, insulin resistance, and diabetes mellitus are also risk factors for atherosclerosis on their own. However, their perpetuation caused by obesity, resulting in simultaneous progression of one or more of these diseases, greatly increases that risk [Semenkovich, 2006; Herrington, et al., 2016].

Briefly, atherosclerosis is a disease involving the build-up of plaque within the wall of arteries, which causes a narrowing of the vessel overtime [National Heart, Lung, and Blood Institute, 2018]. Atherosclerosis is the leading cause of death worldwide and can cause peripheral artery disease, ischemic stroke, and ischemic heart disease [Murray and Lopez, 2013; Herrington, et al., 2016]. Plaques mature within the intimal wall of an artery and may rupture at their luminal surface and can block circulation to cause an ischemic event, such as a heart attack [Bentzon, et al., 2014].

Hypercholesterolemia, specifically pro-atherogenic lipoproteins like LDL, are the main cause of atherosclerosis [National Heart, Lung, and Blood Institute, 2018]. For a plaque to form, apo-b containing lipoproteins migrate into the intima of the artery (sub-endothelium) and become trapped [Klop, et al., 2013]. Small dense LDL has an increased affinity for the proteoglycans within the arterial wall, which leads to elevated lipoprotein retention [Tabas, et al., 2007]. Within the intima, the lipoproteins aggregate and oxidize, with small dense LDL being more susceptible to oxidation due to less anti-oxidative content and free cholesterol [Bentzon, et al., 2014; Subramanian and Chait, 2012]. The now oxidized lipid fractions, which originated from the modified lipoproteins, chronically stimulate the immune system, leading to an increase in pro-inflammatory cytokines as well as monocyte differentiation and migration [Bentzon, et al., 2014]. The resulting macrophages become deposits for the oxidized moieties and develop into foam cells. Eventually, these foam cells gather in great enough layers to become visible, yellow, fatty streaks, which can develop into an atherosclerotic lesion [Bentzon, et al., 2014].

These lesions mature when non-cellular lipids accumulate and form a lipid core underneath the foam cell layers [Stary, et al., 1994]. These lipid cores can become necrotic when macrophages invade and apoptose, leaving oxidized lipid debris and cellular remnants, causing an irreversible change to the intima and resulting in a fiberoatheroma [Betzon, et al., 2014; Tabas, 2010]. The normal connective tissue of the intima is replaced by collagen-rich fibrous tissue by the smooth muscle cells, which can create a fibrous cap. The extracellular matrix, apoptotic cells, and necrotic core act as locations for calcium deposits, which can expand throughout the entire necrotic core [Betzon, et al., 2014]. Further, plaques can cause arterial remodeling, causing the arterial segment to expand in such a way that the lumen area is not compromised until the plaques are too large [Glagov, et al., 1987]. A plaque can also rupture, usually at the thinnest point along the fibrous cap, leading to thrombus formation and acute coronary syndrome (ACS) [Betzon, et al., 2014].

Treatment modalities for atherosclerosis encompass both lifestyle changes and statins. Before pharmaceutical intervention, clinical recommendation is based around reducing adiposity and returning metabolism to normal. This involves heart-healthy lifestyle changes, such as increasing physical activity, managing stress, and a balanced diet [National Heart, Lung, and Blood Institute, 2018]. However, lifestyle changes can be difficult to implement, and may not be effective, as such pharmaceutical intervention is required. The statin therapy for treatment and prevention of atherosclerosis is widely used and effective [Bergheanu, et al., 2017].

STATIN THERAPY OVERVIEW

Statins act primarily by targeting hepatocytes and inhibiting their synthesis of cholesterol [Stancu and Sima, 2001]. The rate limiting step in cholesterol synthesis involves the enzyme HMG-CoA reductase, which converts HMG-CoA into mevalonate, a cholesterol precursor. Statins are competitive inhibitors of HMG-CoA reductase, binding to HMG-CoA reductase's active site and forcing a conformational change, resulting in a non-functional enzyme [Mangravite, et al., 2006; Stancu and Sima, 2001]. The reduction of cholesterol within hepatocytes also causes an increase in LDL receptor expression, causing a downstream reduction of circulating LDL, IDL, and VLDL lipoproteins [Stancu and Sima, 2001; Sehayek, et al., 1994].

Statins have other impacts on atherosclerosis aside from cholesterol and atherogenic lipoprotein reduction. Both simvastatin and fluvastatin inhibit cholesterol esterification in macrophages, reducing its accumulation [Bernini, et al., 1995]. Additionally, simvastatin and lovastatin have been shown to upregulate expression, and inhibit LDL-mediated down-regulation, of the atheroprotective eNOS in endothelial cells [Laufs, et al., 1998; Simionescu, et al., 2002]. Statins can also prevent coronary events via plaque rupture and thrombus formation. Specifically, simvastatin and fluvastatin have been shown to cause the regression of coronary atherosclerosis and reduce the formation of new lesions [Ballantyne, et al., 1997].

Statins are some of the most prescribed drugs in the US, in part to their general effectiveness as well as the prevalence of atherosclerosis and hypercholesterolemia. Atorvastatin and simvastatin (Lipitor and Zocor, respectively) are the third and fifth most prescribed drugs, respectively [Fuentes, et al., 2018]. With such widespread use of statins, there is increasing evidence to suggest complications may arise from statin therapy. Adverse muscle effects are the most commonly reported complication of statin therapy [Golomb and Evans, 2008]. These include muscle pain, weakness, and fatigue. However, rhabdomyolysis, severe muscle necrosis that can result in renal dysfunction or failure, is a possible adverse effect from statin therapy when combined with a fibrate, another lipid lowering medication [Golomb and Evans, 2008].

Insulin homeostasis can also be affected by statin therapy. Briefly, insulin secretion occurs through the pancreatic β-cell, due primarily from the driving force of glucose. Glucose enters the cell via the GLUT2 transporter, where it increases in concentration and causes a concomitant increase in the ATP/ADP ratio after it undergoes glycolysis. ATP then moves to the K_{ATP} channel, binds to it, and causes it to close. In doing so, the cell membrane depolarizes and an influx of Ca^{2+} rushes into the cell, wherein it causes insulin exocytosis [Fu, et al., 2013].

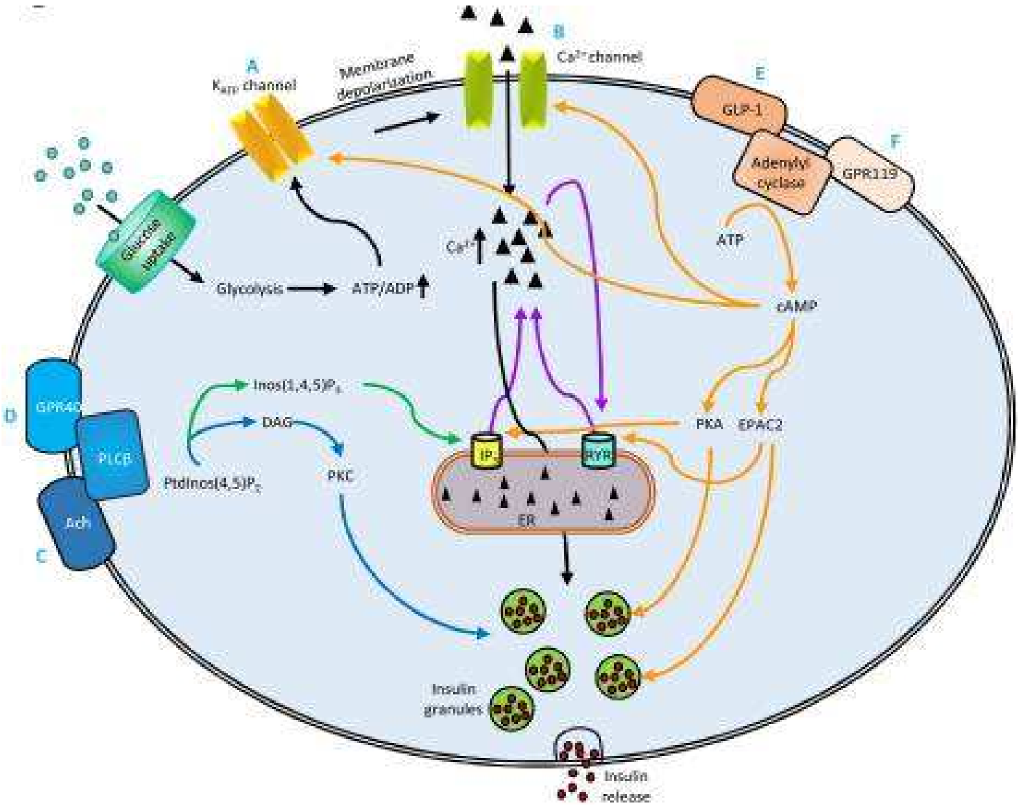


Figure 1: Insulin Secretion Pathways

Although glucose is the primary physiological trigger for insulin secretion, there are many other components of insulin secretion. Dysfunction of one of these components can lead to metabolic imbalance and disease [Image from Fu, et al., 2013].

There are several studies that show some statins may adversely affect insulin secretion. Notably, both simvastatin and atorvastatin have been suggested to inhibit glucose stimulated insulin secretion (GSIS), the latter through inhibition of the Ras/Raf pathway [Scattolini, et al., 2016; Sun, et al., 2016]. However, other statins such as pravastatin have not shown to have a deleterious effect on insulin secretion [Urbano, et al., 2017]. The implications for adverse effects on insulin secretion due to statin therapy are concerning to both clinicians and patients, as type 2 diabetes is closely linked to dyslipidemia and hypercholesterolemia [American Heart Association, 2015]. The nature of statins as a treatment for cardiovascular issues gives these implications great significance, as type 2 diabetes is linked to obesity, atherosclerosis, and dyslipidemia. The patient population that is prescribed statins is already at a greater risk for type 2 diabetes due to existing risk factors. As such, it is pertinent to study a wide array of statins and their effects on the pancreatic β-cell.

SPECIFIC AIMS AND OBJECTIVES

This study looks to elucidate the effects statins have on glucose stimulated insulin secretion and β-cell insulin content. Specifically, pitavastatin, pravastatin, simvastatin, and lovastatin effects.

METHODS

STATIN PREPARATION

Pitavastatin and pravastatin were dissolved in DMSO to a concentration of 10 mM in order to separate statin ions bound by Ca^{2+}. Statins were then further diluted in DMSO to 200 uM, aliquoted and then stored in -20°C.

Simvastatin and lovastatin structure contain a lactone and must first be activated. 4 mg of each statin was dissolved in 100 µl of ethanol. 150 µl of 0.1 N NaOH was added to the solution, which was subsequently incubated at 50°C for 2 hours. The pH was brought to 7.0 using HCl. Statin solution was then dissolved in DMSO to 200 uM, aliquoted, and stored at -20 °C.

INS-1 (832/13) CELLS

INS-1 832/13 clonal pancreatic β-cells were split and then plated onto either 48- and 96-well tissue treated plates (Corning, Corning, NY) to the approximate density of 260,000 and 130,000 cells per well, respectively. Cells were cultured in 11 mM glucose RPMI 1640 media with phenol red with addition of 1 mM sodium pyruvate, 10 mM HEPES and 50 µM β-mercaptoethanol. 24-48 hours after initial plating, original media was exchanged for fresh 11 mM glucose RPMI and statins. Statins were diluted to 200 nM and then serially diluted to 100 nM, 50 nM, and 25 nM within 11 mM glucose media with 0.1% DMSO for addition to cells. Plates contain control wells with 0 nM statin and 0.1% DMSO and experimental wells containing the previously stated statin concentrations. All conditions were cultured in triplicate. Cells were then incubated for 72 hours prior to experiment.

INSULIN SECRETION

Insulin secretion was measured from INS-1 cells grown for 5 days to approximately 85% confluence in 48- and 96-well plates. Media that cells were incubated in were replaced with 2 mM glucose RPMI without FBS. Cells were incubated in that for two hours. Cells were then incubated for 30 minutes in 1 mM glucose Krebs-Ringer bicarbonate buffer (KRB) at 37^{o}C. The KRB buffer contained 119 mM NaCl, 20 mM HEPES, 5 mM NaHCO3, 4.6 mM KCl, 2 mM CaCl2, 1 mM MgSO4, 0.15 mM Na2HPO4, 0.4 mM KH2PO4, 0.05% BSA, pH 7.4. Following the preincubations, INS-1 cells were chilled on ice before 1 mM glucose KRB and 12 mM glucose KRB solutions were added. Cells were incubated for two hours at 37^{o}C, cooled, and then sampled for insulin secretion. Insulin was measured by FRET-based HTRF insulin assay (CisBio, Bedford, MA).

INSULIN CONTENT

INS-1 cells were incubated in trypsin for 5 minutes and then removed. Cells are counted by hand via hemocytometer. Cells are then centrifuged to a pellet and lysed in cold PBS containing 0.1% Triton X-100 and 25 mM NaOH. Insulin content is measured by FRET-based HTRF insulin assay (CisBio, Bedford, MA).

REACTIVE OXYGEN SPECIES MEASUREMENT

Intracellular reactive oxygen species (ROS) accumulation was approximated by fluorescence assay using 2,7-Dichlorofluorescein diacetate (H_2DCF-DA; Life Technologies) for a probe. H_2DCF-DA diffuses into cells, wherein it is enzymatically hydrolyzed to H_2DCF by an intracellular esterase. After which, the H_2DCF is oxidized to DCF, an extremely fluorescent compound while in the presence of ROS, the intensity of which is directly proportional to the amount of intracellular ROS.

After INS-1 cells were incubated with statin (50 nM, 100 nM, 200 nM concentrations) and without, cells were incubated in basal (1mM glucose) KRB buffer, no serum, with 10 uM H_2DCF-DA for 30 minutes at 37^oC. Cells were then washed with basal KRB buffer, no serum, and incubated in the basal KRB buffer without serum and H_2DCF-DA for 10 minutes at 37^oC. After which, cells were chilled and 12 mM glucose KRB buffer with serum was added, and cells were incubated for 2 hours.

A fluorescence assay was taken at timepoint 0 (before incubation), 60 minutes of incubation, and 120 minutes of incubation. Fluorescence was measured in a microplate fluorometer (Tecan, Switzerland) at 488 nm excitation and 525 nm emission.

STATISTICAL ANALYSIS

The data are shown as the mean of independent experiments ±S.E. Comparisons between several means were performed by ANOVA one-way analysis of variance test. The level of significance was set at $p < 0.05$ (*).

RESULTS

Pitavastatin Does Not Decrease Basal Secretion

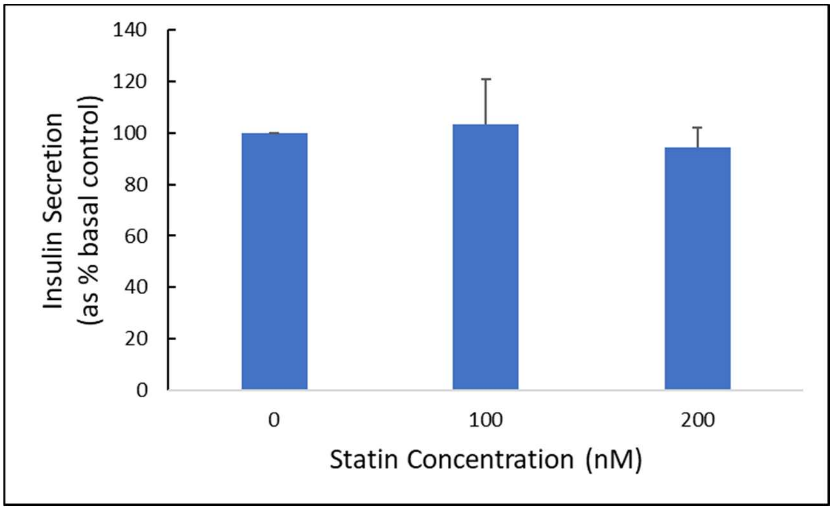
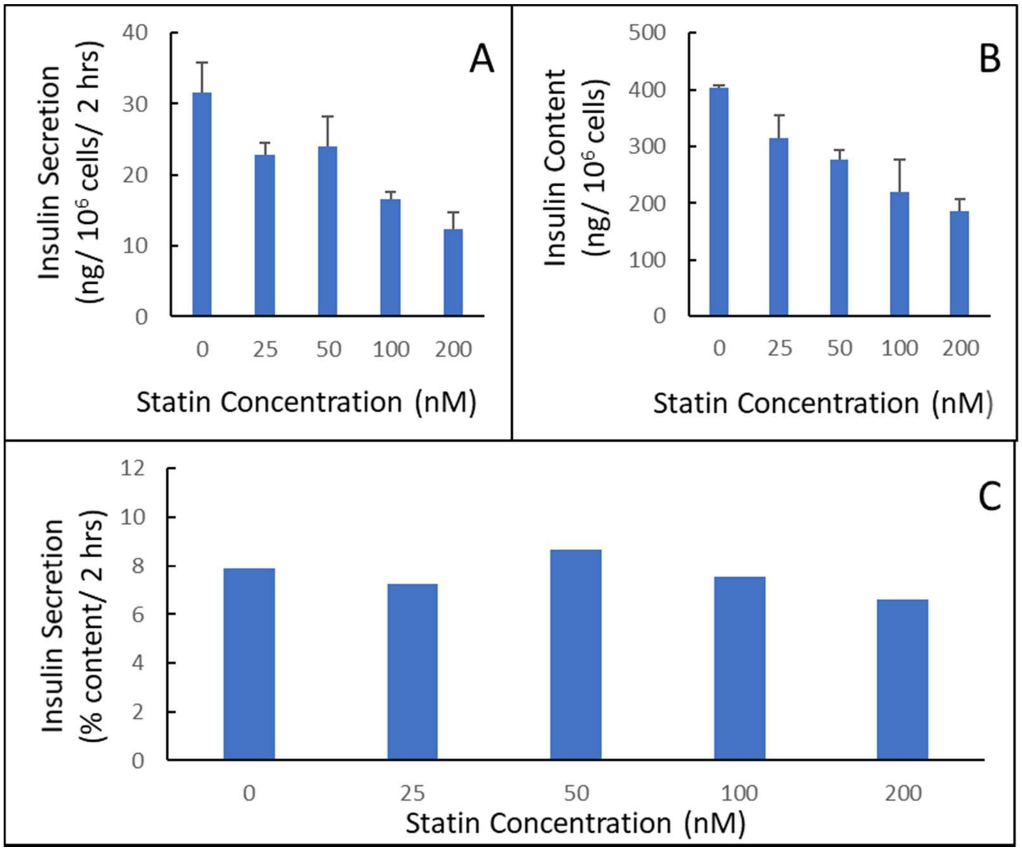


Figure 2: Pitavastatin has no effect on basal secretion

INS-1 cells were cultured in normal 11 mM glucose culture media with or without 25 nM, 50 nM, 100 nM, or 200 nM pitavastatin for 72 h. Basal secretion was *measured after exposure 1 mM glucose KRB buffer. Figure shows basal secretion after exposed to 0, 100, or 200 nM statin. Values were obtained by correcting for basal control. Values are not significantly variable (p>0.05). n=3 experiments.*

Figure 2 shows that pitavastatin does not affect the basal secretion of INS-1 cells. At the highest doses, pitavastatin caused INS-1 cells to basally secrete nearly identical to the basal condition. This data suggests that pitavastatin may not inhibit insulin secretory mechanisms involved in low glucose concentrations. For instance, pancreatic β-cells are known to have Ca^{2+} oscillations for insulin signaling even in basal conditions [Lang, et al., 1979]. Pitavastatin may not affect Ca^{2+} signaling within the β-cell, and thus have no impact on basal levels of insulin secretion.

Pitavastatin Negatively Affects Insulin Content



<u>**Figure 3**: Pitavastatin reduces stimulated insulin secretion and insulin content.</u>

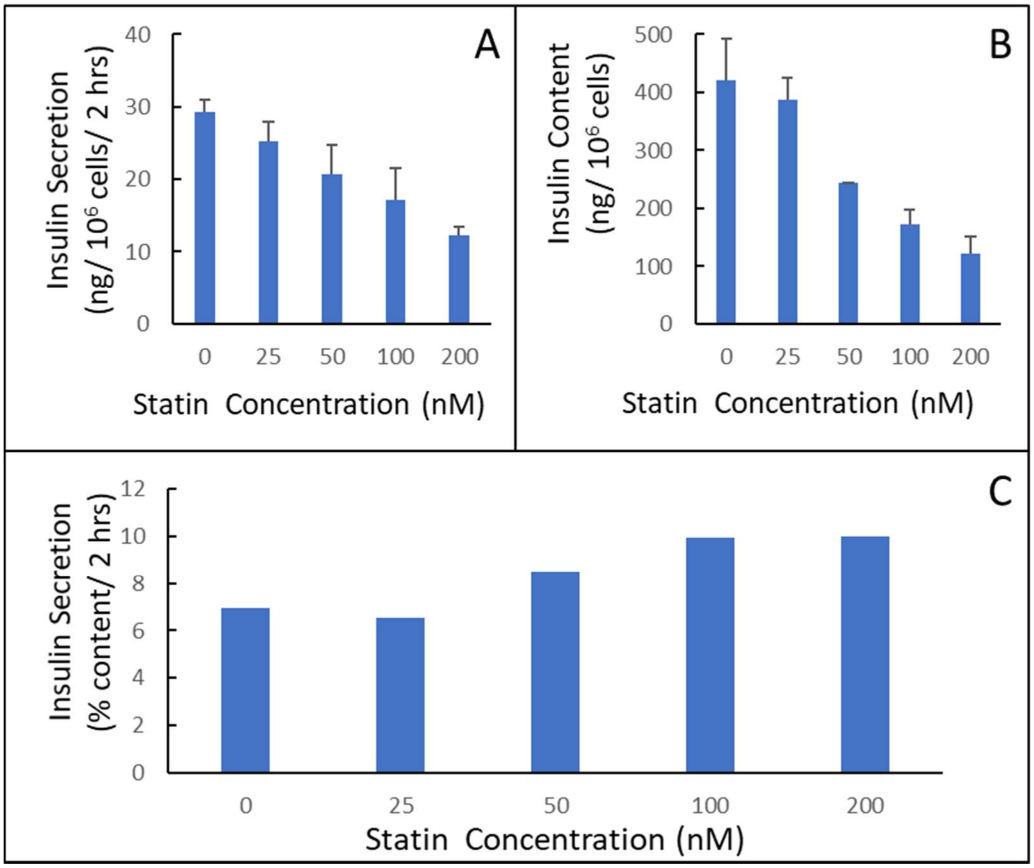
INS-1 cells were cultured in normal 11 mM glucose culture media with or without 25 nM, 50 nM, 100 nM, or 200 nM pitavastatin for 72 h. ***A****, insulin secretion after exposure to these conditions. Values* (p <0.05) are the mean secretion data for each condition after correcting for basal secretion.* ***B****, insulin content measured after exposure to these conditions. Values* (p <0.05) are the mean content data for each condition.* ***C****, mean insulin secretion data as percent of each conditions' respective insulin content. n=3 experiments for A and B.*

Figure 3*A* shows that INS-1 cells incubated in 11 mM glucose culture media with pitavastatin for 72 hours caused an inhibition in insulin secretion. After accounting for basal secretion, the lowest dose caused a decrease of insulin secretion by 33% when compared to control. However, there was no dose-dependent decrease in secretion within the range of concentrations. Between 25 nM and 50 nM there is a small if any difference, which suggests that the two lower concentrations have a similar effect on β-cells. This is unlike the effect seen when comparing 50 nM to 100 nM, as insulin secretion drops markedly between the two. Additionally, the decrease in secretion at 200 nM from 100 nM is a smaller difference than that of 50 nM to 100 nM. While not conclusive, this may suggest that the inhibitory effects of pitavastatin begin to taper off at higher doses.

Similar to its effect on insulin secretion, pitavastatin elicits a decrease in insulin content, within the INS-1 cells, as seen in **Figure 3*B*.** The content decreases incrementally as the concentration of statin increases, signifying a dysfunction in some aspect of insulin storage.

Figure 3*C* represents INS-1 insulin secretion when corrected for total insulin content within the cells. Analysis of secretion as a percent of content analyzes the cell’s ability to both access its insulin stores and secrete insulin properly. Pitavastatin shows a relatively equal percentage of secretion for each concentration’s content, suggesting that as its concentration increases, GSIS and insulin content decrease similarly. As such, pitavastatin may not impair the secretory mechanism involved in insulin secretion, but instead impair insulin synthesis and/or storage mechanisms.

Simvastatin Inhibits Insulin Secretion and Decreases Insulin Content



<u>**Figure 4**: Simvastatin reduces stimulated insulin secretion and insulin content</u>

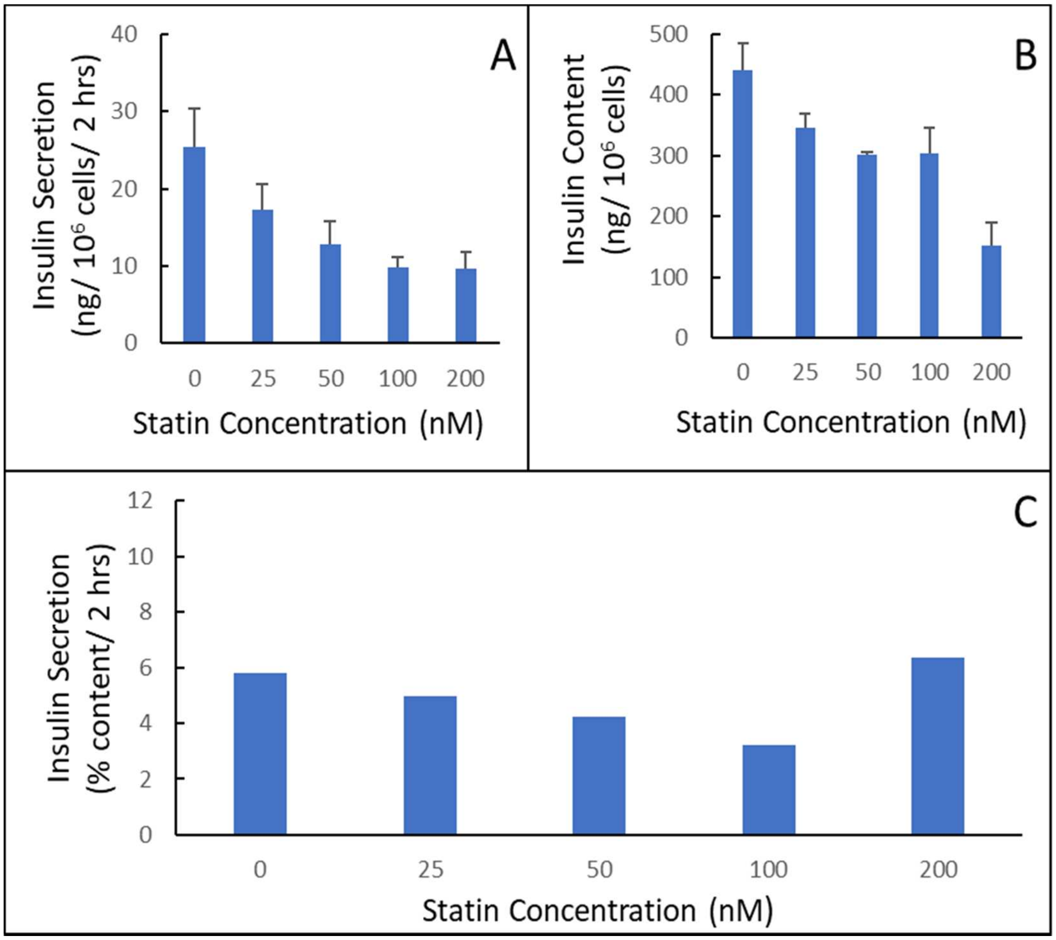
INS-1 cells were cultured with simvastatin in 25, 50, 100, and 200 nM concentrations in conditions stated previously. ***A****, insulin secretion after exposure to those conditions. Values* (p <0.05) are the mean secretion data for each condition after correcting for basal secretion.* ***B****, insulin content measured after exposure to those conditions. Values* (p <0.05) are the mean content data for* *each condition.* ***C****, mean insulin secretion data as percent of each conditions' respective insulin content. n=3 and 2 experiments for A and B*

Simvastatin has been suggested to inhibit insulin secretion in previous studies [Scattolini, et al., 2016]. **Figure 4*A*** demonstrates that in the presence of simvastatin at 25 nM, 50 nM, 100 nM, and 200 nM, INS-1 cells have a reduced ability to secrete insulin in response to stimulatory media (12 mM glucose). Additionally, the decrease seen as concentration increases did not seem to be attenuated, suggesting that even higher doses than 200 nM may cause more severe inhibition of secretion.

Insulin content was also negatively affected by simvastatin, as seen in **figure 4*B***. At the lowest concentration (25 nM), simvastatin hardly decreases insulin content in comparison to control. However, 50 nM nearly halves cellular insulin content, with both 100 nM and 200 nM further establishing a decrease in insulin content without tempering the effect.

While 25 nM simvastatin seems to maintain secretion at a similar % of content to the control, the higher concentrations do not, as seen in **figure 4*C***. With a reduction in both secretion and insulin content, an increase in a larger percentage of content being secreted may imply that the rate at which secretion occurs is similar to the control's rate. However, since the cell has access to a lesser pool of insulin, cell secretion is deceased. It is equally possible that the insulin pool available was disproportionately decreased, such that the decreased rate of secretion was still greater than its access to the cell's insulin stores.

Lovastatin Negatively Impacts Both Insulin Secretion and Storage



<u>**Figure 5**: Lovastatin reduces stimulated insulin secretion and insulin content.</u>

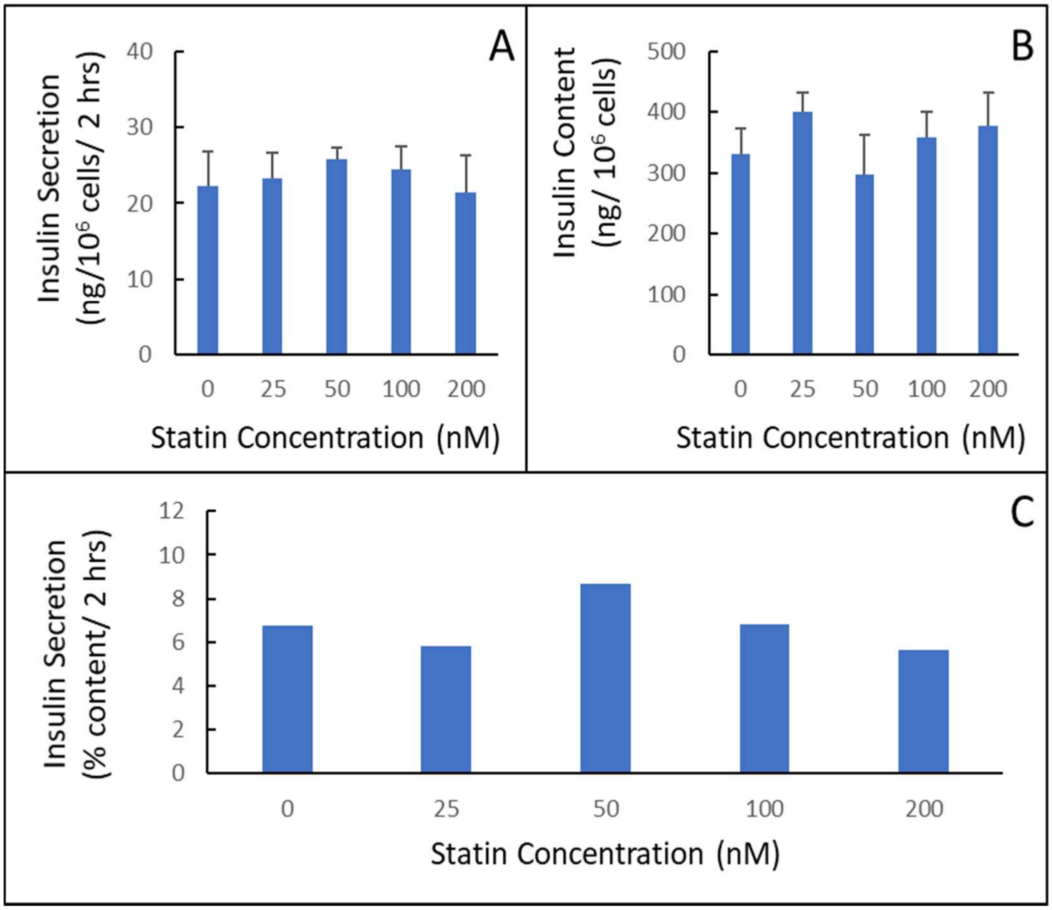
INS-1 cells were cultured with lovastatin in 25, 50, 100, and 200 nM concentrations in conditions stated previously. ***A****, insulin secretion after exposure to those conditions. Values* ($p < 0.05$) are the mean secretion data for each condition after correcting for basal secretion.* ***B****, insulin content measured after* *exposure to those conditions. Values* (p <0.05) are the mean content data for each condition.* ***C****, mean insulin secretion data as percent of each conditions' respective insulin content. n=2 experiments for A and B,*

Lovastatin caused a significant inhibition in insulin secretion even at the lowest dose, as seen in **figure 5*A***. However, the inhibition began to taper between 50 nM and 100 nM. At 100 nM and 200 nM concentrations, the effect on insulin secretion was almost equivalent. This suggests that the inhibitory effect of lovastatin begins at a low dose but quickly reaches its maximum effect.

That effect is not mirrored in lovastatin effect on insulin content, which is to decrease either insulin synthesis or reduce storage function. **Figure 5*B*** shows that all concentrations aside from 200 nM achieve similar inhibitory results on insulin content. The 200 nM concentration nearly decreased the insulin content to a fourth of the control. This suggests that a high dose is required to fully realize lovastatin's insulin content lowering effects. One possibility is that there may be a compensatory mechanism to maintain insulin content through the lower doses of lovastatin that is out competed in 200 nM concentration.

Lovastatin's opposing dose effects on secretion and insulin content are shown in **figure 5*C***. As the dose increased, the percent of content being secreted decreased, similar to the trend seen in GSIS data. In contrast, at 200 nM the % of content that was secreted raised to levels a little higher than the control. The data implies that low doses of lovastatin negatively impact the secretory mechanisms of insulin secretion, but at higher doses the negative impact on insulin storage/synthesis is greater.

Pravastatin does not Inhibit Insulin Secretion or Decrease Insulin Content



<u>**Figure 6**: Pravastatin does not negatively affect insulin secretion or content</u>

INS-1 cells were cultured with pravastatin in 25, 50, 100, and 200 nM concentrations in conditions stated previously. ***A****, insulin secretion after exposure to those conditions. Values are the mean secretion data for each condition after correcting for basal secretion.* ***B****, insulin content measured after exposure to* *those conditions. Values are the mean content data for each condition.* ***C**, mean insulin secretion data as percent of each conditions' respective insulin content. Significance of both secretion and insulin content data variance is null (p >0.05). n=2 experiments for A and B.*

Pravastatin did not show a significant decrease in insulin secretion or insulin content at any concentration, as shown in **figure 6*A*** and **6*B***. **Figure 6*C*** data shows 50 nM pravastatin may cause an increase in the percent of content secreted, but the insulin content value used to determine that was not found to be significantly variant from other data points.

Pitavastatin and, Lovastatin Show a Non-Significant Decrease in ROS Levels

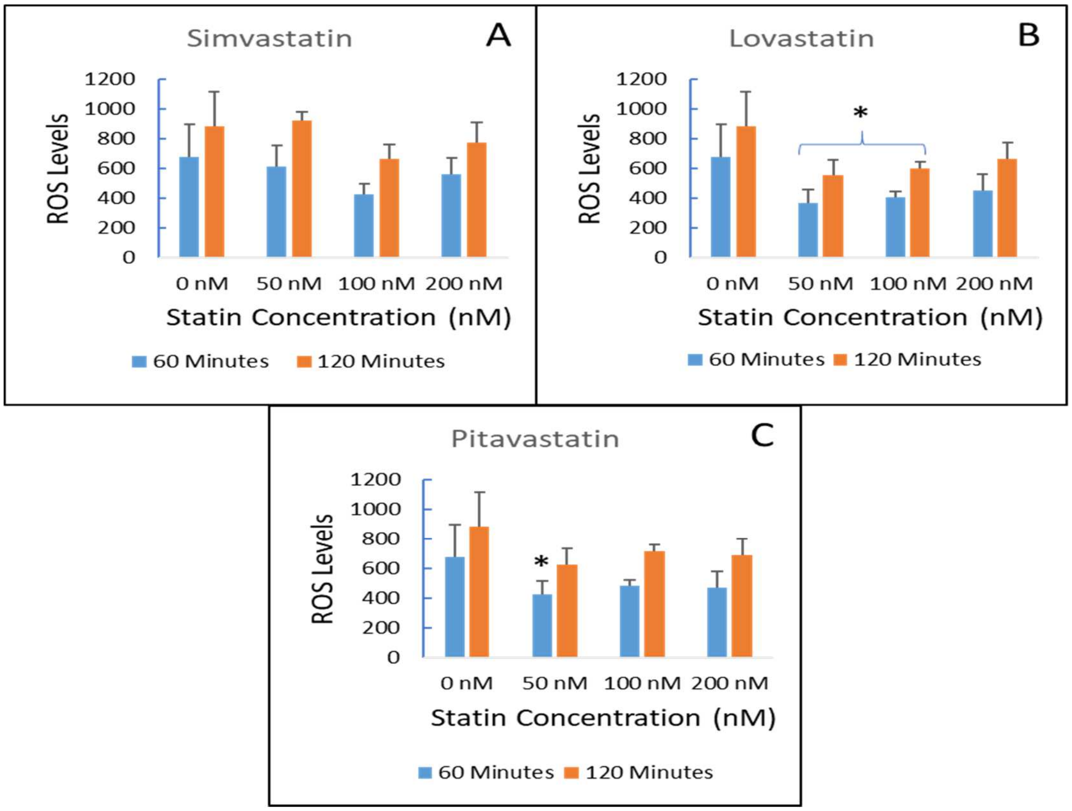


Figure 7: Simvastatin, lovastatin, and pitavastatin effect on ROS levels following glucose oxidation.

*ROS levels in INS-1 cells during a 2 h glucose oxidation period was measured following simvastatin, lovastatin, and pitavastatin exposure at 50, 100, and 200 nM concentrations in normal medium (11 mM glucose) for 72 h. *$p < 0.05$. n=1 experiment for A, B, and C.*

During glucose oxidation, simvastatin at all concentrations had no significant effect on intracellular ROS accumulation, as seen in **figure 7*A***. Lovastatin showed a non-significant overall reduction in cellular ROS levels. At 25 and 50 nM, lovastatin did show a significant reducing effect on ROS levels at both the 60-minute and 120-minute time point, shown in **figure 7*B***. Pitavastatin also showed at 50 nM to reduce ROS levels at the 60-minute timepoint, as shown in **7*C***. More experiments are necessary to determine the effect these statins have on intracellular ROS. Current research shows simvastatin inducing reactive oxygen species in skeletal myotubes, but there has not been research regarding its effect, or the effects of lovastatin and pitavastatin, on pancreatic β-cells [Kwak, et al., 2012].

This study sought to elucidate the effects that different statins had on insulin secretion and insulin content. While mechanistic studies were performed, the relationship between content and secretion created a framework for further understanding of β-cell injury from statins and insulin homeostasis. In this study, pitavastatin, simvastatin, and lovastatin showed significant inhibition of secretion by possibly different mechanisms. Pitavastatin data suggests that insulin content reduction could be the driving force behind a decrease in insulin secretion. In contrast, simvastatin has been verified to effect Ca^{2+} signaling [Yaluri, et al., 2015], hence its decrease in insulin secretion. Similarly, lovastatin appears to negatively impact a component of the insulin secretory machinery, causing its inhibition of secretion. Next steps will include monitoring Ca^{2+} oscillations as well as measuring ATP and K^+ levels to determine secretory defects. While more research will be required to figure out how the insulin content is being reduced, there are various speculations as to what is causing the reduction. As previously mentioned, insulin biosynthesis could be negatively affected, reducing the rate at which insulin content is being created and therefore decreasing the amount of insulin within a secretory granule. Another possibility is that statins may cause an acute hypersecretion of insulin into culture media which depletes insulin content.

DISCUSSION

This study showed that pitavastatin inhibited glucose stimulated insulin secretion through its effect on decreased insulin content. These findings are supported by another similar in *vitro* experiment [Zhao, et al. (2015)]. In which, atorvastatin, pravastatin, rosuvastatin, and pitavastatin, all inhibited insulin secretion when cells were treated with 100 nM of statin. Additionally, these statins were shown to be cytotoxic at 100 nM concentration. However, pitavastatin was shown to elicit the highest insulin secretion range of all 4 statins when stimulated with both physiologic and diabetogenic glucose levels [Zhao, et al., 2015]. Studies performed *in vivo* have shown different results that suggest pitavastatin does not incur risk for developing type-2 diabetes or insulin resistance. Clinically, pitavastatin does not have an effect on insulin secretion at either low or high glucose levels, does not affect endogenous glucose production, and improves insulin sensitivity in patients who are pre-diabetic or currently suffer from type-2 diabetes [Braun, et al., 2018; Wang, et al., 2017; Satoh, et al., 2012]. In conjunction with this study and the *in vitro* study performed by Zhao, et al., data may suggest that pitavastatin has a noticeable impact on insulin secretion, yet negligible in those who suffer from type-2 diabetes.

In contrast, simvastatin has been well studied and shown to be detrimental to those who are pre-diabetic or suffer from type-2 diabetes. Even in the presence of positive control compounds, such as tolbutamide and KCl, simvastatin was shown to decrease insulin secretion [Yaluri, et al., 2015]. Simvastatin inhibits insulin secretion through many pathways, however the most detrimental is its impact on intracellular Ca^{2+}, a key component in signaling for insulin secretion. In pancreatic β-cells, cytosolic Ca^{2+} concentrations oscillate in a pulsatile manner, stimulating insulin secretion. Simvastatin inhibits L-type Ca^{2+} channels, the principle transport protein for Ca^{2+} movement in response to L-arginine, thereby reducing intracellular Ca^{2+} concentrations and preventing insulin secretion [Gilon, et al., 1993; Yada, et al., 1999]. Additionally, simvastatin has been shown to inhibit the K_{ATP} channel, reducing the cell's ability to depolarize and allow Ca^{2+} into the cell to trigger insulin exocytosis [Yaluri, et al., 2015]. This explains this study's data showing simvastatin's ability to decrease insulin secretion but does not explain its ability to reduce insulin content. More research is required.

At the current stage, conclusions surrounding lovastatin are not clear, and more research focused on statin inhibition of insulin homeostasis is needed to elucidate its risk for type-2 diabetes. *In vivo*, lovastatin was found not to influence insulin secretion in patients who currently suffer from, and are treated for, T2DM [Johnson, et al., 1990]. Additionally, it was shown that the pharmacokinetics of the type-2 diabetic drug chlorpropamide was not affected by lovastatin treatment. This study may prove that chlorpropamide treatment offsets any insulin secretion impairment lovastatin will produce, as there is some evidence to suggest lovastatin has a negative impact on insulin secretion. *In vitro*, lovastatin was shown to block the signaling of phospholipase C, a key enzyme that increases cytosolic Ca^{2+} concentration through its creation of IP3 and diacylglycerol [Li, et al., 1993]. Lovastatin selectively reduced the effect of two phospholipase C agonists, bombesin and arginine vasopressin [Li, et al., 1993]. It is apparent that lovastatin merits additional research, as its effects on insulin secretion are not clear.

Pravastatin is also a highly studied statin in terms of its effects on pancreatic β-cells. As with this study, many researchers have reported that pravastatin elicited no effect on insulin secretion [Yada, et al., 1999; Urbano, et al., 2017; Ishikawa, et al., 2006]. Because of this, pravastatin stands out as an effective treatment modality for those who are pre-diabetic or diabetic who suffer from hypercholesterolemia, dyslipidemia, and/or atherosclerosis [McTavish and Sorkin, 1991].

Atorvastatin, the most prescribed statin in the US [Fuentes, et al., 2018], was not included in our study but will be in our future research. Many studies have elucidated the effects that atorvastatin has on insulin secretion. These include downregulation of the phosphorylation of key proteins involved in the Ras pathway [Sun, et al., 2016]. The Ras complex pathway is the major regulator for cell growth and gene transcription [Sun, et al., 2016]. As an HMG-CoA reductase inhibitor, atorvastatin reduces the reduction of mevalonate and therefore cholesterol. However, mevalonate is utilized in creating farnasylated proteins, which are active in perpetuating the signal transduction in the Ras/Raf pathway.

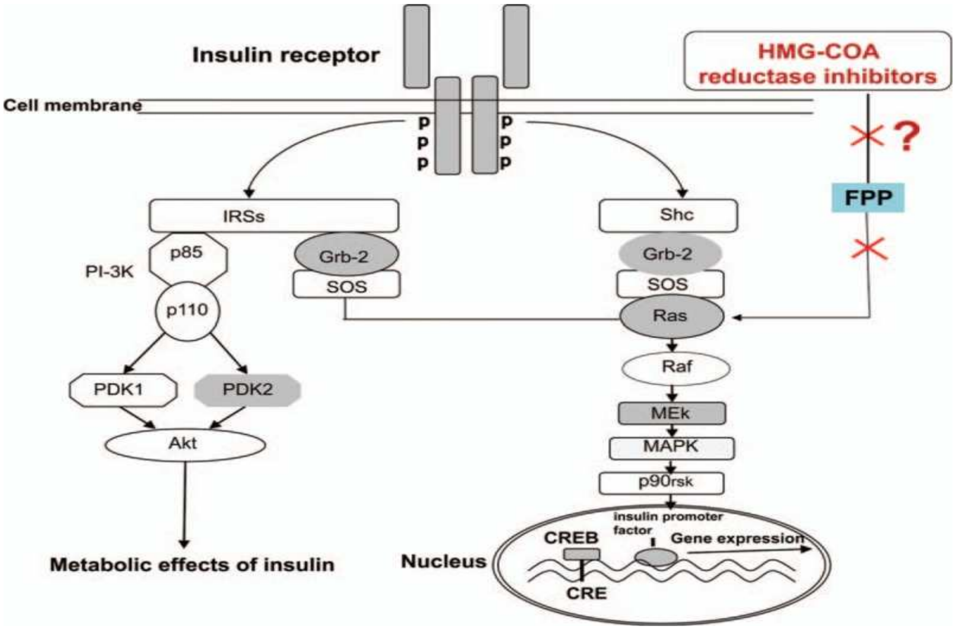


Figure 8: Atorvastatin Causes Dysregulation of Ras/Raf Pathway

Atorvastatin's effect on FPP reduction causes a downstream inhibition of the Ras/Raf pathway. In doing so, CREB, a transcriptional binding protein, is not able to bind to the CRE binding element and induce transcription [Image from Sun, et al., 2016]

Inhibition of this cascade causes a decrease in gene transcription and therefore a decrease in insulin synthesis [Sun, et al., 2016]. This mechanism by which atorvastatin reduces insulin content may be shared by other statins, which will require further studies.

Although statins cause an imbalance in insulin homeostasis and therefore diabetogenic effects, there have been many other aspects of statins that are a benefit to the patient. There is ample evidence to suggest that statins act as anti-inflammatory molecules. For instance, statin inhibition of isoprenoid accumulation can deter vascular inflammation, which assists in preventing atherosclerosis [Antonopoulos, et al., 2012]. Additionally, statins have shown modest antineoplastic properties, specifically in their prevention of colorectal cancer [Bardou, et al., 2010]. Lastly, statins have shown neuroprotective effects. Simvastatin has been shown to induce Bcl-2 expression and 4-hydroxy-2E-nonenal in neurons and protect against neuronal cytotoxicity [Johnson-Anuna, et al., 2007; Lim, et al., 2006]. These recently elucidated effects of statins have shown their far-reaching benefits that have just begun to be understood.

Ultimately, evidence highlights the danger that statins can pose to overall health. This danger is unique and paradoxical, as type 2 diabetic patients are more likely to die from cardiovascular disease [Jackowski, et al., 2008]. However, the efficacy of statins to treat dyslipidemia, hypercholesterolemia, and atherosclerosis will stimulate continued debate about whether treatment outweighs the risks of developing type-2 diabetes. Clinical guidelines for statin therapy use in those who are at risk, or suffer from, diabetes may need to be reevaluated.

Table 1: Summarization Table

A table summarizing the discussed statins as well as their effects on insulin homeostasis.

Statin Chemical Name/Trade Name	Effects on Glucose Metabolism	Safe for T2DM	Currently Testing in Our Projects
Pitavastatin -Livalo™ -Livazo™	-Showed no mean increase in blood glucose in diabetic patients [Yamakawa, et al., 2008] -Decreased insulin secretion and showed cytotoxicity [Zhao, et al., 2015]	Maybe	Yes
Lovastatin -Mevacor™ -Altoprev™	-No effect on insulin secretion in diabetic patients [Johnson, et al., 1990] -Reduced insulin secretion through Ca^{2+} blockage (via Phospholipase C inhibition) [Li, et al., 1993]	Maybe	Yes
Simvastatin -Zocor™ -FloLipid™	-Inhibits Ca^{2+} signaling, the K_{ATP} channel, and blocks L-type Ca^{2+} channels in MIN6 [Yada et al., 1999; McTavish and Sorkin, 1991].	No	Yes
Pravastatin -Pravachol™	-Does not affect insulin secretion [Yada, et al., 1999; Urbano, et al., 2017; Ishikawa, et al., 2006]	Yes	Yes
Atorvastatin -Lipitor™	-Inhibits insulin synthesis via Ras/Raf pathway through its reduction of mevalonate [Sun, et al., 2016].	No	No

Our research will continue to analyze statin alterations in insulin homeostasis. Next steps will include studying things like intracellular lipids and Ca^{2+} signaling in order to analyze the effects statins have on the β-cell.

Table 2: Future steps for our research.

This table highlights the future research that will be done to the previously studied statins in order to gain more insight into their effects on insulin homeostasis.

Future Research	**Description**
Insulin secretion (kinetic studies)	- β-cell perifusion to monitor the oscillatory patterns of insulin secretion after chronic statin exposure.
Intracellular lipid accumulation	- Image analysis of intracellular triglyceride accumulation via fluorescence microscopy using Nile Red. Cholesterol ester kit (abcam) will be used to quantify total cholesterol and cholesterol esters
Membrane potential and intracellular calcium	- Using Fura 2 and bis-oxonol, cellular mechanisms used in insulin signaling will be evaluated.
ROS	- Continue with ROS measurements during glucose oxidation as well as ROS levels due to statins in culture

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