The Pharmacology of Intra-islet GLP-1 in Pancreatic Islets

by

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Abstract

Paracrine signaling within the pancreatic islets is important for the normal secretion of insulin and glucagon. As the secretion of insulin and glucagon are central to whole body glucose homeostasis, paracrine signaling in the islet has a role in glucose control. Genetic animal models have shown that α cell secretion of proglucagon peptides, including GLP-1, are important for normal insulin secretion and glucose homeostasis. I hypothesized that human islets express GLP-1 and that paracrine GLP-1 secretion contributes to insulin secretion in nondiabetic and type 2 diabetic islets. I also tested the effect of increasing GLP-1 levels in the islet on insulin secretion and islet survival.

To investigate my hypotheses, I used several methods known to islet biology and a population-based study. Immunohistochemistry and flow cytometry were used to examine GLP-1 expression. GLP-1 secretion was examined using static incubations and perifusions, while islet cell survival was determined using a dead cell assay. Active GLP-1 levels were increased with the DPP4 inhibitor sitagliptin or adenoviral vectors. Finally, I designed an observational study to investigate outcomes associated with starting sitagliptin early in the therapy of type 2 diabetes.

From my results, I was able to conclude that human islets do express GLP-1 and that GLP-1 expression increases in type 2 diabetic islets. I also show that the dependence of glucose-stimulated insulin secretion on GLP-1 receptor signaling is associated with the amount of GLP-1 expression in nondiabetic and type 2 diabetic islets. These results allow us to consider a GLP-1 signaling axis in human islets. Sitagliptin treatment of human islets does not increase glucose-stimulated insulin secretion, but may increase islet cell survival. Neonatal porcine islets can be engineered to increase GLP-1 secretion, but the functional consequences of this are still unknown. Finally, the results of our observational study suggest that adding sitagliptin to metformin early in therapy may slow diabetes progression and preserve β cell function.

Preface

The research presented in this thesis received research ethics approval from the University of Alberta Research Ethics Board. All animal studies followed the guidelines issued by University of Alberta Animal Care and Use Committee (protocol #s AU00286 and AU01417). De-identified human islets isolated from deceased donors were obtained from the Alberta Diabetes Institute IsletCore and the Clinical Islet Laboratory, University of Alberta, in accordance with institutional human ethics guidelines.

Chapter 2 of this thesis has been submitted for publication and is under peer review. I was responsible for the majority of the data collection, data analysis, and manuscript composition. Dominic Golec performed the flow cytometry and analysis. Koenraad Philippaert performed the calcium imagining and analysis. Matt Hubert collected some of the perifusion data. Nicole Salamon and Janyne Johnson imaged most of the human islet sections, under my supervision. Peter Light is the supervisory author and was involved with concept formation and editing of the manuscript.

Chapter 3 of this thesis has been written for submission for publication. I was responsible for the majority of the data collection, data analysis, and manuscript composition. Donor and islet parameters were provided by Tatsuya Kin at the Clinical Islet Laboratory. Matt Hubert, Nicole Salamon, and Janyne Johnson performed some of the GLP-1 secretion experiments and hormone measurements. Peter Light will be the supervisory author and was involved with concept formation.

For Chapter 4, the NPIs were provided by the Greg Korbutt lab. The adenovirus was provided by the late Stephen Cheley and Amy Barr. I was responsible for data collection and analysis.

Chapter 5 of this thesis has been written for submission for publication. I contributed to the design, wrote part of the Stata code, and wrote the initial manuscript. Scot Simpson wrote the remaining code, guided the design and analysis for the study, and edited the manuscript. Peter Light conceived the idea for the project.

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Abbreviations

A1c	hemoglobin A1c
ACE	angiotensin converting enzyme
ARB	angiotensin II receptor blocker
BMI	body mass index
cAMP	cyclic adenosine monophosphate
CCK	cholecystokinin
CIT	clinical stimulation index
CMV	cytomegalovirus
DPP4	dipeptidyl peptidase-4
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GIP	glucose-dependent insulinotropic peptide
GLP-1	glucagon-like peptide-1
GLP-1R	glucagon-like peptide-1 receptor
GSIS	glucose-stimulated insulin secretion
HFD	high fat diet
IL-6	interleukin-6
KRBH	Krebbs-Ringer bicarbonate HEPES buffer
M3R	muscarinic acetylcholine receptor 3
MACE	major adverse cardiac events
MPGF	major proglucagon fragment
ND	nondiabetic
NOD	non-obese diabetic
NPI	neonatal porcine islets
NSAID	nonsteroidal anti-inflammatory drug
PC1/3	prohormone convertase 1/3
PC2	prohormone convertase 2
PYY	peptide YY
SDF-1 α	stromal cell-derived factor 1 α
SI	stimulation index
SSRI	selective serotonin reuptake inhibitor
STZ	streptozotocin
T1D	type 1 diabetes
T2D	type 2 diabetes
TZD	thiazolidinedione

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Chapter 1

General Introduction

Diabetes

Current global estimates for 2017 indicate that 425 million people have diabetes with 85-95% of these cases being type 2 diabetes (T2D). Strikingly, these numbers are expected to rise to 629 million by 2045 (Federation (IDF), 2017). In Canada, the trend is similar, with rates of diabetes increasing to where 11% of the population is projected to have diabetes by 2028 ("Diabetes in Canada Rounder," 2018). Diabetes is a global disease where the highest rates are now seen in non-western countries. The economic burden of T2D is high with an estimated cost of diabetes worldwide to be US $825 billion (Chatterjee et al., 2017). Furthermore, more than half (60%) of the global costs of diabetes are borne by low income and middle income countries (NCD Risk Factor Collaboration (NCD-RisC), 2016). Clearly, treating diabetes must remain a priority for health care agencies and governments around the world.

Type 2 diabetes

T2D is characterized by insufficient insulin secretion in the face of insulin resistance. The result of this failure to meet insulin demand is high fasting blood glucose and a loss of glucose tolerance. Although obesity is associated with insulin resistance and often contributes to the development of diabetes, ultimately the development of diabetes appears to be due to a loss of insulin secretion from the pancreatic β cells. A sizeable number of the genes identified using genome-wide association studies of type 2 diabetes have identified genes associated with β cell function (Mahajan et al., 2018; Wood et al., 2017), thus confirming the importance of the β cell in the development of T2D.

The importance of tight blood glucose control for the prevention of microvascular complications was established by The United Kingdom Prospective Diabetes Study (UKPDS). Further studies with this patient cohort have also shown that tight glucose control can also reduce cardiovascular risk (Holman et al., 2008). This is an important finding as cardiovascular disease is the leading cause of death in patients with type 2 diabetes (Laakso, 2010).

Pharmacological treatment for T2D has expanded beyond sulfonylureas and insulin therapy to include an array of treatments that enhance blood glucose control. However, these new medications do not imitate physiological control of blood glucose, and other therapies that reduce glucose concentrations in the fasting and postprandial state are still needed.

Type 1 diabetes

Type 1 diabetes (T1D) results from autoimmune destruction of the pancreatic β cells. With the loss of insulin secretion from β cells, glucose homeostasis is lost and blood glucose levels continue to rise with damaging metabolic consequences. Efforts to predict the disease or help with a T1D diagnosis, have used HLA genotypes and autoantibodies with some success. However, emerging biomarkers using T-cell assays or an omics approach may be combined with standard biomarkers to help refine prediction and diagnosis of the disease (Mathieu et al., 2018). Unfortunately, attempts to initiate immunosuppressive therapy at diagnosis with the aim to preserve remaining β cell mass have seen limited success (Skyler, 2018). The mainstay of treatment for T1D is exogenous insulin, either in the form of daily injections or insulin pumps. As with T2D, tight blood glucose control is essential to prevent to the complications associated with high glucose. The Diabetes Control and Complications Trial (DCCT) demonstrated that intensive therapy to control blood glucose in T1D prevents microvascular complications and reduces the risk of macrovascular complications such as myocardial infarction (NathanDCCT/EDIC Research Group, 2014).

Interestingly, the majority of patients with T1D do not have complete destruction of their β cell mass, even after years of living with the disease (Oram et al., 2015). These patients, called microsecretors, have low circulating C-peptide levels that are sensitive to glucose stimulation and indicate residual functioning β cells (Oram et al., 2014). The clinical significance of this finding is not known, but with the prospect of residual β cell mass, the opportunity presents itself for the use of medications that may preserve or expand the remaining β cells.

This suggests that some medications for T2D may be useful in the treatment of T1D. Dipeptidyl peptidase (DPP4) inhibitors and GLP-1R agonists, by activating glucagon-like peptide-1 (GLP-1) signaling in the residual β cells, may help to preserve this residual β cell mass (Suen and Burn, 2012).

The pancreatic beta cell in type 2 diabetes

β cell dysfunction is central to the development of type 2 diabetes. Initially, the β cell is able to adapt to the increasing insulin resistance associated with obesity, but ultimately it is the beta cell that will fail and cause diabetes. Weir et al. proposed five stages of β cell dysfunction in the progression of type 2 diabetes (Weir and Bonner-Weir, 2004). In stage 1, the β cells compensate to increase insulin secretion and β cell mass to meet the increased demand for insulin in the face of insulin resistance. At this stage the beta cells exhibit normal glucose tolerance (NGT). In stage 2, the β cells have stably adapted to the increased demand for insulin secretion, but fasting glucose has increased slightly, and the first phase of insulin secretion is blunted leading to impaired glucose tolerance (IGT). Stage 3 is transient and involves an increase in fasting glucose levels leading to β cell decompensation. This phase often quickly progresses to stage 4 which is overt type 2 diabetes. Stage 4 is described as stable decompensation where patients with type 2 diabetes can stay in this phase for a lifetime. The minimal insulin secretion at this stage prevents the ketosis that is seen in stage 5. Stage 5 is severe decompensation and describes type 1 diabetes where patients would have ketosis if not for daily injections of exogenous insulin.

There are a number of physiologic stressors that contribute and may initiate the β cell dysfunction seen in type 2 diabetes. Five stressors have been identified and postulated by Halban et al. to be important in the pathogenesis of type 2 diabetes (Halban et al., 2014). First, endoplasmic reticulum stress is induced by the increasing demand on the beta cell to produce and secrete insulin. This increased insulin production may trigger the unfolded protein response,

thus promoting β cell secretory dysfunction and if chronic, apoptosis. Secondly, metabolic and oxidative stress, owing to nutritional excess and the ensuing glucolipotoxicity, promotes b cell dysfunction. β cells are more sensitive to reactive oxygen species (ROS) than other cell types, and the increased ROS production during hyperglycemia may promote dysfunction. Thirdly, amyloid plaques, a characteristic of islets in T2D, are formed from the increased production of islet amyloid polypeptide and the formation of oligomers. This induces interleukin-1β production, recruits macrophages, and increases islet inflammation leading to β cell dysfunction. Fourthly, local islet inflammation, whether from infiltrating macrophages or innate inflammation from endocrine cells contributes to β cell dysfunction. Lastly, the organization and cell to cell communication of the islet may be disturbed leading to diminished or dysregulated paracrine signals necessary for normal insulin or glucagon secretion.

The loss of functional β cell mass in the progression of type 2 diabetes may not only be due to beta cell death and very low β cell proliferation rates, but also due to dedifferentiation. Mouse studies have shown that β cell dedifferentiation is a mechanism of β cell failure where β cells revert to a progenitor-like cell or even convert to another endocrine cell type (Talchai et al., 2012). This mechanism for β cell failure has also been reported in islets from patients with type 2 diabetes (Cinti et al., 2016). Furthermore, the concept of stunned β cells with reduced glucose sensitivity fits with the idea that dedifferentiated β cells that have lost the machinery for stimulus-secretion coupling (Ferrannini, 2010). The discovery of dedifferentiation as a mechanism for beta cell failure is important because it may be reversible, and therapies may be developed to rehabilitate the β cells to a fully functional differentiated state.

The pancreatic alpha cell in type 2 diabetes

Dysregulated glucagon secretion is a hallmark of α cell dysfunction in type 2 diabetes. Glucagon secretion in type 2 diabetes is characterized by persistent fasting hyperglucagonemia and a lack of post-prandial suppression (Gromada et al., 2018). It is thought that the loss of

insulin secretion in type 2 diabetes reduces the inhibitory tone of insulin on the α cell, allowing for increased glucagon secretion. This increased glucagon secretion exacerbates the hyperglycemia resulting from insufficient insulin secretion. The effect of persistent hyperglycemia on glucose sensing in the alpha cell is not known, particularly in relation to glucagon secretion. However, glucose does directly control glucagon secretion, but the exact mechanism is not clear (Gromada et al., 2018).

It is not clear if α cell mass is unchanged or increased in type 2 diabetes (Henquin and Rahier, 2011a; Rahier et al., 1983). However, the loss of β cells in type 2 diabetes results in a higher α/β cell ratio. As with reduced insulin secretion from dysfunction β cells, the loss of β cell mass also reduces the inhibitory tone on glucagon secretion from α cells. The loss of β cell mass not only results in reduced insulin secretion, but also a reduction in the secretion of zinc and GABA. Zinc forms the core of insulin crystals and is released with insulin secretion. Although the research is not settled, there is evidence to suggest that zinc may have an inhibitory effect on glucagon secretion (Gromada et al., 2018). GABA is also secreted from β cells and has been shown to contribute to the inhibitory tone on glucagon secretion (Bailey et al., 2007; Rorsman et al., 1989). Clearly, the increased α/β cell ratio has implications for dysregulated glucagon secretion, and possibly GLP-1 secretion, in type 2 diabetes.

The incretin effect and the incretins GIP and GLP-1

The incretin effect refers to the increase in insulin secretion observed with an oral glucose load compared to intravenous administration of the same glucose load. The gut hormones, GLP-1 and glucose-dependent insulinotropic polypeptide (GIP), are secreted in response to nutrient absorption and sensing, and act as endocrine hormones on the pancreatic islets to increase insulin secretion and regulate glucose homeostasis. The effect of the incretin hormones on the stimulation of insulin secretion is substantial and contributes to approximately

two-thirds of the total insulin secretion (Nauck and Meier, 2018). The estimated contribution to insulin secretion from the incretin hormones depends on the dose of oral glucose and may vary from 25% to 75% (Nauck and Meier, 2018). This dose dependence on oral glucose demonstrates that the secretion and action of the incretin hormones are an important mechanism for the regulation of glucose homeostasis.

The incretin hormones, GIP and GLP-1, are products of different genes and are secreted from their own enteroendocrine cells that are primarily located in separate parts of the small intestine. GIP is secreted from the K cells located in the proximal small intestine with particular dominance in the duodenum and jejunum (Brown, 1982). The proximal location of the K cells allows for early nutrient detection and GIP is secreted upon nutrient availability. GIP is expressed from the gene GIP as the prohormone pro-GIP, and post-translationally cleaved to the 42 amino acid polypeptide. On the other hand, GLP-1 is secreted from the L cells located primarily in the distal small intestine and the colon (Eissele et al., 1992). The L cells are predominant in the ileum and also present in the colon. Despite the distal placement of the L cells in the small intestine, GLP-1 is secreted roughly at the same time as GIP, suggesting that a signal from the upper gut to the L cells coordinates secretion. GLP-1 is post-translationally cleaved from proglucagon by the prohormone convertase 1/3 (PC1/3), where proglucagon is expressed from the proglucagon gene. GLP-1 can be truncated from its extended form GLP-1 (1-37), to bioactive GLP-1 (7-37), and further truncated by dipeptidyl peptidase 4 (DPP4) to the inactive GLP-1 (9-37). Active GLP-1 (7-37) may undergo C-terminal amidation, and in humans it predominantly circulates as GLP-1 (7-36amide). Both GLP-1 (7-37) and GLP-1 (7-36amide) are equipotent at the GLP-1 receptor (Nauck and Meier, 2018).

In healthy subjects, GIP is thought to mediate the majority of the incretin effect, while GLP-1 has a minor role to play (Nauck and Meier, 2018). Experiments where GIP and GLP-1 were infused at levels that approximate physiological levels after a glucose load demonstrated that GIP strongly stimulates insulin secretion, while GLP-1 has a weaker effect on insulin

secretion (Nauck et al., 1993a). However, in healthy humans the effects of GIP and GLP-1 are additive. Experimentally, when GIP and GLP-1 are infused the effect on insulin secretion is equal to the sum of the insulin responses when the peptides are secreted alone (Nauck et al., 1993a).

In subjects with type 2 diabetes, the secretion of GIP and GLP-1 is similar to healthy subjects, but the insulin secretory response is diminished. Many studies have been performed on type 2 and healthy subjects, with some studies showing a slight reduction in secretion of incretin hormones in type 2 subjects, while others have not. However, a meta-analysis of these studies suggests that there is no real difference in oral glucose or meal test induced secretion of GIP or GLP-1 between type 2 and healthy subjects (Calanna et al., 2013a; 2013b). However, in type 2 diabetes, the insulin secretory responses are not similar to healthy subjects and the secretory response to GIP is significantly diminished (Mentis et al., 2011). Although, GIP was discovered before GLP-1, the lack of an insulin secretory response to GIP in type 2 diabetes precluded the development of therapies to treat diabetes. Although the magnitude of the insulin response to GLP-1 in subjects with type 2 diabetes is still less than in healthy subjects, the insulin secretory response is still intact (Nauck et al., 1993b). The fact that the insulin response to GLP-1 remains intact in type 2 diabetes has allowed for the successful development of therapies based on GLP-1 biology.

DPP4 inhibitor and GLP-1R agonist pharmacotherapy

Not only was GLP-1 found to be insulinotropic for the pancreatic β cell, it was also shown to increase insulin biosynthesis (Doyle and Egan, 2007). Furthermore, preclinical rodent studies indicated that GLP-1 modulates β cell apoptosis, increases proliferation of β cells, and may even augment β cell neogenesis. Most excitingly, exogenous GLP-1 was shown to increase insulin secretion and improve glucose homeostasis in type 2 diabetics (Drucker, 2003).

However, GLP-1 is quickly degraded in vivo to an inactive form by the proteolytic enzyme DPP4, thus limiting therapeutic use of the native peptide. The development of GLP-1 therapeutics is primarily based on designing compounds that inhibit DPP4 or GLP-1 analogs that are resistant to DPP4 degradation.

DPP4 inhibitors

DPP4 inhibitors are small molecules that inhibit the enzymatic activity of DPP4 and increase circulating levels of active GLP-1 and GIP. Through the action of increased active GLP-1 and GIP they increase insulin secretion and improve glucose homeostasis in T2D. The first DPP4 inhibitor approved for the treatment of diabetes was sitagliptin and many more have followed: linagliptin, alogliptin, saxagliptin, and vildagliptin. As a class, they are effective medicines that reduce fasting glucose and A1c. They are also weight neutral and do not increase cardiovascular risk (Mannucci et al., 2016).

GLP-1R agonists

GLP-1R agonists are GLP-1 peptides modified to prevent degradation by DPP4 and improve pharmacokinetics. Approved therapeutic peptides modify the parent GLP-1 peptide by amino acid substitution and elongation (exenatide, lixisenatide), acylation (liraglutide, semaglutide), albumin conjugation (albiglutide), or antibody Fc domain conjugation (dulaglutide) (Drucker, 2018). Other than initial GI side effects that may be minimized with proper dose titration, GLP-1R agonists are effective and well tolerated agents for glucose control. GLP-1R agonists also increase satiety and reduce weight gain. Furthermore, liraglutide and semaglutide have been show to decrease major adverse cardiovascular events (MACE) in patients with type 2 diabetes (Drucker, 2018).

Paracrine hormone communication in human islets

The human islet is anatomically organized to facilitate paracrine hormone communication. The human islet is not organized like the mouse islet, as the mouse islet is

comprised of a core of β cells surrounded by a mantle of α cells (Caicedo, 2013) (Figure1.2). Paracrine communication in the mouse islet is mostly between like cell types and thought to be dependent on the direction of blood flow through the islets (Caicedo, 2013). In contrast, hormone secreting cells in human islets are interspersed and form heterogeneous connections with different cell types allowing paracrine communication via the interstitium. These cells not only form multiple connections with each other, but are lined up along blood vessels to facilitate coordinated action for hormone secretion. Interestingly, research in non-human primate islets show that capillary diameters and blood flow can be regulated within the islet (Diez et al., 2017). This contrasts with mouse islets where blood flow is not thought to be dynamic within the islet. The possibility of regulated capillary action and blood flow within human islets adds another layer of dynamic regulation of paracrine hormone action.

Paracrine communication in the islet is orchestrated to coordinate hormone secretion for the regulation of glucose homeostasis. A good example of paracrine coordination is the glycemic setpoint of different species. The glycemic setpoint of a species appears to be determined by the α /β cell ratio of the islets, where levels of insulin secretion to control glycemia rely on paracrine α cell signalling (Rodriguez-Diaz et al., 2018). When islets from different species where transplanted into mice, the new glycemic setpoint was determined by the expected glycemic setpoint of the donor islet species. The expected glycemic setpoint is related to the a/b cell ratio, and likely determined by paracrine signaling between the two cell types.

The classic islet hormones insulin, glucagon, and somatostatin serve as paracrine hormones to control endocrine secretion of glucagon and insulin. Insulin has an inhibitory effect on glucagon secretion from α cells. The loss of β cell mass and function in diabetes, and the subsequent decrease insulin secretion, is thought to contribute to the dysregulated and increased glucagon secretion (Kawamori et al., 2009). Glucagon stimulates insulin secretion

from β cells and appears to be required for normal insulin secretion (Huypens et al., 2000; Svendsen et al., 2018). Experiments where the glucagon receptor was overexpressed in β cells have demonstrated the importance of this signaling. In these experiments, β cell function and mass were increased with increased glucagon signaling. Finally, somatostatin from delta cells inhibits both insulin and glucagon secretion (Gerich et al., 1975).

The intriguing aspect of this thesis is that an incretin hormone is expressed in human islets to act in a paracrine manner. GLP-1 is a potent agonist for the β cell GLP-1 receptor and I observe its expression in nondiabetic and diabetic islets. By blocking the GLP-1 receptor and measuring insulin secretion, I test the potential contribution of GLP-1 to regulate insulin secretion. Furthermore, paracrine GLP-1 signaling may regulate or contribute to β cell survival. Not fully investigated in this thesis, but equally important, is the effect of GLP-1 expression in the islet on glucagon secretion.

Early reports of pancreatic GLP-1

Early reports of intra-islet GLP-1 arose from studies on the proglucagon gene and tissue-specific posttranslational cleavage of the proglucagon protein. Genetic analysis of proglucagon predicted two glucagon-like sequences in the segment C-terminal to glucagon itself, known as the major proglucagon fragment (MPGF), with high sequence homology (~50%). These glucagon-like sequences, now known as GLP-1 and GLP-2, were flanked by dibasic peptides, suggesting that proteolytic cleavage and release were likely. Antibodies were raised against synthetic GLP-1 peptides and tested on pancreatic sections and extracts for immunoreactivity. Glucagon was known to be the main product of proglucagon in the α cells of pancreatic islets, while GLP-1 appeared to be the main product of the enteroendocrine L cells of the gut. However, GLP-1 immunoreactants were identified in the islet α cells of human pancreatic sections and small amounts were identified in pancreatic extracts of pigs and humans (Holst et

al., 1994; Uttenthal et al., 1985; Varndell et al., 1985). Another study in fetal and neonatal rats determined that GLP-1 levels decline in the neonate, but are not extinguished at 30 days, suggesting that GLP-1 is present in the adult pancreas (Kreymann et al., 1991). However, Heller et al. were the first to postulate a mode of action for pancreatic GLP-1 that involves direct action of β cells to increase insulin secretion. They were able to show that pancreatic islets contain a releasable source of GLP-1 and that GLP-1 colocalizes with glucagon in the α cell (Heller and Aponte, 1995). However, not all glucagon positive cells were immunoreactive to GLP-1, suggesting a population of α cells that do not synthesize GLP-1.

Proglucagon processing in L cells and α cells

Tissue-specific proglucagon gene expression

There is evidence that transcriptional regulation of proglucagon may be different between pancreatic α cells and intestinal L cells. The promoter for proglucagon has a cAMP response element and four enhancer elements (Sandoval and D'Alessio, 2015). Proglucagon expression is increased by elevations of cAMP and may be regulated by a number of homeodomain protein (HD) transcription factors (Jin, 2008). In islet α cells, the HD transcription factors Pax6 and MafB promote proglucagon gene expression (Artner et al., 2006; Gosmain et al., 2011). Interestingly, insulin inhibits proglucagon gene expression in islet α cells, while in intestinal L cells insulin inhibits gene expression (Jin, 2012; 2008). Inhibition of proglucagon gene expression in the L cells is mediated by effectors of the canonical Wnt signaling pathway, and there is thought to be cross-talk between the insulin and Wnt pathways in the regulation of proglucagon gene expression (Yi et al., 2008). These examples of tissue-specific regulation of proglucagon gene expression parallel the tissue-specific nature of post-translational cleavage of proglucagon.

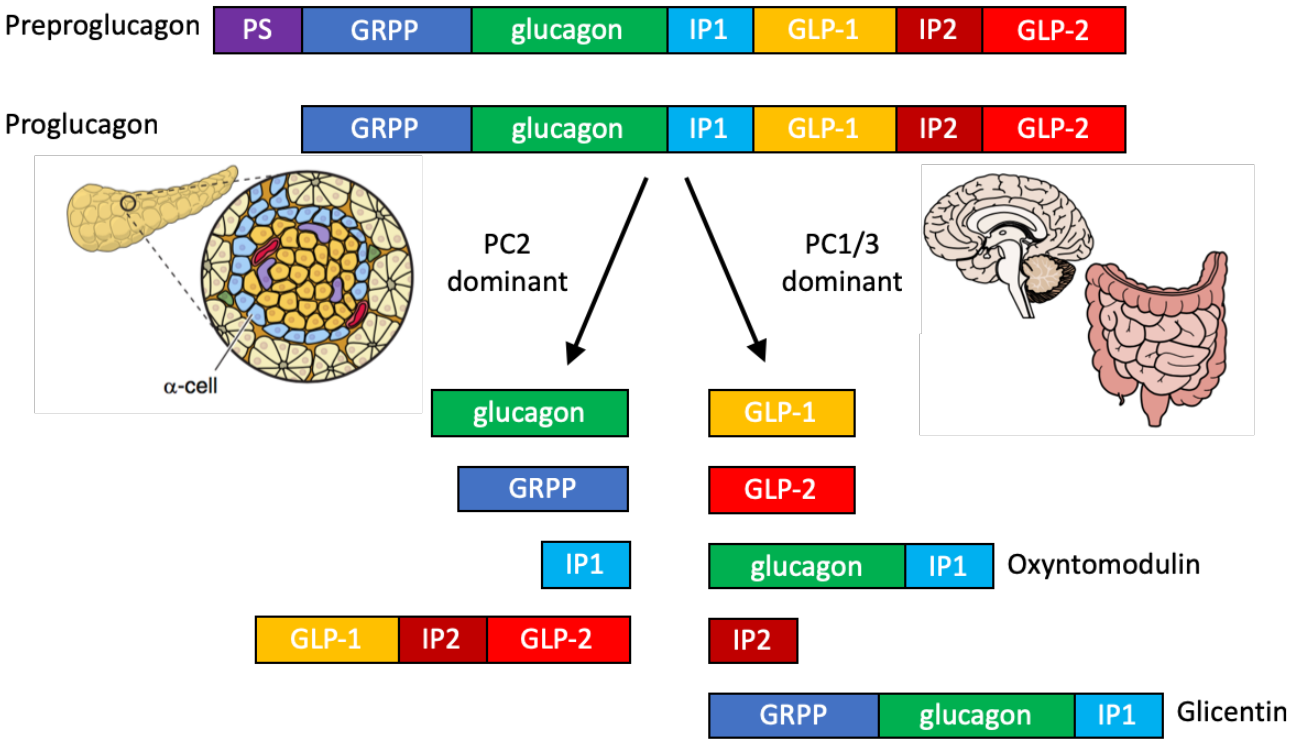
Tissue-specific post-translational modifications

The difference in post-translational cleavage of proglucagon from either the L cell or pancreatic α cell is the prohormone convertases. PC1/3 is the dominant prohormone convertase in the L cell, producing GLP-1, GLP-2, oxyntomodulin, and glicentin; while PC2 is the dominant prohormone convertase in the α cell, producing glucagon, the glicentin-related pancreatic peptide, and the major proglucagon fragment (Figure 1) (Sandoval and D'Alessio, 2015). Experiments with α cell and L cell lines have verified the tissue-specific dominance of the prohormone convertases (Tucker et al., 1996). However, alternate prohormone convertase expression is observed in either cell type. PC1/3 expression does occur in islet α cells, although the expression is low, with the release of GLP-1 (Marchetti et al., 2012). Circulating glucagon has been identified in pancreatectomized patients indicating PC2 expression in the gut and plasticity in proglucagon expressing cell types (Lund et al., 2016).

PC1/3 in islet α cells

The observation that regulation of prohormone convertase 1/3 (PC1/3) in α cells was associated with increased GLP-1 suggested a mechanism for the proper cleavage and secretion of GLP-1 in islets (Nie et al., 2000). A proof of concept experiment used transplanted mouse islets that were engineered to constitutively express PC1/3. These engineered islets secreted significantly more GLP-1, and performed better in a transplant model, achieving euglycemia sooner and having better glucose tolerance than controls (Wideman et al., 2006). The same group experimented with a PC1/3 expressing α cell therapy approach to deliver GLP-1 in a low dose STZ mouse model, demonstrating better glucose homeostasis and β cell survival (Wideman et al., 2007). Also, it has been shown that culturing α cells and rodent islets in high glucose increased PC1/3 gene expression and increased GLP-1 secretion (Hansen et al., 2011; Whalley et al., 2011). Subsequent studies have shown PC1/3 expression, and consequently GLP-1 secretion, increased in the context of metabolic stress (IL-6) and β cell injury (SDF-1 α) (Ellingsgaard et al., 2011; Z. Liu et al., 2011).

Figure 1.1



Adapted from Sandoval and D'Alessio, Physiological Reviews (2014)

Tissue-specific proglucagon processing to glucagon or GLP-1

Mouse models and intra-islet GLP-1

The power of recent experiments using genetic mouse models is they demonstrate a requirement for intra-islet GLP-1 to maintain glucose homeostasis. The experiments of Chambers et al. provided direct evidence that intra-islet GLP-1 is required for normal glucose homeostasis in their mouse model (Chambers et al., 2017). Using selective reactivation of proglucagon in the pancreas on a proglucagon null background mouse and Exendin-9, the authors were able to demonstrate a direct requirement for intra-islet GLP-1. Interestingly, when the converse experiment was performed, Exendin-9 had no effect on glucose homeostasis when proglucagon was reactivated in the gut. Another group did not exclude GLP-1 from the gut, but did show that intra-islet GLP-1 is required to maintain glucose homeostasis in aged and HFD fed mice, demonstrating an adaptive role for intra-islet GLP-1 in metabolic stress (Traub et al., 2017).

The presence and function of intra-islet GLP-1 has been demonstrated in numerous animal models of metabolic disease and β cell regeneration. In the Psammomys obesus, a nutritionally induced animal model of type 2 diabetes, GLP-1 secretion from α cells was increased in the context of hyperglycemia (Hansen et al., 2011). Furthermore, when the progression of diabetes was followed in the db/db mouse, GLP-1 and PC1/3 expression in the islets progressively increased with a rise in hyperglycemia (O'Malley et al., 2014). Ellingsgaard et al. demonstrated that IL-6 mediates organ cross talk between skeletal and adipose tissue to increase PC1/3 expression and GLP-1 secretion from α cells in conditions of metabolic stress, resulting in increased insulin secretion (Ellingsgaard et al., 2011). Another report shows that GIP secretion from the gut can increase GLP-1 secretion from α cells, also using IL-6 as an intermediary (Timper et al., 2016). Finally, in an obesity model, GLP-1 appears to be involved in a feed-forward mechanism to increase cholecystokinin (CCK) secretion from β cells for an autocrine mechanism to increase and maintain β cell mass (Linnemann et al., 2015).

Mice genetically deficient in the glucagon receptor not only exhibit lower glucose levels and improved glucose tolerance, but also hyperglucagonemia, α cell hyperplasia, and high circulating levels of GLP-1 (Gelling et al., 2003).The association of α cell hyperplasia with high circulating GLP-1 suggests that GLP-1 secretion from α cells is related to dysregulated glucagon signaling. Pharmacological antagonism of the glucagon receptor also induces GLP-1 secretion from hyperplastic α cells, along with increasing levels of glucagon (Gu et al., 2009). Although the signal to induce α cell hyperplasia and GLP-1 secretion is not known, the magnitude of the increase in α cell GLP-1 is of therapeutic interest for the control of glucose homeostasis (Jun et al., 2015).

Increased α / β cell ratio in diabetes

The significant increases in GLP-1 secretion from islets described in some animal models of α cell hyperplasia, suggest that intra-islet GLP-1 secretion would also increase in human α cell hyperplasia (Gelling et al., 2003). In fact, large increases in bioactive GLP-1 have been identified, along with hyperglucagonemia, in rare cases of glucagon receptor mutations (Larger et al., 2016; Yu, 2014). However, in patients with diabetes, the increase in α cells is generally relative to a decrease in β cells. Examination of pancreatic sections from patients with type 2 diabetes shows that some pancreata do have an absolute increase in α cell number, but this finding is not consistent, with most pancreata showing an increase in the α / β cell ratio. (Henquin and Rahier, 2011b; MacLean and Ogilvie, 1955; Yoon et al., 2003) As expected, pancreata from patients with type 1 diabetes have a high α / β cell ratio compared to healthy controls. (Orci et al., 1976; Waguri et al., 1997) However, it is plausible that not only α cell hyperplasia, but also a dysregulated islet in terms of cell proportion would lead to increased GLP-1 secretion from α cells.

DPP4 in islets

It is well known that DPP4 plays a major role in glucose metabolism. The foundational paper by Kieffer et al. demonstrated that DPP4 was the enzyme responsible for inactivating GLP-1 in vivo (Kieffer et al., 1995). Other groups extended this finding to human serum and consequently laid a foundation for early clinical trials with DPP4 inhibitors (Deacon et al., 1995; Mentlein et al., 1993). DPP4 is a serine exopeptidase that cleaves the two N-terminal peptides of GLP-1 (7-36amide or 7-37) to produce a truncated peptide that is inactive at the GLP-1 receptor (9-36amide or 9-37). It is the properties of this enzyme that results in the very short half-life of 1-2 minutes for circulating GLP-1. However, DPP4 exists in two forms, the soluble circulating form present in plasma and a membrane bound form that appears to be ubiquitously expressed (Mulvihill and Drucker, 2014).

Although DPP4 expression is ubiquitous, there is evidence for tissue or cell-specific activity of DPP4. A compartmental approach, looking at the intestine and regulation of the secreted incretins GIP and GLP-1, showed that a local intestinal reduction of DPP4 activity, without reducing systemic DPP4, could still improve glucose homeostasis (Waget et al., 2011). A mechanist exploration of DPP4 activity in the intestinal compartment showed distinct roles for DPP4 activity on incretin degradation depending on cell type expression (Mulvihill et al., 2017). Interestingly, inhibition or genetic knockout of enterocyte DPP4 did not alter incretin hormone levels or glucose tolerance. However, when the same techniques were applied to endothelial cell DPP4, incretin levels and glucose control were affected. These findings on selective DPP4 activity in the intestine argue for specific regulation in other tissues, such as the pancreatic islets where DPP4 substrates that regulate glucose metabolism are produced.

DPP4 expression and activity has been identified in human pancreatic islets and the pattern appears to be α cell-specific. In mice, DPP4 expression is predominantly in β cells, while DPP4 expression is constrained to α cells in humans (Liu et al., 2014). More evidence that

DPP4 expression is restricted to α cells in humans comes from techniques that use DPP4 to identify human α cells. DPP4 has been used as a cell surface antigen in FACS to sort α cells from other endocrine cell types in human islet preparations (Arda et al., 2016). Furthermore, DPP4 has been used as an α cell signature gene for RNAseq of human islet cells (Ackermann et al., 2016). Other groups have confirmed the α cell-specific expression of DPP4 and in situ activity in human islet pancreatic sections (Augstein et al., 2015; Busek et al., 2015; Segerstolpe et al., 2016).

It is not known if intra-islet DPP4 regulates paracrine GLP-1 secretion in the islet or how DPP4 expression and activity may be altered in diabetes and obesity. In regards to the genetic mouse model experiments by Chambers et al. mentioned above, Habener et al. suggests adding a DPP4 inhibitor to the experiments to determine if intra-islet DPP4 may be reducing intra-islet GLP-1 concentrations (Habener and Stanojevic, 2017). This would also model a therapeutic scenario where a DPP4 inhibitor is administered to treat type 2 diabetes. Other evidence for the possibility of regulation comes from electron microscopy where GLP-1 and DPP4 have been identified in the same secretory granules of pig α cells (Poulsen et al., 1993). Hyperglycemia in patients with diabetes has been associated with increased DPP4 activity in plasma, but islet DPP4 activity in T2D islets was reported to decrease, although DPP4 mRNA expression was the same as control islets (Mannucci et al., 2005; Omar et al., 2014). Clearly, more research on DPP4 expression and activity is needed in diabetic human islets.

Increasing intra-islet GLP-1 with DPP4 inhibitors in islets

DPP4 inhibitors have the potential to increase bioactive intra-islet GLP-1 concentrations local to the β cells. In fact, this may be an unrecognized mechanism behind the therapeutic success of DPP4 inhibitors for the treatment of type 2 diabetes. The DPP4 inhibitor, linagliptin, has been used to increase active GLP-1 in cultures of human islets with positive benefits on

islet survival and insulin secretion (Shah et al., 2013). Intra-islet DPP4, whether endothelial or endocrine cell derived, may be reducing GLP-1 concentrations, thus limiting GLP-1's capacity to protect β cells and help maintain insulin secretion under metabolic stress. If DPP4 expression or activity is unregulated or increased in diabetic islets, this may further reduce active GLP-1 and limit the ability of the islet to adapt to hyperglycemia or metabolic stress. Inhibition of increased or unregulated DPP4 activity in diabetic islets will increase active GLP-1 and facilitate adaptive paracrine signalling within the islet.

DPP4 inhibition in islets will likely alter paracrine signalling of other islet peptides that are DPP4 substrates, with the potential to increase insulin secretion and islet survival. The islet peptides, peptide YY (PYY) and stromal cell-derived 1alpha (SDF-1 α), are DPP4 substrates (Mulvihill and Drucker, 2014) and secreted by islets cells. Whether they are substrates of intra-islet DPP4 is unknown. PYY has been reported to increase insulin secretion in human islets at high glucose and increased circulating levels in mice have been reported to rescue impaired insulin secretion. (Ramracheya et al., 2016) Elevating active forms of PYY within the islet may also rescue insulin secretion from human diabetic islets. SDF-1 α secretion from β cells is induced as part of a regenerative process in response to β cell injury in animal models. GLP-1 secretion from α cells is also induced during this process. SDF-1 α may be induced in human diabetic islets and DPP4 inhibition would maintain active forms of this peptide to increase islet survival, largely though increased GLP-1 secretion.

Isolated islets from human pancreatic donors are a good tissue source to study the nature of intra-islet GLP-1 secretion and the effect of DPP4 inhibition. With isolated human islets, the confounding GLP-1 secretion from the gut and circulating DPP4 is eliminated, and islet specific questions could be answered with in vitro experimentation. In this thesis, I use the DPP4 inhibitor sitagliptin to test whether inhibiting DPP4 activity in islets will affect glucose-stimulated insulin secretion and islet cell survival.

The therapeutic potential for the effect of DPP4 inhibitors in islets goes beyond the treatment of type 2 diabetes. If intra-islet GLP-1 is relevant to human glucose control, particularly in diabetes, there is an enhanced rationale to use DPP4 inhibitors in prediabetes. Patients with prediabetes may benefit from increased intra-islet GLP-1 levels as the islets adapt to metabolic stress. Furthermore, DPP4 inhibitors may be used in clinical islet transplantation to preserve or increase β cell mass in culture prior to transplant, as a GLP-1 source would be native to the islet. Finally, DPP4 inhibitors may increase GLP-1 secretion from the predominant α cell mass remaining in type 1 diabetes. A number of patients with type 1 diabetes, called microsecretors, have been identified with residual, but functional β cell mass (Oram et al., 2014). These patients may benefit from increased active GLP-1 levels local to the β cell, with potentially less need of exogenous insulin and a reduced risk of hypoglycaemia. With a local islet source of GLP-1, a mechanism to increase active GLP-1 levels, and an excellent safety profile, DPP4 inhibitors are ideally suited to target and concentrate the benefits of GLP-1 therapy to β cell mass in the islet.

Rationale for studying intra-islet GLP-1 in human islets

Clinical studies with Exendin-9

Exendin-9 is a specific antagonist for the human GLP-1 receptor and a very useful tool to interrogate the effects of endogenous GLP-1 in humans. Exendin-9, a peptide fragment of exendin-4, was discovered with exendin-4 in the venom of the Gila monster (*Heloderma suspectum*) (Eng et al., 1992). It was found to antagonize the action of exendin-4 in dispersed guinea pig acini, thus establishing its potential to test for ligands of the GLP-1 receptor in mammals (Eng et al., 1992). Soon after the cloning and identification of the human islet GLP-1 receptor established exendin-4 as an agonist and exendin-9 as an antagonist at the human GLP-1 receptor (Thorens et al., 1993). Some laboratories have reported that exendin-9 is not an antagonist, but rather an inverse agonist; however, these observations have been with the

mouse GLP-1 receptor (Serre et al., 1998; Svendsen et al., 2018). The mouse GLP-1 receptor does have high sequence similarity with the human GLP-1 receptor, but they are not identical and exendin-9 may act differently at either receptor. The described action of exendin-9 at the human GLP-1 receptor in the literature is that of an antagonist (Schirra et al., 1998; Thorens et al., 1993). This species difference becomes important when we interpret the antagonism of the GLP-1 receptor in the presence of active GLP-1. In human islets, we may presume that exendin-9 is reducing GLP-1 receptor activity due to ligand binding and not merely reducing the activity of a constitutively active GLP-1 receptor.

Two clinical studies using Exendin-9 to interrogate the effect of endogenous GLP-1 on insulin secretion observed that the insulin response was decreased even when glucose was only administered intravenously (Salehi et al., 2010; Schirra et al., 1998). This was observed in both healthy and patients with type 2 diabetes with good glucose control. Without oral nutrient ingestion, there was no rise in plasma GLP-1 levels, suggesting that circulating basal levels of GLP-1 can stimulate insulin secretion at high glucose. However, the circulating basal GLP-1 levels were very low in the first study and undetectable in the second. It is debatable whether active GLP-1 secreted from the gut reaches the islet directly to activate β cell GLP-1 receptors (D'Alessio, 2016). Considering this debate, an islet α cell source of GLP-1 should be investigated.

Exendin-9 is highly specific for the GLP-1 receptor and so the effect on insulin secretion seen in these two studies is specific to GLP-1R activation. However, the observation that glucagon can also activate the GLP-1R, although at a much lower potency (400X less), makes a mechanistic interpretation complex (Runge et al., 2003; Svendsen et al., 2018). I have observed GLP-1 expression and secretion in non-diabetic islets and find that expression increases in type 2 diabetic islets. Considering the much higher potency of GLP-1 over glucagon for the GLP-1R, the presence of GLP-1 warrants investigation into its paracrine function within the islet.

Cytoarchitecture of human islets

The structure of human islets suggests a greater role for intra-islet GLP-1 in human islets than mouse islets. Anatomical studies of human islets show that human islets have proportionately more α cells and fewer β cells than mouse islets (Figure 1.2) (Cabrera et al., 2006). The higher proportion of α cells in human islets suggests a more important role for intra-islet GLP-1 in human islets than has been considered in mice. This has been corroborated with GLP-1 secretion experiments, demonstrating that isolated human islets secrete more GLP-1 than mouse islets (Hansen et al., 2011). The α cells are intermingled with β cells in the human islet and their close proximity favours paracrine communication and regulation (Caicedo, 2013). Furthermore, both α and β cells appear to be adjacent to blood vessels, allowing for efficient and appropriate glucose sensing (Bosco et al., 2010).

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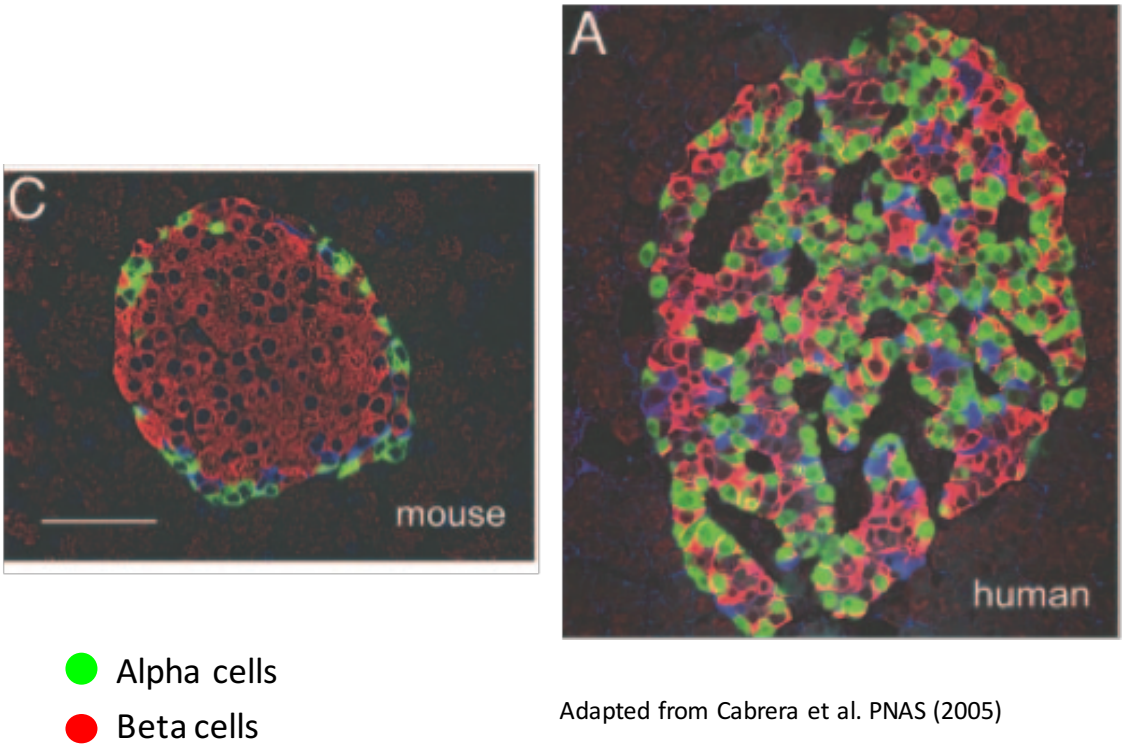
Figure 1.2



Adapted from Cabrera et al. PNAS (2005)

Cytoarchitecture of α and β cells is different between mouse and human islets

Summary of hypotheses and aims for this thesis

General rationale

Paracrine signaling within islets is important for normal islet function. GLP-1 may be expressed and secreted from human islets, but the importance of this paracrine signal for human islet function is not known. Furthermore, it is not known if GLP-1 expression increases in T2D or whether it contributes to insulin secretion and cell survival in the diabetic islet.

Hypothesis 1 – Chapter 2

That human islets have an α cell subpopulation that expresses GLP-1 and that this subpopulation increases in T2D.

That sitagliptin treatment of human islets will increase GSIS by increasing active levels of GLP-1.

Hypothesis 2 – Chapter 3

That active GLP-1 levels in human islets can be increased with the DPP4 inhibitor sitagliptin to increase islet cell survival.

Hypothesis 3 – Chapter 4

That neonatal porcine islets (NPIs) can be bioengineered with adenoviral vectors to increase GLP-1 secretion.

Hypothesis 4 – Chapter 5

That initiating metformin therapy with sitagliptin in T2D patients will be associated with better outcomes as measured by insulin starts and HbA1c.

Specific Aims

1. Characterize and quantify the GLP-1 expressing subpopulation in ND and T2D islets.
2. Determine the contribution of GLP-1R signaling to GSIS in ND and T2D islets.
3. Investigate glucose regulation of GLP-1 secretion in human islets.

4. Determine whether sitagliptin increases active GLP-1 from human islets in long-term cultures and during GSIS.

5. Determine if increased active GLP-1 levels in human islets cultures will increase islet survival.

6. Determine if NPIs can be bioengineered to increase GLP-1 secretion and improve functional maturation in culture.

7. Compare the association between an early versus late sitagliptin start and the outcomes of insulin starts and HbA1c in a T2D population.

Chapter 2

Human islets contain a subpopulation of glucagon-like peptide-1 secreting α cells that is increased and supports insulin secretion in type 2 diabetes.

by

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Chapter 2 of this thesis has been submitted for publication and is under peer review. I was responsible for the majority of the data collection, data analysis, and manuscript composition. Dominic Golec performed the flow cytometry and analysis. Koenraad Philippaert performed the calcium imagining and analysis. Matt Hubert collected some of the perifusion data. Nicole Salamon and Janyne Johnson imaged most of the human islet sections, under my supervision. Peter Light is the supervisory author and was involved with concept formation and editing of the manuscript.

Abstract

Here I show that glucagon-like peptide-1 (GLP-1) is secreted within human islets and may play an unexpectedly important paracrine role in islet physiology and pathophysiology. It is known that α cells within rodent and human pancreatic islets are capable of secreting GLP-1 (Fava et al., 2016; Marchetti et al., 2012), but little is known about the functional role that islet-derived GLP-1 plays in human islets. Our results indicate that human islets secrete ~50-fold more GLP-1 than murine islets and that ~40% of the total human α cells contain GLP-1. Our results also confirm that dipeptidyl peptidase-4 (DPP4) is expressed in α cells (Omar et al., 2014; Segerstolpe et al., 2016). The DPP4 inhibitor sitagliptin increased active GLP-1 levels released from cultured human islets but did not enhance glucose-stimulated insulin secretion (GSIS) in islets from non-diabetic (ND) or type 2 diabetic (T2D) donors, suggesting that β cell GLP-1 receptors (GLP-1R) may already be maximally activated. Therefore, I tested the effects of exendin-9 on the contribution of paracrine GLP-1R signalling to GSIS. Exendin-9 reduced GSIS by 25% in ND islets and by 62% in T2D islets. I also observed significantly more GLP-1+ α cells in T2D islets compared to ND islets that may contribute to the observed differences in GLP-1R signalling on GSIS. In conclusion, I demonstrate that human islets secrete robust amounts of GLP-1 from an α cell subpopulation and that GLP-1R signalling supports GSIS to a greater extent in T2D islets.

Introduction

Using the classical incretin model as a physiological framework, GLP-1 therapies such as DPP4 inhibitors and peptide GLP-1R agonists have been developed and are now widely used clinically (Drucker, 2018). The incretin model describes stimulated GLP-1 release from the entero-endocrine L-cells of the gut to act on pancreatic β cells, thus potentiating insulin secretion in response to nutrient ingestion (Campbell and Drucker, 2013). Early reports showed proglucagon cleavage to be tissue specific with GLP-1 being produced in the L-cells and negligible amounts found in islets. In contrast, glucagon secretion is limited to pancreatic islets with little if any being produced from the L-cells (Holst et al., 1994; Rouillé et al., 1997; 1995). However, there have also been reports of GLP-1 secretion from islets (Heller and Aponte, 1995; Masur et al., 2005) and it has been proposed that GLP-1 may also act as a paracrine factor in islets (D'Alessio, 2016; Donath and Burcelin, 2013; Fava et al., 2016), whereby α cell GLP-1 secretion, dependent on prohormone convertase 1/3 (PC1/3) proglucagon cleavage, acts on adjacent β cells to augment function and insulin secretion. Despite the controversy regarding a functional role for intra-islet GLP-1, this paracrine concept has received recent support from genetic mouse studies showing intra-islet GLP-1 is required for whole body glucose homeostasis (Chambers et al., 2017; Traub et al., 2017). While the physiological relevance of intra-islet GLP-1 in these genetic models has been demonstrated, it is currently unknown whether intra-islet GLP-1 is relevant for human physiology and pathophysiology such as T2D and little information is available as to whether this proposed GLP-1 paracrine axis functionally exists in human islets.

Human islet architecture argues for perhaps a greater functional paracrine role for intra-islet GLP-1 than in mouse islets, as the α cells within human islets are interspersed throughout the islet compared to the mouse islet where α cells form a mantle around the periphery (Cabrera et al., 2006). Consequently, human α cells are organized to facilitate paracrine interactions with other islet cells (Caicedo, 2013). Moreover, α cell glucagon secretion has been reported to

determine the glycemic set-point in mice and humans, suggesting an important integration of α and β cell communication in glucose homeostasis (Rodriguez-Diaz et al., 2018). Due to the paucity of functional information on intra-islet GLP-1 from human tissue, I therefore studied human islets from ND and T2D donors.

Methods

Mouse pancreatic islets

All animal studies followed the guidelines issued by University of Alberta Animal Care and Use Committee (protocol #s AU00286 and AU01417). Pancreatic islets from male C57BL/6 mice (ages 12-24 weeks) were isolated by collagenase Type V (Sigma-Aldrich) digestion of the pancreas and purified by Histopaque density gradient. Mouse islets were cultured in RPMI 1640 media (11.1 mM glucose) supplemented with 10% FBS and 1% penicillin/streptomycin.

Human islets and pancreatic sections

De-identified human primary islets isolated from deceased donors were obtained from the Alberta Diabetes Institute IsletCore and the Clinical Islet Laboratory, University of Alberta, in accordance with institutional human ethics guidelines. If the islet preparation was <90% pure, islets were handpicked to obtain >90% purity. Islets were cultured in DMEM (5.5mM glucose) supplemented with 10% FBS and 1% penicillin/streptomycin for static incubations and perifusions. For flow cytometry and live imaging, islets were cultured in CMRL medium (5.5 mM glucose) supplemented with 0.5% BSA, 1% Insulin-Transferrin-Selenium (ITS), 1% Glutamax, and 1% penicillin/streptomycin. Whole pancreatic sections were obtained from the Alberta Diabetes Institute IsletCore. Sections of isolated human islets and pancreatic sections were formalin fixed (Z-fix), paraffin embedded, and sectioned 5 μm thick. Diagnosis of type 2 diabetes was determined from medical records and a HbA1c > 6.5%.

Antibodies for immunohistochemistry

Anti-glucagon Rab [EP370] (ab92517) and Anti-GLP-1 (amidated) Mab [8G9] (ab26278) were purchased from Abcam. Anti-insulin guinea pig polyclonal DAKO (A056401-2) was purchased from Agilent Technologies. Alexa Fluor goat anti-rabbit 488, Alexa Fluor goat anti-mouse 568, and Alexa Fluor goat anti-guinea pig 647 were used as secondaries and purchased from ThermoFisher Scientific.

Antibodies for flow cytometry

PE mouse anti-glucagon [U16-850] and Alexa Flour 647 mouse anti-insulin [T56-706] were purchased from BD Biosciences. Anti-GLP-1 (amidated) Mab [8G9] (ab26278) and anti-PC1/3 rabbit polyclonal (a154246) were purchased from Abcam. Anti-GLP-1 (amidated) was detected using anti-mouse IgG-biotin (13-4013) and streptavidin-eFluor 450 (48-4317) purchased from eBioscience. Anti-PC1/3 was probed using anti-rabbit IgG-FITC (11-4839) purchased from eBioscience. Mouse anti-human CD26/DPP4-PEcy7 (BA5b) was purchased from Biolegend.

Compounds

Sitagliptin phosphate monohydrate was purchased from Biovision, reconstituted in water, and used at 200 nM concentration for islet culture and perifusion experiments. Exendin-9 (9-39) salt was purchased from Bachem, reconstituted in PBS, and used at 100 nM concentration for perifusion experiments.

Flow cytometry

Human islets were washed in citric saline and dispersed mechanically with a 21G needle and tuberculin syringe. For cell surface antigen detection, dispersed cells were stained with antibody cocktails in FACS buffer (PBS, 1% FCS, 0.02% sodium azide, 1mM EDTA) for 30 min on ice. Cells were washed twice with FACS buffer following staining. For intracellular antigen staining, dispersed cells were treated with BD Cytofix/Cytoperm (BD Bioscience) for 30 min on ice and washed twice with BD Perm/Wash buffer (BD Bioscience). Intracellular antigens were stained with antibody cocktails in BD Perm/Wash buffer for 30 min on ice and cells were washed twice with BD Perm/Wash buffer following staining. Cell events were collected on a BD Fortessa X-20 analyzer and data were analyzed with FlowJo software (Tree Star).

Static incubations and perifusions

Static incubations were performed using 500 µL KRBH buffer containing (mM) 115 NaCl, 5 KCl, 24 $NaHCO_3$, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 0.1% BSA, (pH 7.4) supplemented with the appropriate concentration of glucose. 50 islets were pre-incubated at 2.8 mM glucose buffer for

2 hours, transferred to 2.8 mM glucose buffer for 1 hour, followed by 11.1 mM glucose buffer for 1 hour. A parallel experiment included 200 nM sitagliptin. Supernatant was taken and stored at −20°C for insulin or active GLP-1 analysis. Islet perifusions were performed using a Brandel SF-06 system (Gaithersburg, MD) following a 3 h static preincubation in 2.5 mM glucose buffer. 25 islets and perifusion buffer (same as static incubations) were kept at 37°C where the islets were perfused at 250 µL/min with 2.5 mM glucose buffer for 20 minutes followed by 11.1 mM glucose buffer for 50 minutes. The appropriate treatment was included throughout the whole experiment. Perfusate was collected at timed intervals and stored at -20°C for insulin for analysis.

Calcium imaging

Human pancreatic islets were incubated with 2 µM Fluo-4 AM for 1 h in a humidified incubator at 37 °C and 5% CO_2. They were placed in the recording chamber of an Olympus IX83 inverted fluorescence microscope equipped with an UPLANApo 10X objective. I used a multichannel gravity-fed Warner Instruments TC-324B temperature controlled perfusion system, kept at 37 °C. Excitation was done with a X-Cite 120LEDBoost (Excelitas) light source and image acquisition with an Andor iXon ultra camera using Olympus cellSens software. I measured the fluorescence of whole human pancreatic islets at a frequency of 0.33 Hz. The islets were perfused with a solution containing (mM) 120 NaCl, 4.8 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$ and 10 HEPES (pH 7.4) supplemented with the indicated amount of glucose and Exendin-9. Depolarization of the islets with 30 mM KCl was used as positive control at the end of the measurement. Ca^{2+} events and signal dynamics of active subregions within the islets, before or during the application of Exendin-9, were analyzed.

Hormone secretion assays

Active GLP-1 levels from islet culture media samples (human and mouse) and supernatants from static islet incubations (human) were quantified using the electrochemiluminescent assay Active GLP-1 (v2) Kit (K150JWC-1), MesoScale Discovery. Total GLP levels in human islet culture media samples were quantified with the

electrochemiluminescent assay Total GLP-1(v2) Kit (K150JVC-1), MesoScale Discovery. Insulin levels in supernatants from static incubations and perfusate samples were quantified with Stellux Chemiluminescent Human Insulin ELISA, Alpco.

Immunofluorescence microscopy

Paraffin embedded human islet sections were rehydrated and subjected to antigen retrieval by microwaving the slides for 15 minutes in a citrate buffer (pH 6.0). After cooling, sections were blocked with 20% normal goat serum for 1 hour, incubated with primary antibodies for 1 hour, and then incubated with secondary antibodies and DAPI (1:1000) for 1 hour. Prolong Gold anti-fade mountant was applied and the slide sections were mounted on coverslips. Primary antibodies were as follows: 1:1000, Anti-glucagon Rab [EP370] (ab92517), Anti-insulin guinea pig polyclonal DAKO (A056401-2); 1:2000 Anti-GLP-1 amidated Mab ([8G9] (ab26278). Secondary antibodies were incubated at 1:200 dilution. For epifluorescence microscopy, islet sections were imaged with an Olympus IX83 inverted fluorescence microscope and a UPLanSApo 20X objective. Excitation was with a X-Cite 120LEDBoost (Excelitas) light source with the appropriate filter set for DAPI, AF488, AF568, and AF647. Images were captured with a Hamamatsu Orca Flash4.0 camera operated with Olympus cellSens software. Image analysis was performed using FIJI and a customized macro. DAPI staining was used to identify ROIs (cells) where appropriate thresholds were set to identify positive staining in the green (AF488), red (AF568), or far-red (AF647) channels.

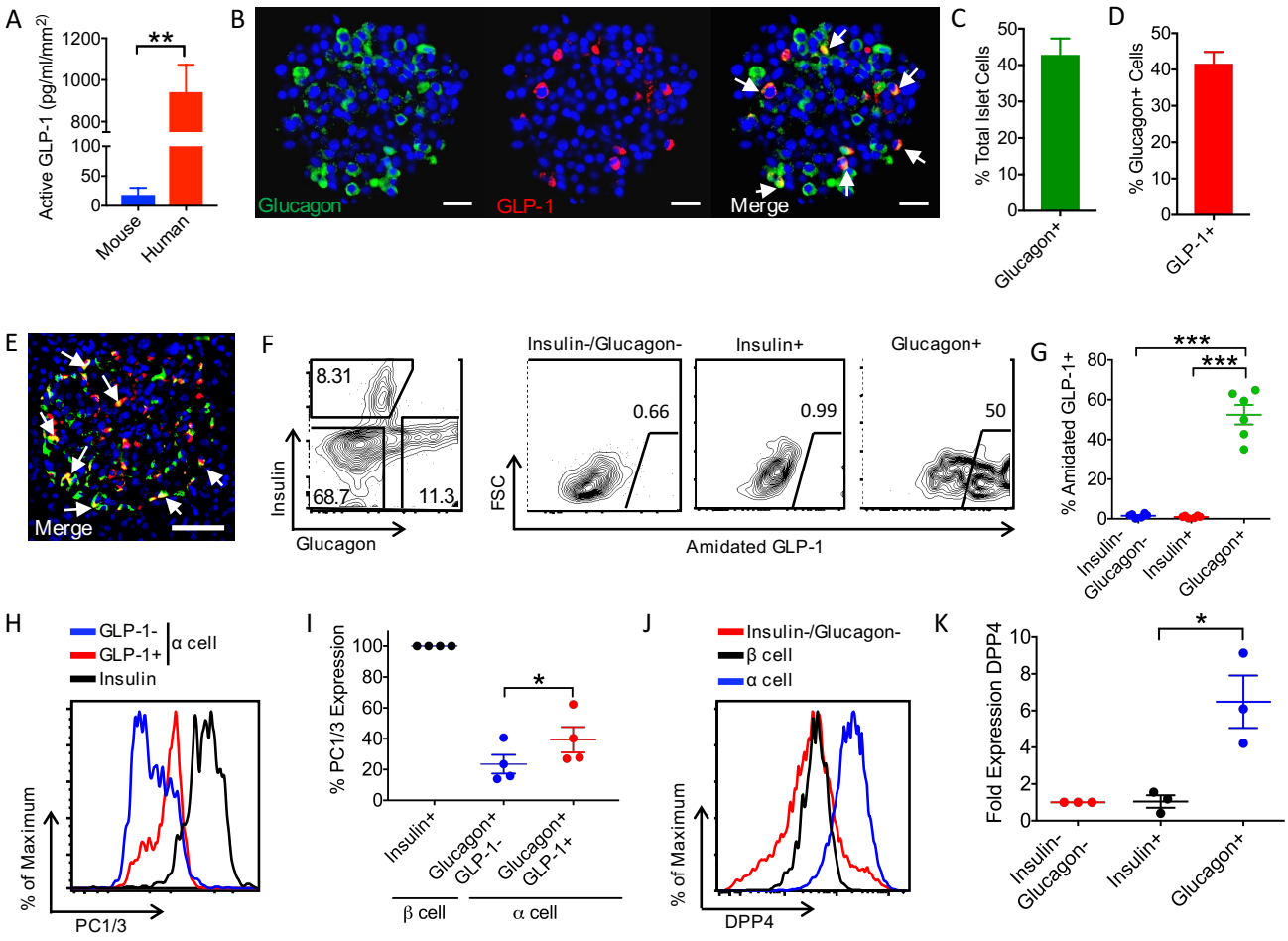
Results

Human islets contain a GLP-1 secreting α cell subpopulation.

As human islets possess ~40% α cells compared to ~20% α cells in mouse islets (Cabrera et al., 2006), it is reasonable to expect that human islets would secrete ~2-fold more active GLP-1 when compared to mouse islets. However, I observed a ~50-fold increase in active GLP-1 in human islet cultures compared to mouse islets suggesting that GLP-1 secretion from α cells may be more important to human islet function (Fig. 2.1A). As the number of α cells and GLP-1 secretion is higher in human islets, I hypothesized that a significant α cell subpopulation would express the post-processed amidated form of GLP-1. Human islet sections were stained with antibodies for glucagon and amidated GLP-1. I determined that 40% of total cells in human islets are α cells and that ~40% of these α cells are a subpopulation that is positive for GLP-1 (Fig. 2.1B-D). Importantly, GLP-1 expression was also found in a subpopulation of α cells in pancreatic biopsies, indicating that the presence of intra-islet GLP-1 is not an artefact of islet isolation and culture (Fig. 2.1E). To study this subpopulation further, flow cytometry was used to segregate three cell populations within human islets: insulin-/glucagon-, insulin+, and glucagon+ (Fig. 2.1F). Our results show that GLP-1 expression is only found in a subpopulation of glucagon+ α cells that constitutes ~50% of the total α cell number (Fig. 2.1F,G), a value that is consistent with the value of 40% observed in human islet sections (Fig. 2.1D). PC1/3 is required for the proteolytic cleavage of GLP-1 from proglucagon and I observed a significant increase in PC1/3 expression in glucagon+/GLP-1+ α cells compared to glucagon+/GLP-1- cells (Fig. 2.1H,I). I further characterized the α cell population using DPP4 expression and confirmed that DPP4 expression is relatively specific to α cells (Omar et al., 2014; Segerstolpe et al., 2016) when compared to β cells (Fig. 2.1J,K). However, I did not observe any differences in DPP4 expression between GLP-1+ and GLP-1- α cells (data not shown).

Figure 2.1. Human islets contain a GLP-1 secreting α cell subpopulation **A.** Human islets secrete ~50-fold more active GLP-1 than mouse islets (normalized to islet area). **B.** Representative images of human islets stained for glucagon, GLP-1, and double positive staining indicating a subpopulation of α cells (white arrows). Scale bar: 20 μm. **C.** Quantification of glucagon+ cells shown as % total islet cells. N=6 donors. **D.** Quantification of amidated GLP-1+ cells shown as % total glucagon+ cells. N=6 donors. **E.** Representative image of glucagon and amidated GLP-1 staining from a section of whole human pancreas. White arrows indicate double positive staining. Scale bar: 50 μm. **F.** Representative flow cytometry contour plots of dispersed islet cells. Left, insulin and glucagon expression were analyzed in total dispersed islets, allowing the identification of insulin-/glucagon-, insulin+, and glucagon+ cell populations. Right, the insulin-/glucagon-, insulin+, and glucagon+ cell populations were further analyzed for forward scatter (FSC) and expression of amidated GLP-1. Amidated GLP-1+ cells were gated within each cell population. **G.** Percentages of amidated GLP-1+ cells in the insulin-/glucagon-, insulin+, and glucagon+ cell populations. N=6 donors. **H.** Representative flow cytometry histograms showing PC1/3 expression levels within GLP-1-/glucagon+, GLP-1+/glucagon+, and insulin+ cells. **I.** Relative expression of PC1/3 expression in GLP-1-/glucagon+ cells, GLP-1+/glucagon+ cells, and insulin+ cells. Median Fluorescent Intensities (MFIs) of PC1/3 staining were calculated within each cell fraction, normalized relative to the insulin+ cell fraction and compiled as percent expression to insulin+ cells. N=4 donors. **J.** Representative flow cytometry histograms showing DPP4 expression in insulin-/glucagon-, insulin+, and glucagon+ cells. **K.** Relative expression of DPP4 expression in insulin-/glucagon-, insulin+, and glucagon+ cells. MFIs of DPP4 staining were calculated within each cell population, were normalized relative to the insulin-/glucagon- cell fraction and compiled as fold expression relative to insulin-/glucagon- cells. N=3 donors. Statistical significance for the data was determined using a paired Student's t test. *, $P<0.05$, ***, $P<0.001$. Error bars indicate SEM.

Figure 2.1


A
Active GLP-1 (pg/ml/mm2)
1200
1000
800
100
50
0
**
Mouse
Human
B
Glucagon
GLP-1
Merge
C
% Total Islet Cells
50
40
30
20
10
0
Glucagon+
D
% Glucagon+ Cells
50
40
30
20
10
0
GLP-1+
E
Merge
F
8.31
68.7
11.3
Insulin
Glucagon
Insulin-/Glucagon-
Insulin+
Glucagon+
0.66
0.99
50
FSC
Amidated GLP-1
G
% Amidated GLP-1+
80
60
40
20
0
***
***
Insulin-
Glucagon-
Insulin+
Glucagon+
H
GLP-1-
GLP-1+
α cell
Insulin
% of Maximum
PC1/3
I
% PC1/3 Expression
100
80
60
40
20
0
*
Insulin+
Glucagon+
GLP-1-
Glucagon+
GLP-1+
β cell
α cell
J
Insulin-/Glucagon-
β cell
α cell
% of Maximum
DPP4
K
Fold Expression DPP4
10
8
6
4
2
0
*
Insulin-
Glucagon-
Insulin+
Glucagon+

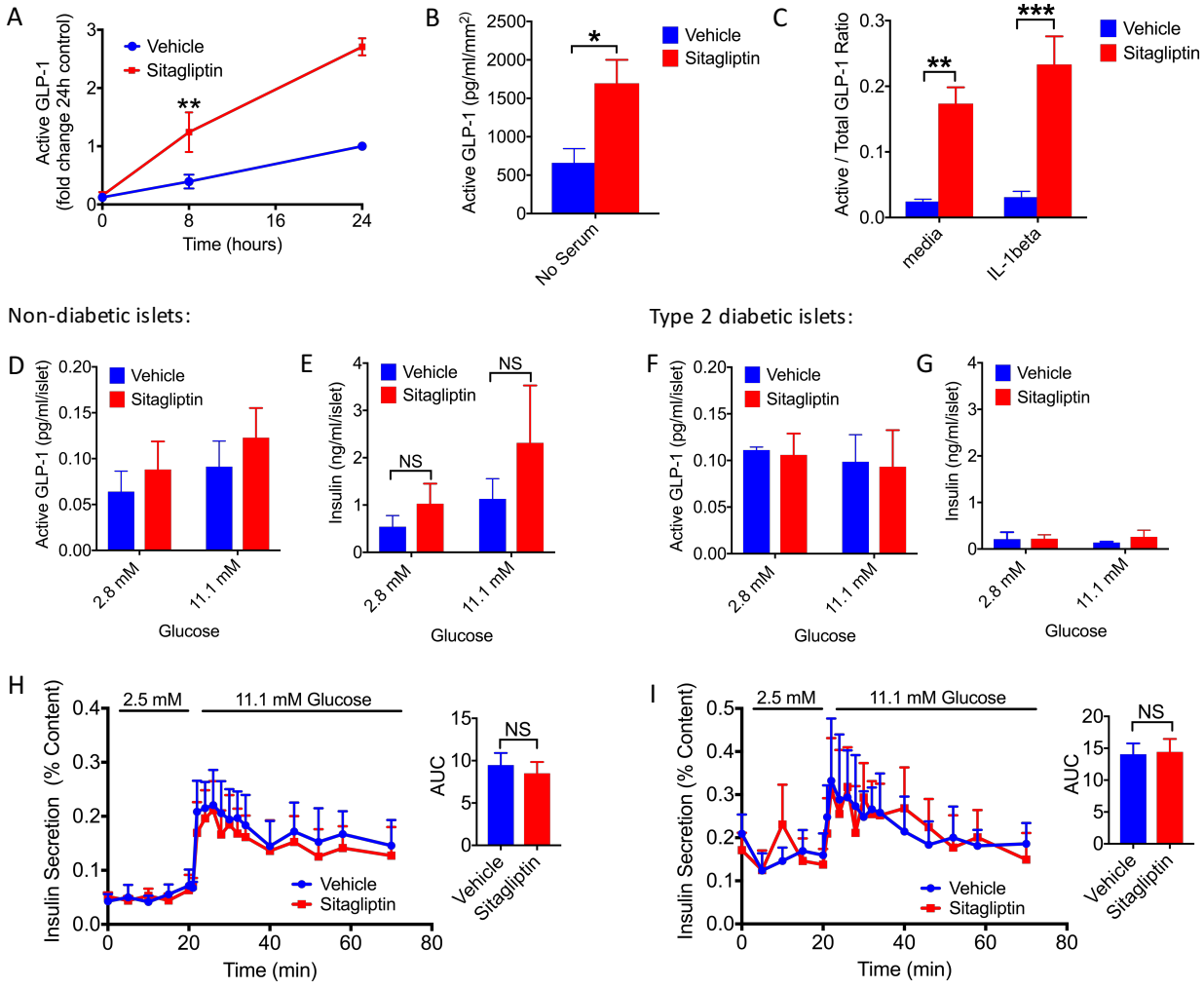
Sitagliptin increases active GLP-1 levels in culture, but does not increase GSIS in ND and T2D islets.

Since human α cells express DPP4, I tested the ability of sitagliptin, a DPP4 inhibitor, to increase active GLP-1 levels in human islet cultures. I observed a significant increase in active GLP-1 levels with sitagliptin treatment in long-term cultures (24 to 48 hours, Fig. 2.2A,B,C). I also tested the ability of sitagliptin to increase active GLP-1 in a serum-free culture and confirmed that intra-islet DPP4 is being inhibited, rather than any DPP4 that may be in the culture media (Fig. 2.2B). As inflammatory cytokines may increase GLP-1 secretion, I tested the effects of IL-1β, and showed that the sitagliptin-mediated increase in active GLP-1 was not affected by this cytokine challenge (Fig. 2.2C). As sitagliptin increased active GLP-1, I hypothesized that sitagliptin would augment GSIS. Unexpectedly, in short-term static incubations, sitagliptin did not significantly increase active GLP-1 or insulin secretion in ND islets at low or high glucose (Fig. 2.2D,E). Furthermore, under perifusion conditions, ND islets with sitagliptin similarly did not show any increase in GSIS (Fig. 2.2H).

As DPP4 inhibitors are successfully used to increase circulating active GLP-1 levels and restore insulin secretion in T2D patients (Mulvihill and Drucker, 2014), I tested whether sitagliptin would increase active GLP-1 and GSIS in T2D islets. No increase in active GLP-1 levels was observed at low or high glucose suggesting that DPP4 activity is low in T2D islets (Omar et al., 2014) (Fig. 2.2F). However, active GLP-1 levels were maintained relative to ND islets, showing that active GLP-1 secretion is not lost in T2D islets. In addition, no increase in insulin secretion was observed in static or perifused GSIS experiments, indicating that DPP4 inhibition in T2D islets does not directly contribute to increased GSIS (Fig. 2.2G,I).

Figure 2.2. Sitagliptin increases active GLP-1 levels in cultured islets, but does not increase GSIS from ND and T2D islets. **A.** Active GLP-1 secretion from human islets in culture showing the inhibitory effects of sitagliptin on DPP4 over 24 hours. N=4 donors. **B.** Active GLP-1 secretion from human islets treated with sitagliptin in serum-free culture (5.5mM glucose) for 48 hours to exclude the effect of serum DPP4. N=3 donors. **C.** Active GLP-1 secretion increases with sitagliptin treatment in control media or IL-1β (50ng/ml) co-incubation. N=3 donors. **D.** Sitagliptin does not increase active GLP-1 levels with glucose-stimulated static incubation of ND islets. 50 islets per group, N=5 donors. **E.** Sitagliptin does not significantly increase insulin levels with glucose-stimulated static incubation of ND islets. 50 islets per group, N=5 donors. **F.** Sitagliptin does not increase active GLP-1 levels with glucose-stimulated static incubation of T2D islets. 50 islets per group, N=2 donors. **G.** Sitagliptin does not increase insulin levels with glucose-stimulated static incubation of T2D islets. 50 islets per group, N=2 donors. **H.** Sitagliptin is unable to increase insulin secretion with glucose-stimulated perifusions of ND islets. 25 islets per lane, N=6 donors. **I.** Sitagliptin is unable to increase insulin secretion with glucose-stimulated perifusions of T2D islets. 25 islets per lane, N=3 donors. For all experiments the sitagliptin concentration was in the therapeutic range at 200nM. Exendin-9 concentration was 100nM. Statistical significance for the data was determined using a paired Student's t test or two-way ANOVA. *, $P<0.05$, **, $P<0.01$, ***, $P<0.001$. Error bars indicate SEM.

Figure 2.2

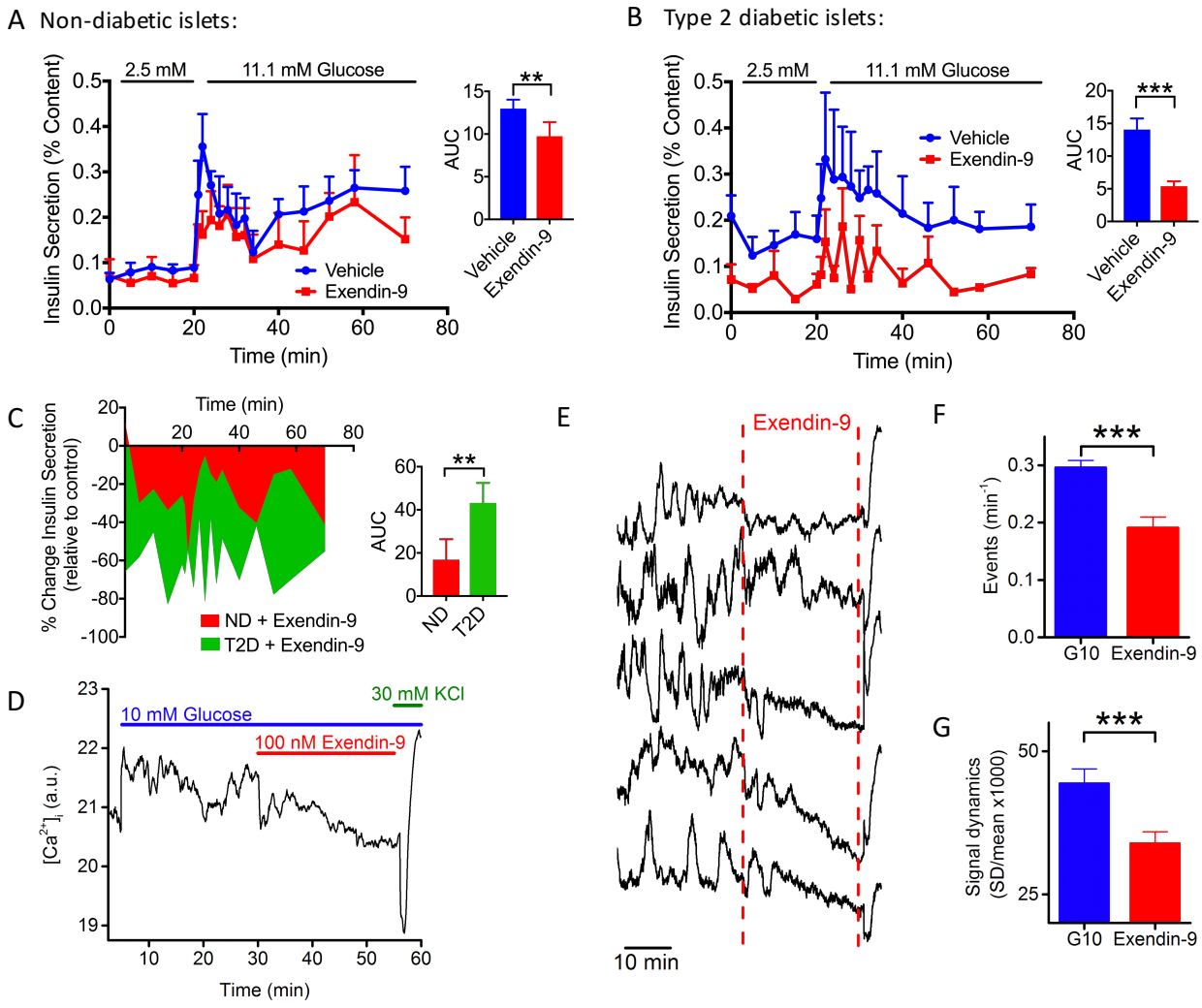

A
Active GLP-1 (fold change 24h control)
Vehicle
Sitagliptin
**
Time (hours)
B
Active GLP-1 (pg/ml/mm2)
*
No Serum
C
Active / Total GLP-1 Ratio
**
***
media
IL-1beta
Non-diabetic islets:
Type 2 diabetic islets:
D
Active GLP-1 (pg/ml/islet)
2.8 mM
11.1 mM
Glucose
E
Insulin (ng/ml/islet)
NS
F
G
H
Insulin Secretion (% Content)
2.5 mM
11.1 mM Glucose
Time (min)
AUC
I

Islet GLP-1Rs contribute to insulin secretion in ND and T2D islets.

Since sitagliptin treatment did not enhance GSIS in ND and T2D islets, I hypothesized that the GLP-1Rs may be maximally stimulated by the robust amount of intra-islet GLP-1 present (Fig. 2.1A). To test this notion I used the GLP-1R antagonist, exendin-9, under the GSIS perifusion conditions and observed a 25% decrease in insulin secretion that occurs primarily at high glucose in ND islets (Fig. 2.3A), suggesting that GLP-1R signalling contributes to insulin secretion in ND islets. In T2D islets, I observed an even larger decrease in insulin secretion of 62% that occurs at both low and high glucose conditions (Fig. 2.3B), suggesting that insulin secretion is strongly supported by GLP-1R signalling in T2D. Indeed, when the change in insulin secretion over time is analyzed for ND and T2D islets, the percent change from control is 61% greater in T2D islets than ND islets (Fig. 2.3C). As fluctuations in intracellular calcium correlate with β cell insulin secretion, I investigated whether treating human islets with exendin-9 would alter calcium dynamics. ND islets were incubated with the calcium sensitive dye Fluo-4 and stimulated with glucose (10 mM). Exendin-9 caused a reduction in calcium dynamics in whole islets as well as dynamic sub-regions of islets (Fig. 2.3D,E). When compared to the high (10 mM) glucose control, calcium events and signal dynamics of sub-regions were significantly decreased in the presence of Exendin-9 (Fig. 2.3F,G).

Figure 2.3. Islet GLP-1Rs contribute to insulin secretion in ND islets and support insulin secretion in T2D islets. **A.** Exendin-9 inhibits GLP-1Rs to reduce insulin secretion at high glucose with glucose-stimulated perifusions of ND islets. 25 islets per lane, N=3 ND donors. **B.** Exendin-9 inhibits GLP-1Rs to reduce insulin secretion at high and low glucose with glucose-stimulated perifusions of T2D islets. 25 islets per lane, N=3 T2D donors **C.** Area under the curve for % change in insulin secretion from control is larger for T2D islets than ND islets. Based on perifusion data from A and B. **D.** Example trace of a human islet perfused with 3 mM glucose (0-5 min) and 10 mM glucose (5-60 min) supplemented with 100 nM Exendin-9 (30-55 min). There are less intracellular calcium dynamics upon perfusion with Exendin-9. **E.** Example traces of the fluorescence in discrete subregions of the islet represented in **D**, individual regions are more dynamic compared to the average signal of the whole islet. **F.** The number of calcium events counted in selected regions during perfusion with 10 mM glucose, either in the presence or absence of 100 nM Exendin-9. **G.** The dynamics of the Fluo-4 calcium signal represented as the standard deviation of the signal over the mean fluorescence in the same conditions as **F**. Data and analysis in **D-F** is based on four experiments with four islets, focusing on five sub-regions per islet. N=1 ND donor. Statistical significance for the data was determined using a paired Student's t test or two-way ANOVA. *, $P<0.05$, **, $P<0.01$, ***, $P<0.001$. Error bars indicate SEM.

Figure 2.3

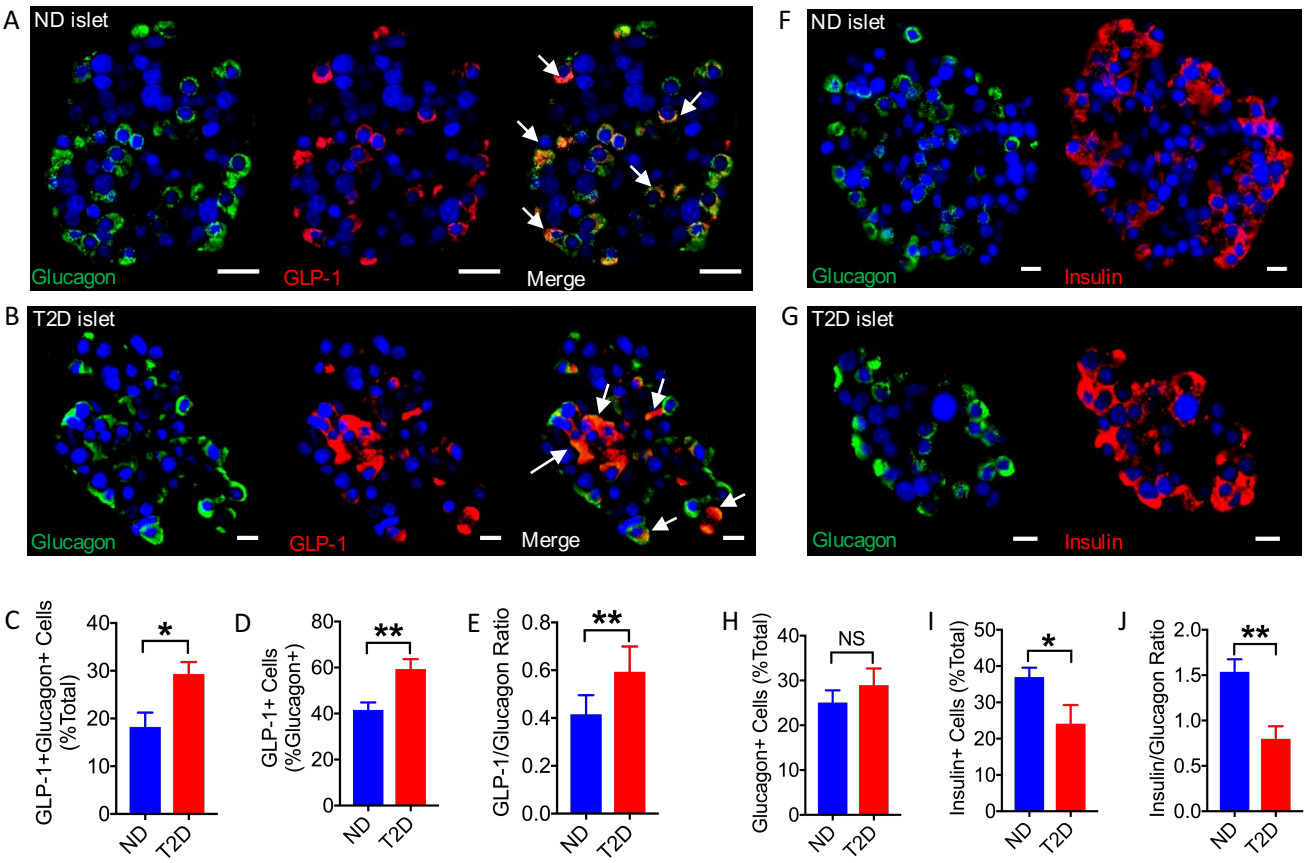

A Non-diabetic islets:
2.5 mM
11.1 mM Glucose
Insulin Secretion (% Content)
Time (min)
Vehicle
Exendin-9
AUC
**
B Type 2 diabetic islets:
***
C
% Change Insulin Secretion (relative to control)
ND + Exendin-9
T2D + Exendin-9
ND
T2D
D
10 mM Glucose
100 nM Exendin-9
30 mM KCl
[Ca2+]i (a.u.)
E
Exendin-9
10 min
F
Events (min-1)
G10
G
Signal dynamics (SD/mean x1000)

The GLP-1 expressing α cell subpopulation is increased in T2D islets.

As GLP-1 expression in pancreatic α cells has been associated with metabolic stress and β cell injury (Ellingsgaard et al., 2011; Z. Liu et al., 2011), I hypothesized that the GLP-1+ α cell subpopulation may be increased in T2D. To test this notion, I obtained islet sections from T2D donors and ND controls and stained for glucagon and amidated GLP-1. Double positive staining for glucagon and GLP-1 was observed in both ND and T2D islet samples (Fig. 2.4A,B). Image analysis revealed that the GLP-1+/glucagon+ subpopulation was significantly increased from 18 to 29% of total islet cells in T2D islets when compared to ND islets (Fig. 2.4C). Importantly, within the α cells of T2D islets, this GLP-1+ subpopulation increased from 40 to 60% (Fig. 2.4D,E). I then immuno-stained islet sections from the same donors for glucagon and insulin, to identify the α and β cell populations respectively (Fig. 2.4F,G). Image analysis of T2D islets showed no change in α cell density, but a significant decrease in β cell density (Fig. 2.4H,I) resulting in a significant decrease in the insulin/glucagon ratio, confirming that the islets are phenotypically diabetic (Fig. 2.4J).

Figure 2.4. The GLP-1 expressing α cell subpopulation is increased in T2D islets. **A.** Representative image of ND islet showing positive staining for DAPI, glucagon and amidated GLP-1. Scale bar: 20 μm. **B.** Representative image of T2D islet showing positive staining for DAPI, glucagon, and increased staining of amidated GLP-1. Scale bar: 10 μm. **C.** GLP-1+/glucagon+ cells increased as % total in T2D islets compared to ND islets. **D.** GLP-1+ α cell subpopulation is increased in T2D islets. **E.** GLP-1+ α cell subpopulation represented as proportion of glucagon+ cells and % total islet cells in ND and T2D islets. **F,G**. Representative images of a ND and T2D islet respectively showing positive staining for DAPI, glucagon and insulin. Scale bar: 10 μm. **H.** Glucagon+ (α) cells as a % of total islets cells does not change in T2D islets. **I.** Insulin+ (β) cells decrease in T2D islets indicating the islets have experienced β cell loss. **J.** The insulin/glucagon ratio decreases in T2D islets indicating they are phenotypically diabetic. N=6 ND donors and N=6 T2D donors matched for age, BMI, and sex. Statistical significance for the data was determined using a paired Student's t test. *, $P<0.05$, **, $P<0.01$. Error bars indicate SEM.

Figure 2.4


A
ND islet
Glucagon
GLP-1
Merge
B
T2D islet
Glucagon
GLP-1
Merge
F
ND islet
Glucagon
Insulin
G
T2D islet
Glucagon
Insulin
C
GLP-1+Glucagon+ Cells (%Total)
*
ND
T2D
D
GLP-1+ Cells (%Glucagon+)
**
E
GLP-1/Glucagon Ratio
**
H
Glucagon+ Cells (%Total)
NS
I
Insulin+ Cells (%Total)
*
J
Insulin/Glucagon Ratio
**

Discussion

The existence of intra-islet GLP-1 within rodent and human islets has been previously documented (Fava et al., 2016; Heller and Aponte, 1995; Masur et al., 2005), yet the presence of GLP-1 within islets does not necessarily provide evidence for any functional role. Despite several recent studies using genetic mouse models providing support for a functional role of intra-islet GLP-1 (Chambers et al., 2017; Traub et al., 2017), the concept of a paracrine role for GLP-1 within human islets remains controversial and requires more evidence from live tissue. Our routine access to high quality human islets from ND and T2D donors has enabled us to investigate the putative paracrine role for GLP-1 in more detail than has been undertaken previously.

Our results confirm previous findings (Hansen et al., 2011) that isolated human islets secrete robust amounts of active GLP-1 and that this GLP-1 is likely originating from a significant subpopulation of α cells. Moreover, the key prohormone convertase PC1/3 required for the proglucagon processing to release GLP-1 (Rouillé et al., 1997) is significantly increased in the GLP-1+ α cell subpopulation. Importantly, I observed an increase in the GLP-1+ α cell population in T2D islets, suggesting that metabolic factors such as hyperglycemia and chemokine action may regulate intra-islet GLP-1 expression. Indeed, hyperglycemia is associated with α cell PC1/3 expression in rodent models of diabetes (Hansen et al., 2011; Nie et al., 2000). In human islets, β cell injury has been shown to increase GLP-1 expression that involves the expression and secretion of the chemokine, SDF-1α (Z. Liu et al., 2011), whereby β cells secrete SDF-1α that acts upon α cells to increase PC1/3 expression and GLP-1 production. Furthermore, the cytokine IL-6, produced from skeletal muscle and adipose tissue, has been shown to function as a metabolic signal that triggers islet α cells to increase islet GLP-1 production (Ellingsgaard et al., 2011).

The possibility remains that intra-islet GLP-1 expression is increased as a result of the use of genetically modified models and/or the peri- and post-isolation procedure of human islets

that may result in β-cell injury described above. However, I observed the presence of a significant subpopulation of GLP-1+ α cell in islets from whole pancreas sections that did not undergo any isolation stress. Furthermore, I observed a significant increase in the number of GLP-1+ α cells in islets from T2D donors when compared to ND islets, despite the isolation procedure and culturing conditions being identical. Taken together, our results argue for a physiological role for GLP-1 in human islets rather than an epiphenomenon resulting from isolation.

As I observed a strong GLP-1 secretory phenotype coupled with a high percentage of GLP-1+ α cells in human islets, the potential for a functional paracrine role for GLP-1 in humans is of obvious importance. Using the GLP-1R antagonist exendin-9, I demonstrate that GLP-1R signalling plays a significant role in modulating intracellular calcium and GSIS and that islets from T2D donors are dependent on GLP-1R activation for >60% of insulin secretion. This observation is further supported by a significant increase in GLP-1+ α cells in T2D islets. As exendin-9 is a specific antagonist of GLP-1Rs, the most plausible explanation is that exendin-9 is competitively inhibiting the actions of intra-islet GLP-1 on β cell GLP-1Rs (Marie et al., 1996). Although, as glucagon is known to stimulate insulin secretion, an alternative explanation may be that glucagon also activates GLP-1Rs. Indeed, a very recent report using gcgr-/- and GLP-1R-/- genetic mouse models suggests that intra-islet GLP-1 levels are very low in wild-type mice and that glucagon can regulate insulin secretion via GLP-1R signalling, albeit at lower potency than GLP-1 (Svendsen et al., 2018). However, previous reports indicate that glucagon is acting via the glucagon receptor (Huypens et al., 2000; Kawai et al., 1995). Moreover, the lack of an inhibitory effect of exendin-9 on insulin secretion at low glucose in our study, when glucagon secretion would be highest, does not support this notion (Fig. 3A) and GLP-1 is a ~400-fold more potent agonist at the GLP-1 receptor than glucagon(Runge et al., 2003). In this study I have determined that there are inherent differences in both the levels of intra-islet GLP-1 secretion and α cell population density between human and mouse islets that further highlights

the controversy surrounding a paracrine role for GLP-1 within human islets. In the absence of difficult to obtain *in vivo* human data to further support the concept of intra-islet GLP-1, our study provides good evidence for a functional paracrine GLP-1R signalling axis in human islets via the localized high levels of GLP-1 secretion, although a possible role for glucagon/GLP-1R signalling cannot be discounted.

The role for DPP4 and the clinically used DPP4 inhibitors on this intra-islet GLP-1 axis is also of interest. Here I tested the effects of the DPP4 inhibitor sitagliptin to evaluate whether some of the clinical efficacy of this class of drugs can be attributed to a direct intra-islet effect. Our flow cytometry analysis showed that DPP4 expression is relatively restricted to α cells arguing for a regulatory role for DPP4 of α cell substrates such as GLP-1. As shown previously, I was also able to increase active GLP-1 in long-term human islet cultures (Omar et al., 2014; Shah et al., 2013). However, short-term static incubation and the perifusion of human islets with sitagliptin did not significantly increase active GLP-1 or GSIS in either ND or T2D islets. These results argue against a direct stimulatory effect of DPP4 inhibitors on GSIS via the enhancement of active GLP-1 levels in an isolated human islet model.

In summary, our results provide evidence of a functional paracrine role for GLP-1 secreted from a subpopulation of α cells in human islets. Moreover, this subpopulation is increased in T2D and is associated with a greater dependency on GLP-1R signalling for insulin secretion, suggesting that the α and β cells within human islets have adapted in T2D to amplify this paracrine pathway in an attempt to support insulin secretion.

Acknowledgments

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Chapter 3

The DPP4 inhibitor sitagliptin increases GLP-1 levels from human islets and may increase islet cell survival prior to transplantation

by

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Chapter 3 of this thesis has been written for submission for publication. I was responsible for the majority of the data collection, data analysis, and manuscript composition. Donor and islet parameters were provided by Tatsuya Kin at the Clinical Islet Laboratory. Matt Hubert, Nicole Salamon, and Janyne Johnson performed some of the GLP-1 secretion experiments and hormone measurements. Peter Light will be the supervisory author and was involved with concept formation.

Abstract

Background

One of the goals of clinical islet transplantation is a single-donor transplant that is dependent on obtaining enough quality β cell mass from one donor pancreas. Human islets are routinely cultured prior to transplantation, and growth factors such as GLP-1 analogues have been reported to maintain β cell mass and survival. Interestingly, human islets also secrete GLP-1 and express the proteolytic enzyme DPP4 that degrades GLP-1 to an inactive form. The aim of this study is to investigate GLP-1 secretion from human islets and to test if the DPP4 inhibitor sitagliptin can increase levels of active GLP-1 to promote islet survival in culture.

Methods

Active GLP-1, glucagon, and insulin were measured from non-diabetic and type 2 diabetic islets using a glucose suppression test. Human islet cultures were treated with or without sitagliptin, and active GLP-1 levels were taken at 48 hours. These levels were then correlated with islet isolation parameters (SI, viability, culture time, cold ischemia time, and DNA content) and donor parameters (age, HbA1c, and BMI). A dead cell assay using Sytox Green was used to measure cell death in human islet cultures treated with sitagliptin.

Results

I found that both non-diabetic and type 2 diabetic islets secrete active GLP-1. Active GLP-1 levels from human islet cultures negatively correlated with the stimulation index for insulin and this correlation was maintained when active GLP-1 levels were increased with sitagliptin. No strong correlations were found for other islet or donor parameters. The fold change in active GLP-1 from sitagliptin treated cultures correlated with islet survival.

Conclusion

Human islets have a local paracrine source of the prosurvival peptide, active GLP-1. Although low levels of active GLP-1 are associated with greater β cell function, these levels may

be increased with the DPP4 inhibitor sitagliptin. The increase in active GLP-1 levels may confer protection from islet cell death and has the potential to improve islet culture prior to transplantation.

Introduction

Clinical islet transplantation (CIT) is a successful treatment for T1D patients with impaired awareness of hypoglycemia and severe hypoglycemic events who have failed to manage their symptoms with insulin therapy (Shapiro et al., 2016). Recent studies show that CIT can restore hypoglycemia awareness, eliminate or reduce severe hypoglycemic events, and improve glycemic control (Hering et al., 2016; Holmes-Walker et al., 2017). Furthermore, insulin independence rates after transplantation have improved significantly since the first Edmonton Protocol and are now nearly 50% (Pepper et al., 2018). Improvements in islet isolation and culture, along with improved donor selection and immunosuppressive agents, have propelled human islet transplantation from an experimental procedure to a clinical treatment.

One of the goals of CIT is a single-donor transplant that is dependent on obtaining enough quality β cell mass from one pancreas (Shapiro, 2011a). Islet preparations are routinely cultured for 24-72 hours to allow for quality control and initiation of inductive immunosuppressive treatment of the recipient (Shapiro et al., 2016). Although this culture period can further purify the islet preparation, it may also result in a loss of β cell mass, falling below the minimum 5000 IEQ per kg of recipient body mass required for transplant (Shapiro, 2011b). During this pre-transplant culture period there is an opportunity to retain β cell mass and precondition the islets for survival immediately post-transplant. The loss of β cell mass immediately post-transplant in the hepatic portal vein is estimated at >50% (Biarnés et al., 2002), suggesting that improvements in islet survival would reduce β cell mass requirements. Agents or techniques that maintain β cell mass and improve β cell survival would further the goal of a single-donor transplant.

GLP-1 receptor agonists and DPP4 inhibitors, medications for the treatment of T2D, have shown promise in islet transplantation. Liraglutide, a long-acting GLP-1 receptor agonist, improved engraftment in mice and was shown to increase survival of human islets in culture

(Merani et al., 2008; Toso et al., 2010). DPP4 inhibitors increase circulating levels of active GLP-1 and have been shown to increase β cell mass in animals. In a clinical pilot study, the DPP4 inhibitor sitagliptin was combined with pantoprazole to successfully restore insulin independence from failing grafts, although insulin independence was lost after treatment was stopped (Senior et al., 2016). However, in a randomized, placebo-controlled study using sitagliptin in islet autotransplantation after total pancreatectomy, sitagliptin was well tolerated, but surprisingly there was no metabolic benefit from treatment (Bellin et al., 2016).

Human islets secrete bioactive GLP-1 from α cells and thus have a paracrine source of GLP-1 localized to β cells. Furthermore, DPP4 is also expressed in the α cells of human islets allowing for the use of DPP4 inhibitors to increase active GLP-1 levels within the islet. DPP4 inhibitors have been reported to increase active GLP-1 levels in human islet cultures (Omar et al., 2014; Zhang et al., 2017), with one group reporting increased β cell survival (Shah et al., 2013). However, little is known about intra-islet GLP-1 secretion and the role it may have in maintaining β cell mass, particularly in the context of DPP4 inhibition.

In this study, I characterize GLP-1 secretion in the context of human islet isolation and donor parameters. I also investigate the potential to improve islet survival by increasing active GLP-1 levels with the DPP4 inhibitor sitagliptin. Here I show that nondiabetic and type 2 diabetic islets secrete active GLP-1. I also observe that GLP-1 levels negatively correlate with the stimulation index for insulin secretion, suggesting that low GLP-1 levels are associated with improved β cell function. Sitagliptin treatment of human islets increased active GLP-1 levels ~7 fold. Increased active GLP-1 levels with sitagliptin also negatively correlate with the SI for insulin secretion. However, when human islets are treated with the sitagliptin, the increased active GLP-1 levels correlate with islet cell survival, suggesting a protective effect against cell death.

Methods

Human islets

De-identified human primary islets isolated from deceased donors were obtained from the Alberta Diabetes Institute IsletCore and the Clinical Islet Laboratory, University of Alberta, in accordance with institutional human ethics guidelines. If the islet preparation was <90% pure, islets were handpicked to obtain >90% purity. Islets were cultured in DMEM (5.5 mM glucose) supplemented with 10% FBS and 1% penicillin/streptomycin for static incubations and islet culture experiments. Diagnosis of type 2 diabetes was determined from medical records and a HbA1c > 6.5%.

Compounds

Sitagliptin phosphate monohydrate was purchased from Biovision, reconstituted in water, and used at 200 nM concentration for islet culture experiments and dead cell assay. IL-1β (Genscript, Z02922-10) was reconstituted in PBS and used at 50 ng/ml for islet culture experiments.

Static incubations

Static incubations were performed using 500 µL KRBH buffer containing (mM) 115 NaCl, 5 KCl, 24 $NaHCO_3$, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 0.1% BSA, (pH 7.4) supplemented with the appropriate concentration of glucose. 50 islets were pre-incubated at 37°C with 2.8 mM glucose buffer for 2 hours, transferred to 2.8 mM glucose buffer for 1 hour, followed by 11.1 mM glucose buffer for 1 hour. Supernatant was taken and stored at −20°C for insulin, glucagon, or active GLP-1 analysis.

Islet culture experiments

40 human islets were cultured in 200uL of Ham's F10 (6.1 mM glucose) supplemented with 10% FBS and 1% penicillin/streptomycin for 48 hours with or without 200 nM sitagliptin at 37°C. A parallel group was coincubated with 50 ng/ml IL-1β. Media samples were taken after 48

hours of culture, spiked with 100 uM sitagliptin to prevent further degradation by DPP4, and frozen at −20°C for active GLP-1 analysis. For the serum-free 8 hour experiment, 50 islets were cultured in 500uL of Ham's F10 supplemented with 10 mM glucose, 10 mM nicotinamide, 2 mM L-glutamine, 1.6 mM calcium chloride, 0.5% BSA, 50 uM IBMX, and 0.5% penicillin/streptomycin.

Hormone secretion assays

Active GLP-1 levels from islet culture media, the dead cell assay, and supernatants from static islet incubations were quantified using the electrochemiluminescent assay Active GLP-1 (v2) Kit (K150JWC-1), MesoScale Discovery. Insulin levels in supernatants from static incubations were quantified with Stellux Chemiluminescent Human Insulin ELISA, Alpco. Glucagon levels in supernatants from static incubations were quantified with HTRF Glucagon Assay, Cisbio.

Dead cell assay

Using a 96 well plate with optical bottoms, 40 islets were cultured at 37°C in 200uL of Ham's F10 (6.1 mM glucose) supplemented with 10% FBS and 1% penicillin/streptomycin. The remaining wells of the 96 well plate were filled with PBS to reduce evaporation. The islets were treated with or without 200 nM sitagliptin for 48 hours. The time in culture was used to challenge the islets and promote cell death. After 48 hours, media samples were taken and Sytox Green (Molecular Probes, Eugene, OR) was added to each well (final concentration 100 nM). The islets were imaged with an AMG EVOS fluorescent microscope, 10X objective, using the brightfield and green channels. Images were analyzed using ImageJ where the dead cells (green spots) were counted using a threshold technique and the area of the islets was quantified. Dead cell density was calculated by dividing the dead cells by the area of the islets.

Immunoblotting

Islets were washed three times with PBS and then lysed with a lysis buffer containing (mM) 50 Tris-HCL, 1 EDTA, 1 EGTA, 1 sodium orthovanadate, 50 sodium fluoride, 5 sodium

pyrophosphate, and 0.27M sucrose. This lysis buffer was supplemented with a protease cocktail (BS387, Bio Basic Inc., 1:100 dilution), 0.1% Triton X 100, and 1 mM DTT. Islet lysates were subjected to SDS-PAGE on 8% gels transferred to nitrocellulose membranes and probed with the primary antibody, hDPPIV polyclonal Goat IgG, AF1180, R & D Systems (1:5000). Detection was with peroxidase-conjugated secondary donkey anti-goat, sc-2020, Santa Cruz (1:1000), and visualization by chemiluminescence with ECL-Plus (GE Healthcare). Images were acquired using a Kodak In-Vivo Multispectral Imaging System (Carestream MI software) and analyzed using ImageJ.

Islet isolation parameters

For assessment of in vitro function of islets, static glucose stimulation was performed sequentially on the same sample. After overnight culture, triplicate islet samples were incubated with 2.8 mM glucose and then with 28.0 mM glucose in Connaught Medical Research Laboratory 1066 medium. Stimulation index was calculated as the ratio of stimulated to basal insulin release during 60 minute incubation intervals. Islet preparations were assessed for cell viability using membrane exclusion dyes. A sample of islet preparations was stained using SYTO Green (SYTO-13; Molecular Probes, Eugene, OR) and ethidium bromide. Following one minute incubation at room temperature, preparations were analyzed under fluorescent microscopy for proportion of live and dead cells, which then was expressed as a percentage. Culture time is the time islets were in the CO_2 incubator prior to release. Cold ischemia time was defined as time from cross-clamp of aorta to initiation of islet isolation. DNA content was measured using the Quant-iT DNA quantification assay.

Donor parameters

Age is obtained from the donor chart. HbA1c is assayed from a donor whole blood sample. BMI is calculated from the height and weight recorded in the donor chart.

Results

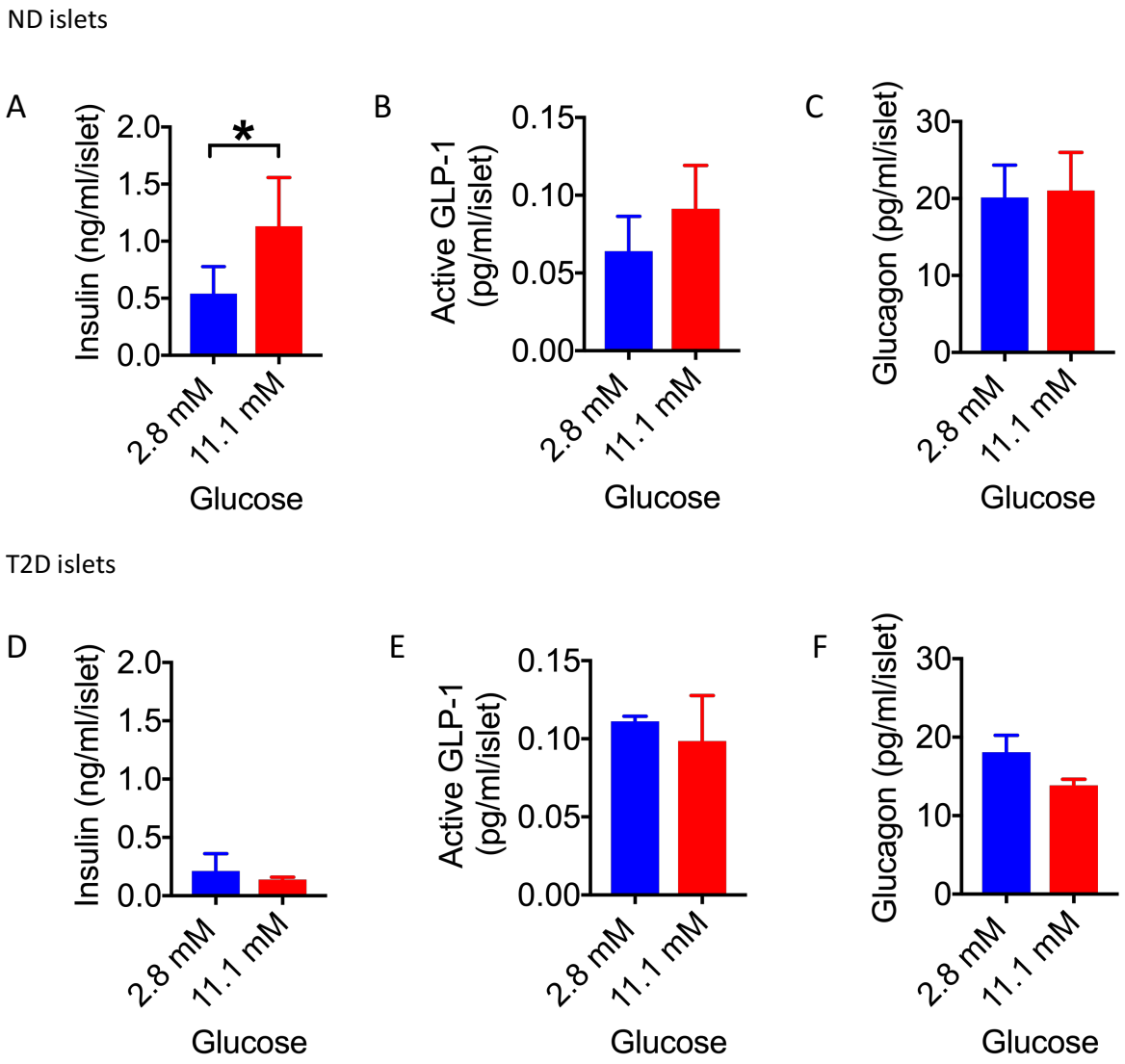
Nondiabetic and type 2 diabetic islets secrete active GLP-1.

As α cell-derived peptides are important for normal insulin secretion and contribute to glucose homeostasis, I first measured active GLP-1 and glucagon from nondiabetic islets (Fig. 3.1A). I performed a glucose suppression test where I observed robust amounts of active GLP-1 secreted at low glucose that surprisingly were not suppressed at high glucose (Fig. 3.1B). To test if the lack of suppression was unique to GLP-1 I also measured glucagon at low and high glucose and found no suppression at high glucose as well (Fig. 3.1C). This indicates that in terms of suppression of secretion at high glucose, GLP-1 and glucagon secretion is similar in our experiments. However, this contrasts with the expected decrease in glucagon secretion from islets at high glucose.

It has been proposed that failing transplanted islets resemble the dysfunction seen in type 2 diabetic islets (Potter et al., 2014). To understand the effect of prolonged islet dysfunction on secretion of α cell-derived peptides, I repeated the glucose suppression test on type 2 diabetic islets. Although glucose-stimulated insulin secretion was reduced compared to nondiabetic islets (Fig. 3.1D), I observed robust amounts of GLP-1 secreted at low glucose that were not suppressed at high glucose, indicating that islet dysfunction does not reduce GLP-1 secretion (Fig. 3.1E). Moreover, glucagon secretion was comparable with nondiabetic islets (Fig. 3.1F).

Figure 3.1. Nondiabetic and type 2 diabetic (T2D) islets secrete active GLP-1. **A.** Glucose-stimulated insulin secretion confirming secretory phenotype of nondiabetic islets. **B,C.** Glucose suppression test of active GLP-1 and glucagon secretion from nondiabetic islets. **D.** Glucose-stimulated insulin secretion confirming secretory phenotype of T2D islets. **E,F.** Glucose suppression test of active GLP-1 and glucagon secretion from T2D islets. Nondiabetic islets, N=5 donors. T2D islets, N=2 donors. Statistical significance for the data was determined using a paired Student's t test. *, $P<0.05$. Error bars indicate SEM.

Figure 3.1


ND islets
A
Insulin (ng/ml/islet)
2.0
1.5
1.0
0.5
0.0
*
2.8 mM
11.1 mM
Glucose
B
Active GLP-1 (pg/ml/islet)
0.15
0.10
0.05
0.00
2.8 mM
11.1 mM
Glucose
C
Glucagon (pg/ml/islet)
30
20
10
0
2.8 mM
11.1 mM
Glucose
T2D islets
D
Insulin (ng/ml/islet)
2.0
1.5
1.0
0.5
0.0
2.8 mM
11.1 mM
Glucose
E
Active GLP-1 (pg/ml/islet)
0.15
0.10
0.05
0.00
2.8 mM
11.1 mM
Glucose
F
Glucagon (pg/ml/islet)
30
20
10
0
2.8 mM
11.1 mM
Glucose

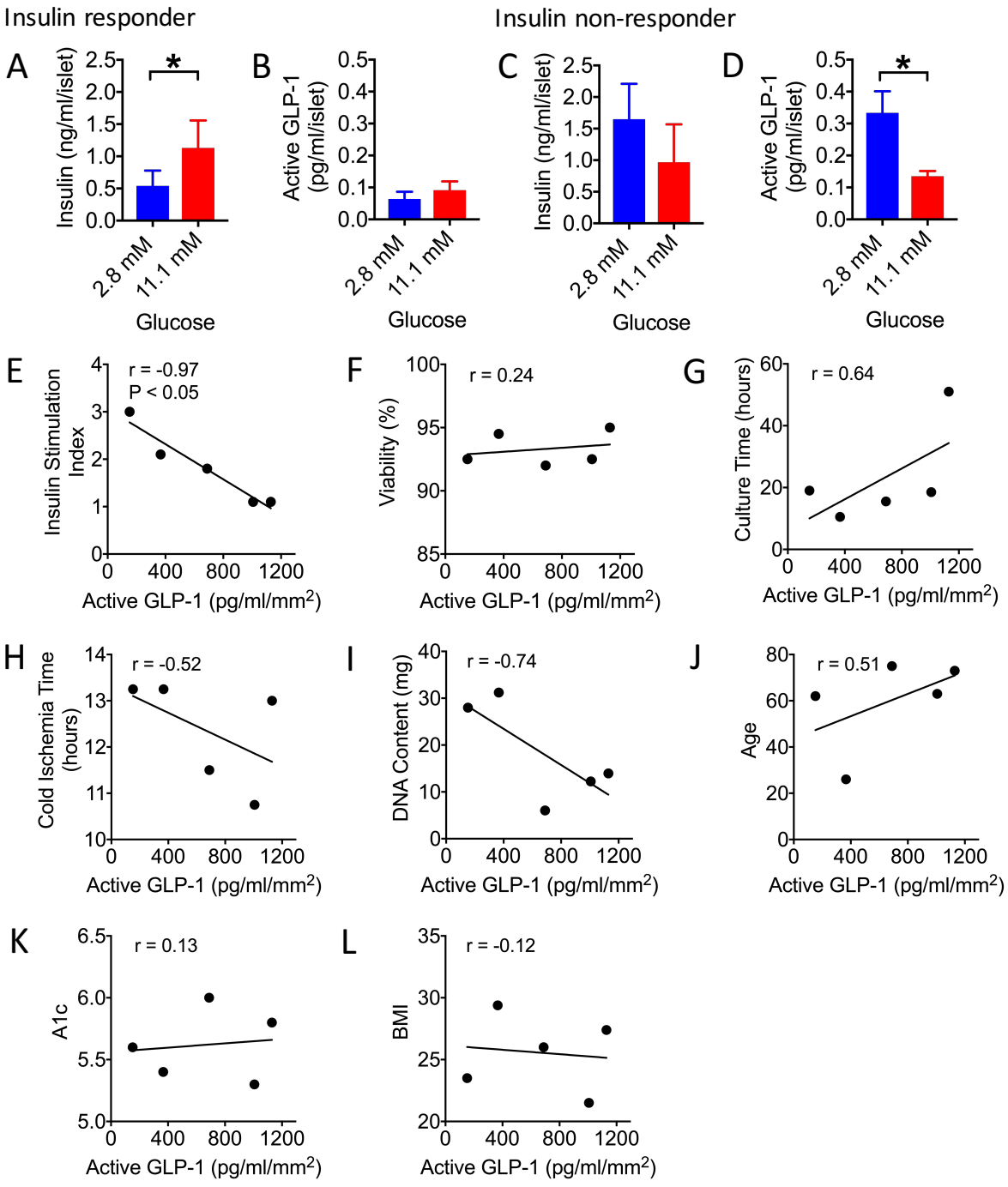
Low active GLP-1 levels associate with greater β cell function in human islets.

The insulin response to glucose varies among isolated islets (Lyon et al., 2016), so I compared GLP-1 secretion from insulin responders and insulin non-responders. As shown in Figure 1, insulin responders secrete GLP-1 that is not suppressed at high glucose (Fig. 3.2A,B). In contrast, insulin non-responders show increased GLP-1 secretion that is suppressed at high glucose (Fig. 3.2C,D). Surprisingly, insulin non-responders show the expected α cell secretion pattern with GLP-1, while insulin responders do not.

I then examined the relationship between the stimulation index (SI) for insulin at isolation and active GLP-1 levels measured after 48 hours of culture for five donors. Consistent with the GLP-1 secretion measured in the glucose suppression tests, I observe a strong negative correlation between SI and active GLP-1 levels (Fig. 3.2E). In other words, low GLP-1 levels associate with normal β cell function, while high GLP-1 levels associate with compromised β cell function. I them examined the relationship between GLP-1 levels and the islet isolation parameters viability, culture time, cold ischemia time, and DNA content (Fig. 3.2F-I). However, GLP-1 levels did not strongly correlate with these parameters. Also, the donor parameters of age, A1c, and BMI did not strongly correlate with active GLP-1 levels (Fig. 3.2J-L).

Figure 3.2. Low active GLP-1 levels associate with greater β cell function in human islets. **A.** Glucose-stimulated insulin secretion of insulin responder. **B.** Active GLP-1 secretion is not suppressed at high (11 mM) glucose. N=5 donors. **C.** Glucose-stimulated insulin secretion of insulin nonresponder. **D.** Active GLP-1 secretion is suppressed at high (11 mM) glucose. N=3 donors. **E-I.** Active GLP-1 levels from human islets after 48 hours of culture plotted against islet isolation parameters. **E.** Insulin stimulation index **F.** Viability. **G.** Culture time before islet preparation release. **H.** Cold ischemia time. **I.** DNA content of islet preparation. **J-L.** Active GLP-1 levels are plotted against donor parameters. **J.** Age (years). **K.** HgA1c (%). **L.** BMI (kg/m^2). N=5 donors. Statistical significance for the data was determined using a paired Student's t test or Pearson's r. *, $P<0.05$. Error bars indicate SEM.

Figure 3.2


Insulin responder
Insulin non-responder
A
Insulin (ng/ml/islet)
2.8 mM
11.1 mM
Glucose
B
Active GLP-1 (pg/ml/islet)
C
D
E
Insulin Stimulation Index
r = -0.97
P < 0.05
Active GLP-1 (pg/ml/mm2)
F
Viability (%)
r = 0.24
G
Culture Time (hours)
r = 0.64
H
Cold Ischemia Time (hours)
r = -0.52
I
DNA Content (mg)
r = -0.74
J
Age
r = 0.51
K
A1c
r = 0.13
L
BMI
r = -0.12

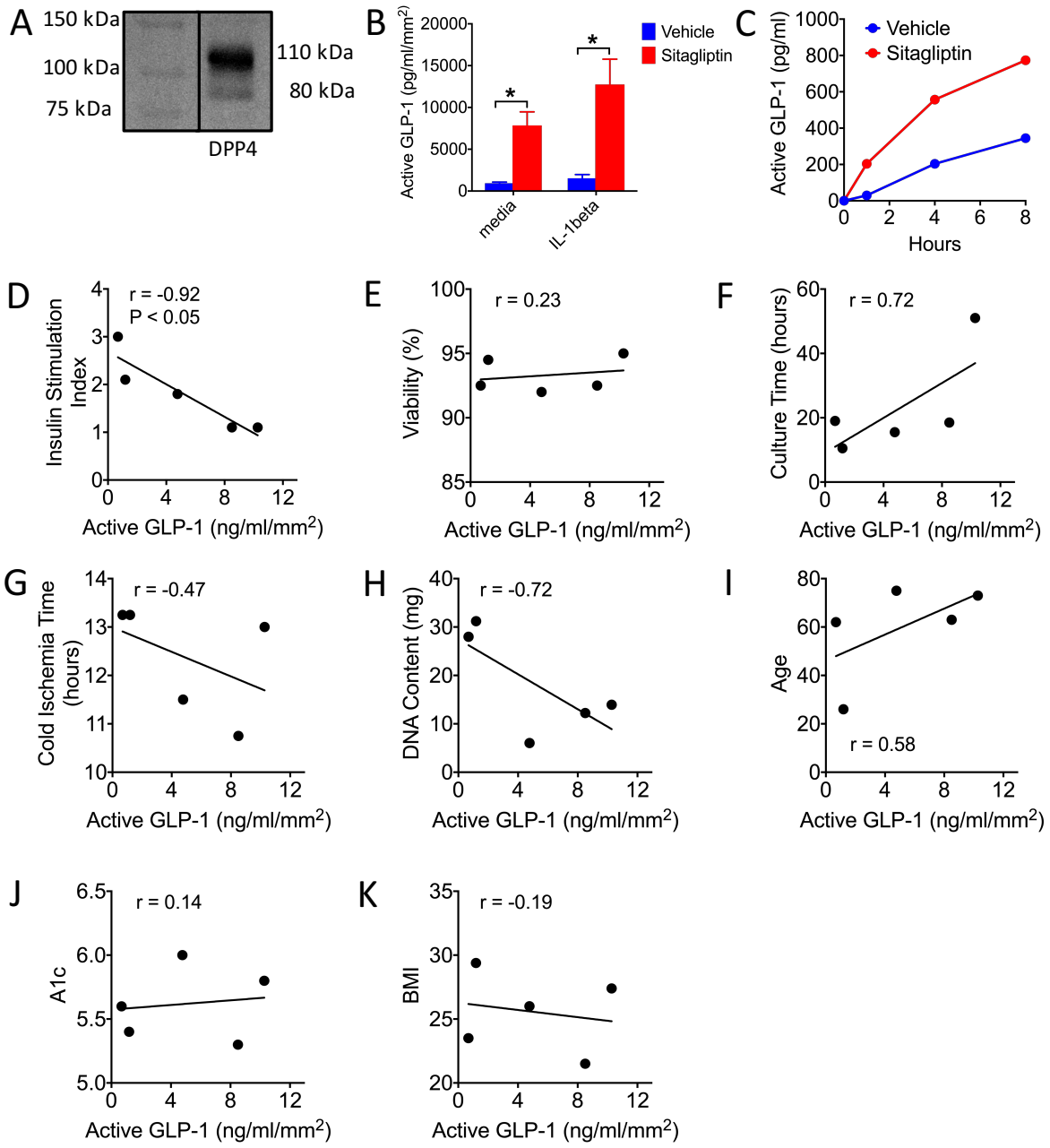
Active GLP-1 levels are increased with sitagliptin and negatively correlate with β cell function.

To test if sitagliptin can increase active GLP-1 levels in human islets, I first probed for DPP4 protein in human islet lysates. As others have shown (Omar et al., 2014), I confirmed with western blot that human islets express DPP4. DPP4 is variably glycosylated and as expected I observe multiple bands (Fig. 3.3A). I then treated human islets with sitagliptin and measured active GLP-1 levels after 48 hours. Active GLP-1 levels increased ~7 fold with sitagliptin treatment and there was no significant increase in the presence of the pro-inflammatory cytokine IL-1β (Fig. 3.3B). To establish that sitagliptin is inhibiting islet DPP4 and not DPP4 present in serum, I treated islets in serum-free culture with sitagliptin where I also observe an increase in active GLP-1 levels (Fig. 3.3C).

With islets from the same five donors as before, I examined the relationship between the increased levels of active GLP-1 with sitagliptin and the initial SI for insulin at the time of isolation. Active GLP-1 levels with sitagliptin treatment should approximate total GLP-1 levels and give an indication of GLP-1 secretion without the modifying effect of DPP4 enzymatic activity. Here too I observe a strong negative correlation between active GLP-1 levels and the SI for insulin (Fig. 3.3D), suggesting that total GLP-1 secretion negative correlates with β cell function. Consistent with active GLP-1 levels at baseline, increased active GLP-1 levels did not strongly correlate with islet isolation or donor parameters (Fig. 3.3E-K).

Figure 3.3. Active GLP-1 levels are increased with sitagliptin and negatively correlate with β cell function. **A.** Western blot of DPP4 in human islet lysates. The two main bands correspond with different levels of glycosylation. **B.** Human islets treated with sitagliptin alone or in the presence for IL-1 β. N=3 donors. **C.** Time course of active GLP-1 secretion from human islets treated with sitagliptin in serum-free culture. N=1 donor **D-H.** Active GLP-1 levels after 48 hours from human islets treated with sitagliptin plotted against islet isolation parameters. **D.** Insulin stimulation index. **E.** Viability. **F.** Culture time before islet preparation release. **G.** Cold ischemia time. **H.** DNA content of islet preparation. **I-K.** Active GLP-1 levels are plotted against donor parameters. **I.** Age (years). **J.** HgA1c (%). **K.** BMI (kg/m^2). N=5 donors. Statistical significance for the data was determined using a paired Student's t test or Pearson's r. *, $P<0.05$. Error bars indicate SEM.

Figure 3.3

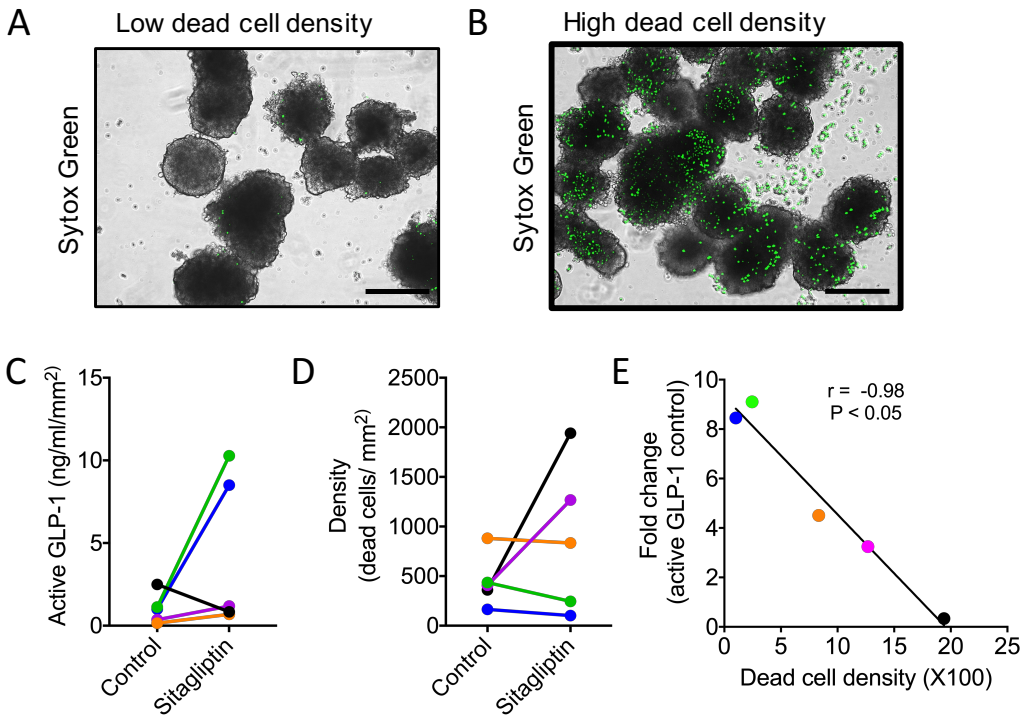

A
150 kDa
100 kDa
75 kDa
110 kDa
80 kDa
DPP4
B
Active GLP-1 (pg/ml/mm2)
Vehicle
Sitagliptin
media
IL-1beta
C
Active GLP-1 (pg/ml)
Vehicle
Sitagliptin
Hours
D
Insulin Stimulation Index
r = -0.92
P < 0.05
Active GLP-1 (ng/ml/mm2)
E
Viability (%)
r = 0.23
Active GLP-1 (ng/ml/mm2)
F
Culture Time (hours)
r = 0.72
Active GLP-1 (ng/ml/mm2)
G
Cold Ischemia Time (hours)
r = -0.47
Active GLP-1 (ng/ml/mm2)
H
DNA Content (mg)
r = -0.72
Active GLP-1 (ng/ml/mm2)
I
Age
r = 0.58
Active GLP-1 (ng/ml/mm2)
J
A1c
r = 0.14
Active GLP-1 (ng/ml/mm2)
K
BMI
r = -0.19
Active GLP-1 (ng/ml/mm2)

Increase in active GLP-1 with sitagliptin correlates with islet cell survival.

As GLP-1 has been reported to decrease apoptosis and increase cell survival in mouse islets (Y. Li et al., 2003), I investigated the effect of increasing active GLP-1 levels with sitagliptin on cell survival in human islets. Although active GLP-1 levels did not correlate with initial viability testing at isolation (Fig. 3.2C), I hypothesized that a sustained increase in active GLP-1 levels may increase islet cell survival. Using Sytox Green to identify dead cells, I developed a dead cell assay to measure cell death in whole islets after 48 hours (Fig. 3.4A,B). Active GLP-1 levels were increased except for one unhealthy donor preparation, but the effect on cell survival was variable (Fig. 3.4C,D). However, when the relationship between the increase in active GLP-1 levels and the dead cell count is examined I observe a strong negative correlation (Fig. 3.4E). This suggests that the increase in active GLP-1 levels with sitagliptin treatment confers a protective effect from cell death.

Figure 3.4. Increase in active GLP-1 with sitagliptin correlates with islet cell survival. **A,B.** Representative images of low and high dead cell density using the cell impermeable DNA binding dye, Sytox Green. Scale bar: 200 μm **C.** Difference in active GLP-1 levels after 48 hours with or with sitagliptin treatment. **D.** Dead cell density after 48 hours with or without sitagliptin. Dead cell densities correspond with active GLP-1 levels in C. **E.** Fold change in active GLP-1 levels with sitagliptin plotted again dead cell densities. N=5 donors. Statistical significance for the data was determined using Pearson's r. *, $P<0.05$.

Figure 3.4


A
Low dead cell density
Sytox Green
B
High dead cell density
Sytox Green
C
Active GLP-1 (ng/ml/mm2)
15
10
5
0
Control
Sitagliptin
D
Density
(dead cells/ mm2)
2500
2000
1500
1000
500
0
Control
Sitagliptin
E
Fold change
(active GLP-1 control)
10
8
6
4
2
0
r = -0.98
P < 0.05
0
5
10
15
20
25
Dead cell density (X100)

Discussion

The observation that GLP-1 levels negatively correlate with β cell function suggests an adaptive response for GLP-1 to maintain insulin secretion. Although GLP-1 potentiates insulin secretion and a positive correlation may be expected, an islet not responsive to glucose may increase GLP-1 secretion in a failed attempt to maintain insulin secretion. This is suggested by the high GLP-1 secretion at low glucose with insulin nonresponder islets. β cell injury has been shown to increase GLP-1 secretion (Z. Liu et al., 2011) and isolated islets are inflamed due to cold ischemia of the pancreas and the islet isolation process (Cowley et al., 2012). However, I did not observe a strong correlation between GLP-1 levels and cold ischemia time. Furthermore, I did not observe a correlation between the donor parameters age, sex, BMI, and GLP-1 levels, suggesting that the SI for insulin secretion is a more important determinant of GLP-1 secretion from human islets.

I used dithizone staining of sample aliquots to ensure the islets were ≥90% pure. Although dithizone staining is a standard technique to assess islet purity, there are limitations with its use. Research in islet transplantation has shown that dithizone staining is a poor indicator of β cell mass and that β cell mass is often overestimated (Kitzmann et al., 2014; Papas et al., 2009). Furthermore, dithizone staining is also a poor indicator of islet cell composition (Ichii et al., 2005). However, in my study the purity of islet preparations for treated and control groups were treated in the same manner. The islet preparations were sometimes cultured for 24 to 48 hours prior to handpicking to ≥90% purity. These cultures of impure (much less than 90%) may have influenced insulin secretion and survival in my experiments. In theory, a healthy acinar tissue may secrete growth factors that influence insulin secretion and survival.

I have shown that sitagliptin treatment of islets increases active GLP-1 levels and that the increase (fold change) in active GLP-1 correlates with islet cell survival in whole islets. This suggests that increased activation of the GLP-1 receptor on islet cells increases survival under

post-isolation culture conditions. Linagliptin, another DPP4 inhibitor, has been show to increase GLP-1 levels and reduce apoptosis in human islet cultures (Shah et al., 2013). Our correlation results are also consistent with the effect of the long-acting GLP-1 receptor agonist liraglutide on human islet survival. In human islets cultures, liraglutide has been shown to reduce apoptosis and preserve islet mass (Toso et al., 2010).

It is possible that other DPP4 substrates, namely the chemokine SDF-1 α, may be responsible for the correlation between sitagliptin treatment and increased cell survival in our data. SDF-1 α signaling has been show to protect and preserve function β cell mass (Alagpulinsa et al., 2018). When β cells are injured, a regenerative process is induced where SDF-1 α is expressed and secreted from β cells. Indeed, the process of islet isolation may be enough to induce SDF-1 α expression. The model proposed by Liu et al. for islet survival describes SDF-1 α acting at β cell CXCR4 receptors to increase survival and at α cell CXCR4 receptors to induce GLP-1 secretion by PC1/3 expression (Z. Liu et al., 2011). GLP-1 would then activate the GLP-1 receptor on β cells to increase survival. Interestingly, both GLP-1 and SDF-1 α are physiological substrates of DPP4 and inhibiting DPP4 may increase levels of both hormones for a greater impact on islet cell survival.

Sitagliptin may not only benefit islet culture, but could improve engraftment and islet cell survival in the hepatic portal vein. DPP4 has recently been identified as a hepatokine indicating that DPP4 is expressed and active in the liver (Ghorpade et al., 2018; Varin et al., 2018). This raises the possibility that islet-derived and circulating GLP-1 may be degraded within the hepatic portal vein, thus reducing levels local to the islet graft. SDF-1 α levels may also be lowered, thus curtailing an adaptive mechanism for β cell survival. Therefore, inhibition of hepatic and islet DPP4 with sitagliptin may promote survival of the islet graft.

In summary, I show that human islets secrete GLP-1 and thus have a local paracrine source of this prosurvival hormone. Although a reduction in glucose-stimulated insulin secretion

may result in increased GLP-1 secretion, the prosurvival properties of GLP-1 may be harnessed to increase islet survival during pre-transplant culture. I show that sitagliptin treatment of human islets inhibits islet DPP4 and increases active levels of GLP-1. The increase in active GLP-1 may confer protection from cell death and has the potential to improve post culture yields prior to islet transplantation.

Acknowledgements

P.E.L. holds the Dr. Charles A. Allard Chair in Diabetes Research. This research was supported by grants from the Canadian Institutes of Health Research (CIHR, to P.E.L. and P.EM.) and the Dr. Rod Eidem Diabetes Research Fund (P.E.L.). Trainee funding was provided by the Alberta Diabetes Foundation (S.C., J.J.), and the Faculty of Medicine and Dentistry (S.C.).

Chapter 4

Bioengineering neonatal porcine islets to increase GLP-1 secretion

For Chapter 4, the NPIs were provided by the Greg Korbutt lab. The adenovirus was provided by the late Stephen Cheley and Amy Barr. I was responsible for data collection and analysis.

Abstract

Background

Neonatal porcine islets (NPIs) are potentially an unlimited source of islets for clinical islet transplantation. However, NPIs are functionally immature at isolation, requiring maturation in culture or after transplantation. Recently, GLP-1 analogues have successfully been used to functionally mature NPIs in culture. I propose that bioengineering GLP-1 secretion from NPIs should increase levels of GLP-1 to the islet and augment a possible GLP-1 paracrine system present in NPIs. To engineer the NPIs, I investigate the use of adenoviral vectors to increase GLP-1 secretion from NPIs and improve functional maturation before transplantation.

Methods

NPIs were transduced with our GLP-1 expressing adenoviral construct, AdGLP-1mCherry. Active GLP-1 secretion was measured at low and high glucose from transduced NPIs. Transduced NPIs were tested for glucose-stimulated insulin secretion (GSIS). Gene-specific primers were used to identify the GLP-1 receptor using PCR. DPP4 specific antibodies were used to probe for the presence of DPP4 in NPI lysates.

Results

Non-transduced NPIs secrete active GLP-1, suggesting that NPIs have a paracrine GLP-1 system. AdGLP-1mCherry increased active GLP-1 levels at day 7 in culture, but not earlier at day 2. Although active GLP-1 was increased in AdGLP-1mCherry NPIs, GSIS was not enhanced. The GLP-1 receptor transcript was identified in NPIs at day 7 and day 12 of culture. Finally, NPIs were found to express DPP4, the enzyme responsible for inactivation of GLP-1.

Conclusions

Having identified GLP-1 secretion in non-transduced NPIs, the GLP-1 receptor transcript, and DPP4 protein, I establish the components of a potentially functional GLP-1 paracrine system in NPIs. Active GLP-1 secretion can be enhanced from NPIs with adenoviral-

mediated GLP-1 secretion. However, the increase in active GLP-1 does not increase GSIS, suggesting the NPIs have not functionally matured.

Introduction

Neonatal porcine islets (NPIs) are potentially an unlimited source of islets for clinical islet transplantation (Ellis and Korbutt, 2015). Clinical islet transplantation is currently limited to patients with hypoglycemic unawareness, severe hypoglycemic episodes, or glycemic liability that cannot be stabilized with exogenous insulin (McCall and Shapiro, 2012). However, when safe immune tolerance is achieved in islet transplantation, the patient pool will expand beyond the current limitations. At this time, a consistent and reliable source of islets cells will be needed. The current practice of isolating islets from cadaveric human donor pancreases will not be able to meet the demand and alternate islet sources, such as stem cell derived β cells or xenogeneic islet cells will be needed (Shapiro et al., 2016).

NPIs are a good alternate islet source for transplantation because they secrete insulin in the same physiological range as human islets and porcine insulin has been used for years in the management of type 1 diabetes (Rayat et al., 1999). NPIs are less fragile than adult porcine islets and easier to maintain in culture, making them a better porcine islet candidate for transplantation (Salama and Korbutt, 2017). They have the advantage of being resistant to the stress of hyperglycemia, protected from apoptosis due to low oxygenation, and resistant to the effects of proinflammatory cytokines (Emamaullee et al., 2006; Harb and Korbutt, 2006; Harb et al., 2007). NPIs are also protected from amyloid deposition, a common attribute of a failing human islet graft (Potter et al., 2010). A reliable protocol has been developed for the isolation of large numbers of NPIs that have the capacity for growth and function both in vitro and in vivo (Korbutt et al., 1996). Transplantation of NPIs successfully reversed diabetes in immunosuppressed non-human primates in 2 to 3 weeks, demonstrating successful control of blood glucose in vivo (Cardona et al., 2006). However, NPIs are functionally immature at isolation and methods to improve maturation in culture may improve engraftment and function in transplantation (Kemter and Wolf, 2018).

Numerous studies have demonstrated that GLP-1 contributes to the differentiation of pancreatic β cells. In β cells or β cell progenitors, GLP-1 increases the expression of proinsulin, insulin biosynthesis, and the machinery for glucose sensing (Egan et al., 2003). GLP-1 has been shown to increase PDX-1 expression in human islet β cell progenitors resulting in a more mature phenotype (Abraham et al., 2002). Finally, GLP-1 increased the differentiation of porcine fetal islet cell clusters in culture that resulted in enhanced glucose-stimulated insulin secretion (GSIS). Upon transplantation in nondiabetic SCID mice, the GLP-1 treated islet cell cluster grafts appeared to be functionally mature as assessed by glucose-induced insulin release (Hardikar et al., 2002).

GLP-1 analogues such as exendin-4 are now being used to promote differentiation and functional maturation of NPIs in culture. Exendin-4 is a GLP-1 analogue that is resistant to degradation by dipeptidyl peptidase-4 (DPP4), making it useful in serum culture conditions. It has been shown to improve β cell mass, viability and insulin secretion with NPIs in culture (Hassouna et al., 2018; Mancuso et al., 2006). In fact, the addition of Exendin-4 to the culture media has become routine for some groups conducting experiments with NPIs (Hassouna et al., 2018; Wolf-van Buerck et al., 2017).

This study aims to increase GLP-1 secretion from NPIs to improve functional maturation in culture with the potential to improve transplantation outcomes. Interestingly, I observe that NPIs already secrete active GLP-1 as I have seen with human islets (chapter 2 & 3). I use an adenoviral GLP-1 minigene to bioengineer NPIs to increase their total secretion of GLP-1 and test the effect on insulin secretion. I also identify the GLP-1 receptor transcript and DPP4 protein in NPIs, establishing the presence of two components that are important to a functioning GLP-1 system in NPIs.

Methods

Adenoviral construct and transduction

Recombinant adenoviruses producing red fluorescent protein reporter alone (AdmCherry) or with our GLP-1 minigene (AdGLP-1mCherry) were created using pAdtrackCMV and the AdEasy system (get Luo et al). NPIs were estimated to be 1000 cells and transduced with 100 multiplicity of infection (MOI) for 4 hours. After 4 hours, the media was changed to remove the adenovirus.

NPI culture

Animal use was in accordance with the guidelines approved by the Canadian Council on Animal Care. Porcine pancreata were obtained from 1- to 3-day Duroc neonatal piglets from the University of Alberta Swine Research Center (1.5-2.0 kg body weight). NPIs were isolated and cultured in Ham's F10 media (Sigma-Aldrich, St. Louis, MO) supplemented with 10 mM glucose (final concentration), 10 mM nicotinamide, 2 mM L-glutamine, 1.6 mM calcium chloride, 0.5% BSA, 50 uM IBMX, and 0.5% penicillin/streptomycin.

Imaging

Whole islets were imaged with an AMG EVOS fluorescent microscope, 10X objective, using the red channel. Confocal visualization was on a Zeiss AxioObserver Z1 with a Zeiss-Colibri light source at 594 nm, a X40/1.3 NA lens, and an AxioCam HRm camera. Images were acquired with Axiovision 4.8 software (Carl Zeiss MicroImaging).

PCR

RNA was extracted from NPIs using TRIzol Reagent (Life Technologies). Primers were: porcine GLP-1R forward 5'-TGT GGC TGC ACA AGG ACA ACT CCA-3'; GLP-1R reverse 5'-ACA GGG CCA GCA GCG TGT ACA GGT-3'; porcine GAPDH forward 5' GCA AGT TCC ACG GCA CAG TCA AG-3'; and porcine GAPDH reverse 5'-GGT AGA AGC AGG GAT GAT GTT CTG G-3'. cDNA was generated using qScript cDNA SuperMix (QuantaBio). PCR was

performed with Q5 Hot Start High-Fidelity DNA polymerase (New England Biolabs) using 35 cycles and bands were stained with ethidium bromide for visualization.

Static incubations.

Static incubations were performed using 500 µL KRBH buffer containing (mM) 115 NaCl, 5 KCl, 24 $NaHCO_3$, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 0.1% BSA, (pH 7.4) supplemented with the appropriate concentration of glucose. 50 NPIs were pre-incubated at 37°C with 2.8 mM glucose buffer for 2 hours, transferred to 2.8 mM glucose buffer for 1 hour, followed by 11.1 mM glucose buffer for 1 hour. Supernatant was taken and stored at −20°C for insulin or active GLP-1 analysis.

Hormone secretion assays.

Active GLP-1 levels from static incubations were quantified using the electrochemiluminescent assay Active GLP-1 (v2) Kit (K150JWC-1), MesoScale Discovery. Porcine insulin levels from static incubations and neonatal porcine islets were quantified using the electrochemiluminescent assay Mouse/Rat Insulin (K152BZC-1), MesoScale Discovery.

Immunoblotting.

Islets were washed three times with PBS and then lysed with a lysis buffer containing (mM) 50 Tris-HCL, 1 EDTA, 1 EGTA, 1 sodium orthovanadate, 50 sodium fluoride, 5 sodium pyrophosphate, and 0.27M sucrose. This lysis buffer was supplemented with a protease cocktail (BS387, Bio Basic Inc., 1:100 dilution), 0.1% Triton X 100, and 1 mM DTT. Islet lysates were subjected to SDS-PAGE on 8% gels transferred to nitrocellulose membranes and probed with the primary antibody, hDPPIV polyclonal Goat IgG, AF1180, R & D Systems (1:5000). Detection was with peroxidase-conjugated secondary donkey anti-goat, sc-2020, Santa Cruz (1:1000), and visualization by chemiluminescence with ECL-Plus (GE Healthcare). Images were acquired using a Kodak In-Vivo Multispectral Imaging System (Carestream MI software) and analyzed using ImageJ.

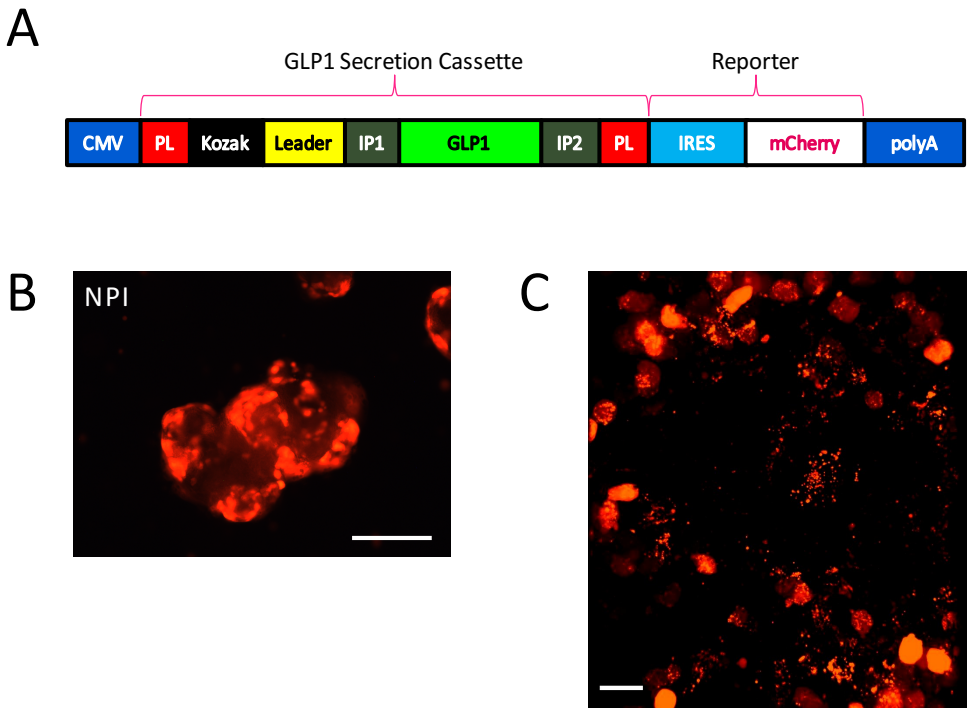
Results

Adenoviral construct and transduction of neonatal porcine islets.

To increase GLP-1 secretion from NPIs, I designed an adenoviral construct containing a GLP-1 secretion cassette and a mCherry reporter (Fig. 4.1A). For effective processing and secretion, the GLP-1 sequence was flanked by proglucagon intervening peptides, preceded by a signal peptide, and led by a Kozak sequence for efficient translation. The GLP-1 secretion cassette was followed by an IRES element and mCherry sequence, and the whole cassette was put under the constitutively active CMV promoter. The full adenovirus was called AdGLP-1mCherry and the adenoviral control, AdmCherry. NPIs were readily transduced with AdGLP-1mCherry as demonstrated by the presence of fluorescence in the red channel (Fig. 4.1B). To further characterize AdGLP-1mcherry transduction of NPIs, I imaged the midplane of the islet and, as suspected, only the outer cells of the NPIs were transduced with the virus (Fig. 4.1C).

Figure 4.1. Adenoviral construct for GLP-1 secretion and transduction of neonatal porcine islets. **A.** Adenoviral construct under the CMV promoter. The GLP-1 secretion cassette: polylinker (PL), Kozak sequence, signal peptide (Leader), intervening peptide (IP1, IP2), and GLP-1. The reporter: IRES element and mCherry, followed by a polyA tail. **B.** Whole NPIs transduced with AdGLP-1mCherry. Scale bar: 200 μm. **C.** Midplane image of AdGLP-1mCherry transduced NPI. Scale bar: 20 μm.

Figure 4.1

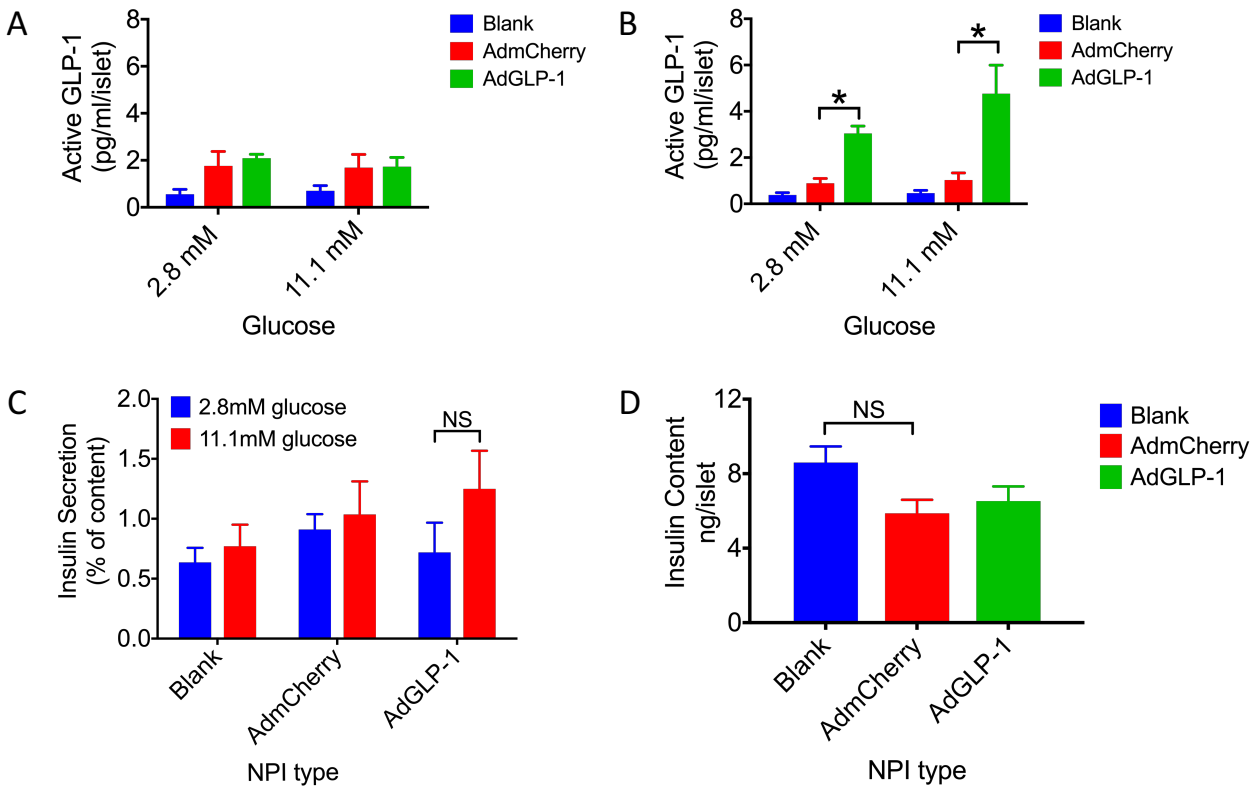

A
GLP1 Secretion Cassette
Reporter
CMV
PL
Kozak
Leader
IP1
GLP1
IP2
PL
IRES
mCherry
polyA
B
NPI
C

Active GLP-1 secretion is increased from AdGLP-1 neonatal porcine islets, but does not increase GSIS.

To assess GLP-1 secretion I measured active GLP-1 from transduced NPI cultures at day 2 and day 7 (Fig. 4.2A,B). Interestingly, I observed active GLP-1 secretion in non-transduced control NPI cultures indicating that NPIs secrete GLP-1 as shown with human islets (chapter 2). At day 2 after adenoviral transduction, AdGLP-1mCherry NPIs did not secrete more active GLP-1 than control; however, at day 7 AdGLP-1mCherry NPIs secreted substantially more active GLP-1 (~3-5 fold) at low (2.8 mM) and high (11.1 mM) glucose. Since GLP-1 is insulinotropic and known to functionally mature NPIs (Hassouna et al., 2018), and increased active GLP-1 levels would be local to the islets, I then tested the AdGLP-1mCherry NPIs for GSIS. Surprisingly, I did not see an increase in GSIS; however, I also did not observe any deleterious effects of adenoviral transduction on insulin secretion (Fig 4.2C). This was confirmed with insulin content analysis showing no significant difference between AdGLP-1mCherry and controls (Fig. 4.2D).

Figure 4.2. Active GLP-1 secretion is increased from AdGLP-1mCherry NPIs, but does not increase GSIS. **A.** NPI GLP-1 secretion at low (2.8 mM) and high (11.1 mM) glucose on day 2 post-transduction. **B.** NPI GLP-1 secretion at low (2.8 mM) and high (11.1 mM) glucose on day 7 post-transduction. **C.** NPI glucose-stimulated insulin secretion for control, adenoviral control, and AdGLP-1mCherry. **D.** NPI insulin content for control, adenoviral control, and AdGLP-1mCherry. All experiments, N=3. Statistical significance for the data was determined using a one-way ANOVA and Tukey's multiple comparisons test. *, $P<0.05$. Error bars indicate SEM.

Figure 4.2

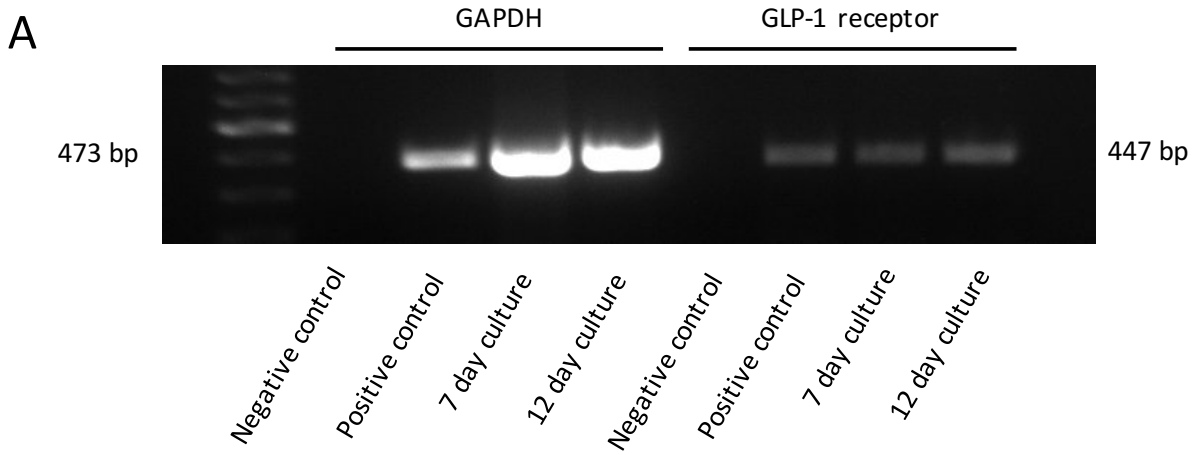

A
Active GLP-1 (pg/ml/islet)
8
6
4
2
0
Blank
AdmCherry
AdGLP-1
2.8 mM
11.1 mM
Glucose
B
Active GLP-1 (pg/ml/islet)
*
*
Blank
AdmCherry
AdGLP-1
2.8 mM
11.1 mM
Glucose
C
Insulin Secretion (% of content)
2.0
1.5
1.0
0.5
0.0
2.8mM glucose
11.1mM glucose
NS
Blank
AdmCherry
AdGLP-1
NPI type
D
Insulin Content ng/islet
12
8
4
0
NS
Blank
AdmCherry
AdGLP-1
NPI type

GLP-1 receptor transcript is present in neonatal porcine islets at 7 and 12 day cultures.

Although the GLP-1R has been identified in adult porcine islets (Kelly et al., 2014), it is not known if neonatal porcine islets express the receptor. I avoided protein identification because antibodies for the GLP-1R are reported to be not specific (Panjwani et al., 2013). Instead, I used primers specific for the porcine GLP-1R to identify the transcript. I identified the GLP-1R transcript at 7 and 12 days of culture, suggesting that the GLP-1R is present in the membrane and able to signal upon binding to GLP-1 (Fig. 4.3A).

Figure 4.3. GLP-1 receptor transcript present in neonatal porcine islets at 7 and 12 day cultures. **A.** Identification of the GLP-1 receptor transcript in NPIs at 7 and 12 day cultures. Housekeeping gene is GAPDH. Positive control is adult porcine pancreatic tissue, while the negative control is no template.

Figure 4.3

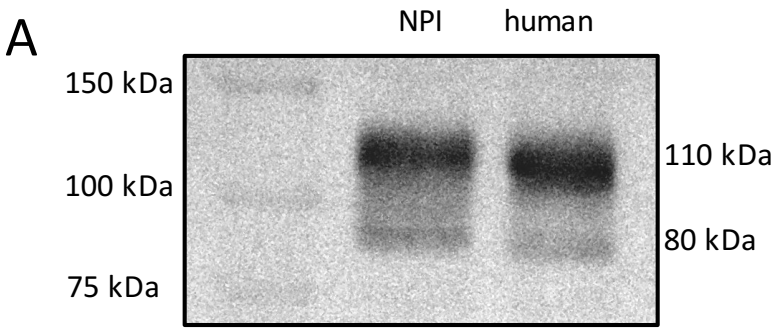

A
GAPDH
GLP-1 receptor
473 bp
447 bp
Negative control
Positive control
7 day culture
12 day culture
Negative control
Positive control
7 day culture
12 day culture

Neonatal porcine islets express DPP4.

To investigate whether intra-islet GLP-1 or adenoviral-mediated GLP-1 secretion from NPIs may be degraded by islet DPP4, I probed NPI lysates for DPP4. I identified DPP4 protein in NPIs and found a similar molecular weight range as human islets, indicating a similar pattern of glycosylation (Fig. 4.4A) (Aertgeerts et al., 2004). The presence of DPP4 in NPIs suggests that GLP-1 may be degraded in our NPI cultures (with or without serum) and warrants the use of a DPP4 inhibitor such as sitagliptin to stabilize active GLP-1.

Figure 4.4. Neonatal porcine islets express DPP4. **A.** Western blot of NPI and human lysates probed for DPP4. The 110-80 kDa range represents various glycosylation states of the enzyme.

Figure 4.4



Discussion

Although AdGLP-1mCherry NPIs increased active GLP-1 secretion ~2-5 fold over controls, I did not observe an improvement in GSIS. There are a number of possible reasons for this, with the first being that an increase in GLP-1 secretion may not have occurred long enough in culture to increase β cell mass or move NPI β cells to a more differentiated state. I did not observe an increase in GLP-1 secretion 2 days after adenoviral transduction and only observed an increase at day 7. The significant increase in GLP-1 secretion may not have occurred until later, day 6 for example, not leaving enough time for sustained GLP-1 action. It is possible that a dynamic perifusion assay of GSIS may reveal an increase in stimulation index over controls, but I did not see a significant increase in insulin secretion at high glucose in our static incubations. Another reason for no improvement in GSIS, is that GLP-1 action alone is not enough to functionally mature or increase GSIS in NPIs. Using adenoviral vectors, Mourad et al. transduced NPIs with a modified GLP-1 and found no increase in GSIS (Mourad et al., 2017). However, when they co-transduced NPIs with GLP-1 and a constitutively active type 3 muscarinic receptor (M3R) they observed significant improvements with GSIS. Amplification of both the GLP-1 and cholinergic pathways increased GSIS, but not activation of the GLP-1R alone. Other growth factors are added to GLP-1 analogues in successful NPI maturation protocols, again suggesting that GLP-1 action alone is not sufficient (Hassouna et al., 2018).

In our study, I identified DPP4 protein expression in NPIs indicating that GLP-1 may be degraded to inactive forms and consequently limiting its action on β cells. Future experiments should include the addition of a DPP4 inhibitor such as sitagliptin to the culture media to stabilized and further increased active GLP-1 levels. Sitagliptin has been shown to increase active GLP-1 levels in human islets (chapter 2) and sustained action my improved β cell mass and contribute to NPI maturation. Sitagliptin has the possible additional benefit of increasing active forms of the chemokine SDF-1 α in NPI cultures (Mulvihill and Drucker, 2014). SDF-1 α

has been show to increase survival of mouse and human β cells and to be required for maturation and proliferation of human fetal endocrine progenitor cells in culture (Kayali et al., 2012; Z. Liu et al., 2011). Furthermore, SDF-1α has been incorporated into alginate encapsulated porcine islets grafts to prolong experimental transplantation without systemic immunosuppression (Chen et al., 2015).

GLP-1 secretion from our AdGLP-1mCherry NPIs has the potential to improve transplantation immediately post-transplant. Transplantation in the hepatic portal vein or in a subcutaneous retrievable site is initially an hypoxic environment for the islets until engraftment occurs. NPIs are inherently resistant to hypoxic conditions (Emamaullee et al., 2006), but increased GLP-1 secretion local to the islets may provide additional protection. GLP-1 is also prosurvival and anti-apoptotic for β cells (Y. Li et al., 2003). I have used adenoviral vectors to increase GLP-1 expression and are therefore limited to transient expression. However, this may be long enough to provide additionally protection immediately after transplantation. Long-term protection would require the use of a lentivirus or adeno-associated virus for genomic incorporation.

I have shown that NPIs can be bioengineered to increased GLP-1 secretion using adenoviral vectors to transduce NPIs with a GLP-1 minigene. Although active GLP-1 secretion is increased from AdGLP-1mCherry NPIs, GSIS is not increased, suggesting the engineered NPIs have not functionally matured in culture. I have also identified the GLP-1 receptor transcript, DPP4 protein, and GLP-1 secretion from non-transduced NPIs, establishing the components of a functioning GLP-1 paracrine system in NPIs that may be enhanced with DPP4 inhibitors.

Chapter 5

Costarting sitagliptin with metformin is associated with a lower likelihood of disease progression in newly treated patients with type 2 diabetes

by

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Chapter 5 of this thesis has been written for submission for publication. I contributed to the design, wrote part of the Stata code, and wrote the initial manuscript. Scot Simpson wrote the remaining code, guided the design and analysis for the study, and edited the manuscript. Peter Light conceived the idea for the project.

Preface

This chapter builds on the observation presented in chapter 3 that in human islets treated with sitagliptin, the increase in active GLP-1 strongly correlates with islet cell survival. As sitagliptin is a drug on the market for the treatment of T2D, I was in a unique position to take our findings at the bench into a population of type 2 diabetic patients. The positive findings presented in this chapter fulfill two of the nine Bradford-Hill considerations for ascribing causation to association (Bradford Hill, 1965). The first is *strength*, as the magnitude of our findings are large. The second is *coherence*, as the findings in this observational study are consistent with our result at the bench.

The observational study presented in this chapter covers the period of April 1, 2008 to March 31, 2015. Sitagliptin (Januvia) was approved for use in Canada in January 2008 and the combination product, metformin and sitagliptin (Janumet) was approved in October 2009. Therefore this study follows the use of sitagliptin in Alberta right after its approval. Sitagliptin is indicated as second-line treatment after metformin, and this was formalized in Alberta when sitagliptin was eligible as a drug benefit for step therapy special authorization in May 2012. A trail of metformin for a minimum of 6 months must be in place before sitagliptin will be approved for special authorization.

Abstract

Aim

Sitagliptin can potentially preserve beta cell function by promoting local action of GLP-1 in the pancreas. This study examined if early addition of sitagliptin to metformin was associated with a delay in the progression of type 2 diabetes.

Methods

Administrative health records from Alberta, Canada between April 1, 2008 and March 31, 2015 were used to conduct a retrospective cohort study. New metformin users were identified and included if they added sitagliptin during follow-up. Patients costarting sitagliptin and metformin were compared to those who added sitagliptin after initiating metformin therapy. Logistic regression models were used to evaluate the association between sitagliptin addition and starting insulin. Change in A1c 1 year after adding sitagliptin was evaluated using linear regression models.

Results

Mean (SD) age of the 8,746 included patients was 52.1 (11.1) years, 5,655 (64.7%) were men, and 1,149 (13.1%) initiated treatment with metformin and sitagliptin. Insulin was added to the therapy of 192 (16.7%) costarters and 1,640 (21.6%) of delayed sitagliptin users (adjusted odds ratio 0.73; 95% CI: 0.62 to 0.86). The A1c change 1 year after starting sitagliptin was greater in the costarters (-2.1%; SD 2.8%) compared to delayed sitagliptin users (-1.0%; SD 1.9%). After adjusting for baseline A1c and other covariables, costarters had a greater reduction in A1c -0.56% (95% CI: -0.74% to -0.38%).

Conclusions

Costarting sitagliptin with metformin is associated with a lower likelihood of disease progression in type 2 diabetes compared with adding sitagliptin to metformin therapy later in therapy.

Introduction

In the management of type 2 diabetes, dipeptidyl peptidase-4 (DPP4) inhibitors are considered a second line agent after metformin. Clinical practice guidelines recommend addition of second line agents if glycemic targets are not being met with metformin alone and after considering a patient's overall clinical state (Lipscombe et al., 2018). However, there may be therapeutic benefits to starting a DPP4 inhibitor early, if not at the same time that metformin is initiated. Although A1c targets and glycemic control may be achieved with metformin alone, costarting a DPP4 inhibitor such as sitagliptin with metformin may improve long-term glycemic control and slow diabetes progression.

Sitagliptin is a safe and effective oral agent to reduce A1c, both as monotherapy and as add-on therapy to metformin (Aschner et al., 2006; Charbonnel et al., 2006; Eurich et al., 2013). As with other DPP4 inhibitors, sitagliptin is weight neutral, does not increase the risk of a major adverse cardiovascular event, and does not contribute to hypoglycemia (Deacon, 2018). Sitagliptin and other DPP4 inhibitors lower glucose by preventing the enzymatic degradation of the incretin hormones, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). By increasing active forms of GLP-1 and GIP, sitagliptin increases post-prandial insulin secretion to improve glucose tolerance and maintain glucose homeostasis (Mulvihill and Drucker, 2014).

Preservation of beta cell mass and function early in therapy is thought to slow progression of type 2 diabetes (Phillips et al., 2014). Evidence for this hypothesis stems from clinical trials where exogenous insulin is initiated early in therapy to improve glycemic control and the benefits persist after treatment has been stopped. This legacy effect slows progression of diabetes and results in fewer medications and a lower risk of complications (Phillips et al., 2014). However, when initial metformin therapy does not meet glycemic targets, there is often a delay in treatment intensification in clinical practice (Desai et al., 2018; Gomes et al., 2013; Phillips et al., 2001). This delay in treatment may be missing a critical window early in the

progression of type 2 diabetes where beta cell mass and function may be partly preserved, thus negating potential long-term benefits. Sitagliptin not only has the ability to increase glycemic control, but also the potential to protect beta cells from cell death through the action of GLP-1. Although not conclusively demonstrated in humans, GLP-1 has been shown to increase beta cell survival by reducing apoptosis in rodents (Y. Li et al., 2003; Yusta et al., 2006). Overt T2D islets show a loss of beta cell mass (Butler et al., 2003) and early sitagliptin use in combination with metformin may contribute to beta cell preservation and delay progression of the disease.

The goal of this study was to determine if starting sitagliptin earlier in the treatment of type 2 diabetes would be associated with a delay in diabetes progression and better glycemic control. The primary objective was to evaluate the need for starting insulin as a surrogate for diabetes progression. A secondary objective was to evaluate change in A1c, since it is an established marker for glycemic control. Sitagliptin was chosen because it was first in class approved for use in Canada and the most commonly dispensed DPP4 inhibitor in our database. We hypothesized that adding sitagliptin early in the treatment of type 2 diabetes would be associated with fewer insulin starts and a greater A1c reduction compared to adding sitagliptin later.

Methods

Population and setting

A population-based retrospective cohort study was conducted using administrative health databases of Alberta Health (Alberta, Canada). Under provincially funded programs, all Alberta residents receive coverage for hospitalizations, emergency department visits, and physician services. The administrative health databases used to manage these programs are linkable and have been used extensively in previous epidemiologic studies because of the high level of accuracy and completeness of data (Abdelmoneim et al., 2014; Gamble et al., 2011; B. Li et al., 2008; Majumdar et al., 2013; So et al., 2006).

In brief, the Ambulatory Care database contains facility-based ambulatory care visits, like emergency department visits; the Inpatient database records information on hospital admissions; the Pharmaceutical Information Network Dispenses database contains information on all prescription drug dispensations in the province; the Population Registry file contains demographic information; and the Practitioner Payments database captures clinician service visits. The Laboratory database from Alberta Health Services contains information on all laboratory tests. The University of Alberta Health Research Ethics Board approved the study protocol.

We used a two-step process to construct our main study cohort (Figure 1). First, we identified new metformin users according to a standard definition (Ray, 2003). Of those patients who were dispensed an oral anti-diabetic drug between April 1, 2008 and March 31, 2015, we excluded those who were never dispensed metformin. We then excluded those patients who were dispensed metformin before 2009, those who had less than 1 year between study entry and first metformin, and those who were dispensed an antidiabetic drug before their first metformin dispensation. We also restricted our study cohort to adults by excluding patients who were less than 18 years old. Secondly, we identified a sitagliptin user by excluding new metformin users who were never dispensed sitagliptin. We further restricted our study cohort to

patients with at least 6 months of follow-up to allow time for our outcomes of interest to occur. Finally, we excluded patients who initiated type 2 diabetes drug therapy with both metformin and insulin on the same day. All patients were then followed until they died, exited the province, or March 31, 2015 (Figure 2).

Exposure assessment

Our exposure of interest was defined in binary terms to identify patients who added sitagliptin early versus later in their drug therapy. We explored the time to sitagliptin start for a natural break point to dichotomize the group of sitagliptin users and found a large number of patients initiated type 2 diabetes drug therapy with both metformin and sitagliptin on the same day (Figure 3). Patients starting sitagliptin on the same day they were identified as a new metformin user were considered costarters, while patients starting sitagliptin on a later date were considered delayed sitagliptin users.

Outcome measures

The primary outcome in our study was initiation of insulin as a surrogate marker for progression of diabetes and failure of oral antidiabetic mediations to provide adequate glycemic control. The requirement of exogenous insulin indicates a reduction in beta cell function and increasing insulin resistance.

The secondary outcome was change in A1c one year after starting sitagliptin. A baseline A1c value was identified as the laboratory result closest to, but no more than 90 days from the first sitagliptin dispensation record. Similarly, a follow-up A1c value was identified as the laboratory result closest to, but no more than 90 days from the date 1 year after the first sitagliptin dispensation record. A1c is an established marker of glycemic control and analysis of change in A1c would allow us to compare the level of long-term glycemic control between patients starting sitagliptin early versus later.

Covariables

To examine the association between sitagliptin start and insulin use, we identified baseline characteristics at the time of new metformin use. In addition to age and sex, other covariables included a predefined list of chronic conditions that includes hypertension, heart failure, obesity, cancer, and hypothyroidism (Elixhauser et al., 1998), as well as prior ischemic heart disease, prior stroke, and hyperlipidemia. Hospitalization records, emergency room visits, and physician visit records prior to starting metformin were reviewed to identify baseline comorbidities using ICD-9 and ICD-10 codes (Quan et al., 2005). The use of cardiovascular medications (diuretics, beta-blockers, calcium channel blockers, ACE inhibitors, ARBs, statins) were identified from prescription drug records prior to starting metformin. To examine the change in A1c one year after starting sitagliptin, we identified baseline characteristics at the time sitagliptin was started. In addition to the covariables used to examine the association between sitagliptin start and insulin use, we included baseline A1c at sitagliptin start.

Statistical analyses

Patients were grouped into either sitagliptin costart or delayed sitagliptin use and baseline characteristics were analyzed with descriptive statistics. Categorical variables were compared by χ^2 tests, while continuous variables were compared using a t-test. A logistic regression model was used to calculate an odds ratio (OR) and 95% confidence interval (CI) to examine the association between sitagliptin user groups (costart versus delayed) and initiation of insulin. Possible confounding variables were added to a multivariable model including sex, age, baseline comorbidities, as well as concurrent statin and antihypertensive drug use at baseline.

Change in A1c was calculated by subtracting the A1c value at the start of sitagliptin from the A1c value at 1 year; therefore, a negative value would indicate A1c decreased over the year. The difference in A1c change between sitagliptin user groups (costart versus delayed) was examined using a linear regression model. A multivariable linear regression model was used to adjust for differences in A1c at the start of sitagliptin, sex, age, baseline comorbidities,

as well as concurrent statin and antihypertensive drug use at baseline. All the analyses in the study were conducted using Stata 14 (StataCorp LP, College Station, TX, USA).

Sensitivity analyses

To assess the robustness of our main results, we conducted several analyses using alternate exposure definitions and different drugs as surrogate markers for diabetes progression. First, we changed the time period used to dichotomize the group of sitagliptin users. A 1 week period was used to provide a grace period in the event sitagliptin and metformin were co-prescribed as initial therapy, but sitagliptin was not immediately available in the pharmacy. A 3 month period was used to allow for adjustments during the initial metformin dispensation, which is usually 90 to 100 days in Alberta. Second, we changed the exposure drug of interest to sulfonylureas and thiazolidinediones (TZDs). We did not consider acarbose, sodium glucose transporter 2 (SGLT2) inhibitors and GLP-1R agonists because there were too few patients using these drugs. Third, we changed the outcome variable to the antidiabetic medications, sulfonylureas and TZDs. Finally, we dropped the exclusion criterion of at least 6 months of follow-up.

Results

Baseline characteristics

Between April 1, 2008 and March 31, 2015, we identified 140,386 patients with at least one dispensation for an oral antidiabetic drug. (Figure 1) From these patients we identified 108,464 adult new metformin users. After excluding patients who did not have a dispensation for sitagliptin, had less than 6 months follow up, and started treatment for type 2 diabetes with insulin and metformin, our final study cohort was 8,746 patients. Of these, 1,149 (13.1%) started sitagliptin on the same day as metformin and 7,597 (86.9%) started sitagliptin later. The mean A1c at the time metformin was started was similar between costarters and delayed sitagliptin users. Costarters were younger and a greater percentage of them were male (Table 1). More costarters were using statins at the time metformin therapy was started. On the other hand, more delayed sitagliptin users were using beta blockers and diuretics. The composite count of Elixhauser chronic conditions such as hypertension, heart failure, or obesity was similar between the groups.

Outcome 1: insulin use

Insulin use occurred in 192 (16.7%) costarters and in 1,640 (21.6%) delayed sitagliptin users ($p<0.001$). The unadjusted odds ratio was 0.73 (0.61-0.87), indicating that costarting sitagliptin with metformin was associated with a lower chance of using insulin later in follow-up. After considering possible confounding variables, the adjusted odds ratio for costarters remained remarkably similar at 0.73 (0.62-0.87) (Table 2).

Outcome 2: change in A1c

There were 2,742 patients with A1c measurements at the start of sitagliptin use and 1 year later in our study sample. While the A1c decreased significantly in both groups (Figure 4), the magnitude of difference was greater in the costarters (-2.1%; SD 2.8%) compared to delayed sitagliptin users (-1.0%; SD 1.9%) ($p<0.001$). Since A1c values at the start of sitagliptin use differ between the groups (Figure 4), we added this variable to the multivariable linear

regression model. When all covariables were considered, the observed difference in A1c reduction was –0.54 (-0.72 to -0.36) in favour of costarters (Table 3).

Sensitivity analyses

Changing the exposure definition to 1 week and 3 months did not change the direction or statistical significance of our main results (Table 4). Substituting sulfonylureas for sitagliptin in the exposure definition reversed the outcomes for insulin use with more insulin starts associated with the costart group. Changing the outcome variable to sulfonylureas or TZDs was consistent with the insulin analysis, whereby costarters were less likely to start a sulfonylurea or TZD. Finally, the analysis with all patients starting sitagliptin, regardless of follow-up duration, was consistent with the main study observations.

Discussion

Among our group of new metformin users, we found that costarting sitagliptin with metformin is associated with fewer insulin starts and a greater degree in A1c reduction compared to delayed sitagliptin use. These observations would suggest that costarting sitagliptin with metformin delays the progression of type 2 diabetes. This study supports the theory that sitagliptin will preserve beta cell function and do so to a greater extent if it is started early in the treatment of type 2 diabetes. Much like early initiation of insulin therapy, costarting sitagliptin with metformin may change the natural history of the disease, ultimately leading to fewer medications and a lower risk of complications (Phillips et al., 2014).

The primary outcome of fewer insulin starts is supported by the greater reduction in A1c observed in the costart group compared to the delayed sitagliptin users. Although the costarters had higher A1c values when sitagliptin was started, the difference between groups was still greater after adjustment for this factor in the multivariable linear regression model. Presumably, the greater glycemic response after initiating sitagliptin contributed to the reduced need to add insulin for glycemic control. These two outcomes, a greater reduction in A1c and fewer insulin starts in the costart group, fit with hallmarks of successful diabetes therapy, improved glycemic control and slowed progression of the disease.

When the interval used to define early sitagliptin use increased, we observed progressively weaker associations with insulin start. Although all of these associations remained statistically significant, this trend suggests that earlier addition of sitagliptin to metformin therapy may provide better management of diabetes progression. The effect of early sitagliptin use compared to later sitagliptin use remained consistent when other antihyperglycemic drugs were used as surrogate markers for disease progression. Consistent with fewer insulin starts in the costart group, we also observe fewer sulfonylurea starts when the outcome for insulin use is substituted with sulfonylurea use. This result confirms that in the

costart group there appears to be less need for treatment intensification, again suggesting a level of beta cell preservation and slowing of diabetes progression.

Our sensitivity analysis revealed the opposite association with insulin use when we substitute sulfonylureas for sitagliptin in our exposure definition. In this case costarting a sulfonylurea with metformin was associated with more insulins starts, indicating a different effect on the progression of type 2 diabetes and beta cell preservation. One explanation for the opposite association is the different mechanisms of action of sitagliptin and sulfonylureas on insulin secretion. Sitagliptin increases active forms of post prandial GLP-1 that circulate and act on GLP-1 receptors on metabolic tissues to increase glucose disposal after a meal. Furthermore, GLP-1 not only increases insulin secretion from pancreatic beta cells, but it also increases insulin biosynthesis, and therefore possibly preventing beta cell exhaustion in the face of increasing glucose demands (Drucker, 2018). Sulfonylureas bind to beta cell KATP channels to depolarize and increase insulin secretion from beta cells, but this does not include a concomitant increase in insulin biosynthesis. Beta cells are then faced with increasing insulin secretion demands that may finally lead to beta cell exhaustion and apoptosis (Alvarsson et al., 2003; Del Guerra et al., 2005).

An important strength of this study is that it compares using sitagliptin when drug therapy is initiated early in diabetes, presumably soon after diagnosis, with adding sitagliptin later after metformin has been started. Studies have shown that sitagliptin is an effective antidiabetic agent with or without metformin (Aschner et al., 2006; Charbonnel et al., 2006). However, our study is novel in that it compares initial combination therapy with later combination therapy in terms of diabetes progression and glycemic control. This observational study also provides evidence for the hypothesis that early intervention with a second antidiabetic agent may slow the progression of the disease. Sitagliptin, other DPP4 inhibitors, and GLP-1 receptor agonists would have the advantage of sustained GLP-1 receptor agonism that, if started early, may modulate the progression of diabetes. Another strength is the magnitude of the difference in A1c

reduction and the stability of the odds ratio when adjusted for potential confounding variables. The adjusted estimate of the difference in A1c in the costart group is greater than the clinically important difference (>0.3%) (Maruthur et al., 2016), indicating a meaningful improvement in glycemic control.

As with other observational studies, there are important limitations inherent to the design and data collection. First, as our costart group is younger and includes more men, there is the possibility of selection bias in our study. For example, costarters may have a different ability to pay for a DPP4 inhibitor and consequently may choose to initiate sitagliptin therapy with metformin. Although we were able to adjust for demographic, clinical, and drug therapy characteristics information on socioeconomic status and other lifestyle choices were not available. Second, we used administrative records and assume that dispensations are a reasonable indicator of medication use. In doing so, we may have overestimated exposure; however, any misclassification would be applied in the same manner to both groups. Third, we cannot exclude the possibility of residual confounding because we were unable to control for some important factors that influence the decision to use DPP4 inhibitors, like patient weight and risk of hypoglycemia. However, we were able to adjust our analysis with cardiometabolic diagnostic codes and medications. Fourth, as our follow-up period is relatively short, we are unable to assess the risk of clinical outcomes, such as onset of microvascular or macrovascular complications. Finally, we did not account for the dose of metformin or sitagliptin in our study and therefore cannot exclude the possibility that a dose-response relationship exists with our outcomes.

We conclude that costarting sitagliptin with metformin is associated with a lower likelihood of disease progression in type 2 diabetics compared with adding sitagliptin to metformin later in therapy. Using sitagliptin, our results provide evidence that initiating metformin therapy with a DPP4 inhibitor may result in better glycemic control and delay the need for exogenous insulin

Acknowledgements

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Figure 5.1. Patient flow diagram

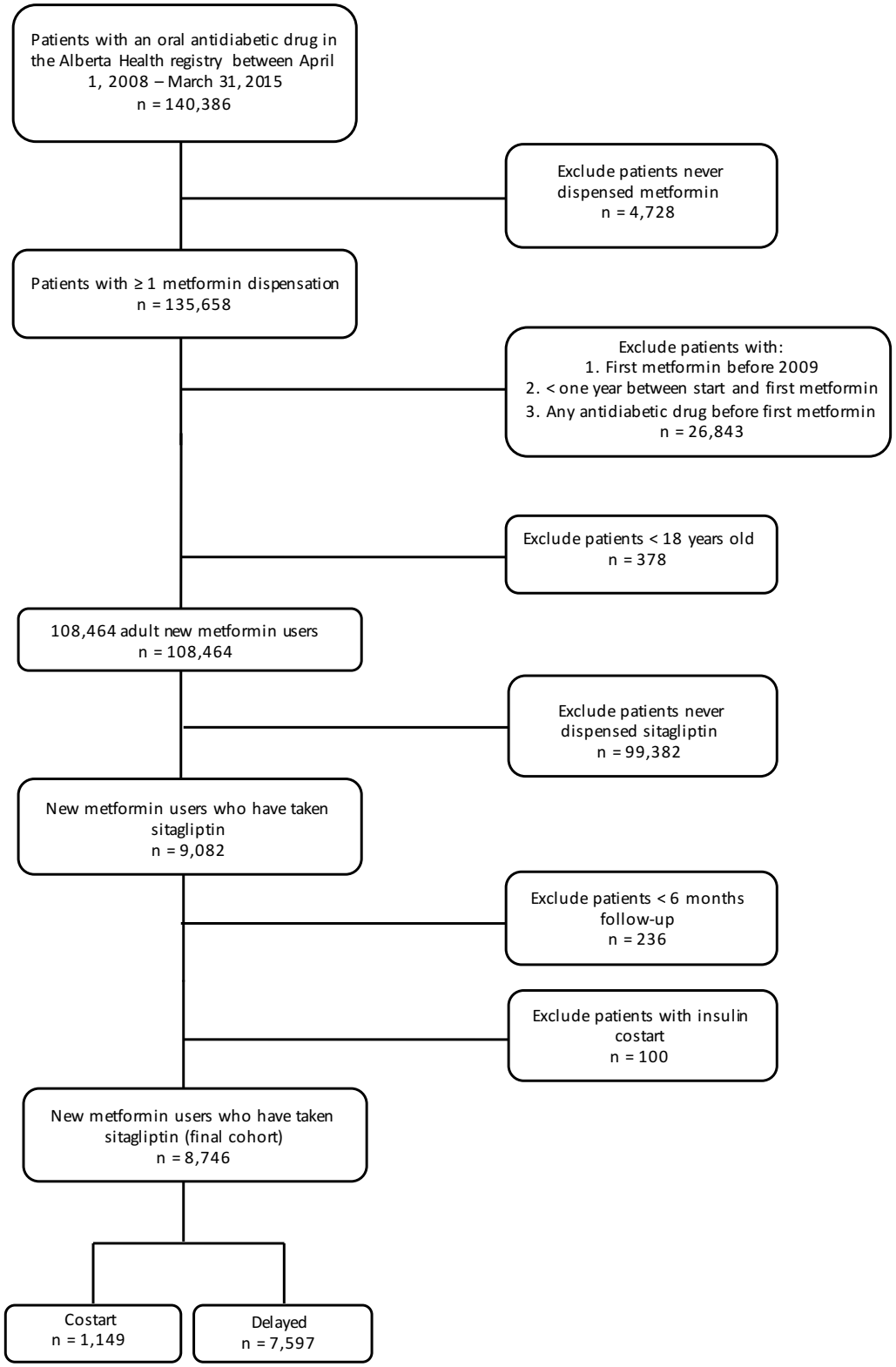


Figure 5.2. Study schematic

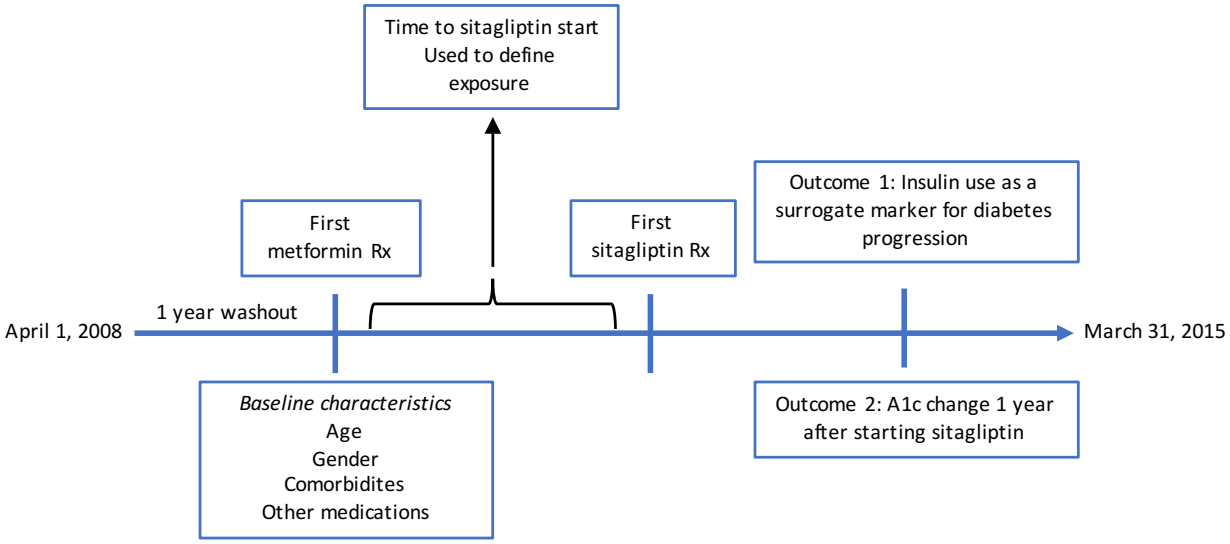


Figure 5.3. Histogram of time to sitagliptin start

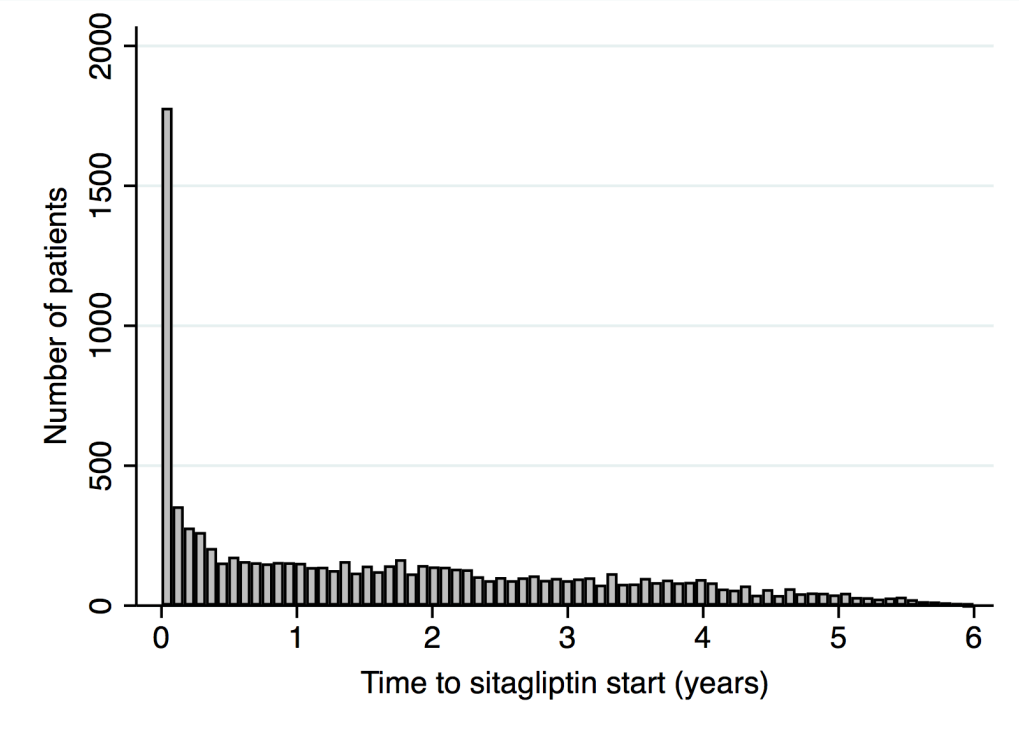


Figure 5.4. A1c measurements at start and 1 year of sitagliptin in new metformin users

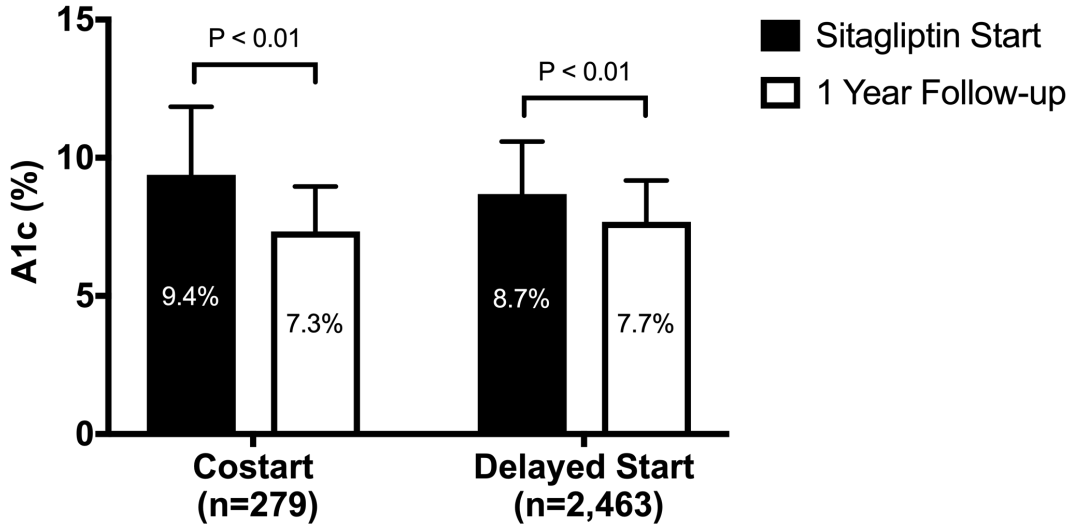


Table 5.1. Baseline characteristics

	Costart n = 1,149 n (%)	Delayed Start n = 7,597 n (%)	Standardized Difference
Age, mean (SD), years	51.2 (10.8)	52.2 (11.1)	0.09
Men	840 (73.1)	4,815 (63.4)	0.21
A1c at metformin initiation, mean (SD), %	9.4 (2.4)	9.3 (2.4)	0.04
Elixhauser chronic conditions, mean (SD)	3.1 (2.0)	3.2 (1.9)	0.05
Hypertension	590 (51.4)	3,903 (51.4)	<0.01
Heart failure	30 (2.6)	241 (3.2)	0.04
Obesity	259 (22.5)	1,566 (20.6)	0.05
Cancer	52 (4.5)	337 (4.4)	<0.01
Hypothyroidism	63 (5.5)	527 (6.9)	0.06
Other Comorbid conditions			
Prior ischemic heart disease	126 (11.0)	949 (12.5)	0.05
Prior stroke	9 (0.8)	129 (1.7)	0.08
Hyperlipidemia	350 (30.5)	2,304 (30.3)	<0.01
Medications			
Diuretics	139 (12.1)	1,224 (16.1)	0.12
Beta blockers	122 (10.6)	1,028 (13.5)	0.09
Calcium channel blockers	145 (12.6)	1,023 (13.5)	0.03
ACE inhibitors and ARBs	551 (48.0)	3,568 (47.0)	0.02
Other hypertensive medications	9 (0.8)	80 (1.1)	0.03
Statins	521 (45.3)	3,119 (41.1)	0.09

Table 5.2. Logistic regression analysis of insulin use*

	OR (95% CI)	P-value
Costart	0.73 (0.62-0.87)	<0.01
Age	0.98 (0.98-0.99)	<0.01
Sex	0.81 (0.72-0.90)	<0.01
Elixhauser chronic conditions	1.02 (0.99-1.05)	0.17
Other Comorbid conditions		
Prior ischemic heart disease	1.14 (0.95-1.37)	0.15
Prior stroke	1.07 (0.70-1.61)	0.78
Hyperlipidemia	0.85 (0.75-0.96)	0.01
Medications		
Diuretics	0.92 (0.78-1.08)	0.32
Beta blockers	1.06 (0.87-1.27)	0.53
Calcium channel blockers	1.14 (0.97-1.35)	0.10
ACE inhibitors and ARBs	0.93 (0.82-1.04)	0.22
Other hypertensive medications	0.87 (0.51-1.48)	0.61
Statins	1.00 (0.88-1.12)	0.97

*All Odds Ratios and 95% Confidence Intervals are adjusted for the other variables in the table

Table 5.3. Difference in A1c reduction – adjusted coefficients from multivariate logistical regression models*

	Coef (95% CI)	P-value
Costart	-0.54 (-0.72 to -0.36)	<0.01
Baseline A1c	-0.75 (-0.78 to -0.72)	<0.01
Age	-0.01 (-0.02 to -0.01)	<0.01
Sex	-0.07 (-0.18 to 0.05)	0.26
Elixhauser codes	-0.01 (-0.04 to 0.02)	0.48
Other Comorbid conditions		
Prior ischemic heart disease	0.09 (-0.07 to 0.24)	0.29
Prior stroke	0.03 (-0.31 to 0.38)	0.85
Hyperlipidemia	-0.13 (-0.24 to -0.01)	0.03
Medications		
Diuretics	-0.15 (-0.29 to -0.01)	0.04
Beta blockers	-0.08 (-0.24 to 0.08)	0.35
Calcium channel blockers	0.16 (0.02 to 0.30)	0.03
ACE inhibitors and ARBs	0.16 (0.03 to 0.28)	0.01
Other hypertensive medications	0.23 (-0.27 to 0.74)	0.37
Statins	0.18 (0.06 to 0.31)	<0.01

*All Coefficients and 95% Confidence Intervals are adjusted for the other variables in the table

Table 5.4. Sensitivity analyses

Model	OR (95% CI)
Main Study (costart sitagliptin)	0.73 (0.62-0.87)
Changes to Exposure Definition:	
Start sitagliptin within 1 week of first metformin	0.79 (0.68-0.93)
Start sitagliptin within 3 months of first metformin	0.84 (0.74-0.95)
Costart sulfonylurea	1.54 (1.43-1.66)
Costart thiazolidinedione	0.94 (0.80-1.10)
Changes to Outcome (Exposure is Costart Sitagliptin)	
Sulfonylurea Start	0.43 (0.37-0.50)
Thiazolidinedione Start	0.34 (0.23-0.51)

Chapter 6

General Discussion

Discussion

What is the mechanism for GLP-1 expression in human islet α cells?

In this thesis, I have shown that human islets have a GLP-1 expressing subpopulation of α cells that secretes significant amounts of active GLP-1. However, the mechanism for GLP-1 expression in α cells is unknown. In α cells that express GLP-1, I observe increased PC1/3 expression and fulfill the basic mechanistic requirements for GLP-1 cleavage from proglucagon. Furthermore, I only observe GLP-1 expression in glucagon positive cells indicating that proglucagon is being expressed, another obvious but essential requirement for GLP-1 expression. I also observe that the GLP-1 expressing subpopulation is increased in T2D islets, suggesting that the mechanism for GLP-1 expressing is linked to the pathophysiology of T2D. Peripheral signals or signals from within the islets that are associated with T2D islets may drive α cell mechanisms to increase GLP-1 expression.

One of the hallmarks of T2D is hyperglycemia, and a number of research groups have investigated the effects of high glucose on PC1/3 and GLP-1 expression in the pancreas. Both β and α cells sense glucose to secrete glucoregulatory hormones, so it seems plausible that glucose may directly or indirectly regulate GLP-1 expression in the islets. Nie et al. reported increased PC1/3 expression in pancreatic extracts and increased PC1/3 positivity with islet sections in their STZ rat model of diabetes (Nie et al., 2000). The hyperglycemia in their model was associated with increased active GLP-1 in pancreatic extracts and plasma. Hyperglycemia can be nutritionally induced in the gerbil, *Psammomys obesus*. Using this model of diabetes, Hansen et al. report increased active GLP-1 levels in the pancreas and plasma of hyperglycemic animals (Hansen et al., 2011). They also observe increased active GLP-1 secretion when the islets are cultured in high glucose (25 mM) conditions. Finally, using the α cell line (αTC1-6), Whalley et al observed increased PC1/3 gene expression after 3 days of culture at 11 mM glucose (Whalley et al., 2011).

Although hyperglycemia appears to increase PC1/3 and GLP-1 expression in rodent islets or cell lines, it is not known if hyperglycemia increases PC1/3 and GLP-1 expression in human islets. Using bulk α cells, and not specifically analyzing GLP-1+ α cell subpopulations, Marchetti et al. show that T2D islets have increased PC1/3 gene expression compared with ND islets (Marchetti et al., 2012). To test the hypothesis that hyperglycemia increases PC1/3 expression in human islet α cells, I cultured ND human islets at 11.1 mM and 2.8 mM glucose for 48 hours, dissociated the islets into single cells, and analyzed them using flow cytometry. Glucagon+ cell populations were analyzed for amidated GLP-1 and PC1/3 expression. Surprisingly, and in contrast to results in rodent islets, there is no increase in amidated GLP-1 or PC1/3 expression after culture at high glucose compared with culture at low glucose (Figure 6.1). It is possible that long-term culture, beyond 48 hours, is required to see an increase in PC1/3 and GLP-1 expression. However, our results suggest that PC1/3 and GLP-1 expression in human islets does not rapidly respond to hyperglycemia.

I have observed that increased GLP-1 expression may be correlated with increased glucagon expression in human islets. Taking flow cytometry data from one islet donor, I observe in GLP-1+ cells that both PC1/3 and glucagon expression is increased (Figure 6.2A). However, when the data is analyzed in terms of PC1/3 expression, glucagon expression is not different between PC1/3+ and PC1/3- α cells (Figure 6.2B), suggesting no association between glucagon and PC1/3 expression. Insulin has been shown to inhibit proglucagon transcription in α cells (Jin, 2008). As β cell loss is often observed in T2D islets, and insulin inhibits glucagon secretion, it is possible that reduced insulin secretion from T2D β cells could be responsible for increased proglucagon transcription in α cells and ultimately GLP-1 expression. This would fit with the observation that T2D islets secrete more glucagon than ND islets. Furthermore, Kawamori et al. demonstrate with their αIRKO mouse model that insulin signaling regulates proglucagon gene expression at low and high glucose (Kawamori et al., 2009). This idea could be tested with

human islets in culture using S961, a peptide insulin receptor antagonist (Jiao et al., 2014). Human islets, or sorted and purified α cells, could be cultured with S961 and then analyzed with flow cytometry for GLP-1 expression. Certainly, more research into the effect of insulin signaling on proglucagon gene expression in the α cell would be helpful to validate this idea.

Figure 6.1. High glucose alone does not increase GLP-1 expression in human islets. **A.** Representative flow cytometry contour plots of dispersed islet cells. Islets were either cultured with low glucose (2.8 mM) or high glucose (11.1 mM) for 48 hours. Left, insulin and glucagon expression were analyzed in total dispersed islets, allowing the identification of insulin+, and glucagon+ cell populations. Right, the insulin+, and glucagon+ cell populations were further analyzed for side scatter (SSC) and expression of amidated GLP-1. Amidated GLP-1+ cells were gated within each cell population. **B.** Percentages of amidated GLP-1+ cells in the GLP-1+/glucagon+ cell populations at low and high glucose. N=3 donors. **C.** Representative flow cytometry histograms showing PC1/3 expression levels within low glucose/glucagon+, high glucose/glucagon+, and insulin+ cells.

Figure 6.1

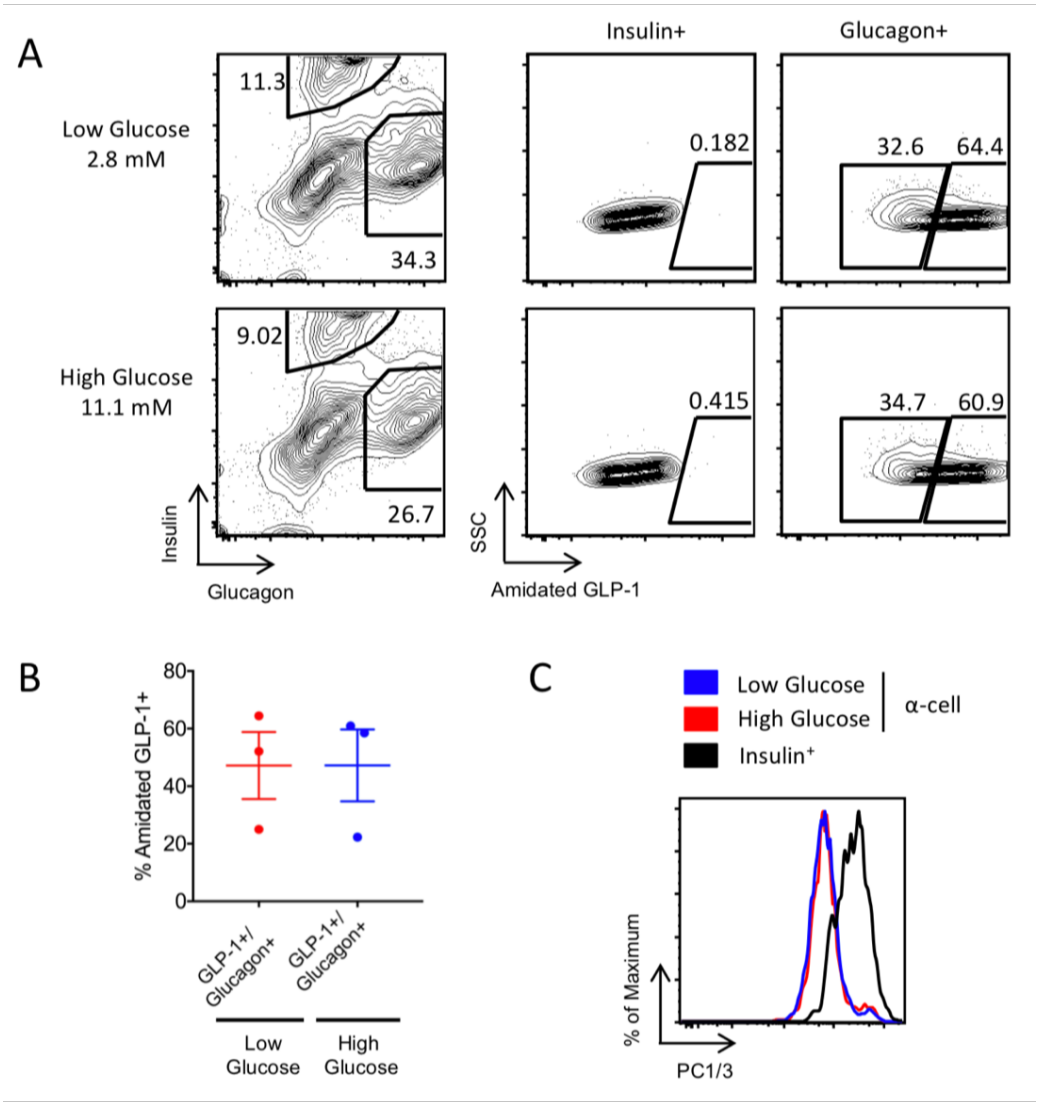
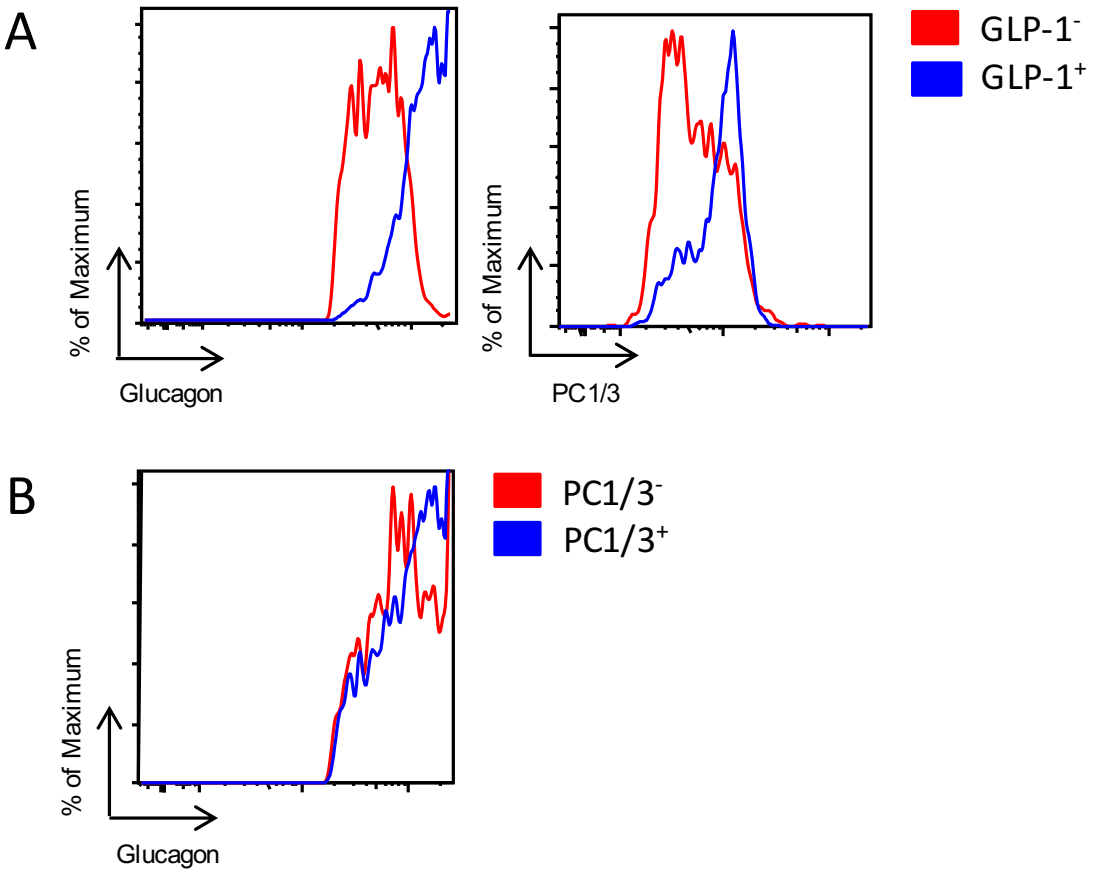

A
Insulin+
Glucagon+
Low Glucose
2.8 mM
11.3
34.3
0.182
32.6
64.4
High Glucose
11.1 mM
9.02
26.7
0.415
34.7
60.9
Insulin
Glucagon
SSC
Amidated GLP-1
B
% Amidated GLP-1+
80
60
40
20
0
GLP-1+/ Glucagon+
GLP-1+/ Glucagon+
Low Glucose
High Glucose
C
Low Glucose
High Glucose
Insulin+
α-cell
% of Maximum
PC1/3

Figure 6.2. Glucagon and PC1/3 expression is increased in GLP-1 expressing α cells from one human islet donor. **A.** Flow cytometry histograms showing glucagon and PC1/3 expression levels within GLP-1- and GLP-1+ cells. B. Flow cytometry histograms showing glucagon expression levels within PC1/3- and PC1/3+ cells.

Figure 6.2.


A
% of Maximum
Glucagon
% of Maximum
PC1/3
GLP-1⁻
GLP-1⁺
B
% of Maximum
Glucagon
PC1/3⁻
PC1/3⁺

What role does intra-islet GLP-1 have in the development of T2D?

An examination of the role of GLP-1 in the development of T2D would provide a better understanding of the pathophysiology of the α cell during the disease process. I characterized GLP-1 expression in ND and T2D human islets and to ensure overt diabetes in the T2D islet sections, I chose sections from donors with an average HbA1c of 7.8%. Control islet sections were from nondiabetic donors with an average HbA1c of 5.5%. However, it would be helpful to know what happens to GLP-1 expression between these two points, between health and overt diabetes. Various questions regarding the role of α cell GLP-1 expression could then be asked. Does GLP-1 expression in α cells have a role to play in prediabetes, or in β cell compensation and decompensation? Does GLP-1 expression associate with BMI or cardiovascular risk? Our data suggests that GLP-1 does have a role to play in the prediabetic or diabetic islet as it adapts, or attempts to adapt, to maintain insulin secretion. In T2D islets, I observe an increase in the GLP-1 expressing α cell subpopulation that is associated with a greater dependency on GLP-1R signaling for insulin secretion. This suggests that α cell secretion of proglucagon-derived peptides has adapted to the needs of β cell signaling for secretion of insulin.

α cell GLP-1 expression has been tracked in vivo using the db/db mouse, a mouse model of type 2 diabetes (O'Malley et al., 2014). Here GLP-1+ staining increased in pancreatic sections taken progressively at 6, 8, 10, and 12 weeks. This progressive increase in GLP-1 expression paralleled the increase blood glucose. PC1/3 expression was observed in pancreatic sections in the db/db mouse, but not in normal control mice. The results from this experiment suggest that GLP-1 progressively increases with development of T2D.

Studies into the role of the proinflammatory cytokine IL-6 in glucose homeostasis connect the development of T2D with GLP-1 secretion from α cells. Elevated levels of the proinflammatory cytokine IL-6 are associated with obesity and an increased risk of developing T2D (Wang et al., 2013). Also, the pancreatic α cell is a target for IL-6 action and IL-6 regulates

α cell mass expansion (Ellingsgaard et al., 2008). Furthermore, IL-6 is an adipokine that is thought to contribute to the induction of insulin resistance in metabolic tissues (Lazar, 2005). Ellingsgaard et al. show that GLP-1 release from human islets and FACS enriched human α cells increases with IL-6 incubation (Ellingsgaard et al., 2011). These experiments suggest that GLP-1 secretion from pancreatic α cells and enteroendocrine L cells is the mechanism whereby insulin secretion is potentiated to match insulin demand. Our GLP-1 secretion data from human islets does not suggest that BMI correlates with GLP-1 secretion. However, when isolated islets from a broader range of BMIs are examined, GLP-1 secretion has been shown to positively correlate with BMI (Linnemann et al., 2015).

DPP4 inhibitors do not increase GSIS in isolated human islets.

Using isolated human islets, I have investigated the physiological mechanism at the islet level for the effectiveness of DPP4 inhibitors in T2D. Our results show, using the DPP4 inhibitor sitagliptin, that in the context of GSIS, DPP4 inhibitors do not significantly increase active GLP-1 and do not increase insulin secretion. This result was surprising considering the 7-fold increase in active GLP-1 levels found in human islet cultures. However, in the context of a static incubation or islet perifusion, active GLP-1 levels could not be increased enough to further potentiate insulin secretion. There also remains the possibility that in some islets the GLP-1 receptors are maximally stimulated and consequently will not respond to further stimulation.

A detailed physiological mechanism for the increase of insulin secretion by DPP4 inhibitors remains elusive (Andersen et al., 2017). It has been proposed that DPP4 tissue beds may work in a compartmental manner to regulate the local availability of DPP4 substrates. DPP4 inhibitors would increase active forms of the substrates to alter tissue biology. The possibility of this in human islets was intriguing as GLP-1 and DPP4 are present in the islet. Furthermore, doubts remain whether the small increases (2 to 4 fold) in circulating active GLP-1

(Andersen et al., 2017) are able to increase activation of the β cell GLP-1 receptor. However, our negative results point away from direct action on the islets by increasing local active GLP-1. Instead, our data point to an alternative DPP4 tissue bed for the primary mechanism for the action of DPP4 inhibitors. As detailed in the introduction of this thesis, evidence has accumulated that portal sensing in the gut is a likely target for increased GLP-1 and GIP levels resulting from DPP4 inhibition (Mulvihill et al., 2017; Waget et al., 2011).

Although I show that sitagliptin treatment of human islets does not increase GSIS, I do show that sitagliptin treatment of long-term cultures (48 hours) correlates with increased cell survival. Our data raises the possibility that DPP4 inhibitors may influence β cell preservation. As shown in chapter 5, I explore this using an observational study of a cohort of T2D patients taking metformin and sitagliptin. Here fewer insulin starts and a greater A1c reduction are associated with costarting sitagliptin with metformin. Our results suggest that inhibition of DPP4 in the islets, along with increased circulating GLP-1 and GIP, early in the pathogenesis of T2D will improve therapy outcomes.

Glucose regulation of GLP-1 secretion

Marchetti et al. describe a GLP-1 system in human islets that appears to include alternative glucose regulation for the secretion of GLP-1 and glucagon (Marchetti et al., 2012). Although both hormones are released from α cells, their data shows increased GLP-1 levels at high glucose, while glucagon secretion is decreased at high glucose, as expected. The action of GLP-1 secretion from the gut is to potentiate insulin secretion at high glucose. Therefore, the observation that GLP-1 secretion is increased from islets at high glucose, fits with the action of GLP-1 as an incretin. However, their data does not fit with the observation that glucagon and GLP-1 are present in the same secretory granules in mice (Kilimnik et al., 2010). Their results

suggest either alternate coupling for GLP-1 and glucagon secretion in regard to glucose sensing or a mixed population of α cells that respond differently to glucose.

Our data is not consistent with Marchetti et al., and suggests a different mode of glucose regulation for GLP-1 secretion. I do not observe an increase in GLP-1 secretion at high glucose, but rather no change in secretion from low glucose levels. Furthermore, GLP-1 secretion at low and high glucose in our experiments mirrors that of glucagon. Interestingly, when insulin non-responders are analyzed for GLP-1 secretion there is a significant decrease in GLP-1 secretion at high glucose that is not seen with insulin responders. Unfortunately, I did not have enough T2D islets to confirm that GLP-1 secretion is higher than in ND islets, due to lack of tissue availability. However, the trend in our low sample size (N=2) suggests that GLP-1 secretion may be higher in T2D islets. Finally, our experiments used a glucose suppression test (low to high glucose) that would also capture glucose stimulation of insulin. However, a true glucose stimulation test for α cells (high to low glucose) would be useful for understanding glucose regulation of glucagon and GLP-1 secretion.

Future directions

GLP-1 expression and secretion in type 1 diabetic human islets

The expression of GLP-1 in the α cells of T1D islets has immense potential for the management and treatment of type 1 diabetes. However, it is not known if the α cells of type 1 diabetic human islets express and secrete GLP-1. α cell GLP-1 expression and secretion could benefit T1D patients with detectable C-peptide levels or in the investigation of α cell to β cell conversion therapy. Clearly, the concept of an insulinotropic and potentially prosurvival peptide that is secreted locally to residual β cell mass is appealing.

GLP-1 expression has been identified in mouse models of type 1 diabetes supporting the idea that GLP-1 expression may be present in the α cells of T1D islets. Plesner et al. compared

islet remodeling in the NOD mouse with the STZ model and found GLP-1 expression in the α cells of both models (Plesner et al., 2014). Furthermore, α cell GLP-1 expression was associated with increased GLP-1 plasma levels suggesting increased pancreatic GLP-1 secretion. However, in terms of α cell mass the models differed. α cell mass increased in the STZ model, but not the NOD model, showing a different response under conditions of immune infiltration versus chemical β cell destruction. O'Malley et al. compared GLP-1 expression in the islets of NOD mouse with the db/db mouse over the development of diabetes (O'Malley et al., 2014). They found less GLP-1 expression in the NOD mouse compared with the db/db mouse, suggesting that GLP-1 expression would be less in T1D islets.

Increasing islet GLP-1 levels in T1D has the potential for β cell preservation in those patients with detectable C-peptide. If PC1/3 expression could be increased in α cells, GLP-1 secretion could potentially act on the residual β cells to increase survival. However, clinical studies using the GLP-1R agonist exenatide in patients with long-standing T1D have been disappointing with no improvement in β cell function (Drucker, 2018). To be effectual, increased GLP-1 secretion may have to happen at the time of diagnosis.

Plasticity between α and β cells has been reported and there is interest in α to β cell reprogramming as a potential treatment for T1D (Gromada et al., 2018). In T1D, the α cell mass is preserved and a potential source of new β cells. GLP-1 expression is potentially a part of this process and may be an important secreted factor to speed up maturation and increase the function of new β cells (Gromada et al., 2018). Habener et al. have proposed a model where pro-α cells express PC1/3, the GLP-1R, and secrete GLP-1 in an autocrine manner to make the pro-α cells competent to become β cells (Habener and Stanojevic, 2012). Using lineage tracing and a recombinant AdGLP-1 virus to induce very high circulating GLP-1 levels, Lee et al show increased α to β cell transdifferentiation in their STZ treated mouse model over controls

suggesting that GLP-1 promotes α to β cell transdifferentiation (Lee et al., 2018). Whether GLP-1 levels in the T1D human islet are high enough for similar effects remain to be investigated.

α cell subpopulations and GLP-1 expression

Research into α cell heterogeneity is still in its infancy, making the task of characterizing α cell subpopulations challenging. This contrasts with the amount of research generated over the last few years regarding β cell heterogeneity (Bader et al., 2016). However, interest in the regulation of glucagon secretion and the potential for α cells to be a source of new β cells has generated interest in α cell heterogeneity (Gromada et al., 2018). As with β cells, identification of proliferative or phenotypically plastic cells would help to understand the role of the α cell in islet biology and the ability to adapt to disease (Gromada et al., 2018; Lam et al., 2018). Gromada et al. have proposed investigating "hub-like" α cells, in a similar manner to β cells, to better understand cellular connectivity for glucagon secretion (Gromada et al., 2018). Single-cell analysis of α cells will help to understand the heterogeneity present in the endocrine pancreas (Tritschler et al., 2017).

The concept of β cell heterogeneity was first introduced by Pipeleers where he argued that although β cells are morphologically similar under the microscope, they are often different in their response to glucose (Pipeleers, 1992). Since this report, β cell heterogeneity has been researched with a number of subpopulations being identified. Markers have been discovered and used to separate β cells into two for more populations, thus identifying β cells in terms of high/low or positive/negative expression of the marker. A number of the subpopulations may be classified as low or high insulin expression and defined in terms of β cell maturity (Benninger and Hodson, 2018). High-throughput single-cell technologies, using mass-spectrometry or RNA sequencing to measure protein abundance or gene expression, have also been useful to identify new β cell subpopulations. However, markers used to identify subpopulations do not

necessarily ascribe functional relevance (Benninger and Hodson, 2018). Furthermore, given that dissociated cells are used for many of the analyses, identification of functional subpopulations within intact islets is of importance.

Johnston et al. investigated β cell heterogeneity in the intact islet by focusing on the coordination of electrical activity and calcium responses among the β cells. They discovered that a subpopulation (5-10%) of β cells act as hub cells to coordinate calcium responses and insulin secretion. Transcriptional analysis of these hub cells reveals that they are immature, but highly metabolically active (Johnston et al., 2016). The authors argue that impairment of these hub cells may lead to reduced insulin secretion and loss of glucose homeostasis. My study also focuses on intact islets and I have identified a GLP-1 expressing α subpopulation in situ. Applying the techniques of Johnston et al. to α cells in the intact islet may reveal functional differences in glucagon secretion that overlap with GLP-1 expression. Furthermore, direct evidence for functional relevance of the GLP-1 expressing α cell subpopulation is important and may benefit from further single-cell analysis of markers unique to α cell function.

Potentially our GLP-1 expressing subpopulation could be mapped with newly identified α cell populations. This would, in turn, provide insights into the mechanism for GLP-1 expression in α cells. Proglucagon gene expression would be a good marker to start with as I have observed increase proglucagon in GLP-1+ cells. PC1/3 gene expression may be helpful too, although the expression would be quite low. The transcription factors CDX2/3 and PAX6 have been associated with the regulation of proglucagon gene expression and may define an α cell subpopulation (Jin et al., 1997; Trinh et al., 2003). As cAMP has been shown to increase proglucagon gene expression, effectors of this second messenger such as Epac, may be a good marker as well (Jin, 2008).

I provide evidence that the GLP-1 expressing α cell subpopulation is dynamic and increases in T2D. This implies that potential markers for the subpopulation are regulated by

factors and signals associated with diabetes. Potentially information from β cell heterogeneity could help identify α cell subpopulations, particularly from β cells that reside next to α cells in the islets. This in situ information from a neighboring cell may provide understanding into why some α cells express GLP-1 and others do not. Paracrine factors from β cells, under autoimmune attack or functional stress, may regulate α cell subpopulations and GLP-1 expression.

A prospective study of costart and delayed start sitagliptin users

A prospective study of costart and delayed start sitagliptin users where variables could be controlled, instead of adjusted, would address many of the limitations described in our observational study. First, I would minimize selection bias by matching patients by age, sex, A1c at metformin start, and BMI. If reasonable, I could also match patients by postal code and attempt to control for socioeconomic factors. This would address the concern in our observational study that costarts are younger and male, and more able to pay to start sitagliptin with metformin. Second, with regular medication visits throughout the study, I would be able to more accurately assess if the patients were taking their medications or not. In doing so, I would not overestimate exposure of metformin or sitagliptin use. Third, residual confounding would be less of a concern because more variables would be controlled through patient matching. Matching for BMI would allow me to control for patient weight, a variable that I was unable to adjust for in the observational study as I did not have the information. Matching or exclusion criteria set to heart disease, blood pressure, or renal disease would also help to more accurately control for confounding cardiometabolic conditions. Fourth, the length of a prospective study could be longer than the 7 years of my observational study, thus allowing for the assessment of more clinical outcomes. A longer study may include the onset of microvascular and macrovascular complications as outcomes. The onset of these complications could then be

used to assess the possible benefits of costarting sitagliptin with metformin. This would potentially support results from insulin use or A1c that relate to the reduction of disease progression in diabetes. Finally, we would be able to account or control for the dose of medication and determine if there is a dose-response relationship with our outcomes.

The sensitivity analysis performed in my observational study revealed that substituting sulfonylureas for sitagliptin in our exposure definition reversed the risk profile for costarting with metformin. In other words, costarting a sulfonylurea with metformin increased the risk of starting insulin. In a prospective study, a sulfonylurea could be used as a comparator to sitagliptin for treatment intensification. In the delayed start arm of the study, treatment intensification with either sitagliptin or a sulfonylurea could be set to a prescribed A1c target, thus matching glucose control for either medication. With this design A1c values could be regularly measured and potentially the time of divergence in glucose control could be tracked. This valuable clinical trial data would presumably support the results of my observational study.

Conclusions

In this thesis, I provide evidence that human islets have a GLP-1 expressing α cell subpopulation that increases in T2D. Active GLP-1 secretion is around 50 times higher from human islets compared with mouse islets, indicating that GLP-1 secretion may be of more importance in the human islets than previously thought. I also show that GLP-1R signaling in human islets contributes to insulin secretion at high glucose and that the increase in α cell GLP-1 expression is associated with a greater dependency on GLP-1R signaling for GSIS. From these results, I conclude that a GLP-1 signaling axis can be considered in human islets and that the human islet is more dependent on this signaling in T2D.

I show that GLP-1 levels can be significantly increased in human islets with the DPP4 inhibitor, sitagliptin. However, in the context of GSIS, active GLP-1 levels are not significantly increased and insulin secretion is not increased with sitagliptin treatment. I conclude that DPP4 inhibition at the level of the islet is not part of the pharmacological mechanism for increased insulin secretion with DPP4 inhibitors in humans. However, the increase in active GLP-1 levels in long-term culturing of human islets with sitagliptin does correlate with cell survival and does suggest an effect on β cell preservation.

I show that NPIs secrete GLP-1 in culture and they can be engineered to increase GLP-1 secretion with adenoviral vectors. However, the adenovirally mediated GLP-1 increase did not result in improved insulin secretion in culture. I conclude that NPIs can be engineered to increase GLP-1 secretion with adenoviral vectors, but functional improvements may only be seen after transplantation.

Finally, I show in an observational study of new metformin users that early sitagliptin treatment is associated with better outcomes: fewer insulin starts and greater reductions in A1c. I used an insulin start as a surrogate for worsening beta cell function. I conclude that treatment

intensification with sitagliptin has the potential to slow progression of type 2 diabetes and preserve β cell function.

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