Role of G protein-coupled receptor-associated sorting protein 1 (GASP1) in post-endocytic trafficking of Glucagon-like peptide 1 (GLP-1) receptor and its effect on receptor function

By

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DAVIS

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For my …

Parents (Papa and mummy), for bringing me where I am today.

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Table of Contents

Abstract

The increasing prevalence of type 2 diabetes (T2D) and obesity poses substantial concerns and economic challenges to the healthcare system. T2D is characterized by disruption of glucose homeostasis. Insulin is the primary driver of glucose homeostasis and released by pancreatic β-cells in response to nutritional and endocrine signals. Glucose stimulated insulin secretion is augmented by the actions of incretin hormones including Glucagon-Like peptide 1 (GLP-1), which activates the GLP-1 receptor (GLP-1R) in islets producing the “incretin effect”. Because of their insulinotropic properties, incretin drugs, such as GLP-1 receptor agonists are used as therapeutic agents to maintain glucose homeostasis in T2D. However, concerns regarding the long-term effects of these drugs and development of tolerance to GLP-1R agonist persist. In this study, we investigate the role of G protein-coupled receptor-associated sorting protein 1 (GASP1), a critical regulator involved in post-endocytic trafficking of GLP-1R, on development of tolerance to GLP-1R agonists. By combining CRISPR-Cas9 technique, Homogenous Time Resolved Fluorescence (HTRF) biochemical assay, transgenic mice and *in vivo* and *ex vivo* animal studies, we found the following:

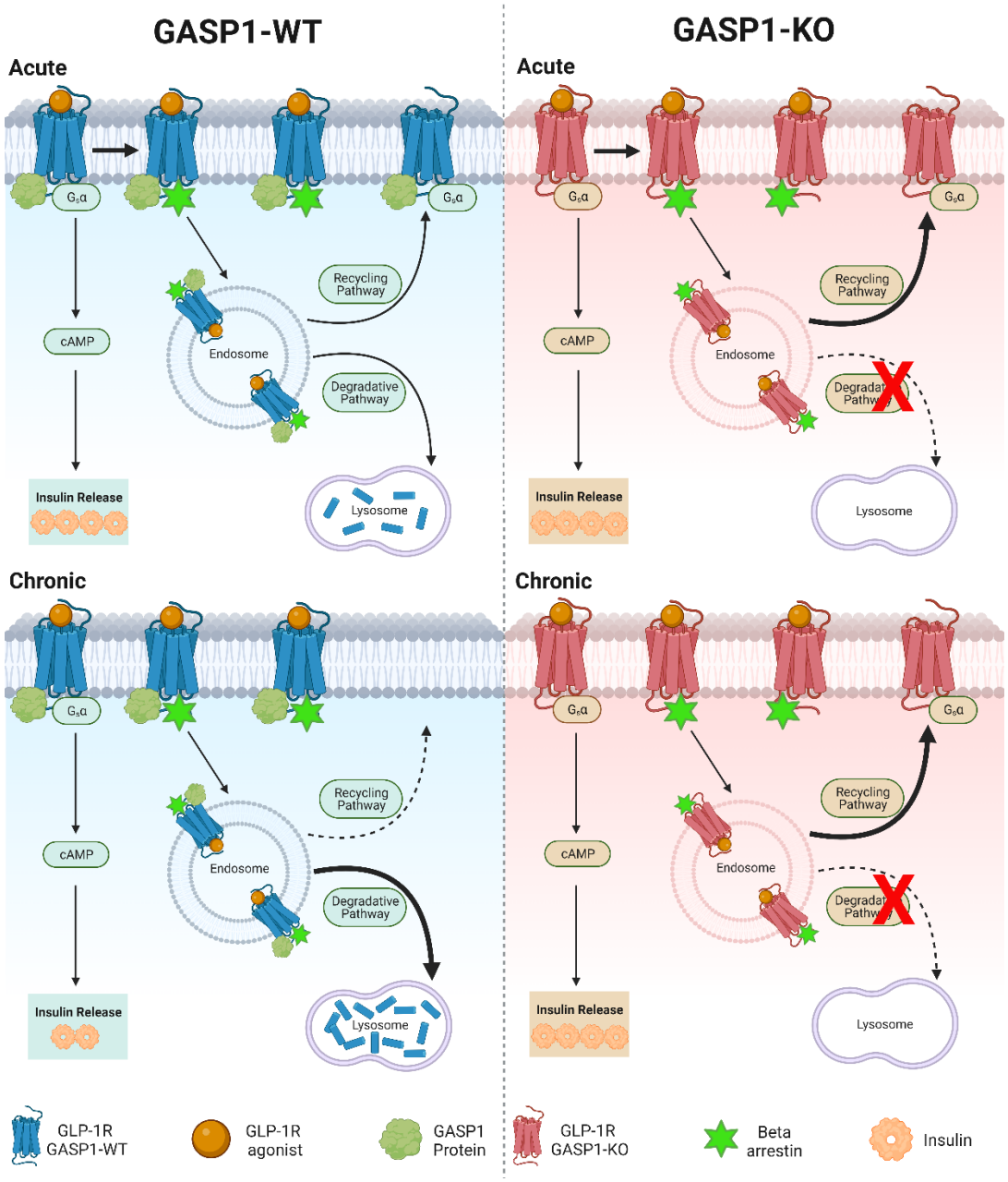
- GASP1 is expressed in both human embryonic kidney (HEK 293) and rat insulinoma derived insulin producing INS-1 cells.
- Following acute treatment with the GLP-1R agonist Exendin-4 (Ex-4), both HEK 293 and INS-1 cells demonstrate a dose-dependent increase in intracellular cyclic adenosine monophosphate (cAMP) levels.
- Prolonged Ex-4 pretreatment of HEK 293 and INS-1 cells with Ex-4 results in loss of responsiveness of the receptor (i.e “Tolerance”).
- CRISPR-Cas9-mediated removal of GASP1 in both HEK 293 and INS-1 cells did not impact acute GLP-1R signaling. However, GASP1 knockout prevented the development of tolerance in response to prolonged Ex-4 pretreatment, indicating that GASP1 plays a role in regulating the post-endocytic trafficking of GLP-1R.

- Similarly, in INS-1 cells, deletion of GASP1 has no effect on incretin-mediated insulin secretion in response to Ex-4. However, upon prolonged Ex-4 pretreatment wild-type (WT) INS-1 cells show reduced incretin response, whereas GASP1 knockout (KO) INS-1 cells retained the incretin effect.
- Furthermore, in a longitudinal mouse islet insulin secretion assay, both GASP1 wild-type and beta-cell specific GASP1 deleted (β-GASP1-KO) islets show robust incretin effect and increase in insulin secretion when stimulated with Ex-4 acute treatment.
- Interestingly, after an Ex-4 pretreatment, GASP1-WT islets show reduced insulin secretion indicating development of tolerance while β-GASP1-KO islets maintained their incretin effect.
- Importantly, both GASP1-WT and β-GASP1-KO islets displayed a substantial incretin effect after a 24-hour recovery period, suggesting that the observed tolerance effect is not due to any inherent unhealthiness of the islets.
- In WT mice which are chronically treated with Ex-4 for six weeks show development of tolerance to glucose-stimulated insulin secretion effect of Ex-4.
- Furthermore, in WT mice treated chronically with Ex-4, there is not only the development of tolerance to exogenous Ex-4 treatment but also to their endogenous incretins.
- In mice with selective disruption of GASP1 in pancreatic beta cells (β-GASP1-KO mice), the acute treatment of oral glucose and Ex-4 are indistinguishable from WT mice.
- However, chronic Ex-4 treatment of β-GASP1-KO mice with Ex-4 does not develop tolerance to either exogenous Ex-4 or endogenous incretins.

This study highlights the pivotal role of GASP1 in regulating the post-endocytic trafficking of GLP-1R in pancreatic β-cells and its impact on receptor function during prolonged drug administration. These findings also emphasize the critical role of GASP1-mediated GLP-1R trafficking in the development of tolerance to incretin drugs and offers potential novel strategies for improving therapeutic efficacy. Therefore, gaining a deeper understanding of the molecular mechanisms governing GASP1-mediated

GLP-1R trafficking could help in the development of improved therapies utilizing GLP-1R agonists to effectively combat T2D and obesity.

Graphical Abstract



List of Abbreviations

ACE2	Angiotensin-Converting Enzyme 2
ANOVA	Analysis of Variance
AP2	Adapter Protein 2
ATP	Adenosine Triphosphate
BK1R	Bradykinin-1 Receptor
BK2R	Bradykinin-2 Receptor
β-GASP1-KO	Beta cell specific GASP1 Knockout
CALCR	Calcitonin Receptor
cAMP	Cyclic Adenosine Monophosphate
CB1R	Canabinoid 1 Receptor
CIE	Clathrin-Independent Endocytosis
CME	Clathrin-Mediated Endocytosis
CREB	cAMP Response Element-Binding protein
CRHR2	Type 2 CRH Receptor
DA	Dopamine
DAG	Diacylglycerol
DPP-4	Dipeptidyl Peptidase 4
DPP-4i	Dipeptidyl Peptidase 4 Inhibitors
DOR	δ-Opioid Receptors
D1R	Dopamine D1 Receptor
D2R	Dopamine D2 Receptor
ESCRT	Endosomal Sorting Complex Required for Transport Machinery
Ex-4	Exendin-4 (Exenatide)
fl-GASP1	Flox-GASP1

GASP1	G-protein coupled receptor Associated Sorting Protein 1
GCGR	Glucagon Receptor
GIP	Glucose-dependent Insulinotropic Polypeptide
GIPR	Glucose-dependent Insulinotropic Polypeptide Receptor
GLP-1	Glucagon-Like Peptide 1
GLP-1R	Glucagon-Like Peptide 1 Receptor
GLP-1RAs	Glucagon-Like Peptide-1 Receptor Agonists
GPCR	G-Protein Coupled Receptor
GRK	G protein-coupled Receptor Kinase
GSIS	Glucose-Stimulated Insulin Secretion
HEK293	Human Embryonic Kidney 293 cells
HTRF	Homogenous Time-Resolved Fluorescence
IACUC	Institutional Animal Care and Use Committee
INS1	Insulinoma 1 cells
IP3	Inositol Triphosphate
IR	Insulin Receptor
IRKO	Insulin Receptor Knockout
IRS2	Insulin Receptor Substrate 2
KO	Knockout
KRB	Kreb’s-Ringer Bicarbonate buffer
LTP	Long-Term Potentiation
MOR	μ-Opioid Receptor
NAc	Nucleus Accumbens
OGTT	Oral Glucose Tolerance Test
PDX-1	Pancreatic and duodenal homobox gene-1

PEI	Polyethylenimine
PIP2	Phosphatidylinositol 4,5-Bisphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
PP	Pancreatic Polypeptide cells
qPCR	quantitative real-time PCR
RAMPs	Receptor Activity-Modifying Proteins
sgRNA	Single-Guide RNA
SNX1	Sorting Nexin 1
SNX27	Sorting Nexin 27
SST	Somatostatin
SSTR	Somatostatin Receptor
SSTR2	Somatostatin Receptor Subtype 2
T2D	Type-2 Diabetes
T2DM	Type 2 Diabetes Mellitus
VDCC	Voltage-Dependent Calcium Channels
VTA	Ventral Tegmental Area
WT	Wild Type

Chapter 1: Introduction

Diabetes is a chronic disease that affects glucose metabolism[1]. The increase in the incidence of diabetes has become a major worldwide problem that is almost certain to worsen in the coming years [2,3]. In U.S., more than 133 million individuals are living with either diabetes (37.3 million) or prediabetes (96 million) out of which 90-95% people have type-2 diabetes (T2D).[4,5] T2D is characterized by the disruption of glucose homeostasis and impacts millions of people worldwide and is closely associated with lifestyle factors such as unhealthy diets, sedentary behavior, and obesity.[6,7] Insulin is the primary driver of glucose homeostasis and released by pancreatic β-cells in response to nutritional and endocrine signals. Unlike type 1 diabetes, which is an auto-immune disorder resulting from the immune-mediated destruction of pancreatic beta cells leading to insulin deficiency, T2D primarily involves insulin resistance and impaired insulin secretion. This complex interplay of factors contributes to elevated blood glucose levels, leading to a host of complications affecting various organ systems.[8,9] T2D not only places an immense economic burden on the healthcare systems but also significantly impacts an individual's quality of life. Its long-term complications, including cardiovascular diseases, kidney dysfunction, neuropathy, and vision impairment, highlight the urgent need for effective management and prevention strategies for T2D patients.[10,11] Over the years, many advancements in medical research and therapeutic approaches have been made to improve the management of T2D. Lifestyle modifications, oral medications including sulfonylureas, glinides, thiazolidinediones and most importantly insulin therapy are among the primary treatment options.[12,13] However, these conventional antidiabetic therapies also have a range of adverse effects including hypoglycemia, gastrointestinal adverse events, weight gain, and increased risk of bone fracture.[14] Hence there is a need for developing novel therapies and improved antidiabetic medications that ideally improve these adverse effects and risk factors. Recent advances in our understanding of the metabolic role of incretin system have led to the development of Incretin-based medications and GLP-1 receptor agonists, as promising new avenues for management of T2D.[15-18] The incretin drugs

such as Exenatide® (exendin-4), Victoza® (liraglutide), and Ozempic® (semaglutide), target the incretin system, a crucial hormonal pathway involved in regulating insulin secretion and glucose metabolism.[19-24] The incretin hormones, such as Glucagon-Like Peptide 1 (GLP-1) and Glucose-Dependent Insulinotropic Polypeptide (GIP), play a vital role in enhancing glucose stimulated insulin release in response to food intake, thereby maintaining glucose homeostasis. The introduction of incretin drugs has revolutionized diabetes management by offering several advantages, such as lower risk of hypoglycemia, potential weight loss, and improved cardiovascular outcomes.[25-27]

Despite the progress made in advancement of incretin drugs for the treatment of T2D, there are still several aspects that remain unknown. The long-term effects and safety profiles of these medications represent a gap in our knowledge and requires further investigation. Another important concern associated with the long-term use of incretin drugs is the development of tolerance. A tolerance to incretin drugs refers to a reduction in their effectiveness over time, leading to a diminished therapeutic response. Over a long-term period of treatment, individuals may experience a reduction in the efficacy of the incretin drugs to insulin release and the magnitude of their glycemic response. As a result, the glucose-lowering effects may become less pronounced, thus requiring higher doses of the drug or the addition of other antidiabetic medications to maintain adequate glycemic control.[28-31] Addressing the development of tolerance to the drugs is crucial to maintaining the effectiveness of incretin drugs in the management of T2D. The development of tolerance to incretin drugs is a matter of concern, our understanding of the molecular mechanism(s) underlying the development of tolerance to incretin drugs remains limited, leaving a significant gap of knowledge. This dissertation seeks to contribute to our fundamental understanding of the development of tolerance to incretin drugs (GLP-1R agonist - Exendin-4, Ex-4) by investigating the effect of GLP-1R agonists on the post-endocytic trafficking/sorting of the receptor, which plays a critical role in regulating the effectiveness of GLP-1R agonist. By focusing on this specific molecular mechanism, we aim to shed light on how this mechanism influences the efficacy of incretin drugs and their impacts

on their therapeutic effects. We will begin with a comprehensive analysis of GLP-1R subcellular cAMP signaling, investigating into the impacts of prolonged Ex-4 treatment on GLP-1R signaling within HEK 293 and rat INS-1 cells. We then focus on determining the significance of post-endocytic trafficking in the development of tolerance. In this pursuit, we closely examined the role of a G protein-coupled receptor-associated protein 1 (GASP1), a pivotal regulator influencing the post-endocytic sorting of numerous GPCRs, including GLP-1R. GASP1 is a protein that modulates the lysosomal degradation and functional down-regulation of variety of GPCRs. We assessed the influence of GASP1 on cAMP signaling and insulin secretion in both HEK 293 and INS-1 cells. Moving forward, we extended these investigations to mice and utilized mouse pancreatic islets to assess GASP1 role in tolerance development after chronic Ex-4 exposure. Ultimately, we outlined future directions for the research aimed at enhancing the efficacy of incretin drugs for the management of T2D.

Type 2 Diabetes Mellitus: Insulin Resistance, Pathophysiology, and clinical implications

In healthy individuals, the insulin secretion from pancreatic β-cells is responsive to the body's metabolic demands. Ingestion of a meal causes an increase in blood glucose levels, which triggers a rapid increase in insulin levels. This transition from fasting to post-prandial state initiates the suppression of glucose production from the liver and prepares body tissues for efficient glucose utilization.[32] Patients diagnosed with T2D show irregularities of both basal and meal triggered insulin release. While their fasting plasma insulin levels are generally elevated, they seems relatively low when compared to their fasting glucose levels.[33] Moreover, the release of insulin post an oral glucose load is delayed and reduced, which contributes to prolonged and elevated rise in plasma glucose levels, a characteristic feature of T2D.[34] These irregularities in insulin response within individuals affected by T2D are attributed to the well-being of β-cells, which resulted in limited insulin secretion and diminished β-cell population.[35] The decrease in β-cell population is hypothesized to be due to increase in β-cell apoptosis, which is triggered by factors such as glucotoxicity and escalated levels of

proinflammatory cytokines.[36,37] This decline in β-cell function advances over time, which progressively worsens glucose control and homeostasis. T2D patients also show insulin resistance phenotype, where the cells of muscles, liver, and fat show impaired response to insulin. As a result, these cells cannot easily take up glucose from the blood, leading to an increase in plasma glucose level.[38,39] In a healthy individual, because of this irregular insulin response, the pancreas makes more insulin to overcome the tissue resistance and help glucose enter the cells. However, this adaption eventually causes exhaustion of β-cell and individuals suffering from insulin resistance eventually progress to diabetes over time.[40,41] In T2D patients, maintaining the high levels of insulin secretion from β-cell becomes challenging, leading to an expedited deterioration of β-cell function. This causes a disruption in the regulation of pancreatic islet biology where pancreatic α-cells increase glucagon secretion and glucose production during meals. This further contributes to fasting hyperglycemia, glucose intolerance and onset of T2D.[42]

The development and advancement of T2D carry significant clinical implications. Anti-diabetic medications that induce insulin release like sulfonylureas or enhance insulin sensitivity like thiazolidinediones will be most effective to yield optimal results during the initial phase of the T2D, when some β-cells remain viable.[43,44] Alternatively, anti-diabetic medications that inhibit hepatic glucose production like metformin can be beneficial in the early phase of the disease, but its effectiveness diminished over time due to declining β-cell function that cause a decrease in insulin level, hence there is a struggle to manage hepatic glucose production.[45] These findings highlight the limited ability of traditional oral medications, which do not prevent loss of β-cell mass and function to sustain glycemic objectives for long term care. Hence, patients who are using these oral medications may ultimately need exogenous insulin supplementations for maintaining their glycemic objectives. Therapies that preserve β-cell health could delay progression of disease, but such therapies are not yet available. It was suggested that thiazolidinediones can fully prevent loss of β-cell function and halt the progression of T2D, but data from a diabetes outcome progression trial (ADOPT) did not support

this hypothesis.[46,47] Therefore, there is a need for novel therapeutics for the better treatment and management of T2D.

The Incretin System

It is well known that glucose triggers a much greater insulin response when ingested orally rather than when administered intravenously (Figure 1.1A). This phenomenon is known as “Incretin effect” and attributed to the incretin system.[48] The incretin system is a crucial physiological regulator of blood glucose levels after a meal. It primarily operates through the actions of specific gastrointestinal hormones called incretins, which are released in response to the ingestion of nutrients, especially carbohydrates and fats. The main function of incretins is to enhance insulin secretion from pancreatic beta cells in a glucose-dependent manner, meaning they stimulate insulin release only when blood glucose levels are elevated.[49] The incretin system accounts for 70% of total insulin secretion in healthy individuals in response to oral glucose.[48] The best known incretins are Glucagon-Like Peptide 1 (GLP-1) and Glucose-Dependent Insulinotropic Polypeptide (GIP). Both GLP-1 and GIP have similar effects on insulin secretion, although GLP-1 is generally considered to have more potent and sustained effects. Once released, incretins travel through the bloodstream to the pancreas, where they bind to specific receptors on the surface of beta cells that result in increased insulin secretion and inhibition of glucagon release from alpha cells, thus lowering blood.[50]

GIP, also known as gastric inhibitory peptide, is a 42 amino acid hormone (Figure 1.2A) secreted by the K cells in the upper small intestine in response to glucose and fat intake.[51] It significantly enhances the release of insulin from β-cell and is deactivated through enzymatic cleavage by dipeptidyl peptidase-4 (DPP-4).[52] It has a short half-life of 5-7 minutes, but despite its short half-life it exerts a prominent effect in healthy individuals.[53] Additionally, GIP also regulates fat metabolism and have shown to enhance the viability of pancreatic β-cell lines *in vitro* (Figure 1.3). Studies have shown that various tissues, including the central nervous system, adipose tissue, and bone respond to GIP, however, the clinical significance of those effects remain unclear.[52,54,55]

Notably, GIP does not induce delays in gastric emptying in humans but stimulates glucagon release under certain conditions.[56,57] GIP mediates its effect through a G protein-coupled GIP receptor (GIPR) located on islet β-cells. Because of its insulinotropic effect, GIPR is a promising target for drug development for management of T2D.[58,59,60,61] Unfortunately, in T2D patients, the insulinotropic sensitivity of GIP is greatly reduced, may be due to reduced GIPR expression or reduced β-cell sensitivity to GIP (Figure 1.1B).[62]

GLP-1, in humans, is secreted by enteroendocrine L cells in distal jejunum, ileum and colon in response to food intake (Figure 1.2B).[63] GLP-1 is released in two biologically active forms with equal potency: Amidated form, GLP-1 (7-36) amide and glycine-extended GLP-1 (7-37).[64,65] Within minutes of meal ingestion, the plasma concentration of GLP-1 increases. This suggests that endocrine and neural cues trigger the secretion of GLP-1 prior to direct nutrient-mediated stimulation of L cells. Like GIP, DPP-4 quickly breaks down GLP-1 peptide, with half-life < 2 minutes, limiting its duration of action, and reducing the overall efficacy.[67] GLP-1 mediates its action through GLP-1 receptor (GLP-1R), which is expressed in pancreatic islet cells, the stomach, heart, and hypothalamus. Activation of GLP-1R initiates different intracellular signaling pathways, including those increasing insulin secretion, β-cell proliferation and regeneration, and inhibiting β-cells apoptosis.[68,69,70,71] Additionally, GLP-1 plays an important role in modulating glucose metabolism. GLP-1 infusion to healthy individuals stimulates insulin secretion in a glucose-dependent manner. For T2D patients, GLP-1 has been shown to increase fasting and meal-stimulated insulin levels, therefore preventing after meal hyperglycemia and without causing hypolglycemia.[73,74] GLP-1 promotes glucose-stimulated insulin gene transcription and synthesis.[75] Additionally, studies have shown that GLP-1 hormone is associated with restoring glucose-resistance β-cells, protecting β-cells health and apoptosis, and promoting differentiation of islet progenitor cells *in vitro*.[76] Moreover, GLP-1 also delays gastric emptying in a dose-dependent manner thus slowing the entry of nutrients into the circulation.[77,78] Both GLP-1 and GIP promote satiety and weight loss and reduce energy intake.[79,80] GLP-1 also inhibits the

release of glucagon from pancreatic α-cells in a glucose-dependent fashion, therefore it is unlikely to produce hypoglycemia. Preclinical studies have shown that GLP-1 reduces glucose production, increase glycogen synthesis in liver, increases glucose metabolism in muscle, and regulates fat metabolism in adipocytes.[81,82] Different studies have also reported the potentially beneficial effect of GLP-1 on the cardiovascular system, nervous system, and renal system (Figure 1.3).[83,84,85] Unlike GIP, GLP-1 preserve their insulinotropic activity in T2D patients. Therefore, therapeutic attention has been focused on the GLP-1 and GLP-1R.[86]

The incretin system, particularly, GLP-1/GLP-1R has gained significant attention in the treatment of T2D. Researchers have developed medications that either mimic the action of incretins or inhibit their breakdown, leading to prolonged incretin effects. These medications include GLP-1 receptor agonists like exenatide (exendin-4), liraglutide, semaglutide, and dipeptidyl peptidase-4 (DPP-4) inhibitors like Januvia® (Sitagliptin) and Tradjenta® (Linagliptin). These two classes od medications have demonstrated favorable effects on glycemic control in people with T2D.[79,84,87,88]

G protein coupled receptors canonical pathway

GPCRs are membrane proteins comprised of seven transmembrane domains. In eukaryotic cells, GPCRs are the largest and most diverse group of receptors, with more than 800 GPCR genes identified in the human genome. They play a vital role in a wide range of physiological responses to environment signals, neurotransmitters, and hormones.[89-91] The importance of GPCRs and their signaling as therapeutical targets is underscored by the fact that 475 drugs, representing ~34% of FDA approved drugs, act on 108 unique GPCR targets.[92,93]

A classical GPCR consisting of seven transmembrane α-helices (helices 1-7) with three extracellular (E2-E4) and intracellular (C1-C3) loops, the ligand binding N-terminus (E1) on the outside of the cell and the cytoplasmic C-terminus (C4) on the inside of the cell (Figure 1.4). The loops between α helices 5 and 6, and 3 and 4 are important for interaction with the heterotrimeric G proteins. Ligand binding causes movement of the helices 5 and 6 of the GPCRs, changing the

conformation of the cytosolic loop in between leading to G protein binding. G protein contains three subunits α, β, and γ. Depending on their α-subunits, G proteins are classified into four categories. The G-stimulatory proteins (Gαs) and G-inhibitory proteins (Gαi) regulate membrane bound effector enzyme adenylyl cyclases, while the Gαq and $G\alpha_{12/13}$ protein activate phospholipase C enzymes and small GTPase family, respectively. Their active/turned “on” state is GTP-bound and inactive/turned “off” state is GDP-bound.[89-93]

For GPCRs that bind to Gαs proteins, when no ligand is bound to the receptor, the α subunit of G protein (Gαs) is bound to GDP and complexed with Gβ and Gγ subunits. Ligand binding changes the conformation of the receptor, allowing it to bind to Gαs and displace GDP with GTP.[94,95] This process results in the dissociation of Gαs-GTP from the Gβγ portion of the trimer, which then binds to and activates adenylyl cyclase. Adenylyl cyclase converts adenosine triphosphate (ATP) to cAMP, leading to an increase in intracellular cAMP levels. The increase in intracellular cAMP levels activates cAMP-dependent serine/threonine protein kinase A (PKA), which modulates the activity of many other enzymes through phosphorylation. Due to the G protein's intrinsic GTPase activity, GTP-bound Gαs hydrolyzes GTP to GDP within seconds. This results in the reassociation of Gαs with Gβ and Gγ subunits, leading to the inactivation of adenylyl cyclase. PKA activation, in turn triggers various downstream effects that can lead to different physiological responses (Figure 1.5).[96]

After the activation of G protein via a GPCR, the activated GPCR is involved in another major pathway, including β-arrestin mediated internalization of the receptor.[97] Upon ligand binding, the GPCR undergoes conformational changes that facilitate phosphorylation of serine and threonine residues within the third intracellular loop and carboxyl-terminal tail domain of the receptor by G protein-coupled receptor kinase (GRK). GRK plays a central role in regulating the desensitization of GPCR-G protein signaling, GPCR endocytosis to endosomes, and GPCR signaling via G protein-independent mechanisms.[98] GRK-mediated phosphorylation of the GPCR alone is not sufficient to mediate receptor desensitization, therefore, the GRK-mediated phosphorylation of the GPCR

promotes the recruitment and high-affinity binding of β-arrestin to the receptor. β-arrestins are a class of proteins important for regulating GPCR signaling. β-arrestin binding has three distinct functions. First, its “arreststst” the G protein signal and desensitizes the receptor to GLP-1 signaling by uncoupling the receptor from heterotrimeric G protein. Second, as the receptor binds to β-arrestin, it interacts with adapter protein-2 (AP2), dynamin and clathrin to induce clustering of the receptor into clathrin-coated pits and subsequent clathrin-mediated internalization.[99,100,101,102] This places the receptor into the endosomes where it is thought to be either recycled and sent back to the plasma membrane or targeted for degradation via lysosomal pathway (Figure 1.6). This post-endocytic sorting of the receptor is an important regulatory mechanism for the regulation of many GPCRs signaling pathways.[103] β-arrestin also interacts with other proteins, including E3 ubiquitin ligases like mdm2, which help in regulating clathrin-mediated endocytosis.[104] Third, β-arrestin also acts as a scaffolding protein to facilitate the recruitment of other signaling molecules to the activated receptor that can trigger alternative signaling pathway distinct from classical G protein-mediated signaling. These G protein-independent pathways are referred to as arrestin-mediated signaling or biased signaling. In addition to functioning as an adapter protein, β-arrestin serves as a scaffold for variety of signaling complexes. Studies have shown that β-arrestin interacts with a wide variety of kinases particularly Src family kinases and can couple the GPCR to MAPK ERK1/2 pathways.[105-107]

Post-endocytic trafficking and sorting of GPCRs

Following endocytosis, the individual receptors can be sorted between recycling or degradative pathways.[108,109] This post-endocytic fate of the GPCR plays a crucial role in regulation of GPCR signaling. It is generally believed that GPCRs that do not internalize as a complex with β-arrestin are dephosphorylated, resensitized and recycled back to the plasma membrane. Receptors that bind strongly with β-arrestin and internalize as a complex are either inefficiently recycled back or not recycled at all and undergo degradation instead.[110] Therefore, for recycling receptors, endocytosis serves as a mechanism for receptor resensitization in which internalized receptors are recycled back

to the plasma membrane in fully active form.[111,112] For receptors that are degraded, rapid endocytosis may cause receptor downregulation as after endocytosis the receptor is targeted to lysosomal degradation. This sorting of the receptor post-endocytosis is referred to as receptor trafficking (Figure 1.7). [110]

Receptor phosphorylation is necessary for receptor endocytosis, but it has also been suggested that receptor phosphorylation influences the post-endocytic fate of the receptor by regulating its interactions with sorting proteins including PDZ domain-containing proteins, Receptor Activity-Modifying Proteins (RAMPs), EBP50, sorting nexin 1 or inducing posttranslational modification like ubiquitination which involved the covalent attachment of ubiquitin to lysine residues of the cytoplasmic c-tail and promotes lysosomal targeting.[113-116] Recycling of the GPCR receptor post-endocytosis was traditionally thought to occur by default since the membrane is continuously recycled by itself and disruption of lysosomal signaling increase GPCR recycling.[117,118] However, studies have shown that specific recycling sequences are present in the cytoplasmic tails that aid in recycling of the receptor. For example, β2-adrenergic receptor contains a DSLL sequence that has been shown to be important for its recycling to the plasma membrane via interaction with PDZ-domain containing proteins.[119,120] The sorting of receptor to lysosomal degradation pathway is facilitated by its interaction with one or more sorting proteins.[110] For ubiquitinated proteins, the highly conserved endosomal sorting complex required for transport machinery (ESCRT) directs the receptor for degradation.[121-123] However, for some GPCRs like δ-opioid receptors (DOR) ubiquitination is not required for post-endocytic degradation.[124] This data suggests that ESCRT machinery is also able to recognize non-ubiquitinated receptors and transport them for lysosomal degradation. One possible mechanism by which ESCRT can recognize non-ubiquitinated receptors is with sorting proteins that serves as a linker between non-ubiquitinated GPCRs and ESCRT machinery. Sorting nexin-1 (SNX-1) is one such sorting protein that downregulates protease-activated receptor -1 GPCR binds to Hrs (a part of ESCRT) *in vitro*.[125,126] Another cytoplasmic protein Dysbindin, is implicated in post-

endocytic sorting of DOR and dopamine D2 receptor to lysosomal degradation and interacts with both Hrs proteins and the GPCR-associated sorting protein 1 (GASP1).[127,128]

Role of G protein-coupled receptor- Associated Sorting Protein 1 (GASP1) in post-endocytic sorting of GPCRs

GPCR-Associated sorting proteins (GASPs) are GPCR-interacting proteins that regulate receptor signaling and trafficking. The GASP family is comprised of 10 proteins (GASP1 – GASP10) with significant sequence similarity particularly in the C-terminus domain.[129-132] Out of 10 family members, GASP1 is shown to interact with 30 different GPCRs including delta opioid receptor, dopamine D2 and D4 receptor, mGluR1a receptors among others.[133-135] GASP1 was discovered in a yeast two-hybrid screen by Whistler et al. GASP1 is a large protein with ~180 KDa molecular weight and comprised of 1,394 amino acids.[132] It is expressed in a few tissues including brain, pancreas, and hepatocytes.[133] Since its discovery, GASP1 has been reported to target different GPCRs to lysosomal degradation pathway including canabinoid 1 receptor (CB1R), dopamine D2 (D2R), calcitonin receptor (CALCR), delta opioid receptor among others.[129-135] GASP1 also shows selective binding for certain GPCRs. For example, the DOR binds to GASP1 are targeted to lysosomal degradation but μ-opioid receptor (MOR) do not interact with GASP1 and recycled to plasma membrane. Similarly, D2R after endocytosis undergoes GASP1-mediated lysosomal degradation but dopamine D1 receptor (D1R) does not interact with GASP1 and undergo recycling after endocytosis.[132,134,137] These results show that the post-endocytic trafficking and sorting of the receptor is a highly controlled process that is fundamentally important for the regulation of GPCRs signaling. These data also suggest that GASP1 functions to regulate post-endocytic sorting of GPCRs. No other GASP family member is involved in GPCR sorting but promotes cell survival and differentiation. Inhibition of dopamine D2 receptor and GASP1 interaction through neutralizing antibodies have been shown to reduce the desensitization of agonist activated D2 receptors.[134] These data imply that disrupting GPCR-GASP1 interaction could potentially enhance GPCR signaling when exposed to continuous agonist

stimulation, may be due to prevention of receptor degradation. Additionally, it has been demonstrated that GASP1 is directly involved in modulating GPCR signaling in virally encoded US28 GPCR. Overexpression of GASP1 in HEK293 cells significantly increases the PI3-kinase signaling while shRNA silencing of GASP1 reduces IP3 formation. Furthermore, the cAMP-responsive element-binding protein was also either enhanced or prevented by overexpression or silencing of GASP1.[131] These results implicate that GASP1 not only plays a crucial role in modulating the post-endocytic sorting of the receptor, but also influences its signaling activity, thus pointing toward a more important role of GASP1 in regulation of signal transduction by GPCRs.

Glucagon-Like Peptide 1 Receptor (GLP-1R): Functions and Signaling

GLP-1 hormone targets the GLP-1 receptor (GLP-1R) expressed in pancreatic beta cells, the gastrointestinal tract, and the central nervous system.[83,84,85] GLP-1R plays a crucial role in maintaining glucose homeostasis and insulin regulation. In addition to its role in insulin secretion, GLP-1R has other important functions in the body including[25-27]

1). Appetite Regulation: Activation of GLP-1R in the brain promotes satiety and reduces the feeling of hunger thus regulating appetite and food intake.

2). Gastric Emptying: GLP-1R activation in the gut slows down processing of the food in the stomach which helps to control the rate of nutrient absorption contributing to more stable blood glucose levels.

3). Cardiovascular effects: GLP-1R activation also promotes vasodilation, reduces blood pressure and the risk of cardiovascular events.

4). Neuroprotection: Studies have shown that GLP-1R activation has been implicated in enhancing synaptic plasticity and neuron survival thus offering benefits for neurodegenerative conditions like Alzheimer's disease.

5). Memory and Learning: Studies have shown that GLP-1R signaling in hippocampus and ventral tegmental area (VTA) modulates learning and memory. In preclinical animal models, these drugs

have been shown to protect long-term potentiation (LTP) and to produce improved performance in several diverse cognitive tasks.

6). Renal Effects: GLP-1R activation has been shown to reduce markers of renal injury in experimental kidney disease models to protect kidney function. This suggests a potential role in the treatment of diabetic nephropathy and other renal disorders.

These varied roles of GLP-1R highlight the broad impact of the incretin system on the human body. The focus of this work is on GLP-1 receptor signaling and its post-endocytic trafficking and how it affects the GLP-1R function in maintaining glucose homeostasis.

Glucagon-like peptide 1 receptor belongs to class B GPCR family characterized by seven transmembrane α-helices connected by three intracellular and three extracellular loops and mediates the effect of the incretin hormone GLP-1.[138] Various studies have reported the expression of the GLP-1R mRNA transcripts in different tissues including pancreas, lung, kidney, stomach, brain, and retina. [52] Human GLP-1R is 463 amino acid long protein consisting of a signal peptide sequence at its N-terminal which is cleaved after its delivery to cell membrane. The extracellular N-terminal domain is crucial for ligand binding while the intracellular C-terminal end interacts with and activates G protein for signal transduction.[138]

Canonical GLP-1R signaling (Figure 1.8) occurs through heterotrimeric Gs proteins (as described above) resulting in activation of adenylyl cyclase leading to increase cyclic adenosine monophosphate (cAMP) level.[139] This rapid increase in cAMP causes the activation of protein kinase A and exchange protein activated by cAMP-2 (Epac-2). In pancreatic beta cells, activation of PKA has the following effects: 1) Activation of cAMP response-binding element (CREB) that promotes the expression of pancreatic and duodenal homobox gene-1 (PDX-1) transcription factor and insulin receptor substrate 2 which are essential for insulin synthesis, beta cell growth and survival. 2) Activation of cyclin D1 and MAPK that is crucial for G1/S transition during cell cycle thus enhancing beta cell neogenesis. 3) Phosphorylation of sulfonyl urea receptor 1 and K-ATP channels leading to

beta cell membrane depolarization. 4) Activation of activating transcription factor 4 (ATP4) that prevents the development of ER stress in beta cells in cAMP-dependent manner.[140-142] These findings suggest that sustained GLP-1R signaling in T2D patients may result in preservation of functional beta cells. Activation of Epac-2 closes K-ATP channel thus promoting membrane depolarization, Ca^{2+} influx and release of Ca^{2+} from intracellular stores, causing insulin exocytosis.[143-145] Additionally, GLP-1R can couple to Gαq G protein thus leading to stimulation of phospholipase C (PLC) enzyme that cleaves a phosphatidylinositol 4,5-biphosphate (PIP2) into two secondary messengers: inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 activates protein kinase C (PKC) that phosphorylates different downstream effector proteins. IP3 also binds to its receptor on endoplasmic reticulum and release Ca^{2+} ions into the cytosol. Increase in intracellular Ca^{2+} concentration triggers insulin release from beta cells via fusion of insulin-containing vesicles with cell membrane.[146-148]

After activation, the activated GLP-1R receptor undergoes rapid endocytosis which is either clathrin-mediated (CME) or clathrin-independent (CIE), dynamin-dependent endocytosis pathway.[149,150] In pancreatic beta cells, the GLP-1R follows the canonical GPCR internalization mechanism via clathrin-coated pits involving GRK-dependent phosphorylation of the cytoplasmic domain of GLP-1R, β-arrestin recruitment and interaction of the receptor with adaptor protein 2 (AP2) leading to movement of GPCR-β-arrestin complex into vesicular pits, thus causing endocytosis of the receptor.[151-154] Post-endocytosis, the first organelle receiving receptor are sorting endosomes, also known as early endosomes, characterized by the expression of Rab5.[155,156] At this stage, the GLP-1R may be directed to recycling pathway to the membrane via (1) a short cycle ($Rab5^+$ → $Rab4^+$ endosomes) to (2) long cycle ($Rab7^+$ late endosome → $Rab11^+$ recycling endosome) or toward degradation pathway via transition from early endosome to $Rab7^+$ late endosome and subsequently to the lysosomes (Figure 1.6 and 1.7).[157] This post-endocytic trafficking/sorting of the receptor plays a crucial role in GLP-1R signaling; however, the molecular events governing this process are not well-

understood. After ligand dependent internalization, only 10-30% of internalized GLP-1R returns to the surface.[158] In beta-cells, sorting nexins 27 (SNX27) has been shown to play a crucial role in sorting GLP-1R from early endosome to recycling endosome.[159] Previous data have also shown that ubiquitination of the receptor to target the receptor toward ESCRT-dependent lysosomal fusion does not apply toward the GLP-1 receptor.[160] Another sorting nexin 1 (SNX1) was shown to restrict receptor recycling thus targeting GLP-1R toward lysosomal degradation.[159] These preliminary data do not provide clear mechanisms that govern the fate of endocytosed receptor, nor do they tell us the protein factors involved in this process, thus representing a gap in the knowledge. In this study, we focus to examine the role of a GASP1 protein in post-endocytic degradation of GLP-1R in pancreatic β-cells and how this post-endocytic trafficking affects receptor function and GLP-1R agonist signaling.

Glucagon-Like Peptide 1 Receptor (GLP-1R) agonists and DPP-4 inhibitors in treatment of T2D

In individuals with T2D, there is often a decrease in GLP-1 secretion from the gut. Moreover, studies have shown that T2D individuals have impaired GLP-1R signaling. Additionally, in T2D the insulinotropic effect of GIP is attenuated. In contrast, the insulinotropic effect of GLP-1R is preserved even in T2D patients.[161,162] Due to their ability to enhance insulin secretion from beta-cells upon activation even in T2D patients, the therapeutic approach to incretin-based therapy is focused on GLP-1 and GLP-1R agonists. Researchers and pharmaceuticals company have developed a class of drugs called incretin drugs which harness the body's natural incretin system to enhance insulin secretion and regulate blood glucose levels.[163,164] These drugs are categorized into two categories:

A). Incretin mimetics, GLP-1 agonists (GLP-1R agonists, GLP-1RAs)[165]

Incretin mimetics or GLP-1R agonists are a class of medications that mimic the action of the incretin hormone GLP-1. They are synthetic analogs of GLP-1 peptide. Like GLP-1, they activate the GLP-1R on pancreatic beta-cells which leads to increased insulin secretion and decrease glucagon release. This helps to lower blood glucose after a meal and maintain glucose homeostasis. [165] Some

common GLP-1R agonists include exenatide and liraglutide. Incretin drugs are usually administrated as subcutaneous injections and have several distinct advantages:

1.Glucose-dependent action: GLP-1R agonists enhance insulin secretion in a glucose-dependent manner, meaning they promote insulin release only when blood glucose is elevated. This reduces the risk of hypoglycemia or low blood sugar compared to insulin therapy.[163-165]

2. Weight loss: GLP-1R agonists are associated with weight loss in individuals making them a useful option for an individual who is obese and has T2. As being overweight or obese greatly increases your risk of developing T2D, GLP-1R agonist will be useful to treat both conditions simultaneously.[163-165]

3. Cardiovascular benefits: GLP-1R agonists have also been shown to reduce the risk of heart attacks and strokes in T2D individuals.[163-165]

4. Renal benefits: GLP-1R agonists have demonstrated potential renal benefits like preserving kidney function and reducing the risk of diabetic kidney disease.[163-165]

Exenatide was derived from exendin-4 and is resistance to DPP-4 degradation with a half-life of 2.4 hours following administration.[166] Exenatide is a peptide whose first 30 amino acids have sequence identity with naïve human GLP-1 while the nine amino acid extension has no homology in human.[167] FDA approved exenatide for the treatment of T2D in 2005. Exenatide when given as a monotherapy twice daily has been shown to reduce the HbA1C in drug naïve patients.[168] As an add-on therapy to existing therapy like metformin and sulfonylurea in T2D patients, exenatide reduces HbA1C by 0.5 – 1 %.[169] Exenatide has an excellent safety profile with lower rates of hypoglycemia and significant improvement in weight loss. Studies have shown that when exenatide is given in a formulation to extended release (Exenatide QW), the once weekly administered Exenatide QW lowered HbA1C to greater degree than twice daily administered exenatide. The Exenatide QW shows a similar weight loss profile and fewer patients experience the GI side effects (DURATION-5 trial). [170]

Liraglutide is an analog of human GLP-1 with 97% sequence homology. It has a fatty acid side chain that promotes albumin binding and is resistant to DPP-4 degradation with half-life of 11-13 hours. In contrast to exenatide, liraglutide does not undergo renal clearance.[171] FDA approve liraglutide for the treatment of T2D in 2010. As a monotherapy, liraglutide shows significantly greater reduction on HbA1C compared to glimepiride in a dose-dependent manner (LEAD-3 trail).[172] As an add-on therapy, with existing oral therapy, liraglutide further decrease HbA1C in T2D patients (LEAD-1, LEAD-2, Lead-4, LEAD-5 trials).[173-176] Studies have also shown that liraglutide administration improve the β-cell functions in these trials.[177] Compared to exenatide (LEAD-6 trial), liraglutide decrease HbA1C to a greater extent than exenatide, it has greater effect on fasting glucose but show less postprandial glucose control compared to exenatide. Both treatments are well tolerated, and weight loss is comparable.[178]

The common adverse effects for GLP-1 agonist are GI symptoms including nausea, vomiting, and diarrhea. These symptoms generally improve with time. The risk of hypoglycemia is low with these GLP-1 agonists as they stimulate insulin release in glucose-dependent manner.[166] One early cause of concern with the use of GLP-1R agonist is the higher risk of pancreatitis. However, subsequent studies have shown no increased risk of pancreatitis in patients treated with exenatide.[179]

B). Dipeptidyl peptidase-4 inhibitors (DPP4i)[180]

Dipeptidyl peptidase-4 (DPP-4) inhibitors inhibit the action of enzyme DPP-4 which is responsible for cleaving GLP-1 and GIP hormone. By inhibiting DPP-4, these drugs increase the endogenous levels of incretin hormones GLP-1 and GIP, which in turn enhances insulin secretion.[180] Some common examples of DPP-4 inhibitor drugs include sitagliptin, alogliptin, linagliptin, saxagliptin. These medications are available in tablet form making them more convenient for patients. They have been recently approved by the FDA for treatment of T2D. These agents completely inhibit DPP-4 activity for 24 hours, thus raising GIP and GLP-1 concentration.[181,182] Whether DPP4i is given as a monotherapy or as an add-on therapy, they show reduction in HbA1C levels of 0.5 – 0.8%. Their

modest glycemic reduction compared to GLP-1R agonist is because GLP-1 agonist produces supraphysiological concentration of GLP-1, while in comparison, DPP4i achieved lower GLP-1 concentration.[183-185] DPP4i is weight neutral and does not cause weight loss. Administration of sitagliptin and vildagliptin has been associated with improved β-cell functions. DPP4i are well tolerated with low incidence of hypoglycemia and GI upset.[186-188] Inhibition of DPP4 not only increases the circulatory levels of GLP-1 and GIP but other peptides also like neuropeptide Y, peptide YY that could have hypertensive effect.[189]

C). Dual incretin agonists – Twincretins

A relatively recent advancement in diabetes treatment and research are dual incretin agonists that simultaneously activated both incretin receptors: GLP-1R and GIPR. By targeting both the incretin receptors involved in glucose regulation, these medications aim to amplify the glucose-lowering effects and potentially provide additional benefits for T2D patients. The combined action of GLP-1R and GIPR activation is to synergistically enhance insulin secretion and reduce blood glucose level in glucose-dependent manner.[190] Recently, the FDA has approved the first dual GLP-1R/GIPR co-agonist Tirzepatide (Mounjaro®) for treatment of individuals with T2D and obesity. Tirzepatide is an acylated peptide that activates both GLP-1R and GIPR.[191] Clinical trials have shown that tirzepatide produced significantly greater reduction in the HbA1C levels and body weight as compared to GLP-1Ras single agents (SURPASS 1-5 trails). The adverse effects of tirzepatide is like those of exenatide and liraglutide and include GI symptoms like nausea, vomiting and diarrhea. Tirzepatide also improves insulin sensitivity and insulin secretion to a greater extent than semaglutide.[192-196] However, the mechanisms of action by which tirzepatide promotes its clinical effects are still under investigation. Irrespectively, the clinical advantage of dual incretin agonist over GLP-1Ras has sparked renewed interest in development of novel therapeutic that activates both the incretin receptors. In fact, several such molecules have been reported and shown to produce improved glycemic control in mice.[153]

Biased signaling and the role of receptor trafficking in biased signaling

The activation of GLP-1R has a pleiotropic effect on multiple signaling proteins within the cell. This led to the advancement of a fascinating concept in cellular signaling known as Biased agonism or signal biased. Biased signaling refers to the ability of a ligand to selectively favor and show preference to a particular signaling pathway, thus activating one pathway more strongly than the others, resulting in distinct cellular outcomes (Figure 1.9).[197] Moreover, various agonists can also induce distinct receptor endocytosis and trafficking profiles leading to differential downstream signaling pathways.[198-200] The concept of biased agonism has implications for therapeutics intervention and has opened new avenues for understanding receptor pharmacology and drug development. Studies have shown that preferential activation of one pathway over the other could increase the benefit -to-adverse ration in the therapeutic management of diseases like T2D and obesity. For example, exendin-4 is more biased toward cAMP signaling while oxyntomodulin a natural GLP-1R/GCGR co-agonist is more biased toward ERK1/2 signaling.[198] Compared to GLP-1 peptide, Exendin-4 is more biased at β-arrestin recruitment. Studies assessing the GLP-1RAs and their activation of downstream signaling pathways like cAMP or ERK1/2 show that exendin-4 and liraglutide show reduce efficacy of cAMP response in comparison to endogenous ligand GLP-1.[201] Recent studies have also shown a difference in receptor internalization and insulinotropic effect of the GLP-1R agonist depending on chronic vs acute administration. Chronic administration of exendin-4 derivative GLP-1-Val8 displayed reduce β-arrestin recruitment, weekend receptor endocytosis and improved glucose tolerance in diabetic mice.[202] These data suggest that improved insulinotropic effects of these compounds is due to lower desensitization and decrease in receptor internalization leading to differences in intracellular trafficking over prolonged period.

The Gαs signaling of GLP-1R is attributed to the insulin release effect of GLP-1R while the β-arrestin recruitment and signaling is thought to be responsible for downregulation, desensitization, and reduction in GLP-1RAs efficacy.[203,204] Therefore, compounds that reduce β-arrestin engagement

at GLP-1R show improved anti-diabetic effects. Many compounds are designed to be G-biased for GLP-1R including exendin-F1 that show enhanced insulin release via avoiding GLP-1R desensitization by not recruiting β-arrestin.[205] For GLP-1R agonists those having a fatty acid moiety at the C terminus ends like liraglutide and semaglutide, these fatty acid chains may have the potential to promote bias signaling. Studies have shown that adding a fatty-diacid to C-terminus of exendin-4 (exendin-4-C16) reduces binding affinity and β-arrestin recruitment to the receptor along with altering the postendocytic trafficking of the receptor.[206]

While the exact molecular mechanism underlying biased agonism is not yet known, it is widely believed that biased ligands exhibit a preference for stabilizing distinct GPCR conformations, thus enabling selective engagement with specific signaling pathway.[207] For example, [Sar1, Ile4, Ile8]-AngII ligand selectively activates β-arrestin recruitment and ERK1/2 phosphorylation at the angiotensin 2 type 1 receptor, but is unable to activate G protein signaling, thus showing biased to β-arrestin pathway.[208] Therefore, we can utilize a combination of structural, homology and mutational studies to determine unique structural rearrangements and changes in bias-linked networks. These findings can be used to deduce signaling pathways specific to each ligand. Thus, identifying bias-related structural and confirmational changes for a ligand may help to effectively predict pharmacological similarities or differences among GLP-1R agonists. This provides a significant understanding of the molecular mechanism of GLP-1R signaling as prompted by GLP-1R agonist small molecules.

Glucagon-Like Peptide 1 Receptor (GLP-1R) agonists and tolerance development

Currently there are six FDA approved GLP-1R agonists for the treatment of T2D. All the GLP-1R agonists either as monotherapy or in combination therapy show remarkable reduction in HbAC1, glucose lowering, glycemic control and weight loss.[209] However, the question to what extent tolerance develops with GLP-1R agonist remains. Moreover, which of the many pharmacological effects of the GLP-1R is affected due to development of tolerance is not well studied. Studies have shown that after

continuous prolonged exposure of GLP-1R agonists, there is a diminished effect/tachyphylaxis on the gastric motility. Additionally, in mouse models, studies have shown that some degree of tolerance may developed toward the glucose lowering effect of GLP-1R agonist after prolonged use.[28,30,170, 210] Sedman et al. have shown that tolerance developed toward the glucose-lowering effect GLP-1R agonist in mice. They have shown that for chronically administered exenatide and liraglutide, the glucose-lowering effect is weaker compared to acute administration indicating development of tolerance.[210] However, the exact mechanism for the development of tolerance to GLP-1R agonist needed further investigation and a more focused study to prove whether insulin release and change in insulin sensitivity is the leading mechanism.

In this study, by utilizing a combination of genetically modified cell lines, Homogenous Time Resolved Fluorescence (HTRF) biochemical assay, transgenic mice and *in vivo* and *ex vivo* animal studies, we investigated a novel mechanism for the development of tolerance toward GLP-1R agonist. Here we have shown that post-endocytic trafficking of the GLP-1R within pancreatic β-cells have significant implication in therapeutic response of GLP-1R agonist over prolonged period. Additionally, we have examined the functional implication of GASP1 – a GPCR Associated Sorting Protein, which plays a key role in GLP-1R degradation after receptor internalization in pancreatic β-cells. Finally, we have investigated how post-endocytic trafficking of the GLP-1R in β-cells contributes to the development of tolerance *in vivo.* This work provides an insight into one of the molecular mechanisms by which tolerance is developed toward prolonged use of GLP-1R agonists. Further, this work shows that GASP1-mediated GLP-1R degradation in pancreatic β-cells is responsible for the loss of GLP-1R agonist efficacy over time. These findings underscore the crucial role of GASP1-mediated GLP-1R trafficking in the development of tolerance to incretin drugs and could suggest novel ways to improve therapeutic utility. Additionally, these findings implicate post-endocytic trafficking of the GLP-1R as a mechanism that limits the efficacy of incretin therapeutics during

chronic use. These studies also suggest that an incretin memetic drug that promotes G protein signaling but not arrestin engagement, could provide prolonged therapeutic utility.

Chapter 2: *In vitro* investigation of the role of GPCR-associated sorting protein 1 (GASP1) on GLP-1R signaling.

Introduction

GLP-1 receptor agonists are safe for long-term use and are prescribed to T2D patients as either once-weekly (Exenatide, Dulaglutide) or once-daily (Liraglutide) prescriptions. They exert their molecular effects by activation of GLP-1 receptor (GLP-1R) on the plasma membrane of pancreatic β-cells.[12,15,17,52] GLP-1R is a G-protein coupled receptor, whose activation promotes an increase in cytoplasmic cAMP levels, which in turn activates PKA and exchange protein directly activated by cAMP2. However, there is evidence that GLP-1R couples with Gαq and other G proteins also initiate different downstream signaling pathways. PKA and EPAC-2 trigger the closure of K_{ATP} and K_V channels, which depolarizes the cell membrane, opens voltage-dependent calcium channels (VDCC), and causes Ca^{2+} influx (Figure 1.8). Beyond the traditional functions of cAMP, the cAMP/CREB pathway also exhibits the capacity to induce the expression of insulin receptor substrate 2 (IRS2).[139-148] This induction, in conjunction with its role in promoting β-cell survival, highlights the protective effects of GLP-1 analogs on β-cells.

Insulin secretion occurs in two phases. The early phase lasts for the first 10-15 minutes after feeding. During the early phase, the already synthesized and stored insulin granules are released from the β-cells. The more sustained late phase requires synthesis and processing of new insulin, via signaling through different GPCRs such as incretins, muscarinic, adrenergic, and nutrient receptors. The Gαq pathway directly orchestrates the glucose-triggered release of insulin granules while the GLP-1R-Gαs-cAMP-EPAC-2 pathway is implicated in the early phase of insulin release potentiation by regulating insulin granule maturation, trafficking, and exocytosis. [211]

After agonist stimulation, the GLP-1R is desensitized and removed from the plasma membrane. Previous studies over past decades have improved our understanding of the molecular mechanisms underlying GPCR internalization from the cell surface. Two proteins that play a crucial

role in this process are the GPCR kinases (GRKs) and the β-arrestins (Figure 1.6). GRKs are serine/threonine kinase that phosphorylates the GPCRs c-terminal tail and cause receptor desensitization. For GLP-1R, G-protein coupled receptor kinase 2 (GRK2) phosphorylates the 33 amino acid long C-tail of GLP-1R at specific serine/threonine residues. This leads to recruitment of β-arrestins, thus promoting internalization of GLP-1R and triggering β-arrestin dependent signaling. Two types of β-arrestin, β-arrestin-1 and β-arrestin-2, mediate the GLP-1R downstream signaling in β-cells, and elicit the receptor internalization process. β-arrestin-1 facilitates the phosphorylation of CREB and ERK1/2, subsequently leading to the phosphorylation of Bad, a regulator associated with Bcl-xL/Bcl-2, thereby preventing cellular apoptosis. Meanwhile, β-arrestin-2 plays a crucial role in insulin regulation, as demonstrated by the disruption of insulin secretion in mouse models upon β-arrestin-2 knockout. Additionally, the post-translational ubiquitination of both receptor and β-arrestin play definitive and discrete roles in regulating the life cycle of GPCRs.[149-154]

After being internalized from the cell surface, GPCRs can follow various sorting routes. They may undergo dephosphorylation, resensitization, and be recycled back to the plasma membrane. Another possibility is that GPCRs may be targeted for degradation via the endosomal/lysosomal pathway (Figure 1.7). GLP-1R is a fast-internalized and recycling receptor. After activation, GLP-1R-ligand complexes enter the endosome. A portion of that is eventually transported to lysosomes for degradation, while the other portion returns to the cell membrane.[155-160] Different agonists may show different effects on GLP-1R internalization and recycling. For example, GLP-1 is apt to receptor recycling, while exendin-4 may favor slower recycling and lysosome targeting. Further research has brought to light a set of auxiliary proteins that interact with GLP-1R, GRK and β-arrestin complexes.[212] Recent research is now focused on elucidating the intricacies of these proteins interaction that are inherent to the process of GLP-1R endocytosis and trafficking.

The post-endocytic trafficking of the receptor plays a pivotal role in modulating receptor function, particularly during prolonged drug/agonist use. If the receptor is recycled after endocytosis,

the cell surface expression of the receptor remains constant even after chronic drug administration, hence leaving receptor signaling unaffected. In contrast, if receptor degrades after endocytosis, prolonged agonist exposure may lead to reduction in receptor cell surface expression, thus causing a decline in signaling activity. The protein factors responsible for determining whether internalized GLP-1R is recycled or degraded remain unknown, thus presenting a significant gap in our knowledge of GLP-1R trafficking. We hypothesize that GPCR-associated sorting protein 1 (GASP1) plays a crucial role in dictating the post-endocytic fate of GLP-1R. We postulate, during prolonged exendin-4 (a GLP-1R agonist) treatment, GASP1-mediated post-endocytic degradation of GLP-1R is responsible for decrease agonist response by the receptor. This effect may be attributed to a reduction in the receptors cell surface expression.

We have developed snap-tagged GLP-1R stably expressing HEK293 cell. Additionally, using CRISPR-Cas9 technique, we have generated Snap-GLP-1R HEK293 cells and INS-1 cells with and without GASP1 protein. We examined the role of GASP1 on GLP-1R signaling and function particularly in scenario of prolonged drug use. In this chapter, using Ex-4 dose-response curves for intracellular cAMP increase and insulin release, we will determine if GASP1 is crucial for GLP-1R signaling during chronic drug administration. Our data shows that GASP1 knockdown in snap-GLP1R HEK293 cells and INS-1 cells does not alter the acute GLP-1R signaling suggesting that knocking down GASP1 does not affect receptor affinity or efficacy for Ex-4. However, for chronic Ex-4 exposure, GASP1 wild-type cells (both snap-GLP-1R HEK293 and INS-1 cells) show a decrease in Ex-4 response while GASP1 knockdown cells show a similar response to acute treatment. This diminished agonist response exhibited by the receptor after prolonged drug exposure is defined as tolerance development. These data provide evidence supporting our hypothesis that GASP1 is critical in deciding the post-endocytic fate of GLP-1R and may be contributing to development of tolerance to exendin-4 after prolonged use.

Results

Deletion of GASP1 protein in HEK293 or INS-1 cells does not affect acute GLP-1R signaling.

We first aimed to examine the role of GASP-1 protein on GLP-1 receptor acute signaling. To assess this, we used stably expressing snap-GLP-1R HEK293 cells and INS-1 cells to investigate the impact of GASP1 deletion on GLP-1R signaling. We applied CRISPR-Cas9 technique to successfully delete GASP1 protein from snap-GLP1R HEK293 (HEK-GLP1R GASP1-WT) and INS1 (INS1 GASP1-WT) cells to generate HEK-GLP-1R GASP1 knockout (HEK-GLP1R GASP1-KO) and INS1 GASP1 knockout (INS1 GASP1-KO) cells. To verify the deletion of GASP1 protein, we assess the expression of GASP1 protein in cell lysate from both GASP1 wild-type and knockout HEK-GLP1R and INS1 cells using western blot analysis (Figure 2.1). For both HEK-GLP1R (Figure 2.1A) and INS1 cells (Figure 2.1B), in the GASP1-WT cell lysate, a distinct protein band of 180 KDa corresponding to GASP1 molecular weight was observed, however, in the GASP1-KO cell lysate, the GASP1 protein band was absent. This indicates the efficient knockout of the GASP1 gene in the INS-1 cells. GAPDH expression was also detected as a control to ensure equal protein loading on to the gel.

We further assess the role of GASP1 protein on GLP-1R function and its acute signaling. To examine this, we performed Ex-4 dose-response experiments in HEK-GLP1R (Figure 2.2B and C) and INS1 (Figure 2.3A and B), both with GASP1-WT and GASP1-KO cells and measured the GLP-1R dependent increase in intracellular cAMP levels within these cells. The dose-response curves in the figures are representative of three different experiments performed on different days in triplicates. The E_{max} and EC_{50} data is represented as mean ± S.E.M. As expected, in both HEK-GLP1R and INS1 GASP1-WT cells, Ex-4 stimulation led to an increase in intracellular cAMP levels in dose-dependent manner. The Ex-4 dose-response curves show a characteristic sigmoidal shape where the highest Ex-4 concentrations provide maximum response. The E_{max} value (the maximum effect attributed to a drug) and EC_{50} (the potency of the drug) are calculated from the Ex-4 dose-response curves (Table 1 and 2). For HEK-GLP1R and INS-1 GASP1-WT cells, the cAMP stimulation at a highest Ex-4 dose

(100nM) was normalized to 100% E_{max} value and all other treatment groups were normalized to it. The EC_{50} for HEK-GLP1R and INS-1 GASP1-WT are EC_{50} = 2.09 nM ± 1.06 and EC_{50} = 0.59 ± 0.28 respectively. For both HEK-GLP1R GASP1-KO and INS1 GASP1-KO cells, interestingly Ex-4 treatment also exhibits a similar dose-dependent increase in intracellular cAMP levels as compared to the HEK-GLP1R GASP1-WT and INS1-GASP1-WT cells respectively. For HEK-GLP1R GASP1-KO cells, the E_{max} = 107.13% ± 6.88 and EC_{50} = 0.77 nM ± 0.04, and for INS1 GASP1-KO cells, the E_{max} = 95.14 % ± 3.26 and EC_{50} = 0.55 nM ± 0.34.

The EC_{50} value for HEK-GLP1R GASP1-WT and GASP1-KO cells (2.07 nM ± 1.06 and 0.77 nM ± 0.04 respectively) shows no significant difference, indicating that deletion of GASP1 does not affect the potency of Ex-4 drug towards GLP-1R. Similarly, for INS1 cells, knocking out of GASP1 has no impact on the potency of Ex-4 for GLP-1R (EC_{50} values for INS1 GASP1-WT vs GASP1-KO cells = 0.59 nM ± 0.28 vs 0.55 nM ± 0.26). Furthermore, on comparing the E_{max} value for HEK-GLP1R GASP1-WT vs KO cells (100% vs 107.13% ± 6.88), there is no significant difference between the two, indicates that GASP1 deletion does not attenuate the maximal cAMP response to acute Ex-4 stimulation. Similarly, for INS1 cells, knocking out GASP1 does not affect the efficacy of the acute Ex-4 stimulation (E_{max} values for INS1 GASP1-WT vs GASP1-KO cells = 100% vs 95.14% ± 3.26). These data indicate that deletion of GASP1 does not affect the acute GLP-1R signaling in both HEK-293 and INS-1 cells.

Pretreatment of HEK293 or INS1 cells with Ex-4 reduces GLP-1R-dependent cAMP signaling in GASP1-WT but not in GASP1-KO cells.

We next investigated the role of GASP1 protein on GLP-1R function and signaling after prolonged exposure to GLP-1R agonist Ex-4. To assess this, we again used the GASP1-WT and KO cell lines created above. Both GASP1-WT and GASP1-KO cells were pretreated with 100 nM Ex-4 for 3 hours, washed and then Ex-4 dose response experiments were performed to determine to measure the GLP-1R dependent increase in intracellular cAMP levels within these cells (Figure 2.2A). The

dose-response curves in the figures (Figure 2.2B and C, Figure 2.3A and B) are representative of three different experiments performed on different days in triplicates. The E_{max} and EC_{50} data is represented as mean ± S.E.M. As expected, in both HEK-GLP1R GASP1-WT and INS1 GASP1-WT cells, Ex-4 stimulation led to an increase in intracellular cAMP levels in dose-dependent manner.

The E_{max} value and EC_{50} are calculated from the Ex-4 dose-response curves (Table 1 and 2). For HEK-GLP1R and INS-1 GASP1-WT cells, the cAMP stimulation at a highest Ex-4 dose (100nM) was normalized to 100% E_{max} value and all other treatment groups were normalized to it. We found that following Ex-4 pretreatment both HEK-GLP-1R-WT and INS-1-GASP1-WT cells show reduced response to Ex-4. The E_{max} value for HEK-GLP-1R GASP1-WT cells with or without Ex-4 pretreatment are 100% vs. 33.61% ± 4.18, ***$p<0.001$. The E_{max} value for INS-1-WT cells with or without Ex-4 pretreatment are 100% vs. 44.30% ± 11.53, **$p<0.01$. This reduction in the response to the drug over time is referred to as development of tolerance to the drug. Thus, both HEK-GLP-1R GASP1-WT and INS-1 GASP1-WT developed tolerance to Ex-4 after prolonged exposure to the drug compared to vehicle pretreated cells.

Interestingly, GASP1-KO cells after Ex-4 pretreatment did not show development of tolerance compared to GASP1-KO vehicle pretreated cells. The E_{max} value for HEK-GLP-1R GASP1-KO cells with or without Ex-4 pretreatment is 107% ± 6.88 vs. 111.70% ± 12.8. The E_{max} value for INS-1 GASP1-KO cells with or without Ex-4 pretreatment are 95.14% ± 3.26 vs. 92.17% ± 4.32. These data suggest that GASP1 plays a crucial role in GLP-1R development of tolerance to Ex-4 response in HEK293 and INS-1 cells.

Moreover, for INS-1 GASP1-WT and GASP1-KO cells, there was no change in the potency of the drug with or without Ex-4 pretreatment as shown by the EC_{50} values. The EC_{50} value for INS-1 GASP1-WT cells with vehicle or Ex-4 pretreatment are 0.59 nM ± 0.28 vs. 0.78 nM ± 0.42. The EC_{50} value for INS-1 GASP1-KO cells with vehicle or Ex-4 pretreatment are 0.55 nM ± 0.26 vs. 0.76 nM ± 0.34. However, for HEK-GLP1R GASP1-WT and GASP1-KO cells, there is a change in the potency

of the drug. with or without Ex-4 pretreatment as shown by the EC_{50} values. The EC_{50} value for HEK-GLP1R GASP1-WT cells with vehicle or Ex-4 pretreatment are 2.09 nM ± 1.06 vs. 34.06 nM ± 4.19, **p<0.01. The EC_{50} value for HEK-GLP-1R GASP1-KO cells with vehicle or Ex-4 pretreatment are 0.77 nM ± 0.04 vs. 4.7 nM ± 2.19. This change in the EC_{50} value may be due to a change in the receptor number present on the cell membrane.

GLP-1R mediated insulin release is decreased in GASP1-WT cells upon Ex-4 treatment but not in GASP1-KO INS1 cells.

We next explore the functional significance of GASP1 protein on GLP-1R signaling and its function. To assess this, we measure the Ex-4 dependent insulin release from INS-1 cells (Figure 2.3C and D). We performed Ex-4 dose-response experiments in INS-1 GASP1-WT and INS-1 GASP1-KO cells with or without pretreatment with Ex-4 and measured the insulin release. The dose-response curves in the figures are representative of three different experiments performed on different days in triplicates. The E_{max} and EC_{50} data is represented as mean ± S.E.M. For vehicles treated INS-1 GASP1-WT cells, Ex-4 stimulation led to an increase in insulin release in a dose-dependent manner. The Ex-4 dose-response curves show a characteristic sigmoidal shape where the highest Ex-4 concentrations provide maximum insulin release. The E_{max} value and EC_{50} are calculated from the Ex-4 dose-response curves (Table 3). For INS-1 GASP1-WT cells, the insulin release at the highest Ex-4 dose (100nM) was normalized to 100% E_{max} value and all other treatment groups were normalized to it. The EC_{50} value for INS-1 GASP1-WT is 0.044 ± 0.01. The INS-1 GASP1-KO cell pretreated with vehicle also show a similar dose-dependent increase in insulin levels as compared to INS1-GASP1-WT cells (E_{max}: 99.98 ± 4.76 and EC_{50}: 0.034 ± 0.02). This suggests that knocking out GASP1 in INS-1 cells does not attenuate the E-4 maximal response to acute Ex-4 treatment nor does it affect the potency of the EX-4 toward GLP-1R in INS-1. INS-1 GASP1-WT cell pretreated with Ex-4 (100nM) for 3-hrs show reduced Ex-4 dependent insulin secretion response compared to vehicle pretreated INS-1 GASP1-WT cells (E_{max} value INS-1 GASP1-WT vehicle vs. Ex-

4 pretreatment: 100% vs. 56.65% ± 13.14, *p<0.01). This diminished response to Ex-4 is development of tolerance to the drug. Therefore, INS-1 GASP1-WT cells developed tolerance to Ex-4 after chronic exposure to the drug compared to vehicle pretreated cells. The $EC_{50\ values}$ for INS-1 GASP1-WT vehicle and Ex-4 pretreated cells: 0.044 nM ± 0.01 and 0.050 ± 0.02 respectively. However, INS-1 GASP1-KO cells pretreated with Ex-4 do not show reduced insulin secretion response compared to INS-1 GASP1-KO cells (E_{max} value INS-1 GASP1-KO vehicle vs. Ex-4 pretreatment: 99.97% ± 4.76 vs. 92.49% ± 3.34). Thus, INS-1 GASP1-KO cells do not develop tolerance to the Ex-4 even after chronic treatment indicating that GASP1 plays a crucial role in GLP-1R signaling and development of tolerance to Ex-4.

Limitation of the study and alternative interpretation

It is important to note limitations and alternative interpretations of the data presented herein. We have shown that GASP1 mediates the post-endocytic trafficking of GLP-1R in INS-1 cells and this GASP1-mediated post-endocytic degradation of the receptor is responsible for development of tolerance to prolonged treatment of GLP-1R agonist exendin-4. However, several limitations exist. While the data obtained from GASP1 knock out of INS-1 cells assert that GASP1 is necessary for development of tolerance, this study does not explain how GASP1 interacts with the GLP-1R and how this interaction affects receptors within the cytoplasm. Based on the proposed role of GASP1 in literature for other GPCR,[133-135] we hypothesized that GLP-1R interaction with GASP1 causes GASP1-mediated GLP-1R degradation via lysosomal degradative pathway. However, further investigation needs to be done to determine if GASP1 targets GLP-1R to lysosomal degradation or not and if some other mechanism involving GASP1 is in play here for the development of tolerance to exendin-4. Previous studies have shown that the number of receptors present on the cell membrane do affect the potency and the EC_{50} value of the drug.[153] Since GASP1 cause degradation of the GPCR receptor through lysosomal pathway, prolonged exposure of the drug may cause change in the cell-surface expression of the receptor which may impact the result.

Additionally, it is important to note that GASP1-mediate degradation is specific to certain GPCRs and not all GPCRs are affected in the same way.[132,134,137] Therefore, it may be possible that GASP1 is not directly involved in receptor degradation but serve as a linker protein to facilitate GLP-1R degradation. Indeed, GASP1 is known to interact with dysbindin, a member of ESCRT protein complexes that are well characterized for their role in post-endocytic receptor degradation.[213] Hence, we should be cautious in saying if GASP1 directly mediates GLP-1R degradation or does it serve only as a linker between GLP-1R and ESCRT machinery. Further research needs to be done to assess the compete role of GASP1 in GLP-1R degradation. Conversely, it is also not known if GASP1 interacts with the receptor at the plasma membrane or only transiently complexed with the receptor and may not be present at all in the endosomes. While it could be the case that transient interaction of GASP1 with GLP-1R is sufficient to drive receptor to lysosomal degradation, it remains unclear if GASP1 is present with the receptor in the endosome and how this interaction helps in prompting the receptor to lysosomal degradation.

This study focuses on GLP-1R in HEK293 and INS-1 cells that are β-cells derived from rat insulinoma. We have generated the GASP1-KO cells using CRISPR-Cas9 technique. Interpretation of data derived from CRISPR-Cas9 gene knockout experiments comes with several limitations. The major limitation is the off-target effect of CRISPR-Cas9 modification that may introduce unintended genomic modification in regions like target regions. These off-target effects can confound the observed phenotype. Another challenge is that cells might activate compensatory mechanisms in response to gene knockdown to maintain cellular homeostasis. In GASP1-KO cells, knocking down GASP1 may affect internalization of the GLP-1R that leads to accumulation of lots of dead/desensitize receptors on the plasma membrane. To maintain cellular homeostasis, there might be an increase in the transcription of GLP-1R mRNA and translation of the GLP-1R protein. This could influence the observed phenotype and make it difficult to isolate the direct effect of the

knocked-down gene. Experimentally, this hypothesis could be easily tested using qPCR technique to determine the mRNA expression level in GASP1-WT and GASP1-KO cells.

Methods

Cell Culture

Human Embryonic Kidney cells

Human embryonic kidney 293 (HEK293) cells were originally obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (Corning, MA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis MO). HEK293 cells were cultured under standard aseptic tissue culture conditions and maintained at 37°C in 5% CO_2 humidifier incubator. HEK293 cells were transfected with SNAP-GLP-1R plasmid (CisBio) using polyethylenimine (PEI) according to manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). The transfected cells were serially diluted and selected for G418 resistance marker. Individual cell colonies were picked up, expanded and screened for the expression of SNAP-GLP-1R on the surface via immunofluorescence staining.

Rat Insulinoma cells (INS-1 cells)

Rat insulinoma derived insulin producing INS-1 cells were purchased from AddexBio (C0018007). Cells were cultured using AddexBio optimized RPMI -1640 media (C0004-02) containing 11mM glucose and L-glutamine, supplemented with 10% fetal bovine serum (Corning, 35-010-CV) and 0.05 mM 2-mercaptoethanol (Sigma-Aldrich, M6250). INS-1 cells sub-cultured using 0.25% trypsin/EDTA (Corning, 25-053-CI) when 70-80% confluency was reached. INS-1 cells were cultured under standard aseptic tissue culture conditions and maintained at 37°C in 5% CO_2 humidifier incubator.

Human Embryonic Kidney 293T cells (HEK293T)

HEK293T cells were purchased from ATCC and cultured in DMEM media (Gibco, 11965-092) containing L-glutamine and 4.5 g/L D-glucose, supplemented with 10% FBS. HEK293T cells were

cultured under standard aseptic tissue culture conditions and maintained at 37°C in 5% CO_2 humidifier incubator.

CRISPR/Cas9 mediated gene editing

Single guide RNAs (sgRNA) targeting rat GPRASP1 (GASP1) gene were originally purchased from Applied Biological Materials (ABM, 22601116). The sgRNAs target three distinct sites of GASP1 gene and were cloned into a lentiviral vector system (pLenti-U6). Lentiviruses expressing Cas9 and sgRNAs were produced by co-transfecting HEK293T cells with sgRNAs and packaging plasmids (ABM, LV003) as per manufacturer's protocol. Supernatant containing lentivirus were harvested after 48hrs of transfection. The viral supernatant was filtered using 0.45-micron filter (Nalgene, 190-2545) to remove any HEK293T cells and concentrated by centrifuging at 25,000 RPM for 100 min. at 37°C. The viral concentrate was resuspended in complete RPMI – 1640 media and used for transduction of INS-1 cells. Hexadimethrine bromide (Polybrene, sc134220) is also added to the cells at 2 µg/mL concentration to enhance lentiviral infection efficiency. After 48hrs post-transduction, the cells were selected with puromycin (Invitrogen, A11138) at 2 µg/mL concentration for 7-10 days. After selection, the cells were allowed to grow until visible colonies were formed. Colonies derived from single cells were picked, expanded, and examined for GASP1 expression.

Validation of CRISPR/Cas9 editing proficiency

CRISPR/Cas9 editing efficiency was evaluated using immunoblot analysis (Figure 2.1). Proteins lysates were extracted from the single cell derived colonies by lysing the cells in RIPA lysis buffer (Thermofisher Scientific, 89900) containing one EDTA-free protease inhibitor mini tablet (Roche, 11836170001). Protein was quantified using a Pierce BSA assay (ThermoFisher, 23225) and read on a Flexstation-3 (Molecular Devices). Equal amounts of protein (60 µg) were resolved on an 10% acrylamide SDS-SDS-PAGE gel and transferred to PVDF membrane (Bio-Rad, 1620177) as per standard procedure. The membrane was probed for GASP1 expression with rabbit polyclonal anti-GASP antibody (1/1000 dilution, design, developed and produce by Whistler)[214]. GAPDH was used

as a loading control (Invitrogen, MA5-15798). Immune complexes were detected using IRDye® conjugated secondary antibodies-goat anti-rabbit 800CW (Odyssey, 926-32211) and goat anti-mouse 680LT (Odyssey, 926-68020) respectively. The images were developed using the Odyssey CLx Li-Cor system.

Intracellular cAMP homogenous time-resolved fluorescence (HTFR) Assay

HEK-GLP-1R and INS-1 cells were seeded in a 384-well white, low volume, flat bottom plate (Fisher scientific, 781981) at 7 x 10^3 cells/well and incubated overnight in AddexBio optimized RPMI-1640 media. After overnight incubation, cells were washed twice with PBS and pretreated either with vehicle (media) or 100 nM exendin-4 (Tocris, 1933) for 3 hrs. Following pretreatment, cells were stimulated with either vehicle (PBS) or increasing concentration of exendin-4 diluted in stimulation buffer (PBS) containing 100 µM of IBMX (Sigma-Aldrich, I5879) for 30 minutes at 37°C. After stimulation, cells were lysed and intracellular cAMP was measured using HTRF immunoassay (CisBio - cAMP Gs dynamic kit, 62AM4PEB) according to manufacturer's instructions and measured using Flexstation-3 (Molecular Devices) (Figure 2.2A). The concentration of cAMP (nM) in samples were extrapolated from cAMP standard curve. The results were expressed as cAMP dose-response curves fitted using non-linear regression – three parameter curves in GraphPad Prism 9.

Insulin secretion assay in INS-1 cells

INS-1 cells were seeded in a 384-well white, low volume, flat bottom plate (Fisher scientific, 781981) at 7 x 10^3 cells/well and incubated overnight in AddexBio optimized RPMI-1640 media. After incubation, cells were washed twice with KRB buffer (130 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 Mm KH_2PO_4, 25 mM $NaHCO_3$, 20 Mm Hepes pH7.4) and glucose starved with RPMI media containing 10% FBS and 1 % penicillin/streptomycin for 3 hrs. During starvation, cells were either pretreated with vehicle (media) or 100 nM exendin-4 (Tocris, 1933) for 3 hrs. After glucose starvation and pretreatment, cells were washed with KRB buffer and recovered in RPMI media containing 5.5 mM glucose for 15 min. Following recovery, cells were stimulated with 11 mM glucose

± increasing concentration of exendin-4 in Kreb's-Ringer Bicarbonate buffer (KRB buffer) containing 0.1% w/v BSA for 30 min. After treatment, supernatant containing secreted insulin was collected and transferred into a new 384-well plate. The insulin concentration was determined using insulin HTRF assay kit (CisBio, 62IN1PEG) according to manufacturer's instructions and measured using Flexstation-3 (Molecular Devices). The amount of insulin (ng/mL) secreted in samples were extrapolated from insulin standard curve. The results were expressed as insulin dose-response curves fitted using non-linear regression – three parameter curves in GraphPad Prism 9.

Figures

Diagrams were created with BioRender.com.

Data Analysis

Data were analyzed using GraphPad Prism9 software (GraphPad Inc., San Diego, CA) and expressed as mean ± S.E.M. as indicated in figure legends. Differences between the two groups were assessed by appropriate two-tailed unpaired Student's *t*-test. Differences among three or more groups were assessed by one-way ANOVA with Tukey's post hoc test. $P < 0.05$ was considered statistically significant. For the E max and EC 50 determination, vehicle pretreated WT-cells treated with 100nM Ex-4 was set at a reference value of 100%. All other samples were normalized to this treatment. The normalized data is then used to determine the Emax and EC50 value using GraphPad prism version 9.

Chapter 3: *Ex vivo* investigation of the role of GPCR-associated sorting protein 1 (GASP1) on GLP-1R signaling.

Introduction

Pancreatic islet is a mini endocrine organ responsible for glucose metabolism, regulation, and control in the body. They are composed of a variety of endocrine and support cells, including the insulin-secreting β-cells, glucagon (GCG)-secreting α-cells, somatostatin (SST)-secreting δ-cells, ghrelin-secreting epsilon (ε) cells and pancreatic polypeptide (PP) cells. In human islets, these cells are in heterogenous mixture in varying proportions. In contrast, in rodent islets, β-cells are predominantly present at the core surrounded by α- and δ- cells in the mantle.[215-217] Each of these cell types is responsive to many endocrine, paracrine, nutritional, and neural inputs, that shape the final insulin output of the islet (Figure 3.1). Full insulin secretion *in vivo* requires glucose, but the effect of glucose is potentiated by the combined actions of other nutrients, endocrine and paracrine factors. Disruption of this highly regulated paracrine network or dysfunction of any cell type contributes to impaired glucose regulations and development of diseases like T2D.[218,219]

In response to high blood glucose levels, β-cells uptake glucose and metabolize it to generate ATP. This increases the ATP/ADP ratio with the cells causing the closure of ATP-sensitive-K^+ channels leading to membrane depolarization. This stimulates the voltage-dependent calcium channels (VDCC) leading to firing of an action potential and rise in intracellular calcium, thus causing insulin exocytosis.[142] Additionally, GLP-1 release from gut post-prandially, also activates the GLP-1R on the β-cells to further potentiate insulin synthesis and release. GLP-1 exerts its action only under the condition in which glucose-mediated Ca^{2+} influx trigger insulin secretion, thus preventing incretin to promote insulin secretion in hypoglycemic state (Figure 3.2).[72]

During hyperglycemia, glucagon releases moderately from α-cells such that it does not raise glucagon blood circulation level but still can potentiate GSIS from β-cells without increasing hepatic glucose production.[220] Additionally, α-cells also release GLP-1, corticotropin-releasing hormone, and

acetylcholine which stimulate β-cells via their cognitive receptor to potentiate insulin secretion. β-cells express both glucagon receptor (GCGR) and GLP-1R and glucagon can activate either of them to stimulate insulin release. Both GCGR and GLP-1R are GPCR that activate Gαs-mediated downstream signaling cascade leading to potentiation of insulin release. An important point to note is that this potentiation only occurs under hyperglycemic conditions where β-cell membrane depolarization led to influx of Ca^{2+} that trigger insulin release. This ensures that during hyperglycemia, glucagon should not trigger glucose production from liver, instead it only stimulate β-cells to enhance insulin secretion to decrease glucose levels.[221] Studies by different groups have shown that either deleting GCGR or GLP-1R from β-cells or using antagonist to block GCGR/GLP-1R activation led to significant reduction in insulin release in response to nutrient stimulations, thus suggesting the paracrine stimulation of β-cells by α-cells.[222,223] Insulin secreted from β-cells also acts on nearby alpha cells and activate the insulin receptor (IR) on them to stimulate PI3K-Akt pathway and GABA leading to Cl-ion influx and inhibition of action potential firing (Figure 3.2). This decreases glucagon secretion to suppress hepatic glucose production and maintain an anabolic state after feeding. A study with glucagon-cre driven insulin receptor knockout (IRKO) mice show that during fed state, α-cell IRKO mice developed hyperglucagonemia, while in fasting state the glucagon response in attenuated showing that insulin have a role in regulation of glucagon secretion.[224-226] During hypoglycemia, glucagon released enhances as a part of the counterregulatory response and it will not stimulate insulin secretion. In addition, glucagon stimulates hepatic glucose production to fend off hypoglycemia via gluconeogenic and glycogenolytic effects on liver.

In response to glucose, δ-cells release somatostatin like insulin secretion from β-cells. Somatostatin is a robust inhibitor of both insulin and glucagon secretion.[227] It has been shown that the response of β-cells and δ-cells to glucose is pulsatile and near-synchronous suggesting that the coordination between β-cells and δ-cells is mediated by gap-junctions.[228] However, glucose stimulated Ca2+ response is asynchronous between β-cells and δ-cells suggesting a less immediate

paracrine crosstalk between the two cell types.[229] One such mechanism is the co-production of urocortin-3 hormone by β-cells along with insulin release. UCN3 activates type 2 CRH receptor (CRHR2) on pancreatic δ-cells. CRHR2 are also class B GPCR that stimulates release of somatostatin from δ-cells.[230] Somatostatin release within the islet activates a somatostatin receptor subtype 2 (SSTR2) on β-cells that inhibit release of insulin from β-cells. SSTR2 is a GPCR. Upon ligand-dependent activation, SSTR2 activates Gi-protein which inhibits adenylyl cyclase activity leading to inhibition of Ca^{2+} release. This cause membrane hyperpolarization and reduction in exocytosis. The negative feedback mechanism provided by somatostatin is critical to restrain insulin release to counterbalance nutrient stimulation. This negative feedback inhibition ensures that insulin release is attenuated in a timely manner once normal glycemia is achieved thus preventing hypoglycemia (Figure 3.2).[231] In addition to inhibition of insulin release, somatostatin also inhibits glucagon release. During low glucose condition, glucagon is released from α-cells to increase hepatic glucose production. Somatostatin exhibits paracrine inhibitory effect on α-cells under hypoglycemic conditions. Somatostatin receptor antagonists have been shown to increase glucagon release in low glucose condition thus showing paracrine crosstalk between α-cells and δ-cells. During hyperglycemic conditions, glucagon is released in modest quantity and stimulates GSIS from β-cells. Somatostatin dampens this glucagon-dependent GSIS response by suppressing glucagon release from α-cells. [232-235]

Taken together, it is well established that there is rich paracrine crosstalk between major cell types within pancreatic islets that modulate glucose homeostasis. Under hyperglycemic condition, β-cell responds to increase glucose uptake by release of insulin and urocortin-3 hormones. This glucose stimulated insulin secretion helps in decreasing blood glucose levels by uptake of glucose from other cells. Alpha cells modestly produce glucagon which do not increase hepatic glucose production but stimulate β-cells for GSIS. The urocortin-3 released by the β-cells stimulates δ-cells to secrete somatostatin that provides a negative feedback inhibition to insulin and glucagon secretion by

β- and α-cells respectively. This paracrine crosstalk has potential pathophysiological relevance for T2D patients. The hyperglycemic condition during T2D could cause excess release of glucagon which in turn contributes to hyperglycemia. Recent studies have demonstrated that in T2D the paracrine inhibition of α-cells by somatostatin is compromised suggesting a malfunction of this complex interplay of paracrine signals.[236]

Most of the hormones released in pancreatic islet exert their paracrine effect via G protein coupled receptors (GLP-1R, GCGR, SSTR etc).[237] Despite such in-depth knowledge of the various crosstalk's between islet cell-type as well as how the signaling of these GPCRs affect GSIS, very little is known about the post-endocytic trafficking of these receptors within the cells. GPCRs signaling and its functions are regulated by its pos-endocytic trafficking. In the previous chapter, we have elaborated a novel mechanism by which GASP1-mediated post-endocytic degradation of GLP-1R in INS-1 cells affect receptor signaling and efficiency. In this chapter, we extend this hypothesis to pancreatic islets. We aim to investigate whether GASP1 mediates post-endocytic degradation of GLP-1R in intact islets. We further wanted to explore how this GASP1-mediated post-endocytic trafficking of GLP-1R in intact isles affects GSIS in response to acute vs. chronic Ex-4 drug administration. To assess this, we generated beta-cell specific GASP1 knockout mice (β-GASP1-KO) to determine the role of GASP1 in GSIS from intact islets. Our data shows that GASP1 knockdown from pancreatic β-cells does not change either glucose-dependent insulin secretion or incretin-mediated GSIS after acute treatment in intact islets. However, after chronic Ex-4 treatment, GASP1-WT islets show tolerance development to Ex-4 while β-GASP1-KO islets do not so such tolerance suggesting that GASP1 play a significant role in post-endocytic trafficking of GLP-1R in pancreatic islets β-cells.

Results

Deletion of GASP1 protein in pancreatic beta (β) cells alters the relative mRNA expression of GLP-1 receptor and Insulin (Ins) gene in islets.

Next, we investigate the role of GASP1 protein in GLP-1R signaling *ex vivo* within mouse islets. To evaluate this, we generate two mouse lines: Flox-GASP1 mouse and beta-cell-specific GASP1 knockout mice (β-GASP1-KO) using the Cre-LoxP system.[239] To generate Flox-GASP1 mouse, a targeting vector was constructed using the GASP1 locus on mouse X chromosome in which G418 antibiotic resistant sequence was flanked by lox P sites (Lox P site 1 and 2) and was inserted in the intron upstream of GASP1 ORF. Additionally, a third lox P site (Lox p site 3) was inserted downstream of the GASP1 ORF. The targeting vector electroporated into the ES cells of C57/BL6 mice and clones were selected by G418 resistance. Homologous recombination into the GASP1 locus was detected by southern blotting and individual clones were incubated with Cre recombinase (Figure 3.3). Clones where only lox P sites 1 and 2 were recombined were identified by PCR screening and were used to create flox-GASP1 mice. To generate a β-GASP1-KO mouse we use the following two mouse lines: Flox-GASP1 (fl-GASP1) mouse and UCN3-Cre mouse.[240] We crossed the homozygous female fl-GASP1 mouse to heterozygous male mouse with Cre-recombinase driven by the beta-cell specific urocortin-3 gene promoter (UCN3-Cre), producing mice with islet β-cells specific knock out of the GASP1 (β-GASP1-KO). The offspring were genotyped and mice carrying both the UCN3-Cre and flox-GASP1 gene were used in the experiments (Figure 3.4). Mouse carrying either *UCN3-Cre* or *floxed* GASP-1 gene were used as WT controls. The successful deletion of GASP1 gene was confirmed through qPCR analysis of isolated islets from GASP1-WT and β-GASP1-KO mice. The qPCR analysis revealed a significant reduction in GASP1 mRNA expression in β-GASP1-KO islets (**$p<0.01$) compared to GASP1-WT islets. Moreover, the qPCR shows a significant increase in insulin gene mRNA expression in β-GASP1-KO islets compared to GASP1-WT islets, indicating increase in insulin synthesis in β-GASP1-KO islets. Interestingly, the deletion of GASP1

also led to a significant reduction in GLP-1R gene expression in β-GASP1-KO islets (*p<0.05) compared to GASP1-WT islets, possibly because GLP-1R synthesis needs are reduced due to prevention of post-endocytic GLP-1R degradation (Figure 3.5).

β-GASP1-KO islets but not GSAP1-WT islets retain their GLP-1R mediated insulin secretion after chronic exposure to Ex-4.

Following GASP1 knockout confirmation, we performed a longitudinal islet insulin secretion assay using isolated islets from GASP1-WT and β-GASP1-KO mice. The islets were subjected to different conditions: acute treatment, a 3-hour pretreatment with Ex-4, and a 24-hour recovery period (Figure 3.6A). Both GASP1-WT and β-GASP1-KO islets displayed a robust increase in insulin secretion when stimulated with a high glucose concentration of 11mM compared to basal glucose level of 5.5 mM (Figure 3.6B and C, white vs black bars, white vs magenta bars, respectively, left panel). Furthermore, stimulation of both GASP1-WT and β-GASP1-KO islets with high glucose and 100nM Ex-4 (acute) show significant enhancement of insulin release compared to high glucose alone (Figure 3.6B and C, black vs teal bars, magenta vs orange bar, left panel). This enhanced insulin release following Ex-4 stimulation is commonly known as the "Incretin effect." After a 3-hr Ex-4 pretreatment, GASP1-WT islets displayed a reduced insulin secretion response indicating the development of tolerance to prolonged Ex-4 exposure (Figure 3.6B, middle panel, black vs teal bar). In contrast, β-GASP1-KO islets maintained their incretin effect even after 3-hrs of Ex-4 pretreatment as shown by a significant enhancement of insulin release upon Ex-4 treatment (Figure 3.6C, middle panel, magenta bar vs orange bar). Importantly, both GASP1-WT and β-GASP1-KO islets displayed a substantial incretin effect after a 24-hour recovery period, suggesting that the observed tolerance effect is not due to any inherent unhealthiness of the islets (Figure 3.6B and C right panel). These findings indicate that GASP1 plays a crucial role in regulating GLP-1R signaling and the development of tolerance to Ex-4 stimulation in mouse islets.

Limitation of the study and alternative interpretation

In pancreatic islets, a complex web of paracrine interactions exists between the endocrine cells, thus regulating the activities of β- and α-cells in both hypoglycemia and hyperglycemia conditions. This interplay is so intertwined with nutrient stimulating insulin and glucagon release, it becomes challenging to separate nutrient-driven responses in β-cells from the concurrent activation of paracrine pathways. The main take home message and one limitation of this data is to acknowledge the fact that GSIS is a net result of not only glucose stimulation of β-cells but also an amalgamation of paracrine actions involving stimulation by α-cells and inhibition by δ-cells. Therefore, it is hard to isolate the contribution of α- and δ-cells from β-cells using this data set. Furthermore, detailed investigations are needed to be done to truly determine the effect of GASP1 knock out in pancreatic β-cells only and how this knockout affects the response of β-cells toward GSIS.[240]

Moreover, although using Cre-Lox knockout mice is a powerful tool to study gene function, there are limitations associated with this technique. The effectiveness of the Cre-lox system depends on complete deletion of the gene (as opposed to some exons) or deletion of the gene in all cells. In our data, we use qPCR to analyze GASP1 mRNA deletion from whole islet rather than isolated beta-cell. Within the islet, the UCN3 promoter is robustly activated in pancreatic β-cells,[241] therefore it is unlikely but incomplete gene deletion can lead to residual gene activity or truncated proteins which make it difficult to ascertain the true impact of the gene knockout. This limitation can be overcome if we isolate the β-cells from WT and KO islets and compare the relative expression of GASP1 between them. Additionally, the Ucn3-Cre is a BAC transgenic, which essentially acts as a large, randomly inserted piece of DNA that consists of a 200kb piece of the chromosome that contains the Ucn3 promoter, with Cre inserted instead of the Ucn3 coding region. The BAC transgene is integrated somewhere randomly at an unknown site. This can lead to an off-target effect which may cause deletion of genes that are not intended to be knocked out. This can complicate interpretation of the data thus obtained.

In our study, qPCR analysis data also show that deletion of GASP1 in islets decreases the GLP-1R mRNA expression. This makes sense because if the cells are recycling the receptors and are not able to degrade them, they do not need to synthesize more receptors as they can recycle and re-use the same receptors. However, an alternative interpretation of the data is since GASP1 KO alters the transcription of GLP-1 receptor within pancreatic β-cells, this decrease in GLP-1R expression may lead to compensatory mechanisms by other related genes like GLUT and insulin synthesis gene thus masking the true phenotype of the GASP1 KO in β-cells. Indeed, in our qPCR analysis we see an increase in mRNA expression of insulin gene. This could be due to enhanced and sustained GLP-1R signaling as by knocking out GASP1, we abolish GASP1-mediated degradation of the GLP-1R or could be a homeostatic adaptation of the cell due to alteration in the GLP-1R transcription.

Ex vivo islets studies involve isolating pancreatic islets from animals and studying their function and behavior outside of their natural environment. While these studies offer valuable insights into islet biology, one should acknowledge the limitations of the procedure. The process of isolating the islets from pancreas can be stressful and potentially lead to cell damage, changes in gene expression and altered physiology which do not represent their native state accurately. Isolated islets are cultured for experimental procedures that can lead to short-term culture effects including dedifferentiation, loss of cell-cell contact and change in gene expression all of which can impact islet biology and function. Moreover, *ex vivo* studies capture a snapshot in time and do not represent the full dynamic changes that occur in response to chronic conditions. Finally, the *in vivo* environment of the pancreases is more complex than the *ex vivo* settings. Factors like neural inputs, blood flow and hormonal fluctuations can impact the GSIS in ways that might not be fully replicated in isolated islets. Therefore, to mitigate these limitations, we combine ex *vivo* studies with in *vivo* experiments. Therefore, in our next chapter, we investigate the role of GASP1 on GLP-1R signaling *in vivo* using mouse model.

Methods

Mice

Mice were bred in-house in UC Davis AAALAC certified vivarium and handled according to National Institute of Health guidelines for the care and use of laboratory animals. All the protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at University of California, Davis. Adequate measures were taken to minimize animal suffering and discomfort. Mice were housed in cages (typically 3-5 per cage) in temperature and humidity controlled rooms with 12:12 hrs light:dark sleep cycle and provided with food and water *ad libidum* all the time. Only male mice were used for the study. Animals were grouped without blinding but were randomized during experiments. Groups were spread across multiple cages to minimize cage effects.

Generation of Flox-GASP1 (fl-GASP1) conditional knockout mice

Gprasp1 locus on the mouse X chromosome. A targeting vector was designed with a neomycin (G418)-resistance gene flanked by loxP sites inserted into intron 4 upstream of the GPRASP1 gene and a third loxP site inserted downstream of the GASP1 open reading frame (Supp Fig. 1B). The linearized targeting vector was electroporated into ~10^7 C57BL/6 ES cells and clones were selected with 200 µg/ml G418. ES cells with homologous recombination of the targeting vector (Supp. Fig. 1C) were determined by southern blot. These were treated with Cre recombinase and clones where loxP sites 1 and 2 were recombined were identified by PCR (Figure 3.3).[238] Clones were implanted into C57/Bl6 mothers and germline transmission of the fl-GASP1 conditional KO gene was confirmed by breeding. Genotyping is performed with a set of 3 primers:

WT forward primer: 5’ – GAGTGACTACTGTGAGACTTGG – 3’

GASP1-KO forward primer: 5’ – GTGAACTGAGCCGTTGTAAATAAGATGC – 3’

Common reverse primer: 5’ – CATCTCTTCGATTTATAGTTCTCCCACC – 3’

Generation of β-cell specific GASP1 knockout mice

To generate pancreatic β-cell specific GASP1 knock out mice (β-GASP1-KO) we bred floxed GASP1 mice (supp fig. 1) to *UCN3-Cre* driver mice. GASP1 is an X-linked gene. Female homozygous floxed GASP1 mice ($GASP1^{fl/fl}$) were crossed with male heterozygous *UCN3-Cre* driver mouse ($UCN3^{Cre/+}$). Male littermates from this cross were genotyped for the floxed GASP1 allele and the *UCN3-cre* transgene using tail DNA. Mice with and without *UCN3-Cre* transgene were used in all experiments (Figure 3.4).[239] The following primers were used for genotyping of these mice (5' – 3' sequence):

GASP1:

WT forward primer: 5' – GAGTGACTACTGTGAGACTTGG – 3'

GASP1-KO forward primer: 5' – GTGAACTGAGCCGTTGTAAATAAGATGC – 3'

Common reverse primer: 5' – CATCTCTTCGATTTATAGTTCTCCCACC – 3'

UCN3:

Forward: 5' – CGAAGTCCCTCTCACACCTGGTT – 3'

Reverse: 5' – CGGCAAACGGACAGAAGCATT – 3'

Pancreatic Islet isolation

Mouse pancreatic islets were obtained from 10-12 weeks old C57BL/6 WT or β-GASP1-KO mice. Islets were isolated by euthanizing the mice and clamping the duodenum with a bulldog clamp where the pancreatic duct terminated in the ampulla of Vater. Using a 30G needle, 2ml per mouse of with HBSS (no Ca^{2+} or Mg^{2+}, Gibco, 14170-112) containing 0.8 mg/mL collagenase P (Roche, 11249002001) were infused in the pancreatic duct. The pancreata were dissected out in a 15 ml tube and digested at 37°C for 13 min in a water bath. The tubes were shaken >=10x to complete the digestion. The digested pancreas is then washed 2-3 times with cold HBSS + 5% NCS + 1 mM $CaCl_2$. The islets were subsequently isolated using a Histopaque gradient (Sigma, 10771). The isolated islets were further purified by picking twice into fresh HBSS + 5% NCS (Gibco, 16010-167) +

1mM $CaCl_2$ (Sigma-Aldrich, C5080) and then a third time into a fresh petri dish with 15 ml of RPMI + pen/strep + 10 % FBS + 5.5 mM glucose. After a few hours the islets were picked again into a fresh of RPMI + pen/strep + 10 % FBS + 5.5 mM glucose to reduce lamping. Isolated islets were allowed to be recovered overnight in RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin (Gibco, 15140-122) before using them for experiments.

mRNA extraction and cDNA synthesis

Total RNA were extracted from isolated WT or β-GASP1-KO islets using phenol:chloroform:isopropanol extraction method. Briefly, islets were collected into an RNase-free microcentrifuge tube containing 500 µL Trizol reagent (Invitrogen,15596018) followed by addition of 100 µL of chloroform (Fisher Scientific, C298-500). The tubes were shaken for 20 sec and centrifuged at 12,000g for 15 min at 4°C. The clear top aqueous phase containing RNA was collected into a new RNase-free tube. RNA was precipitated by adding 250 µL of isopropanol (Fisher Scientific, BP2618500) and centrifuging the tube at 12,000g for 10 min at 4°C. The RNA pellet was then washed with ethanol, air dried and re-suspended in RNase free water. The cDNA was synthesized from extracted RNA by reverse transcription using high-capacity RNA-to-cDNA™ kit (Applied Biosystems,4368813) according to manufacturer's instructions.

Quantitative real-time PCR

cDNA synthesized using RNA extracted from WT or β-GASP1-KO islets were used to determine GASP1, GLP-1R and Ins gene expression in WT or β-GASP1-KO islets using quantitative real-time PCR (qPCR). The cDNA was amplified using PowerUp™ SYBR™ green super mix (Applied Biosystems, A25742) through 30-40 cycles of qPCR. For qPCR reaction we used 0.5 µM forward and reverse primer. The following primers were used for the qPCR reactions.

For GASP1,

Forward primer: 5' – TGGTTCTGGGCAGATGATGAAGAGA - 3'

Reverse primer: 5' – TTGTTGCTTTTGTAGATGCCGACC - 3'

For GLP-1R,

Forward primer: 5’ – CCCTGGGCCAGTAGTGTG – 3’

Reverse primer: 5’ – GCAGGCTGGAGTTGTCCTTA – 3’

For Ins,

Forward primer: 5’ – GCAGCCTTTGTGAACCAACA – 3’

Reverse primer: 5’ - CGTTCCCCGCACACTAGGTA – 3’

For HPRT – Housekeeping gene

Forward primer: 5’ – TCCTCCTCAGACCGCTTTT – 3’

Reverse primer: 5’ – GCAGGCTGGAGTTGTCCTTA – 3’

Quantification was performed using the 2^(ΔΔCt) method. Data were normalized to endogenous housekeeping gene control (hypoxanthine phosphoribosyltransferase 1, HPRT gene) and expressed as relative gene expressions compare to WT control islets.

Mouse islets glucose stimulated insulin secretion (GSIS) assay

Following overnight recovery, mouse islets were picked twice in KRB buffer (130 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 Mm KH_2PO_4, 25 mM $NaHCO_3$, 20 Mm Hepes pH7.4) supplemented with 0.1% BSA and 5.5 mM glucose. The islets were placed in a 48-well plate and glucose starved for 1 hr at 37°C in KRB + 0.1% BSA + 5.5 mM glucose. The islets were then stimulated for 1 hr at 37°C with KRB buffer supplemented with low glucose (5.5 mM), high glucose (11 mM) and high glucose + 100 nM Ex-4 (Tocris, 1933). After 1hr, supernatants were collected for insulin detection (Acute, Figure 3.6A). The islets were then treated with 100 nM Ex-4 diluted in complete RPMI-1640 media for 3 hrs at 37°C. Following Ex-4 treatment, the islets were washed before stimulation with KRB buffer supplemented with low glucose, high glucose and high glucose + Ex-4 for 1 hr. After an hour the supernatants were collected for insulin detection (Tolerance, Figure 3.6A). After testing for Ex-4 tolerance, the islets were placed in RPMI – 1640 complete media and allowed to recover overnight to test for islets dysfunction. After overnight recovery, the islets were

again glucose starved for an hour followed by stimulation with KRB buffer supplemented with low glucose, high glucose with or without Ex-4 for 1 hr. The supernatants were collected after incubation for insulin detection (Recovery, Figure 3.6A).

Insulin measurement from GSIS islet study and mouse plasma

The plasma insulin secreted from islets were measured using Lumit Insulin Immunoassay kit (Promega, CS3037A05) as per the manufacturer's instructions and measured using Flexstation-3. The concentration of insulin in each sample was extrapolated from an insulin standard curve. The results were expressed as insulin concentration using GraphPad Prism 9.

Figures

All the figures were created with Biorender.com.

Statistics

Data are presented as means ± SEM and the number of experiments is indicated in every case. GraphPad prism version 9 was used to perform all statistical analysis. The student's t-test or two-way analysis of variance (ANOVA) with Tukey's multiple comparison test was performed in GraphPad prism to detect statistical differences. $P < 0.05$ was considered statistically significant.

Chapter 4: *In vivo* investigation of the role of GPCR-associated sorting protein 1 (GASP1) on GLP-1R signaling.

Introduction

Glucose is a simple carbohydrate used to store energy in mammalian cells. Its molecular formula is $C_6H_{12}O_6$. It is a monosaccharide (a sugar monomer) that can form glycosidic bonds with other sugar molecules. Glucose has a hexose structure in the form of a pyranose ring with an aldehyde group and several hydroxyl groups.[242] It is a small molecule that is rich in chemical energy due to which it is well suited to store and move potential energy between the cells. The chemical rich nature of glucose is due to the stability of carbon atoms and their ability to bond with many other elements. The ring structure of glucose is optimal for energy storage in humans due to the large number of C-H bonds.[243] The glucose is converted into energy within the cell by the process of cellular respiration, where cell break down glucose into energy in form of ATP, NADH and pyruvate. Since glucose is central for energy needs of the body, glucose concentration is highly regulated and is known as glucose homeostasis.[244] Maintaining glucose homeostasis within the body is critical for overall homeostasis and dysregulation of glucose homeostasis can lead to development of diseases like T2D.

While liver and kidney synthesize some glucose, humans require glucose ingestion to fulfill most of the demand of the molecule needed for survival. All carbohydrates we ingested break down into simple sugar like glucose in the gut from where it is rapidly absorbed in small intestine and circulated though the blood stream. Normally, plasma glucose levels are maintained within a relatively narrow range of 4.0 – 9.0 mM.[245] However, post prandial (after meal ingestion), the plasma glucose level increases rapidly. Hyperglycemia is not good and should be avoided because of its potential macro and microvascular complications including retinopathy, neuropathy, nephropathy, and increased risk of cardiovascular diseases.[246] Conversely, our body should also not have low blood glucose (hypoglycemia) because it can cause injury to the brain. Glucose is the main energy source

to the brain. The brain cannot produce or store glucose, therefore depends on plasma glucose for its survival. If plasma glucose levels decrease as little as 1.2 – 3.8 mM, this will affect brain glucose uptake and may cause brain injuries.[247]

One way of maintaining plasma glucose homeostasis to avoid either hyper or hypoglycemia is through endocrine hormones secreted in response to low/high glucose concentration (Figure 4.1). After fasting for 12-16 hours, plasma glucose concentration reaches a steady state where the rates of release of glucose into the blood stream are approximately equal to rates of glucose removal. [248] However, if fasting is prolonged, by 20-24 hours plasma glucose levels gradually decrease to 10-15 %. This causes a hunger response and signals to hypothalamus via hunger hormones ghrelin. Ghrelin is a gut hormone that regulates energy homeostasis by sending information to hypothalamus about the nutritional needs of the body. It primarily signals hypothalamus in response when food intake needs to be increased, thus increasing hunger and appetite.[249] Once the nutritional need of the body is fulfilled, another hormone leptin is released by adipocytes and signal hypothalamus via neuropeptide Y to promote satiety and maintain energy homeostasis.[250] Because of their role in maintaining energy homeostasis and maintaining satiety and hunger balance, leptin and ghrelin and their receptor are therapeutic targets for drug development in managing diseases like obesity and T2D.

Post prandial, the assimilation of the nutrients is completed within 5 hours. Various factors affect the plasma glucose concentration after meal including time, degree of physical activity, meal composition, rate of gastric emptying, digestion, and absorption within the gut and most importantly the inhibition of glucagon hormone and release of insulin hormone by the pancreas.[251] Through the release of insulin and glucagon, pancreas regulates blood glucose levels. After meals, when blood glucose level is high, pancreatic β-cells release insulin in glucose-dependent manner. Insulin decreases blood glucose by increasing uptake of glucose by skeletal muscles, adipose tissue, splanchnic organs, blood cells and other cells in the body via glucose transporters GLUTs where

glucose is oxidized and used as a fuel for energy.[252-254] Additionally, it also promotes triglyceride synthesis by stimulating glucose and fatty acid uptake by adipocytes.[255] Insulin also increases glycogenesis where glucose is stored as glycogen in liver and muscles. Glycogen is a highly branched molecule, insoluble in nature and does not interfere with other cellular reactions. Because of these characteristics, glycogen is the primary form in which glucose is stored within the body.[256,257] When blood glucose levels are low (< 4 mM/L), glucagon release from pancreas promotes hepatic glycogenolysis and gluconeogenesis, which results in increase blood glucose.[258]

Glucose homeostasis is a critical function that involves complex nervous and endocrine system interaction. This is accomplished through the release of pancreatic and gut hormones to target cerebral, hepatic, renal, and adipose tissue. All these players complex interaction is essential for the final blood plasma glucose concentration and impairment of these hormones results in metabolic diseases like T2D. In this chapter, we will investigate the role of GASP1 on GLP-1R signaling in pancreatic β-cells and how it affects the glucose homeostasis in the mouse model. We will be using the β-GASP1-KO mice generated above for a 9 weeklong longitudinal study. In this study, the mice were given chronic Ex-4 treatment for 7 weeks and examined for their oral glucose clearance following oral glucose gavage and plasma insulin levels to determine if knocking out GASP1 in pancreatic β-cells influence glucose clearance and insulin release in whole animal.

Results

Wild-type mice develop tolerance toward incretin drugs.

We next investigate the role of GASP1 protein in the development of tolerance to chronic Ex-4 treatment in both WT and β-GASP1-KO mice *in vivo* (Figure 4.2 – 4.6). We used the GASP1-WT and β-GASP1-KO mice generated above. We implemented a 9-week long longitudinal paradigm (Figure 4.2A) and assayed both glucose clearance post-oral gavage (Oral glucose tolerance test, OGTT, Figure 4.3 and 4.6) and glucose-stimulated insulin secretion (GSIS, Figure 4.2 and 4.5) at multiple timepoints in WT mice treated with chronic saline or Ex-4. The timeline for the longitudinal paradigm

and different time points at which glucose monitored and blood was collected is shown in figure 4.2A. The mice were housed in their home cages in groups of 3-5 animals per cage and allowed to have access to water and food *ad libitum*. The mice were entered into the paradigm when they were 8 weeks old.

At week 1 (Baseline), we performed glucose-stimulated insulin secretion (GSIS) and OGTT following glucose gavage (2g/kg) in WT mice. We observed significant increases in plasma insulin concentration in response to oral glucose gavage, indicating WT mice showing robust GSIS (Figure 4.2B, solid vs hatched bars, **p<0.01). Blood glucose levels were also measured at different time-points (as shown in figure 4.4) after oral glucose administration (2g/kg body weight). The glucose was also cleared within 120 minutes as shown in figure 4.3B. Following week 1(baseline), the mice were categorized into two groups based on their treatment: WT Saline (black, n=4) and WT Ex-4 (teal, n=4).

In week 2 (Acute), we assessed the acute effect of Ex-4 treatment (200 µg/kg) on insulin secretion. The WT- saline mice received a saline injection 15 minutes prior to oral glucose gavage while the WT Ex-4 group mice were given an Ex-4 (200 µg/kg) subcutaneous injection 15 minutes prior to oral glucose gavage. After glucose gavage, both WT saline and WT Ex-4 treated mice show robust GSIS (Figure 4.2C, solid vs hatched bars, ***p<0.001). However, mice treated with Ex-4 show enhanced insulin secretion compared to WT saline treated mice (Figure 4.2C, glucose + saline vs. glucose + Ex-4, black vs teal solid bars, $^{\#\#}$p<0.01). This increase in insulin secretion after Ex-4 treatment indicates the presence of the incretin effects. Additionally, blood glucose levels were also measured after oral glucose challenge in WT mice following saline and Ex-4 treatment. Ex-4 treated mice show significantly faster clearance of glucose compared to saline treated mice (Figure 4.3C, glucose + saline vs. glucose + Ex-4, black vs. solid teal bar, ****p<0.0001).

Following week 2 (acute), mice were injected twice daily either with saline or Ex-4 for an additional week. In week 3 (Chronic Ex-4 Week 1), we evaluated the impact of one week of

continuous saline or Ex-4 treatment on GSIS in WT mice. After glucose gavage, both WT saline and WT Ex-4 treated mice show robust GSIS (Figure 4.2D, solid vs hatched bars, ***p<0.001). Moreover, we found that Ex-4 treated mice continue to exhibit sustained incretin effect compared to the saline-treated group after one week of chronic Ex-4 treatment. (Figure 4.2D, glucose + saline vs. glucose + Ex-4, black vs teal solid bars, $^{\#\#}$p<0.01). We also measure glucose clearance in WT saline and Ex-4 treated mice. Ex-4 treated mice show significantly faster clearance of glucose compared to saline treated mice (Figure 4.3D, glucose + saline vs. glucose + Ex-4, black vs teal solid bars, ****p<0.0001).

After week 3 (chronic Ex-4 Week 1), mice were injected twice daily either with saline or Ex-4 for an additional six weeks. On week 8 (Chronic Ex-4 week 6), we examined the effect of six weeks of chronic Ex-4 or saline treatment on GSIS in mice. After glucose gavage, both WT saline and WT Ex-4 treated mice show robust GSIS (Figure 4.2E, solid vs hatched bars, ****p<0.0001 and **p<0.01 respectively). Following six weeks of chronic Ex-4 treatment, WT mice displayed reduced insulin response to Ex-4 treatment, leading to comparable insulin production as that of the saline-treated mice (Figure 4.2E, glucose + saline vs. glucose + Ex-4, black vs. teal solid bars, ns), indicating the development of tolerance to Ex-4. For OGTT, Ex-4 treated mice continue to show significantly faster clearance of glucose compared to saline treated mice (*p<0.05), however, WT Ex-4 treated mice exhibit significantly lower AUC values at week 2 vs week 8 (Figure 4.3G, $^{\#}$p<0.05, Panel G) showing moderate development of tolerance to Ex-4.

Lastly, to determine the response of endogenous incretins after seven weeks of chronic Ex-4 treatment, we evaluated the long-term impact of chronic Ex-4 or saline treatment on GSIS in mice at week 9 (Glucose only). On the test day, plasma insulin concentration was measured after oral glucose administration without either saline or Ex-4 treatment injections. WT mice treated with Ex-4 exhibited similar GSIS as the saline-treated mice (Figure 4.2F, black vs. teal solid bars, ns), indicating a loss of response to endogenous incretins and to oral glucose alone.

We also determine the percentage increase in insulin secretion from basal insulin release upon Ex-4 treatment for all the mice (Figure 4.2G). Interestingly, the percentage increase in insulin secretion from basal levels in response to glucose at week 9 (glucose only, after seven weeks of Ex-4 treatment) was lower compared to week 1 (baseline). Additionally, the GSIS for Ex-4 treated WT mice was lower compared to saline-treated mice at week 9 (Figure 4.2G), indicating a loss of response to endogenous incretins. This data shows that mice chronically treated with Ex-4 show both a reduction in their response to exogenous incretin (Figure 4.2G, week 3 vs. week 8) and to oral glucose alone which includes the endogenous incretin effect (Fig. 4.2G, week 1 vs. week 9).

β-GASP1-KO do not develop tolerance to exendin-4

To determine whether GASP1 is critical for the development of tolerance to EX-4, we repeated the same longitudinal paradigm as above (Figure 4.5A) in β-GASP1-KO mice. We measured glucose stimulated insulin secretion (GSIS) and OGTT in β-GASP1-KO mice (n=9) at different time-points in the paradigm. The mice were housed and treated in the same way at the WT mice above. The mice were entered into the paradigm at 8-weeks old.

At week 1 (baseline) following oral glucose gavage administration (2g/kg body weight), β-GASP1-KO mice show robust GSIS in response to glucose gavage (Figure 4.5B hatched vs solid bar, ***p<0.001). Blood glucose levels were also measured at different time-points (as shown in figure 4.4) after oral glucose administration. The glucose was also cleared within 120 minutes as shown in figure 4.6B. Following week 1(baseline), the mice were categorized into two groups based on their treatment: β-GASP1-KO Saline (black, n=4) and β-GASP1-KO Ex-4 (teal, n=5).

In week 2 (Acute), we assessed the acute effect of Ex-4 treatment (200 µg/kg) on insulin secretion. The β-GASP1-KO - saline mice received a saline injection 15 minutes prior to oral glucose gavage while the β-GASP1-KO Ex-4 group mice were given an Ex-4 (200 µg/kg) injection 15 minutes prior to oral glucose gavage. Both β-GASP1-KO saline and Ex-4 treated mice show robust GSIS (Figure 4.5C, solid vs hatched bars, ****p<0.0001). However, β-GASP1-KO mice treated with Ex-4

show significantly increased insulin secretion (Incretin effect) compared to saline treated mice (Figure 4.5C, glucose + saline vs. glucose + Ex-4, purple vs orange solid bar, $^{###}p<0.001$). The glucose clearance in β-GASP1-KO mice treated with Ex-4 is significantly faster compared to saline treated mice (Figure 4.6C, glucose + saline vs. glucose + Ex-4, magenta vs. orange bar, $*p<0.05$).

On week 3 (Chronic Ex-4 week 1), after one week of chronic Ex-4 treatment, we observed both β-GASP1-KO saline and β-GASP1-KO Ex-4 treated mice maintain their glucose-dependent Ex-4 mediated incretin effect compared to saline treated mice (Figure 4.5D, glucose + saline vs. glucose + Ex-4, purple vs. orange solid bar, $^{###}p<0.001$), this showing robust GSIS. β-GASP1-KO Ex-4 treated mice clear glucose significantly faster as compared to β-GASP1-KO saline treated mice in an oral glucose challenge (OGTT) (Figure 4.6D, glucose + saline vs. glucose + Ex-4, magenta vs. orange bar, $*p<0.05$).

On week 8 (Chronic Ex-4 week 6), after six weeks of chronic Ex-4 treatment, we observed that β-GASP1-KO mice treated with Ex-4 maintain their incretin effect compared to saline treated mice (Figure 4.5E, glucose + saline vs. glucose + Ex-4, purple vs. orange solid bar, $^{#}p<0.05$). Hence, unlike WT mice (Figure 4.2E, saline + glucose vs saline vs. Ex-4, black vs. teal solid bars, ns), β-GASP1-KO mice did not developed tolerance to chronic Ex-4 treatment. For OGTT, Ex-4 treated mice continue to show significantly faster clearance of glucose compared to saline treated mice ($*p<0.05$). Additionally, Ex-4 treated β-GASP1-KO mice also do not show significantly lower AUC values at week 2 vs week 8 (Figure 4.5G, ns, Panel G) showing β-GASP1-KO mice did not developed tolerance to Ex-4.

On week 9 (Glucose only), we determine the response of endogenous incretins after seven weeks of chronic Ex-4 treatment in β-GASP1-KO mice, to check if, like WT mice, they also show loss of response to glucose only which include the endogenous incretin response also. We observed that β-GASP1-KO mice treated with Ex-4 do not develop tolerance to endogenous incretins (Figure 4.5F).

We also determine the percentage increase in insulin secretion from basal insulin release upon Ex-4 treatment for all the mice (Figure 4.5G). Ex-4 treated β-GASP1-KO mice exhibited a higher percent increase in insulin from basal levels after glucose gavage at week 9 compared to week 1 (Figure 4.5G). Furthermore, GSIS in Ex-4 treated β-GASP1-KO mice was significantly higher than that of saline-treated mice (Figure 4.5G and 4.5I), indicating increased sensitivity to GSIS at week 9. Overall, our results demonstrate that chronic Ex-4 treatment developed tolerance to Ex-4 dependent GSIS in WT mice but not in β-GASP1-KO mice.

These data summarized in figure 4.5G, 4.5H and 4.5I, show that β-GASP1-KO mice treated with Ex-4 do not develop tolerance to either exogenous Ex-4 (Figure 4.5G, week 3 vs. week 8) or endogenous incretin (Figure 4.5G, week 1 vs. week 9). By comparing the responses of WT and β-GASP1-KO mice, we found that the acute response of WT and β-GASP1-KO mice to both oral glucose (Fig. 4.5I, week 1) and Ex-4 (Fig. 4.5H, I week 2) are indistinguishable, while chronic treatment with Ex-4 produces substantial tolerance to Ex-4-stimulated insulin secretion in WT but not β-GASP1-KO mice (Fig. 4.5H, week 2 vs. 8). In addition, β-GASP1-KO treated chronically with Ex-4 show significantly greater GSIS compared to WT mice even in the absence of exogenous incretin drug (Fig. 4.5I, week 9).

Limitation of study and alternative interpretation

OGTT is the most common method to assess how the body handles a standard oral glucose challenge. However, response to glucose can vary between individuals due to different factors like genetics, health, diet, physical activity, stress, and even circadian rhythms. While planning, designing, and executing the experiments, every effort was made to reduce the discrepancies that may arise due to these factors, but one should be mindful that however care is taken there can still be inter-individual and intra-individual variability that may affect the outcomes of the experiments. The outcome of an OGTT is influenced by the food (carbohydrate) content of the meal preceding the test. In our experiments, before doing an OGTT test the mice were fasted for 12-16 hours, however, in

their home cage they have access to food and water *ad libidum*. Thus, we did not control for the amount of food intake in mice before the OGTT experiment as we wanted to mimic what usually happens in humans. Additionally, mice were housed in groups of 3-5 mice per cages, so it may be possible due to social hierarchical nature of mice living in same cages; some of them can eat more as compared to the others. We have gavage the glucose bolus and injected Ex-4 based on the body weight of the mice on the test day to take into consideration their potential varying food intake habits, but it may be possible that different food intake can introduce variability in the result and affect the reproducibility of the results. Moreover, it is difficult to monitor and control the physical activity of the mice without introducing a lot of stress to the animal. Hence, it is difficult to interpret how physical activity of the mice affects the data obtained in our experiments.

The mice used in these experiments are inbred isogenic mice that have nearly identical genetic makeup. Even among genetically identical mice, it has been known that variations in response to a particular drug can occur. These variations may arise due to number of factors including but not limited to epigenetic differences that can influence gene expression without altering the DNA sequence, the gut microbiome variation that may affect how medication is processed, physiological variations of organ size blood flow and cellular responses and metabolic difference in terms of drug metabolism and general metabolism. All these factors may affect the response of the mice toward twice daily exendin-4 injections and therefore may introduce variability in the results. These variations in drug response even among genetically identical mice emphasize the complexity of biological systems. Hence, while conducting *in vivo* experiments, we have used multiple individuals and trials along with appropriate controls (as described in the result section) to account for inherent variability in the mouse data and obtained more robust reproducible results.

For the OGTT data obtained in our experiments, we have calculated the total area under the curve from the baseline. The first limitation of such analysis is the choice of baseline value from which AUC is calculated. In our data, we found a huge amount of baseline variability exists between mice as

well as between same mice on different test days. This makes it difficult to select a single baseline value using which we can calculate AUC. We can calculate incremental area under the curve so that we take into consideration the baseline variability. We have noticed small changes in the baseline value might lead to difference AUC values affecting the interpretation of the data. We have chosen to used baseline of 0 in our analysis, however, it is important to acknowledge that relying solely on total area under the curve from zero may not fully encapsulate the intricacies of glucose response but can mask important temporal variations during the entire duration of the test. This type of data is needed to be analyzed in multiple ways to capture the full complexity of the glucose response and dynamics. Additionally, calculating AUC assumes that the relationship between time and glucose levels is linear, which is not always the case in our data especially during dynamic changes.

From the plasma insulin measurement data, we found that in WT mice after 6 weeks of chronic Ex-4 treatment, the GSIS is lower than the saline treated mice. This tolerance phenotype could be due to either their loss of response to glucose or loss of response to incretin or both. In our longitudinal paradigm, after 7 weeks of chronic Ex -4 treatments, we performed an OGTT without Ex-4 injection prior to glucose gavage to examine if there is loss of response to glucose or incretins or both. We found that after 7 weeks of chronic Ex-4, the insulin produce is still lower than saline treated mice indicating that chronic Ex-4 affects glucose sensitivity as well as endogenous and exogenous incretin sensitivity to pancreatic β-cells. Alternatively, it is entirely possible that due to chronic exposure to Ex-4 for such a long period, there is islets dysfunctionality leading to loss of glucose as well as incretin sensitivity. In either case, β-GASP1-KO mice do not show any such tolerance development or loss of sensitivity to either glucose or incretins.

Methods

Mice

Mice were bred in-house in UC Davis AAALAC certified vivarium and handled according to National Institute of Health guidelines for the care and use of laboratory animals. All the protocols

were approved by the Institutional Animal Care and Use Committee (IACUC) at University of California, Davis. Adequate measures were taken to minimize animal suffering and discomfort. Mice were housed in cages (typically 3-5 per cage) in temperature and humidity controlled rooms with 12:12 hrs light:dark sleep cycle and provided with food and water *ad libidum* all the time. Only male mice were used for the study. Animals were grouped without blinding but were randomized during experiments. Groups were spread across multiple cages to minimize cage effects.

Oral glucose tolerance test (OGTT)

OGTT was performed on age-matched WT and β-GASP1-KO mice. A total of 8 WT and 9 β-GASP1-KO mice were randomly assigned into two experimental groups - control (saline) and treatment (Ex-4): WT saline (n=4), WT Ex-4 (n=4), β-GASP1-KO saline (n=4) and β-GASP1-KO Ex-4 (n=5). The mice were bred in-house and handled gently to minimize stress. Mice were entered into the longitudinal study paradigm (Figure 4.2A) at 8 weeks old. At week 1, the baseline glucose tolerance and plasma insulin were measured. Mice were fasted overnight (~14-16 hrs) and then administered a 2g/kg oral bolus of glucose. Blood glucose was measured using standard glucometer (TrueFocus, Walgreen) at 0,5-,15-,30-,60-,90- and 120-min. post glucose gavage. The mice were returned to their home cages for a week where they had access to water and food *ad-libidum*. At week 2, after overnight fasting, mice were injected either with saline or 200 µg/Kg Ex-4 (MedChemExpress,HY-13443) 15 min. prior to oral glucose tolerance test. After 15 min, mice received oral glucose gavage at 0 time-point. Blood glucose levels were measured at 5-,15-,30-,6-,90- and 120-min after glucose administration (Figure 4.4A). The mice were returned to their home cages and injected twice daily with either saline or Ex-4 (200 µg/Kg) for up to additional seven weeks. Oral glucose tolerance test was performed at week 3 and 8 (one and six weeks after chronic Ex-4 treatment respectively) similarly as in week 2 (Figure 4.4B). On week 9 (seven weeks after chronic Ex-4 treatment), the mice were fasted overnight and administered a 2g/Kg oral bolus of glucose

without prior saline or Ex-4 injection. Oral glucose tolerance was measured over a period of 120 min. (Figure 4.4A).

Blood collection for plasma insulin measurements

Blood was also collected from the tail vein of each mouse during the OGTT paradigm. For week 1 (baseline), ~100µl of blood was collected at 0 and 15 min after glucose administration in a microvette capillary tube (Thermofisher, 16.444.100). To extract plasma, the blood in the microvette tubes was centrifuged at 1500xg for 10 min at 4°C. The plasma samples were stored at -80°C until needed for further analysis. For week 2 (Acute), week 3 (Chronic Ex-4 week 1) and week 8 (Chronic Ex-4 week 6), the blood samples were collected before saline or Ex-4 injection (-15 minute) and 15 minutes after glucose gavage. For week 9 (Chronic Ex-4 week 9), the blood was collected for plasma preparation at 0- and 15-min. post oral gavage.

Insulin measurement from GSIS islet study and mouse plasma

The plasma insulin secreted from islets were measured using Lumit Insulin Immunoassay kit (Promega, CS3037A05) as per the manufacturer's instructions and measured using Flexstation-3. The concentration of insulin in each sample was extrapolated from an insulin standard curve. The results were expressed as insulin concentration using GraphPad Prism 9.

Figures

All the figures were created with Biorender.com.

Statistics

Data are presented as means ± SEM and the number of experiments is indicated in every case. GraphPad prism version 9 was used to perform all statistical analysis. The student's t-test or two-way analysis of variance (ANOVA) with Tukey's multiple comparison test was performed in GraphPad prism to detect statistical differences. $P < 0.05$ was considered statistically significant.

Chapter 5: Discussion

GLP-1R agonists have revolutionized the treatment of type 2 diabetes mellitus (T2DM), offering improved glycemic control and other beneficial effects including weight loss. These billion-dollar drugs also have a remarkable efficacy in reducing cardiovascular risk in T2D patients. [3,259,260] However, prolonged use of GLP-1R agonists can lead to reduced therapeutic response due to the development of tolerance to these important medications.[29,170,210,262] Several factors contribute to the development of tolerance including disruption in GLP-1 receptor trafficking and post-endocytic sorting resulting in decreased receptor surface expression and reduced drug responsiveness.[262] Therefore, understanding the molecular mechanisms involved in the development of tolerance is crucial for optimizing the long-term effectiveness of incretin drugs.

The present study shows that tolerance to the incretin drug Ex-4 is dependent on GASP1, which is a crucial mediator in the post-endocytic sorting of GLP-1 receptor to lysosomes. We found that GASP1-dependent GLP-1R post-endocytic trafficking directly influences the responsiveness of receptor signaling and development of tolerance to GLP-1R agonists. GASP1 disruption in either stably expressing GLP-1R HEK293 cells or INS-1 cells did not affect the Ex-4 dependent acute GLP-1R signaling or insulin secretion *in vitro*. However, we found that prolonged exposure to Ex-4 resulted in loss of incretin response in both WT HEK-GLP-1R or INS-1 cells, while GASP1 knockout HEK-GLP-1R or INS-1 cells maintained robust GLP-1R signaling or incretin-mediated insulin responses even after chronic Ex-4 treatment, thus preventing the development of tolerance toward incretin drug Ex-4. Similarly, in *ex vivo* islets experiments, we have shown that acute Ex-4 treatment does not affect incretin-mediated glucose stimulated insulin secretion in islets from WT mice or mice with beta-cell specific deletion of GASP1 (β-GASP1-KO). However, prolonged exposure to Ex-4 showed diminished insulin secretion in WT islets, while β-GASP1-KO islets again maintained robust incretin-mediated insulin secretion. Furthermore, prolonged treatment with Ex-4 resulted in the development of tolerance to the glucose-stimulated insulin secretion effect of Ex-4 in WT mice. In contrast, mice

with a selective disruption of GASP1 in pancreatic beta-cells maintained the incretin effect even after chronic treatment period and did not developed tolerance to Ex-4 drug. These findings underscore the crucial role of GASP1-mediated GLP-1 receptor trafficking in the development of tolerance to incretin drugs and can have significant implications for developing novel therapeutic approaches and improving therapeutic utility in the field.

Based on the findings of this study, we can potentially target GASP1 and its interaction with GLP-1R to develop therapeutic interventions for humans with an aim to prevent the development of tolerance to the GLP-1 receptor effects and enhancing the effectiveness of GLP-1R agonist drugs. While GASP1 inhibition could potentially prevent development of tolerance to GLP-1R agonists, caution should be exercised in pursuing this approach. GASP1 plays an important role in post-endocytic trafficking of several other GPCRs including dopamine receptor (D2R) in brain. Developing drugs which inhibit GASP1 could have a negative impact on these other receptors, thus disrupting their signaling. This highlights the complexity of targeting GASP1 as a therapeutic strategy due to its involvement in multiple receptor systems. Additionally, developing a selective inhibitor that targets the interaction between GASP1-GLP-1R might be difficult. Given these challenges, an alternative approach to capitalize on this study finding for therapeutic purposes is instead of directly targeting GASP1 or its interaction with GLP-1R, we can prevent the post-endocytic degradation of the GLP-1 receptor. GLP-1R agonists - GLP-1, Ex-4 and liraglutide, all stimulate both G protein activation from the GLP-1R and recruitment of arrestins, leading to GLP-1R endocytosis. However, for many GPCR targets, various ligands show distinct potencies and efficacies in activating the G protein versus the arrestin signaling pathways. A ligand-specific signaling bias for the G protein versus the arrestin effector has been demonstrated for many classes of GPCR including the GLP-1R.[153]

For example, tirzepatide (Mounjaro®), a dual GLP-1R and glucose-dependent insulinotropic polypeptide (GIP) receptor agonist, has been shown to have improved therapeutic utility. Tirzepatide addresses multiple aspects of diabetes management. It activates both GLP-1 and GIP receptor, thus

combines the actions of the two important hormones involved in glucose homeostasis- GLP-1 and GIP hormone. Therefore, the therapeutic advantage of tirzepatide could be attributed to its dual agonism at both GLP-1 and GIP receptor.[192,263,264,265] Additionally, tirzepatide is also distinguished from other GLP-1R agonists by displaying a bias toward G protein signaling from the GLP-1R, as it doesn't promote arrestin recruitment and receptor endocytosis.[266,267] This selective G-biased signaling of tirzepatide at GLP-1R has potential implications for its therapeutic effects and safety profile. By favorably activating G protein signaling, tirzepatide promotes insulin release, and therefore, improved glycemic control, while avoiding any unwanted effects due to arrestin recruitment. Moreover, due to its ability to minimize arrestin recruitment, tirzepatide may be allowing the GLP-1R to evade GASP1-mediated post-endocytic degradation and thereby better sustain the efficacy of treatment. Since tirzepatide is not only biased for G protein at the GLP-1R but also strongly activates GIP receptors, it is not possible to determine whether bias or dual agonism is key to its improved therapeutic effects. Tirzepatide precise mechanism of action and the extent of its's ability to G-protein bias is currently under investigation but its development underscores complexity of signaling pathways involved in maintaining glucose homeostasis and highlights the potentials of drugs that offers targeted effects. Therefore, tirzepitide provides proof-of-concept that G-biased ligands at the GLP-1R are an achievable goal. In fact, several such molecules have been reported and shown to produce improved glycemic control in mice.[153]

In recent years, GLP-1R agonists have gained attention as potential therapeutics for weight loss. Due to their ability to effectively regulate appetite, delay gastric emptying and impact central appetite control, GLP-1R agonist drugs have been approved recently for weight management in individuals who do not have T2D. Two GLP-1R agonists have been approved by FDA to treat obesity including semagluide and liraglutide.[268-270] Additionally, research on the efficacy of the GLP-1R/GIPR dual agonists and GLP-1R/GIPR/GCGR triple agonists pave the way for therapeutic interventions for the treatment of obesity, suggesting that multi-target drugs may be more advantageous than single

target drugs. However, the long-term safety of using the GLP-1R agonists for weight loss in non-diabetic subjects is still under evaluation, as the use of these medications in the sole context of weight loss is relatively recent. In this study, we observed that prolonged administration of Ex-4 to healthy non-diabetic WT mice resulted in a significant decrease in their responsiveness to glucose-stimulated insulin secretion during an oral glucose challenge when they were not on drug (Figure 4.2). These data indicate an altered response to their endogenous incretin hormones as well as a change in the glucose sensitivity of WT mice chronically treated with Ex-4. This effect was not observed in the GASP1 knock-out mice (Figure 4.5). Therefore, caution should be exercised when considering the use of GLP-1R agonists as weight loss medications for healthy individuals, as prolonged treatment with these agonists has the potential to modify the body's insulin secretion profile and disrupt glucose homeostasis.

Within pancreatic islets, paracrine signaling between different islets cells including α-, β- and δ-cells, is crucial for the coordinated regulation of insulin secretion, maintenance of glucose homeostasis and overall physiological responses.[42] Preliminary data from our lab have shown that GASP1 is not only expressed in pancreatic β-cells but selectively distributed within different islet cells. Among the three islet cell types, δ-cells exhibit the highest expression of GASP1, surpassing α and β-cells. The selective distribution of GASP1 suggests its involvement in regulating cell-to-cell communication, influencing the fine-tuning of GLP-1R function and hormone secretion within islets. Pancreatic isles have other GPCR proteins including glucagon receptor on β-cells, somatostatin receptors on α- and β-cells and urocortin-3 and GLP-1 receptor on δ-cells.[42,236] GASP1 could interact with these GPCRs after they have been activated and can affect the sorting and trafficking of these GPCRs within endosomes, potentially influencing their signaling outcomes. Although the exact role of GASP1 in α-, β- and δ-cells, is still being investigated and how GASP1 affect islet paracrine signaling is not completely understood, it is possible that GASP1-mediated modulations of the receptors on islets cells could impact the release of hormones from different islets cell types. Alteration of GASP1-

mediated GPCRs signaling in different islets cell types could influence the intricate balance of hormone secretion within the islet, thus disrupting and maintenance of glucose homeostasis. Therefore, understanding the role of GASP1 in modulating paracrine signaling may shed light on the complex regulation of glucose metabolism and aid in the development of targeted therapies for T2DM.

Moreover, it is worth noting that GASP1 is an X-linked gene in both mice and humans.[135] This genetic characteristic may have implications for the expression and regulation of GASP1, potentially contributing to differences in GASP1 levels between males and females.[271] It is well known that men are considered to have higher risk of developing T2D compares to females. The risk of diabetes can be influenced by a combination of genetic, lifestyle, and environmental factors. This disparity in GASP1 expression between genders may contribute to gender-specific differences in GLP-1R signaling and its associated metabolic effects, including glucose control and insulin secretion. Being an X-linked gene, variations or mutations in GASP1 may have differential impacts on disease susceptibility between males and females, potentially leading to variations in the progression of diabetes or development of tolerance to GLP-1R agonists. Understanding the potential interplay between GASP1 genetic variants and gender-related factors may provide insights into the mechanism underlying gender disparities in diabetes prevalence and outcomes. It is important to note that while the X-linked nature of GASP1 presents intriguing possibilities, further research is needed to fully understand its implications in diabetes leading to personalized treatment approaches and optimize therapeutic outcomes.

In conclusion, my thesis work highlights the crucial role of GASP1 in regulating GLP-1 receptor trafficking and the development of tolerance against GLP-1R agonists. By disrupting GASP1 in INS-1 cells or mouse pancreatic islets, the study demonstrated preserved incretin effects and prevention of tolerance against chronic treatment of Ex-4. Furthermore, tirzepatide, a novel dual GLP-1 and GIP receptor agonist with G-biased signaling, offers a promising approach to sustaining the efficacy of

treatment by avoiding GASP1-mediated post-endocytic degradation. Understanding the role of GASP1 in paracrine signaling within pancreatic islets and its tissue-specific distribution can provide further insights into the complex regulation of insulin secretion and glucose metabolism. Additionally, the X-linked nature of GASP1 and potential gender-specific differences in its expression may contribute to disparities in GLP-1R signaling, diabetes prevalence, and treatment outcomes. Moreover, while GLP-1R agonists show potential as weight loss medications, their long-term safety profile and potential effects on insulin secretion and glucose homeostasis should be carefully considered. Overall, continued investigation into the molecular mechanisms underlying the mechanism by which GASP1 influences GLP-1R functions provides a foundation for developing novel therapeutics strategies and optimizing the long-term effectiveness of GLP-1R agonists in the treatment of T2DM. Further research in these areas is essential to unlock the full potential of GASP1 as a target for enhancing GLP-1R agonist therapy.

Chapter 6: Future perspectives on GLP-1R/GASP1 interaction – What's Next?

Glucose homeostasis is critical for human health, and its disruption can cause diabetes-associated complications.[272] In 2017, diabetes affected approximately 30 million people in the US, which poses a substantial economic burden on society.[273] Glucose homeostasis is maintained by the combined action of insulin and glucagon hormone secreted by pancreatic beta and alpha cells.[274] The amount of insulin secreted after eating is also regulated by incretin gut hormones (incretin effect). There are two known incretin hormones: Glucagon-like peptide-1 (GLP-1) and Glucose-dependent insulinotropic polypeptide (GIP). GLP-1 and GIP stimulate insulin release from beta cells by their action on their cognate G protein-coupled receptors (GPCRs) GLP-1R and GIP-R.[275] Due to their insulinotropic effect, incretin receptor agonists are used as therapeutic agents to maintain glucose homeostasis in T2D.[276] The therapeutic benefits of these medications, including improved glucose control and weight loss, require continued usage and wane with time. This loss of effect to incretin drugs over time is known as "development of tolerance" to the drug. Despite all the beneficial advantages of incretin therapies, very little is known about the specific mechanisms which cause development of tolerance to incretin drugs over a long period of usage.

The work presented in this thesis project aimed to provide a small mechanistic contribution to how tolerance developed to incretin drugs after prolonged use. To that end this work highlights a novel mechanism in which GPCR-associated sorting protein 1 (GASP1) regulates the post-endocytic sorting of the GLP-1 receptor and causes lysosomal degradation of the receptor after endocytosis. The post-endocytic fate of a GPCR has profound implications for signal transduction, especially under conditions, such as exogenous drug use, where there are high concentrations of ligand and therefore a large degree of receptor endocytosis. For receptors that are recycled, endocytosis serves to rapidly re-sensitize signal transduction while for receptors that are degraded, endocytosis will promote prolonged loss of signaling, thus reducing their effectiveness.[110-112] Here we demonstrate that tolerance to incretin drug was prevented at the cellular, tissue and whole animal level in mice with a

selective disruption of the GASP1 protein in beta cells of the pancreatic islet. These studies implicate post-endocytic sorting of the GLP-1R in the loss of effectiveness of incretin therapeutics with prolonged use. These findings also suggest a novel strategy to prevent tolerance by biasing incretin drugs for G protein and away from arrestin engagement. Over the course of this thesis project as we gathered more data supporting the proposed mechanism, interesting questions began to emerge regarding other roles of GASP1 and how it affects the post-endocytic sorting of GLP-1R in other tissues. GASP1 is not ubiquitously expressed in all body tissue but specifically expressed in a few including pancreatic islets, brain, and kidney cells. Additionally, within the pancreatic islet, GASP1 is differentially expressed. Preliminary data in our lab have shown that GASP1 is more abundantly expressed in pancreatic δ-cells compared to β- or α-cells. Moreover, both GLP-1 receptor and GASP1 have common genetic variations that are known to alter insulin secretion in humans. These naturally occurring receptor variations do not affect the affinity or the efficacy of GLP-1 at their cognate receptor, but still alters the insulin secretion. Taken together, these data points toward a more diverse role of GASP1-mediated post-endocytic trafficking of GLP-1 receptor. This chapter will discuss what comes next for this line of research and GLP-1R signaling in health and disease.

Genetic variation in GLP-1 receptor

The incretin-based therapies have good tolerability, excellent safety and a low rate of hypoglycemia and weight gain.[277] A critical aspect of incretin therapies that remains unknown is their different sensitivity across individuals. Recent studies have identified GLP-1R and GIP-R gene on Chromosome 6 and 19 as T2D risk alleles.[278] Genetic variations within these loci are known to affect the insulinotropic effect of incretins on beta cells. Studies have shown that two genetic variants in the GLP-1R - R131Q and G168S show altered GLP-1 induced insulin responses in healthy individuals. R131Q increases while G168S decreases insulin responsiveness to infused GLP-1.[279] R131Q genetic variation is present at the extracellular N-terminal ligand binding region, while G168S is located on the intracellular loop. With regards to GIP-R locus, genetic variant E354Q is reported to be

associated with a decrease in fasting serum C peptide concentration.[280] The molecular mechanism that underlies the phenotypes associated with these altered genotypes is not well understood. Therefore, significant gaps in the knowledge remain regarding the impact of genetic variations in incretin receptors on their ability to stimulate beta cells. Preliminary data in the Whistler lab has confirmed that these genetic variations in GLP-1R/GIP-R do not affect ligand affinity or efficacy. This poses a pharmacological mystery that cannot be explained by “classical” pharmacology. Based on the work done in this thesis project, we can attempt to provide a mechanistic explanation for this pharmacological mystery. As mentioned before, following the activation of G protein, GPCRs are trafficked away from the plasma membrane to endosomes. From there, individual GPCRs are then either recycled or degraded in lysosomes. Hence receptor trafficking regulates GPCR signaling and function.[110] We hypothesized that genetic variations in incretin receptors affect the endocytic/post-endocytic trafficking of the receptor, which leads to the differential incretin response in pancreatic beta cells. To examine this, we can use different cellular and molecular biology techniques to define the role of genetic variations in incretin receptor on GSIS and characterize the key players involved in the post-endocytic sorting of incretin receptors. The new knowledge obtained from the study will further enhance our understanding of incretin dependent insulin release in beta cells and could be important for predicting the effect of genetic variations in treatment response toward incretin-based therapies.

Preliminary data generated in our lab in HEK293 cells stably expressing GLP-1R variations show that the G168S variant promotes receptor degradation while the R131Q variant promotes receptor recycling thus supporting our hypothesis. The variants do not affect receptor-ligand binding or receptor ability to activate cAMP signaling. However, the variants do alter trafficking of the receptors in HEK293 cells. Moreover, initial cAMP signaling data generated using HEK 293 cells stably expressing R131Q and G168S GLP-1R variant show that R131Q and G168S variant do not show altered cAMP accumulation with acute agonist treatment. However, after prolonged GLP-1R

agonist treatment, R131Q showed enhanced cAMP response compared to G168S variants. These results suggest that R131Q and G168S genetic variants do not change the affinity or the efficacy of the ligand to the receptor but change the post-endocytic trafficking of the receptor where R131Q promotes recycling and G168S promoted degradation of GLP-1 receptor leading to altered cAMP response. As described in this thesis, the factors that play an important role in post-endocytic sorting of GLP-1R in pancreatic β-cells is GASP1, it will be highly interesting to examine whether these genetic variations also alter GLP-1 receptor binding with GASP1 that leads to altering the post-endocytic trafficking of the receptor. The information thus obtained can have substantial impact on determining the long-term effect on the therapeutic outcome of GLP-1R agonists in an individual. It also proposed a mechanistic explanation of the pharmacological mystery as to why some but not other T2D patients respond well to incretin therapies.

Genetic variation in GASP1 protein

After ligand-dependent activation and G protein signaling initiation, most GPCRs undergo a cascade of events that culminates into receptor endocytosis. Once endocytosed, the receptors are then “sorted” to either a recycling or a degradative pathway. For receptors that are recycled, endocytosis serves as a mechanism for restoring signaling sensitivity by returning receptors to the cell surface, enabling them to bind and respond to ligands once again. For receptors that are targeted for degradation, endocytosis is the first step toward irreversible receptor degradation within the lysosome.[110-112] Years of research have been done to examine the sorting process of the endocytosed receptor, segregating it between recycling and non-recycling membrane-bound compartments. To briefly summarize this, protein complexes known as ESCRT (endosomal sorting complexes required for transport), guides sections of membrane through a sequence of maturation steps, which culminate in the delivery of both the membrane cargo and cytosolic component to the lysosomes.[121-123] GASP1 is a GPCR sorting protein that is shown to be necessary for the sorting of the receptor to the lysosome.[129,132] Furthermore, GASP1 is known to decrease insulin secretion in

murine insulinoma cells.[159] In this study we have shown that GASP1 contributes to the post-endocytic sorting of GLP-1 receptor in INS-1 cells and pancreatic β-cells. Data from our lab and others have shown that GASP1 plays a role in marking receptors for degradation by interacting with an ESCRT protein complex component dysbindin. Dysbindin facilitates endosomal maturation into a multi-vesicular body, which subsequently fuses with the lysosomes. This fusion process ensures delivery of membrane-bound cargo for lysosomal degradation.[213] Like other ESCRT components, dysbindin is also highly conserved across species, while GASP1 is unique to mammals. This distinctiveness of GASP1 might be attributed to the need for further regulating the trafficking of GPCRs.

There are three common genetic variants of the GASP1 gene: A315G, I779V and P1093S and preliminary data from our lab have shown that at least one variant of GASP1 (P1093S) shows altered affinity for dysbindin. Hence genetic predisposition at the incretin receptor as well as at GASP1 could be influencing receptor trafficking and therefore signaling. Therefore, we can assess whether genetic variation of GASP1 alters its affinity for either GLP-1R and/or to dysbindin, which links GASP1 to the ESCRT machinery. The information thus obtained proposes solutions to several pharmacological and cell biological mysteries. Additionally, examining whether common allelic variants in the human population of the GASP1 sorting protein alter receptor traffic help us to determine the therapeutic benefits of some of the most important T2D therapies available today.

GLP-1R-GASP1 interaction in pancreatic α-cells

The GLP-1R, predominantly found on pancreatic β-cells, is also present in α-cells, which is responsible for producing the hormone glucagon that elevates blood glucose levels. GLP-1 receptors have been identified on a subpopulation of alpha cells.[281] However, other investigations and several transcriptomic profiling of α-cells have indicated the absence of GLP-1R on pancreatic α-cells.[282-284] Therefore, it is continuously debated whether α-cells within the islets expressed GLP-1R and further research is needed to confirm the expression of GLP-1R on pancreatic α-cells. Additionally, studies have shown that GLP-1 acts on multiple receptors: the canonical GLP-1R and the glucagon receptor

(GCGR - a GPCR). GLP-1 is also shown to inhibit glucagon secretion from pancreatic islet α-cells. Researchers have found that GLP-1R and GCGR can bound to multiple proglucagon-related peptides like GLP-1 (7-36), GLP-1(9-36) and glucagon. GLP-1 binds to GCGR with a lower affinity compared to GLP-1R.[285] Furthermore, alpha cell-specific deletion of GLP-1R results in impaired glucose response.[286] These data suggest that α-cells-specific GLP-1R signaling play a significant role in maintaining glucose homeostasis and coordinating the dynamics of glucagon release. This aspect of GLP-1R pharmacology may have significant implications. Activation of GLP-1 receptor on α-cells initiates a cascade of that results in PKA-dependent inhibition of P/Q-type voltage-gated Ca^{2+} channels. This reduces intracellular calcium flux, thus inhibiting glucagon granule release from the α-cells. The signaling cascade initiated by GLP-1 binding to GLP-1R on α-cells may also have a paracrine effect on neighboring islet cells. This crosstalk between the α- and β- cells is important for maintaining proper cellular communication which is essential for glucose regulation and insulin secretion.

Furthermore, the proglucagon gene *GCG* is expressed in intestinal L cells, pancreatic alpha cells, and neurons in nucleus tractus. Differential processing of the *GCG* gene yield glucagon in pancreatic alpha cells and GLP-1 and GLP-2 in intestinal L cells. However, GLP-1 expression was in HPLC analysis of human alpha cell extracts. Subsequent research has shown that GLP-1 is co-localized within the glucagon secretory granules of alpha cells in isolated islets or in mouse models after experiencing cellular stress.[287] This stress-induced GLP-1 production may be a potential mechanism to prevent further islet cell damage. Several studies have also confirmed that in isolated intact human islets, alpha cells are capable of secreting GLP-1. Taken together these findings suggest a potential autocrine signaling role of GLP-1 within α-cells.

GASP1 is also expressed in pancreatic α-cells and potentially affects the post-endocytic fate of GLP-1R upon activation by either glucagon or GLP-1. The interaction between GLP-1R and GASP1 and whether it directs GLP-1R toward lysosomal degradation pathway in α-cells remains

subject of further investigation. Examining the impact of GASP1-mediated post-endocytic trafficking of GLP-1R in α-cells on glucose regulation and insulin secretion warranted more research to better understand the role of GASP1 in pancreatic α-cells. Additionally, GASP1 may also cause degradation of GCGR on β-cells, potentially altering glucagon dynamics thus influencing glucose homeostasis. Moreover, assessing the impact of genetic variations in GLP-1R, GASP1 and GCGR on receptor post-endocytic trafficking of the receptor and how it affects the insulin secretion and glucose regulation, is a promising avenue for further research.

GLP-1R-GASP1 interaction in pancreatic δ cells

GLP-1R has been characterized in pancreatic δ-cells where when activated it potentiates somatostatin (SST) release.[288] Somatostatin acts as negative feedback for insulin and glucagon release from β- and α- cells respectively via its interaction with the somatostatin receptors (SSTR - a family of GPCRs). SSTRs are a G-inhibitory protein coupled receptors. Binding of somatostatin to an SSTR activates the Gi protein which result in inhibition of adenylyl cyclase activity and the opening of G-protein coupled K-ion channels leading to negatively polarized non-excitable state of β- and α-cells, thus suppressing hormone release. Hence, GLP-1 potentiates insulin and somatostatin release from β- and δ-cells respectively, somatostatin inhibits insulin release, thus depending on the tone of somatostatin GLP-1 can indirectly inhibit insulin secretion rather than stimulating its release. Similarly, somatostatin may inhibit the release of GLP-1 from α- cells. [231-235]

As mentioned previously, we found that GASP1 is expressed in pancreatic α-, β-, and δ-cells. Preliminary immunohistochemistry data generated in our lab have shown that out of three cell types, GASP1 is highly expressed in δ-cells compared to α- and β-cells. This suggests a potential role of GASP1 in determining the post-endocytic fate of GLP-1R and other GPCR in δ-cells. Activation of the GLP-1R on δ-cells can modulate release of somatostatin which binds to the SSTR receptor on α- and β-cells to prevent release of glucagon and insulin respectively. Additionally, GLP-1R signaling on δ-cells can potentially influence paracrine interaction within the pancreatic islets. This paracrine

communication between different islet cell types is essential for proper glucose homeostasis and GLP-1-mediated modulation of somatostatin release may play a role in maintaining this balance. Chronic activation of GLP-1R may cause GASP1-mediated receptor degradation both in β- and δ-cells, which may result in a decrease in receptor number on the cell surface. The reduction in cell-surface expression of GLP-1R on δ-cells may impact GLP-1R signaling that can potentially disrupt the interplay between δ-cells and other islet cell types including α- and β-cells which is crucial for maintaining glucose homeostasis. Therefore, understanding GLP-1R signaling and its interaction with GASP1 within pancreatic δ-cells could provide insight into the comprehensive network of interaction within pancreatic islets. Further research is needed to fully elucidate the mechanism and implication of GASP1-mediated GLP-1R post-endocytic trafficking and its effect of GLP-1R signaling in δ-cells.

GLP-1R signaling in dopamine dynamics, memory and learning

Cognitive flexibility is a critical executive function, deficits in which are found in multiple neurological and psychological disorders including OCD, bipolar and mood disorders,[289] Parkinson's disease,[290] Alzheimer's, and substance use disorder[291] and declines precipitously with age even in humans with no neurological disease. One of the key challenges in treatment of these diverse neurological disorders is that the existing pharmacological agents, including antidepressants and anti-psychotics are not effective at treating the cognitive deficits and have serious side effects including exacerbation of cognitive deficit, insomnia, nausea, fatigue and risk of addiction.[292-294] Hence there is a need to develop better and safer treatments to replace or supplement existing drugs if we want to stabilize the cognitive impairments associated with disease or age.

Several recent studies have described the neuroprotective effects of the neuroendocrine incretin hormone – Glucagon-Like Peptide 1 (GLP-1) on brain structure and function.[295,296] GLP-1 and its cognate GLP-1 receptor are present in key regions of the brain implicated in memory and decision making, including both the hippocampus and ventral tegmental area (VTA),[297] whose dopamine (DA) neurons are thought to play a critical role in regulating entry of information into long-term memory via

hippocampal-VTA loop.[298] Furthermore, there is significant evidence that incretin signaling in these brain regions modulates learning and memory. For example, over-expression of GLP1R in hippocampus enhances spatial learning in a maze task,[299] while mice with a disruption of GLP1R show impaired special learning and memory and LTP impairments in hippocampus.[300] Incretin analog agonist drugs to the GLP1R are in widespread use for glycemic control in T2D patients and have an outstanding safety profile. Importantly, these agonist drugs, including liraglutide (Victoza®) and extendin-4 (Byetta®), also readily cross the blood brain barrier, and their use has recently been shown to reduce the risk of Alzheimer disease in T2DM patients.[301] In preclinical animal models, these drugs have been shown to protect long-term potentiation (LTP)[302] and to produce improved performance in several diverse cognitive tasks. We were intrigued by the observation that activation of the GLP1R with incretin drug in the VTA modifies not only food intake but also reward seeking behavior,[303] and hypothesized, that GLP1Rs in the VTA could modulate dopamine dynamics to gate memory and learning. Indeed, I have recently found, in preliminary studies using the dLight DA sensor[304] and fiber photometry, that systemic administration of the incretin drug liraglutide alters dopamine dynamics in the medial nucleus accumbens (NAc) in mice. I have further shown that liraglutide can block changes in dopamine release in the NAc produced by the rewarding drug morphine. Cumulatively, our data and that previously reported have led us to hypothesize that incretin drugs could be used to improve cognitive deficits.

Moreover, common genetic variants in the GLP-1R R131Q (rs3765467) and G168S (rs6923761) produce alterations in both amino acid sequence and incretin response to glucose intake[305] but show no change in affinity or efficacy for the ligand GLP1. As described earlier, we have uncovered a mechanism that can explain this pharmacological mystery. We found that the post-endocytic targeting of the GLP-1R variants differs from that of the wild type (WT) GLP-1R. Specifically, the R131Q variant receptor is endocytosed and recycled and therefore remains highly expressed even following prolonged drug treatment, while the G168S variant receptor is endocytosed

and degraded more rapidly than the WT receptor. We therefore could predict that genotype at the GLP-1R could affect the ability of incretin drugs to control not only glucose homeostasis but also learning and memory. We can assess the effects of the incretin drug like liraglutide on dopamine dynamics and cognitive flexibility in WT, R131Q and G168S knock-in mice. Our hypothesis is that activation of the GLP1R will improve cognitive flexibility, and that it will do so with better efficacy in mice and humans with the R131Q mutation. Our goal is the validate incretins as therapeutic treatments or adjuvants for cognitive deficits associated with disease and/or aging.

GLP-1R signaling and cardiovascular effects

GLP-1R has been shown to exert cardioprotective effect in cardiomyocytes through activation of antiapoptotic mechanism.[306] Preclinical studies in mice show liraglutide to improve cardiac function after myocardial infraction. Studies have shown that in mouse cardiomyocytes, liraglutide modulates the expression of cardioprotective genes including Nrf2 and PPAR.[307] These data suggest that GLP-1 may have a possible beneficial myocardial effect and potential therapeutic use in patients with cardiovascular diseases. However, GLP-1R agonists like exendin-4 and liraglutide fail to improve cardiac function in patients with heart failure. One possible explanation for the beneficial action of GLP-1 regarding cardiovascular effect is that the molecular mechanism is different from the classical GLP-1R signaling and requires further new research perspectives. In heart, a dual mechanism of GLP-1 action is proposed. The classical GLP-1R signaling is thought to be responsible for inotropic effects, glucose uptake, improvement of coronary flow and partly mediating the cardia effect, whereas a GLP-1R-independent mechanism is thought to be responsible for the cardioprotective function.[308,309] GASP1 is also known to be expressed in cardiovascular system and it will be interesting area of research to examine how GASP1-mediated GLP-1R trafficking post-endocytosis affect the GLP-1 cardioprotective effects. A thorough understanding of the molecular mechanism and post-endocytic trafficking of GLP-1R in cardiomyocytes will enable us to develop better GLP-1R agonists that have improved therapeutic effect on cardiac functions.

As highlighted in this chapter, post-endocytic trafficking of GLP-1 receptor in the pancreatic β-cells remains understudied but also has great potential for understanding fundamental of β-cells function and pancreatic insulin release as well as the pathogenesis of T2D and obesity. The data presented in this thesis offers a small contribution to our mechanistic understanding of GLP-1R signaling in pancreatic β-cells. In this study, we have introduced a novel perspective that shed light on the development of tolerance to GLP-1R agonist with prolonged use. Having this knowledge will enable future endeavors to design and develop innovative GLP-1R agonists that could circumvent receptor endocytosis, thus effectively bypassing GASP1-mediated receptor degradation and subsequently preventing the development of tolerance. Future studies in our lab and others are anticipated to expand on these insights, thereby providing a greater understanding of the role of GASP1-mediated receptor endocytosis in the realms of therapeutic advancements and overall health management.

GASP1-mediated post-endocytic trafficking of bradykinin receptor and COVID-19

The COVID-19 pandemic is the preeminent health care challenge of our times. Approximately 30 million people in the US have been infected with the virus, out of which more than a million have died. Even with our current understanding of the pathophysiology of the disease we are unable to predict with any defined metric how severe a disease will be in any one individual. Advanced age and several preexisting conditions like T2D and obesity increased the risk of severe COVID-19. Additionally, male gender and African and Hispanic descent exhibit association with serious illness even among healthy individuals, suggesting a potential role of genetic factors contributing to the severity of COVID-19. Recent findings have pointed towards a significant involvement of “bradykinin storm” in patients with severe COVID-19.[310,311] The bradykinin storm involved hyperactivity of bradykinin system, an important mediator of inflammation and is characterized by increases in levels of proinflammatory bradykinin and des-Arg10-kallindin, along with unexpected increase in expression level of the bradykinin-1 receptor (BK1R) which normally is expressed at very low levels. BK1R

upregulation has also been observed in chronic inflammatory conditions such as diabetes, suggesting that altered BK1R levels could underlie risk for severe COVID-19.[312]

SARS-CoV-2 virus gains entry to the cells using the angiotensin converting enzyme 2 (ACE2) receptor, which converts angiotensin II to angiotensin (1-7) and promotes vasodilation. ACE2 also regulates the activity of angiotensin converting enzyme ACE, whose primary function is to convert angiotensin I into angiotensin II. Additionally, ACE/ACE2 enzymes are also critical for controlling the level of bradykinin and its active metabolite des-Arg10-kallidin.[313] Thus, a balance of ACE/ACE2 is critical for proper control of blood pressure through the angiotensin system and inflammation through the bradykinin system. COVID-19 infection wreaks havoc on this balance. Interestingly, this imbalance in ACE/ACE2 is also a “pre-existing” condition in patients with diabetes, myocardial injuries and with age. Bradykinins and its active metabolite activate des-Arg10-kallidin activate two distinct GPCR: bradykinin-1 receptor (BKR1) and bradykinin-2 receptor (BKR2). The BK2R is widely expressed in multiple tissues throughout the body while the BK1R is expressed at very low levels if at all under normal conditions.[314] However, in case of an infection, myocardial ischemia, or pre-existing conditions like diabetes, the BK1R expression is rapidly upregulated all of which are risk factors for severe COVID-19.

Activation of BK2R with endogenous ligand bradykinin leads to activation of coupled Gq protein to mediate its cellular effect followed by recruitment of β-arrestins which arrest G protein signal and cause receptor endocytosis. Following endocytosis, the BK2R receptor recycled back to the plasma membrane. In contrast, in the absence of endogenous ligand, Gq-coupled BK1R is mainly found in endosomes since they constitutively endocytosed and targeted for GASP1- mediated lysosomal degradation in absence of ligand, therefore expressed on the cell membrane at very low levels. However, upon binding with des-Arg10-kallidin, the BK1R stabilizes on the plasma membrane, delaying its endocytosis and therefore preventing receptor degradation.[315] Hence, any biological process that increases the amount of des-Arg10-kallidin like pre-existing conditions or decreases the

breakdown of des-Arg10-kallidin, which is mediated by ACE2, would increase the BK1R expression on cell surface. Thus GASP1- mediated receptor trafficking is an attractive candidate for the mechanism responsible for upregulation of BK1R in different physiological conditions. Additionally, the naturally occurring genetic variation in GASP1 may affect the post-endocytic trafficking of BK1R in an individual which affects the severity of COVID-19 symptoms. Further research needs to be done to characterize the role of GASP1 and its genetic variations in COVID-19 severity and multi-organ inflammation. As shown in this research thesis, GASP1-mediated GLP-1R trafficking have profound implications on the therapeutics efficacy of GLP-1R agonists exendin-4, it may be possible that further understanding of the GASP1-mediated degradation of BK1R will aid in the developments of novel therapeutics like BK1R blockers or drug that promotes BK1R endocytosis and degradation for the treatment of COVID-19.

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