THE ROLE OF SOMATOSTATIN IN THE DEFICIENT GLUCAGON RESPONSE TO ACUTE AND RECURRENT HYPOGLYCEMIA IN TYPE 2 DIABETES.

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Abstract

Treatment-induced hypoglycemia results from impaired glucagon counterregulation in a setting of intensive glucose-lowering therapies and remains the major clinical barrier to achieving optimal glycemic control in type 1 diabetes (T1D) and advanced type 2 diabetes (T2D). This dissertation establishes a role for paracrine somatostatin (SST) signaling in the pathogenesis of glucagon counterregulatory failure in diabetes based on data collected from rodent models *in vivo* and human donor islets *in vitro*. First, building on existing evidence from pre-clinical models of T1D,[1–3] this work demonstrates that pharmacologic SSTR2 antagonism can restore physiologic glucagon counterregulation and resist hypoglycemia onset when administered before insulin overdose in rat models of recurrent hypoglycemia (Chapter 4) and pre-diabetes (Chapter 5). Second, when administered as a rescue agent after the onset of severe hypoglycemia in recurrently hypoglycemic rats, SSTR2 antagonism was shown to provide more gradual but sustained glycemic recovery compared to the relatively transient effect of high-dose exogenous glucagon (Chapter 6). Third, functional analysis of isolated human islets *in vitro* showed that deficient glucagon secretion from pancreatic α-cells under low glucose conditions corresponded with a marked hypersecretion of SST, as in T1D.[4,5] Excess paracrine inhibition by SST may underscore the α-cell defect in T2D islets as evidenced by the restorative effect of SSTR2a antagonism on counterregulatory glucagon secretion (Chapter 7.1). These, and other findings from this thesis, suggest that inhibitory SST tone is typically alleviated under low glucose concentration in non-diabetic islets, providing a permissive paracrine signal for counterregulatory glucagon release that is compromised in T2D. Collectively, this work proposes that targeting SST secretion or action may

represent a novel therapeutic strategy for improving glycemic control and reducing the risk of acute and recurrent hypoglycemia in advanced T2D.

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List of Abbreviations

αTOS	α-Tocopheryl Succinate
10ZT	10 mg/g ZT-01
3ZT	3 mg/kg ZT-01
ACC1	Acetyl-CoA Carboxylase-1
ACC2	Acetyl-CoA Carboxylase-2
Acetyl-CoA	Acetyl Coenzyme A
ADP	Adenosine Diphosphate
AID	Automated Insulin Delivery
Akt	Protein Kinase B
AMP	Adenosine Monophosphate
AMPK	AMP-Activated Protein Kinase
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
AUC	Area Under the Curve
BMI	Body Mass Index
cAMP	Cyclic Adenosine Monophosphate
CPT1	Carnitine Palmitoyl Transferase 1
CFTR	Cystic Fibrosis Transmembrane Regulator
CMRL	Connaught Medical Research Laboratories
CNS	Central Nervous System
C-peptide	Connecting peptide
CREB	Cyclic AMP-Responsive Element-Binding Protein
CRTC2	CREB-Regulated Transcription Coactivator 2
CYN154806	Selective SSTR2 Antagonist (experimental compound)
DHAP	Dihydroxyacetone Phosphate
DIO	Diet-Induced Obesity
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EDL	Extensor Digitorum Longus
EG	Exogenous Glucagon
ELISA	Enzyme Linked Immunosorbent Assay
F(2,6)P2	Fructose-2,6-bisphosphate

FBPase-1	Fructose-1,6-bisphosphatase-1
FBS	Fetal Bovine Serum
Fh1βKO	Fumarate Hydratase β-Cell Knockout
FOXO1	Forkhead Box Protein O1
G-6-P	Glucose-6-Phosphate
G-6-Pase	Glucose-6-Phosphatase
GABA	Gamma-Aminobutyric acid
GcGR	Glucagon Receptor
GDH	Glutamate Dehydrogenase
GE Neuron	Glucose-Excited Neuron
GI	Gastrointestinal
GI Neuron	Glucose-Inhibited Neuron
GIP	Gastric Inhibitory Polypeptide
GIPR	Gastric Inhibitory Polypeptide Receptor
GKRP	Glucokinase Regulatory Protein
GLP-1	Glucagon-Like Peptide-1
GLTX	Glucolipotoxic
GLUT1	Glucose Transporter 1
GLUT2	Glucose Transporter 2
GP	Glycogen Phosphorylase
GPCR	G Protein-Coupled Receptor
GS	Glycogen Synthase
GSIS	Glucose-Stimulated Insulin Secretion
GSK3	Glycogen Synthase Kinase 3
HAAF	Hypoglycemia-Associated Autonomic Failure
HbA1C	Glycated Hemoglobin
HFF	High-Fat Fed
HGP	Hepatic Glucose Production
HK	Hexokinase
IM	Intramuscular
IP	Intraperitoneal
IRS1	Insulin Receptor Substrate-1
K_{ATP} Channel	ATP-Sensitive Potassium Channel
KK-Ay Mice	KK-Ay/Ta Jcl Mice

KOH	Potassium Hydroxide
KRBH	Krebs-Ringer Bicarbonate Hepes
L1	Level 1
L2	Level 2
LDH	Lactate Dehydrogenase
Malonyl-CoA	Malonyl Coenzyme A
MAPK	Mitogen-Activated Protein Kinase
MDH	Malate Dehydrogenase
mRNA	Messenger RNA
mTOR	Mammalian Target of Rapamycin
mTORC1	Mammalian Target of Rapamycin Complex 1
mTORC2	Mammalian Target of Rapamycin Complex 2
NAC	N-Acetyl Cysteine
$NaHCO_3$	Sodium Bicarbonate
ND	Non-diabetic
nNOS	Neuronal Nitric Oxide Synthase
NO	Nitric Oxide
NOD	Non-Obese Diabetic
PBS	Phosphate-Buffered Saline
PC	Pyruvate Carboxylase
PDE3B	Phosphodiesterase 3B
PDH	Pyruvate Dehydrogenase
PDK1	3-Phosphoinositide-Dependent Kinase 1
PEP	Phosphoenolpyruvate
PEPCK	Phosphoenolpyruvate Carboxykinase
PFK-1	Phosphofructokinase-1
$PI(3,4,5)P_3$	Phosphatidylinositol (3–5)-trisphosphate
$PI(4,5)P_2$	Phosphatidylinositol (4,5)-bisphosphate
PI3K	Phosphoinositide 3-Kinase
PK	Pyruvate Kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PP1	Phosphoprotein Phosphatase 1
Pre-T2D	Pre-diabetes

PRL-2903	Selective SSTR2 Antagonist (experimental compound)
PRL-2915	Selective SSTR2 Antagonist (experimental compound)
RIA	Radioimmunoassay
ROI	Region of Interest
ROS	Reactive Oxygen Species
SC	Subcutaneous
SD	Standard Deviation
SEM	Standard Error of the Mean
SGLT2	Sodium-Glucose Co-transporter 2
SST	Somatostatin
SST-14	Somatostatin-14
SST-28	Somatostatin-28
SSTR	Somatostatin Receptor
SSTR1	Somatostatin Receptor 1
SSTR2	Somatostatin Receptor 2
SSTR2a	Somatostatin Receptor 2 Antagonist
SSTR3	Somatostatin Receptor 3
SSTR4	Somatostatin Receptor 4
SSTR5	Somatostatin Receptor 5
SSTR5a	Somatostatin Receptor 5 Antagonist
STZ	Streptozotocin
TA	Tibialis Anterior
TCA Cycle	Tricarboxylic Acid Cycle
T-T2D	Treated-Type 2 Diabetes
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
UCN3	Urocortin 3
UT-T2D	Untreated-Tye 2 Diabetes
Veh	Vehicle
VMH	Ventromedial Hypothalamus
ZDF Rat	Zucker Diabetic Fatty Rat
ZT-01	Selective SSTR2 Antagonist (experimental compound)

Chapter 1: Introduction

Over the past several decades, diabetes has become recognized as a multi-hormonal disorder involving insulin, glucagon, and somatostatin (SST).[6] In type 1 diabetes (T1D), insulin secretion is rapidly lost to autoimmune-selective β-cell destruction[7] whereas in type 2 diabetes (T2D), the delayed onset and gradual progression of insulin deficiency reflect exhausted β-cell efforts to compensate for insulin resistance.[8] In T1D and late-stage (insulin deficient) T2D, the relationship between glucagon secretion and blood glucose levels is inverted, such that plasma glucagon level increases after a mixed-meal but not during hypoglycemia.[9] Early evidence from rodent models of diabetes suggests that the SST response to glucose may also become inverted with progressive insulin deficiency in T2D, resulting in blunted release at high glucose[10–12] and excess release at low glucose[4]. Without restoring glucagon counterregulation to hypoglycemia in T1D and advanced T2D, strategies for improving glycemic control, including the use of rapid-acting insulin analogues[13,14] and continuous glucose monitoring[15,16] fail to eliminate the incidence of clinically significant hypoglycemia.[17] As a result, treatment-induced hypoglycemia remains the major barrier to achieving optimal glycemic control in intensively-treated diabetes.[17]

Since glucagon is the body's chief counterregulatory defense against hypoglycemia, and since glucose counterregulation is eventually lost in diabetes,[18] the study of SST biology led to the discovery that pharmacological antagonism of SST receptor 2 (SSTR2) – the SST receptor that mediates glucagon secretion from the pancreatic α-cell – could augment the plasma glucagon response to clamped hypoglycemia in T1D rats.[1–3] In turn, a new class of therapeutic emerged with the potential to prevent insulin-induced hypoglycemia in adults with T1D.[19] Despite advancing SSTR2

antagonists (SSTR2a) to clinical trials,[19,20] direct *in vitro* evidence for an effect of advanced T2D on SST release by pancreatic δ-cells or its paracrine interactions with α- and β-cells, is lacking. Further, hypoglycemia is associated with the intensive use of insulin and/or sulfonylureas in advanced T2D,[21] but few studies have examined the therapeutic potential of an SSTR2a in preventing and/or correcting insulin-induced hypoglycemia in this setting.[22]

Managing the complications of intensive glucose-lowering therapies in individuals with diabetes is often prioritized over management of the disease (i.e., hyperglycemia) itself. Since there are no pharmacological strategies available to prevent hypoglycemia, clinical treatment guidelines recommend a relaxation in insulin dosing to allow for the recovery of sympathoadrenal counterregulatory mechanisms upon self-report of a hypoglycemic event.[23] However, these neuroendocrine mechanisms cannot entirely compensate for deficient glucagon counterregulation in diabetes,[18] and therefore, strategies directed at restoring glucagon secretion may be more effective at preventing hypoglycemia recurrence. In recent years, pancreatic δ-cells have become recognized as an intra-islet signaling hub, integrating metabolic inputs to fine-tune insulin and glucagon secretion and maintain whole body glucose homeostasis.[24] This PhD thesis uses pharmacologic SSTR2 antagonism as an investigative and therapeutic tool to delineate a role for SST signalling in the pathophysiology of defective glucagon counterregulation during acute and recurrent hypoglycemia in T2D, and to propose a pharmacologic strategy for its reversal.

Chapter 2: Literature Review

2.1 Epidemiology of Treatment-Induced Hypoglycemia in T2D

Treatment-induced hypoglycemia is a pervasive clinical complication of intensive treatment with insulin analogues (T1D and T2D) and/or secretagogues (T2D).[21] Annual rates of hypoglycemia are three to four times higher in adults living with T1D versus T2D; [25,26] however, owing to disease prevalence (~20-fold that of T1D globally), T2D now accounts for the majority of diabetes-related hypoglycemic events requiring hospitalization[27,28]. Despite clinically significant improvements in glycemic control with the use of newer basal and bolus insulin analogues[13,14] and glucose-sensing technologies [15,16], rates of severe hypoglycemia (resulting in emergency room visit or hospitalization) have increased [29,30]. The use of insulin analogues across all age-groups in T2D, along with growing disease prevalence, likely account for these trends.[31] Further, the declining rate of all-cause mortality in diabetes, which was twice that of the general population between 1990 and 2010, has increased patient longevity, and thus, extended disease duration.[28,32] Longer duration diabetes is a major risk factor for hypoglycemia[33] that is further compounded by advancing age[34]. Accordingly, hospital admission rates for hypoglycemia have surpassed admission rates for hyperglycemia/diabetic ketoacidosis in older adults living with diabetes in the United States.[29,30] Three large randomized controlled trials found that frequent inadvertent hypoglycemia, secondary to intensive (HbA1C <7%) versus standard (HbA1C <9%) glycemic control in patients with longstanding T2D largely negated the cardiovascular benefits of intensive glycemic management.[35–37] The recurrence of severe hypoglycemia in T2D is associated with increased risk of macrovascular and microvascular disease,[36] arrhythmias,[38] early

dementia,[39] and major adverse cardiovascular events[40]. In both T1D and T2D, one or more severe hypoglycemic events over five years raises mortality rates ~3.5-fold[41]. To offset this risk, clinical practice guidelines recommend a temporary relaxation of glycemic targets upon self-report of one or more severe hypoglycemic events,[23] which typically precludes the maintenance of normal HbA1C levels in adults with diabetes[42]. As a result, glycemic control in diabetes is largely limited by the trade-off between short-term hypoglycemia avoidance and long-term vascular protection.[43]

2.2 Mechanisms of Glucose Counterregulation

Glucose counterregulation during hypoglycemia is mediated by a hierarchy of seemingly redundant neuro-hormonal responses in healthy individuals. First, a drop in blood glucose levels within the euglycemic range suspends insulin secretion from pancreatic β-cells at a glucose threshold of ~4.4–4.7 mM.[44] The ratio of insulin to glucagon in the hepatic portal vein (pancreatic effluent) dictates glucose output by the liver. Therefore, lower portal vein insulin levels favour higher hepatic glucose output and lower glucose uptake.[45] A drop in blood glucose levels below the euglycemic range (≤3.9 mM, classified as level 1 hypoglycemia)[23] triggers the release of chief counterregulatory hormone, glucagon, from islet α-cells at a glucose threshold ~ 3.6–3.9 mM[44]. Along with epinephrine, glucagon stimulates hepatic glycogenolysis and gluconeogenesis to prevent and correct hypoglycemia.[45] Autonomic and neuroglycopenic symptoms typically begin to appear during level 2 hypoglycemia (<3.0 mM)[23] accompanied by the onset of cognitive impairment at blood glucose levels <2.8 mM[44]. Level 3 hypoglycemia, which is not defined by a glycemic threshold, denotes severe cognitive and/or physical impairment requiring external assistance for glycemic recovery.[23]

2.3 Glucose Sensing in the Central Nervous System (CNS)

Glucose-sensing neurons in the CNS regulate blood glucose levels by modulating parasympathetic and sympathetic outflow to the pancreatic islets.[46] Glucose-sensing neurons are widely distributed in the brain but highly represented in the hypothalamic nuclei and brainstem – regions involved in regulating energy homeostasis and food intake.[47] The ventromedial hypothalamus (VMH) is one of the most studied glucose-sensing regions, largely due to its role in regulating neuroendocrine responses to both hypoglycemia and hyperglycemia.[48–50] Accordingly, the expression of immediate early gene, c-fos, is induced in the VMH by both peripheral glucose administration and insulin-induced hypoglycemia.[51,52] Two broad categories of glucose-sensing neurons have been described in the brain: glucose-excited (GE) and glucose-inhibited (GI), which increase their firing rates in response to increasing and decreasing concentrations of extracellular glucose, respectively.[53] Electrophysiological analyses have confirmed the presence of both GE and GI neurons in the VMH, which express critical components of the glucose-sensing pathway found in β-cells, such as glucose transporter 2 (GLUT2), glucokinase, and ATP-sensitive potassium (K_{ATP}) channels.[53,54]

2.3.1 Mechanisms of Central Glucose Sensing

Like pancreatic β-cells, GE neurons in the VMH typically express glucokinase, the rate-limiting glycolytic enzyme that couples glucose metabolism to insulin secretion.[54] Since glucokinase has a low affinity for glucose and there is no end-product inhibition,[55] its activity is proportional to glucose concentration.[46] As extracellular glucose concentration and neural glucose metabolism increase, so does the intracellular ratio of ATP to ADP, leading to K_{ATP} channel closure, plasma membrane depolarization, and

increased neuronal firing rate.[56] Metabolism-independent mechanisms of glucose-sensing have also been reported. One example is the sodium-glucose cotransporter (SGLT), which couples the inward transport of glucose and sodium ions.[57] As extracellular glucose levels rise and glucose enters the neuron, the corresponding influx of sodium ions depolarizes the cell.[57] GI neurons in the VMH also express glucokinase, but unlike GE neurons, their depolarization depends on the inhibition of chloride conductance through the cystic fibrosis transmembrane regulator (CFTR).[58] These neurons are activated during hypoglycemia by an interaction between AMP-activated protein kinase (AMPK) and the gaseous messenger nitric oxide (NO), which leads to chloride channel closure, membrane depolarization, and increased action potential frequency.[59–61] Specifically, AMPK triggers NO production from neuronal NO synthase (nNOS) and the resulting activation of NO receptor, soluble guanylyl cyclase enables the AMPK-mediated inhibition of chloride conductance.[61] Therefore, hypothalamic AMPK acts as a metabolic fuel sensor regulated by NO in GI neurons of the VMH.[59,60,62]

2.3.2 Role of Central Glucose Sensing in Glucose Counterregulation to Hypoglycemia

Activated by a decrease in extracellular glucose, VMH GI neurons project indirectly, via networks of hypothalamic nuclei, onto sympathetic pathways that innervate the adrenal medulla (stimulating the release of epinephrine) and pancreas (stimulating the release of glucagon and inhibiting the release of insulin).[46] Sympathetic motor neurons secrete norepinephrine locally at the islet level and also increase circulating levels of epinephrine, which both stimulate glucagon secretion from α-cells via β2 adrenergic receptors.[63] Accordingly, glucagon, epinephrine, and norepinephrine

responses are reduced by 50-60% during mild hypoglycemia and by 75-80% during severe hypoglycemia in VMH-lesioned rats.[64]

2.4 Hepatic Glucagon Signaling

Glucagon is the primary driver of hepatic glucose production (HGP) *in vivo* during fasting, exercise, and hypoglycemia.[65] A physiological rise in glucagon rapidly increases hepatic HGP by activating glycogenolysis and gluconeogenesis and inhibiting glycolysis and glycogenesis.[65] Glucagon's biological actions are mediated by the glucagon receptor (GcGR), a transmembrane G protein-coupled receptor (GPCR) predominantly expressed in the liver, but also detected in the kidney, adipose tissue, lymphoblasts, spleen, pancreas, brain, adrenal gland, and gastrointestinal tract.[66] Protein kinase A (PKA) is the major intracellular mediator of glucagon's counterregulatory actions in hepatocytes.[67] An acute shift in metabolism favouring net HGP is dependent on the phosphorylation and allosteric regulation of rate-limiting metabolic enzymes in a time course of seconds to minutes.[68,69] However, some conditions, like prolonged fasting or chronic hyperglucagonemia (as in diabetes), engage transcriptional programs that upregulate enzyme expression to supplement HGP.[68,69]

2.4.1 Acute Signaling Pathways

Binding of glucagon to its receptor in hepatocytes leads to the activation of adenylyl cyclase, increased cAMP production, and the activation of PKA.[70] PKA then phosphorylates glycogen phosphorylase (GP, indirectly via glycogen phosphorylase kinase) and glycogen synthase (GS) to stimulate glycogenolysis and inhibit glucogenesis, respectively.[65,70] In parallel, PKA phosphorylates glucokinase regulatory protein (GKRP), either directly or via adenosine monophosphate-activated protein kinase (AMPK), which

disables the activity of glycolytic enzyme, glucokinase, and confines it to the nucleus.[71] As a result, the glycogenolytic and gluconeogenic intermediate, G-6-P, is converted to glucose by glucose-6-phosphatase (G-6-Pase) for hepatic output without futile cycling back to G-6-P by glucokinase.[67] G-6-Pase activity is increased by PKA-dependent signaling, which also inhibits glycolysis and activates gluconeogenesis by reducing the intra-cellular concentration of fructose-1,6-bisphosphate [F(2,6)P2] – an allosteric inhibitor of the gluconeogenic enzyme, fructose-1,6-bisphosphatase (FBPase-1) and allosteric activator of the glycolytic enzyme, phosphofructokinase-1 (PFK-1).[72–74] Finally, pyruvate kinase, the enzyme that converts phosphoenolpyruvate (PEP) to pyruvate as the final step in glycolysis, is directly phosphorylated and inhibited by PKA, thereby further biasing metabolism toward gluconeogenesis.[73,74]

2.4.2 Chronic Signalling Pathways

The dependence of gluconeogenesis on transcriptional activity explains why it is maximally engaged after ~12 hours of fasting, which also coincides with the depletion of glucagon stores.[75] A well-characterized target of hepatic glucagon signaling is the transcription factor, cAMP-responsive element binding protein (CREB).[76] Direct phosphorylation by PKA enables CREB to act as a scaffold for co-regulators of gluconeogenic gene transcription, such as CREB-regulated transcription coactivator 2 (CRTC2).[77] Glucagon-dependent activation of PKA, in turn, induces translocation of CRTC2 to the nucleus, where it co-activates CREB to upregulate the transcription of key gluconeogenic enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and G-6-Pase.[78] PEPCK catalyzes the first committed step in gluconeogenesis by converting oxaloacetate to PEP, and as described in the previous section, G-6-Pase hydrolyzes G-6-P to glucose

as the final step in both gluconeogenesis and glycogenolysis.[79] Glucagon also downregulates glycolysis in favour of gluconeogenesis by inhibiting the transcription of glycolytic enzyme, pyruvate kinase, and promoting the degradation of pyruvate kinase mRNA.[73]

2.4.3 Gluconeogenic Fuel Sources

Gluconeogenesis is the process of converting non-carbohydrate substrates, such as amino acids and glycerol, into glucose.[67] During a prolonged fast, glucagon shifts hepatic metabolism in favour of protein catabolism via mammalian target of rapamycin (mTOR) and its related complexes, mTORC1/2.[80,81] Namely, PKA-activated AMPK acts as a negative regulator of mTORC1, leading to the inhibition of protein synthesis and the stimulation of protein degradation by autophagy.[80,82] In turn, protein catabolism releases endogenous amino acids for use as gluconeogenic precursors.[70] All amino acids, excluding leucine and lysine, are considered gluconeogenic.[70] By activating PKA and CREB, glucagon can upregulate the hepatic expression of amino acid transporters, leading to higher uptake of gluconeogenic amino acids into hepatocytes.[83] The amine group is then removed via enzymatic oxidative deamination or transamination, generating either α-ketoacids that enter the tricarboxylic acid (TCA) cycle directly or glycolytic intermediates that are first converted to pyruvate and subsequently to acetyl coenzyme A (acetyl-CoA).[70] TCA cycle products malate, oxaloacetate, and pyruvate can then feed into the gluconeogenic pathway.[70,84]

In the fasted state, glucagon can also shift hepatic energy metabolism from lipogenesis to gluconeogenesis by activating lipolysis and β-oxidation, and inhibiting lipogenesis.[85] β-oxidation breaks down fatty acid chains to acetyl-CoA, which condenses

with oxaloacetate to form citrate as the first step in the citric acid (TCA) cycle.[70] As mentioned, TCA cycle products, pyruvate, malate, and oxaloacetate can then be diverted to gluconeogenesis.[70] Further, glycerol liberated by the downstream activation of hormone sensitive lipase or adipose triglyceride lipase, can be converted by hepatic enzymes to the gluconeogenic intermediate, dihydroxyacetone phosphate (DHAP).[86] Glucagon signaling also targets the rate-limiting enzyme in fatty acid synthesis, acetyl-CoA carboxylase-1 and 2 (ACC1 and ACC2) via phosphorylation inhibition mediated by PKA and its substrate, AMPK.[85] ACC1/2 both generate malonyl-CoA, a key substrate for fatty acid synthesis and allosteric inhibitor of carnitine palmitoyl transferase 1 (CPT1), the rate-limiting enzyme in fatty acid oxidation.[87] Localized to the outer mitochondrial membrane, CPT1 facilitates the transport of long-chain fatty acids into the mitochondria.[87] Therefore, beyond inhibiting lipogenesis, lower levels of malonyl-CoA also relieve the inhibition of CPT1, in turn, increasing fatty acid uptake and β-oxidation.[87]

2.4.4 Hepatic Insulin Signaling

Insulin counteracts HGP by stimulating glycogenesis and suppressing glycogenolysis.[88] Insulin binds to hepatic insulin receptors, leading to the activation of insulin receptor substrate 2 to engage and activate phosphatidylinositol 3-kinase (PI3K).[89] PI3K catalyzes the conversion of phosphatidylinositol (4,5)-bisphosphate [$PI(4,5)P_2$] into phosphatidylinositol (3–5)-trisphosphate [$PI(3,4,5)P_3$] in the hepatocyte plasma membrane.[89] The generation of $PI(3,4,5)P_3$ leads to the activation of phosphoinositide-dependent kinase-1 (PDK1) and translocation of protein kinase B (Akt) to the plasma membrane, where PDK1-mediated phosphorylation activates Akt.[89] In turn, Akt inhibits glycogen synthase kinase-3 (GSK3), preventing the inhibitory phosphorylation of GS and

maintaining its active state.[89] Akt can also phosphorylate phosphodiesterase-3B (PDE3B), which dampens PKA signaling by degrading intracellular cAMP.[89] Finally, insulin promotes the activation of phosphoprotein phosphatase-1 (PP1), which dephosphorylates GP (inhibitory) and GS (stimulatory), in turn, promoting glycogenesis and inhibiting glycogenolysis, respectively.[89]

2.5 Somatostatin (SST) Biology

SST-14 is an inhibitory peptide hormone secreted by pancreatic δ-cells, which constitute ~5–10% of the total islet-cell mass[90] (Figure 2-1). Circulating levels of SST are unaffected by pancreatectomy in animals[91,92] and humans[93], suggesting a negligible contribution by the pancreas. The predominant isoform of SST in circulation, SST-28, originates from enteroendocrine cells of the gastrointestinal (GI) tract where it regulates digestive functions (i.e., reduces gastric and intestinal motility) by inhibiting the release of various GI hormones.[94] Islet-derived SST is most concentrated in the portal vein (pancreatic effluent), though detection is hampered by the hormone's short half-life (<1 min)[6] and a lack of commercially available assays that can select for either biological isoform. Consequently, islet secretion of somatostatin cannot be reliably measured *in vivo* and must instead be measured *in situ* from the perfused pancreas or *in vitro* from isolated islets or dispersed δ-cells.

2.5.1 Paracrine Mechanisms Regulating Pancreatic SST Secretion

In isolated rodent islets, SST secretion is stimulated by glucose concentrations as low as 3 mM and increases dose-dependently towards a peak at ~20 mM glucose,[95] with half maximal stimulation at ~5–6 mM glucose in rodents (Figure 2-2A) and ~10 mM glucose in humans.[96] SST release is stimulated by β-cell factors, including γ-Aminobutyric

acid (GABA)[97] and urocortin 3 (UCN3)[10] that are co-secreted with insulin in response to glucose. UCN3 accounts for the majority of SST secretion during hyperglycemia and represents a form of autoregulation by the β-cell that feeds back (on a brief delay) via SST to inhibit insulin secretion by paracrine effect.[98] However, secreted β-cell factors appear less important for the regulation of δ-cell activity under hypoglycemic conditions.[98] Speculation that SST secretion is mediated by glucose alone below the threshold for stimulated insulin secretion[98] (~7 mM; Figure 2-2A) has been challenged by the discovery of electrical coupling between β- and δ-cells[97,99]. Islet cells are electrically excitable cells that secrete hormones in response to membrane depolarization.[97] Mathematical modeling of islet-cell signaling suggests that hyperpolarizing (inhibitory) membrane currents spread from β-cells to δ-cells via gap junction connections under low glucose conditions (≤3.9 mM), in turn, suppressing δ-cell activity and SST release.[97,99] In other words, δ-cells are electrically silenced by neighboring β-cells at low glucose concentrations, independent of diffusible paracrine factors.[97,99] The resulting reduction in inhibitory SST signaling "releases the brake" on glucagon secretion during hypoglycemia.[97,99] This permissive paracrine signal is thought to be dysfunctional in diabetes (see section 2.6, below).

2.5.2 Regulation of Insulin and Glucagon Secretion by Pancreatic SST

2.5.2.1 SST Receptors

Islet-derived SST inhibits glucagon and insulin secretion by activating SST receptors (SSTRs) in α-cells and β-cells, respectively. Both cell types have been co-localized with four (SSTR1-3 and SSTR5) of five (SSTR1-5) known SSTR subtypes in humans, but, selective agonism has revealed that SSTR2 is the functionally dominant receptor in human α- and β-cells, with lesser contributions by SSTR1 and SSTR5 to the regulation of both hormones.[100,101] In rodents, insulin and glucagon secretion are inhibited by SSTR5 and SSTR2, respectively.[102]

2.5.2.2 Intracellular SST Signaling

Glucose metabolism drives ATP production by both α- and β-cells, leading to the closure of ATP-sensitive potassium (K_{ATP}) channels. In β-cells, the resulting depolarization by intracellular potassium accumulation triggers action potential firing and a rise in intracellular calcium that stimulates insulin exocytosis.[103] In α-cells, moderate K_{ATP} channel activity at low glucose concentrations establishes a resting membrane potential that drives conductance through voltage-gated calcium (T- and L-type) and sodium channels. The resulting depolarization triggers action potential firing that opens voltage-gated calcium channels, and in turn, the accumulation of intracellular calcium stimulates glucagon exocytosis.[104]

SSTR signaling suppresses glucagon and insulin secretion from α- and β-cells, respectively, through four main effector pathways: (1) inactivation of inhibitory $G_{\alpha i}$-coupled proteins, which decreases adenylate cyclase activity and cytoplasmic levels of cAMP,[105,106] (2) activation of sodium-potassium pumps (β-cells only)[107] and G protein-

gated inwardly rectifying potassium channels, which hyperpolarizes the plasma membrane and inhibits action potential firing[100,108], (3) inactivation of voltage-gated calcium channels, which reduces depolarization-induced calcium influx,[100,109] and (4) direct inactivation of hormone exocytosis, downstream of calcium signaling, in a cAMP-independent manner[100].

2.5.2.3 Regulation of Counterregulatory Glucagon Secretion

The regulation of glucagon secretion from pancreatic α-cells relies on a complex interplay of humoral (circulating nutrients, hormones, and neurotransmitters), neural (sympathetic, parasympathetic, and sympathoadrenal), autocrine, and paracrine inputs.[96] However, physiologic glucagon secretion is retained in isolated intact islets,[110] suggesting a dominant role for intra-islet factors[111]. As illustrated in Figure 2-2A, glucagon secretion from healthy rodent islets is inhibited by glucose in a concentration-dependent manner with maximal effect at 4–5 mM.[95,112] The onset of glucose-stimulated insulin secretion at ~7 mM glucose (Figure 2-2A) does not support a role for insulin, or co-secreted β-cell factors, in delivering glucose-dependent feedback to the α-cell during hypoglycemia.[96] Alternatively, SST is stimulated by glucose in a concentration-dependent manner beyond a glycemic threshold of ~3 mM in rodent islets, which coincides with the concentration-dependent inhibition of glucagon secretion towards a nadir between 4 and 5 mM glucose.[113] Direct evidence for a tonic inhibitory effect of SST on islet α-cells was established in rodent islets by demonstrating that both basal and stimulated glucagon secretion are increased by immunoneutralization of SST[113,114] and pharmacological antagonism of SSTR2[98,115].

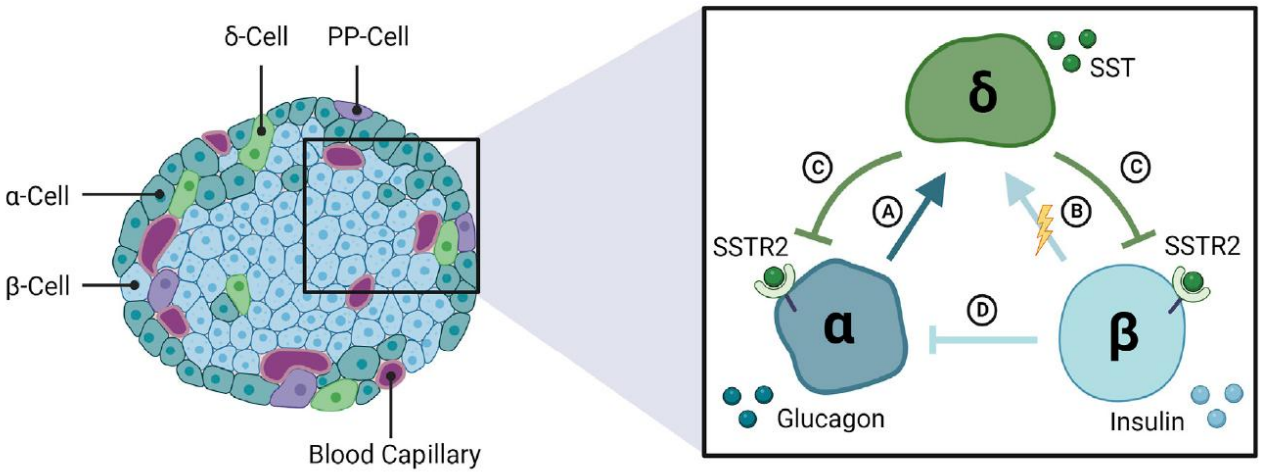

Figure 2-1. The interrelationship among α-, β-, and δ-cells of the endocrine pancreas. The pancreatic islets of Langerhans are composed of three glucose-regulating cell types, α- β- and δ-cells, that secrete glucagon, insulin, and somatostatin (SST), respectively. SST secretion is stimulated by **(A)** glucagon and **(B)** β-cells, likely via electrical coupling with δ-cells rather than secreted insulin. **(C)** SST feeds back to inhibit glucagon and insulin release via SSTR2 in human islets. **(D)** Glucagon secretion is inhibited by insulin and other β-cell secretory products. SSTR2: somatostatin receptor 2; PP: pancreatic polypeptide.

2.6 Glucose Counterregulatory Failure in Diabetes

Hypoglycemia is the most common complication resulting from the intensive use of exogenous insulin therapy (i.e., insulin analogues) and/or endogenous insulin secretagogues (i.e., sulfonylureas and glinides; T2D only) for the treatment of T1D and late-stage (insulin-deficient) T2D.[21] This complication stems from imperfect (non-physiologic) insulin replacement in a setting of defective glucose counterregulation.[21] Since insulin analogues and secretagogues do not act in a glucose-sensitive fashion, they cannot respond to dynamic changes in insulin requirement. As a result, imperfect dosing can lead to a state of relative hyperinsulinemia (i.e., excess systemic insulin levels relative to blood glucose concentration), in which glucose counterregulatory mechanisms become

critical to protecting against hypoglycemia.[116] However, α-cells fail to meet this demand, and become increasingly “blind” (unresponsive) to recurrent hypoglycemia with longer disease duration.[7,18,117,118] Accordingly, hypoglycemia in the range of ~2.5–3.5 mM typically fails to trigger a clinically significant increase in plasma glucagon levels (systemic values fail to increase above ~60 pg/mL when the expected increase is >100 pg/mL) within months of T1D diagnosis,[7] thereby shifting the counterregulatory burden to sympathoadrenal (i.e., epinephrine) and other autonomic mechanisms[18]. However, these neuroendocrine pathways are easily overwhelmed by the glucose-lowering action of insulin analogues and/or secretagogues, leading to inadvertent hypoglycemia, and eventually, the clinical syndrome of hypoglycemia-associated autonomic failure (HAAF).[18] In short, each episode of insulin-induced hypoglycemia lowers glycemic thresholds for sympathoadrenal activation during subsequent hypoglycemia in healthy and diabetic rodents[24,25] and humans[26], delaying the onset of counterregulatory and neuroglycopenic symptom responses. Hypoglycemia unawareness, which is further compounded by other factors that blunt sympathoadrenal activation, such as sleep and advancing age, profoundly impacts quality of life by minimizing the window for restorative glycemic intervention (i.e. carbohydrate consumption).[18] As a result, each episode of hypoglycemia increases the risk of recurrence in a self-perpetuating cycle.[34] Unlike epinephrine, defective glucagon counterregulation, which develops in parallel with HAAF, is not corrected by scrupulous avoidance of hypoglycemia in humans with T1D even though glucagon-secreting α-cells remain responsive to other secretory stimuli, such as certain amino acids.[119,120]

The natural history of α-cell "blindness" to hypoglycemia is less clear in T2D but is thought to mirror the progression of endogenous insulin deficiency, which is much slower in T2D than T1D.[42,121] Of the few *in vivo* studies examining glucagon counterregulation in hypoinsulinemic T2D, all were conducted in human subjects. However, T2D is a heterogeneous condition, and participants tended to differ with respect to age, disease duration, treatment modality, metabolic control, insulin sensitivity, and basal insulin levels, if such details were reported at all. Despite this limitation, basal hypoinsulinemia (relative to control subjects) has emerged as a clinical surrogate of defective glucose counterregulation in T2D.[121–124] The rapid cessation of intra-islet insulin secretion below a glycemic threshold of 4.6 mM is considered a permissive signal for glucagon release,[125] which is reportedly reduced and delayed in late-stage T2D.[122,123] The gradual depletion of functional β-cells in T2D and a corresponding impairment in the glucose-sensing capacity of neighboring α-cells may help to explain why treatment-induced hypoglycemia and HAAF become limiting to glycemic control with longer disease duration.[42,121]

2.6.1 Regulation of Counterregulatory Glucagon Secretion by SST in Diabetes

2.6.1.1 T1D

The paracrine basis of defective glucagon counterregulation in T1D and advanced (insulin-deficient) T2D has eluded islet physiologists for over 40 years. That said, increased islet content (*ex vivo*)[12,126,127] and secretion (*in situ* and *in vitro*)[12,128,129] of SST under low glucose conditions have long implicated SST in the pathogenesis of defective glucagon counterregulation in diabetes. More recently, Vergari et al. measured glucose-dependent SST secretion in a mouse model of T1D lacking the Krebs cycle enzyme fumarate hydratase in pancreatic β cells (Fh1βKO).[4,130] As illustrated in figure 2-3B, SST

secretion at 1 mM glucose was 6-fold higher in Fh1βKO islets relative to control islets, which corresponded with a >75% reduction in glucagon secretion.[4] Consistent with the observation of elevated SST secretion, application of a SST receptor 2 antagonist (SSTR2a, CYN154806) at 1 mM glucose increased glucagon release by 143%±11% in Fh1βKO islets but only by 13%±14% in control mouse islets.[4] Since then, Hill et al. has repeated these experiments in a non-obese diabetic (NOD) mouse model of T1D.[5] Female NOD mice began to develop T1D at >11 weeks of age, associated with an abrupt increase in plasma glucose concentration to >30 mM.[5] However, up until 18 weeks of age, 30–50% of these NOD mice remained normoglycemic and were considered non-diabetic (ND).[5] Experiments conducted at 1 mM glucose in isolated islets and perfused pancreas preparations showed that glucagon secretion decreased sequentially between young ND NOD mice (<7 weeks old), adult ND NOD mice (>12 weeks old), and adult T1D NOD mice (>12 weeks old), which correlated with a sequential increase in SST secretion.[5] Application of the SSTR2a, CYN154806, to isolated islets or the perfused pancreas of adult T1D NOD mice restored glucagon secretion to at least the level observed in age-matched ND NOD controls.[5] In isolated islets from human donors with T1D, lowering the glucose concentration from 6 to 1 mM only stimulated glucagon secretion in the presence of CYN154806, corresponding with markedly elevated SST secretion at 1 mM glucose in T1D versus ND islets.[5]

2.6.1.2 Pre-diabetes

Unlike reports of impaired counterregulatory glucagon secretion in T1D and advanced T2D, glucagon output in the islets of normoglycemic, high-fat-fed (HFF) mice was found to be 2-fold higher than control islets at 1 mM glucose.[131] The observed hypersecretion of glucagon at low glucose was attributed to a 30% reduction in SST secretion and acquired α-cell resistance to SST signaling that could not be explained by changes in α-cell expression of SSTR2.[131] While not tested at lower glucose concentrations, application of the SSTR2a, CYN154806, at 6 mM glucose had little to no effect on glucagon secretion in HFF islets, unlike the marked stimulatory effect observed in control islets.[131]

2.6.1.3 T2D

Hypersecretion of glucagon within and above the euglycemic range (4.0-7.0 mM), a metabolic hallmark of diabetes, has been linked to reduced inhibitory signalling by islet-derived SST.[4,110,131] Recent studies report deficient SST secretion and/or α-cell resistance to exogenous SST at 6-20 mmol/L glucose in intact islets from pre-diabetic[131] and T1D mice[4] and T2D humans[4,110]. Early evidence points to the opposite effect under *low* glucose conditions in islets from T1D mice and T2D humans, whereby a pathological elevation in SST signalling may underscore the characteristic deficiency in counterregulatory glucagon release.[4] In intact isolated islets from humans with T2D, glucagon secretion at 1 mM glucose was 65% lower than from non-diabetic donors, though a reciprocal trend towards elevated SST secretion failed to reach statistical significance as it did in T1D mouse islets.[4] Application of a SSTR2 antagonist (CYN154806) increased glucagon output at 1 mM glucose in two of three islet

preparations with natively impaired glucagon secretion compared to non-diabetic controls.[4] These preliminary findings suggest that the suppression of SST secretion from islet δ-cells may act as a permissive signal for counterregulatory glucagon release, and this signal may be compromised in T1D and advanced T2D.

As mentioned above, islet δ-cells are electrically silenced by neighboring β-cells via gap junction connections at low glucose, which restrains SST at basal levels (i.e., ~<3.0 mM glucose) in healthy rodent islets.[97,99] Release of this hyperpolarizing brake in conditions of significant β-cell loss or dysfunction, as in T1D and late-stage T2D, is thought to increase the electrical excitability of the δ-cell, and in turn, raise SST secretion above normal levels.[97,99,132] In support of this theory, δ-cell dispersion appears to induce δ-cell hyperactivity and increase SST secretion at low glucose concentrations.[133] While this thesis literature review focuses on SST-dependent mechanisms of glucagon counterregulatory failure in insulin-deficient diabetes, other causative mechanisms, such as changes in the anatomical and paracrine relationships between α- and β-cells, may also play a role.[134]

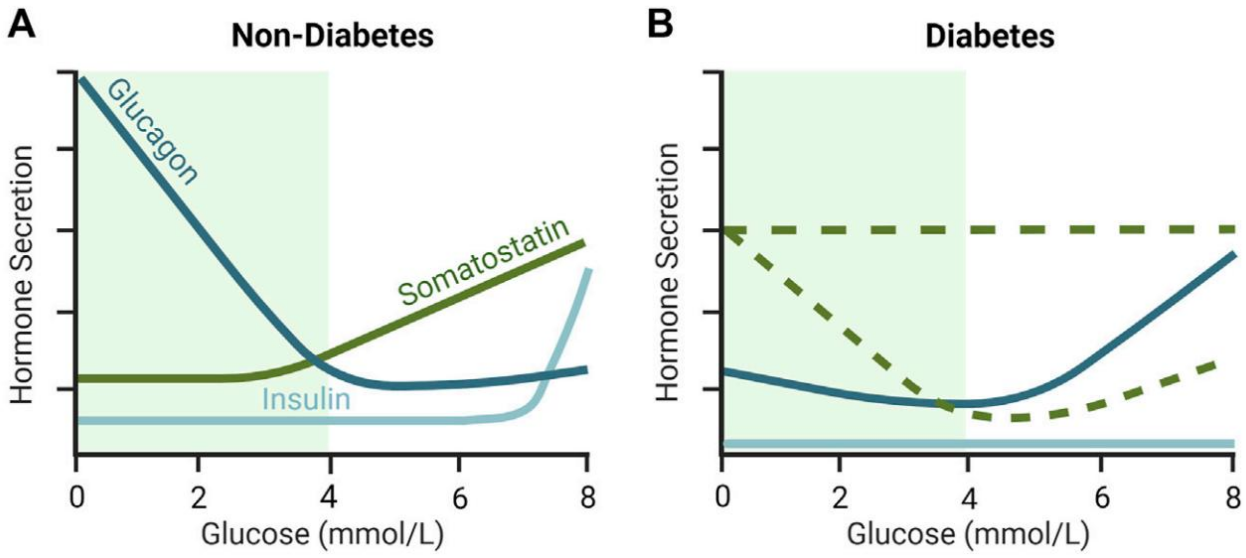

Figure 2-2. Glucose-dependent secretion of insulin, glucagon and somatostatin (SST) in **(A)** non-diabetic and diabetic **(B)** islets. In non-diabetic rodent islets, glucagon secretion is maximally stimulated at low glucose and declines to a nadir at an islet glucose concentration of ~4–5 mmol/L. SST secretion is low during hypoglycemia and increases as glucose rises above 3 mmol/L. In contrast, in diabetes, counterregulatory glucagon secretion is supressed during hypoglycemia and SST is elevated. The loss of β-cell mass in T1D and late-stage T2D rodent islets results in low insulin secretion, but also increased SST secretion, at least during hypoglycemia, and elevations in glucagon secretion in euglycemia and hyperglycemia. As represented by the dashed green lines, the glucose-dependent secretion of SST in diabetes in not completely understood and different animal models provide conflicting views. More specifically, it is unclear whether SST secretion remains relatively stable (i.e., unresponsive) with increasing glucose concentration, or whether it drops below non-diabetic levels during hyperglycemia in diabetes. Green shaded region indicates the glycemic range over which the majority of counterregulatory glucagon secretion occurs in non-diabetic islets. Inspired by Huising et al. (2018); Noguchi and Huising (2019)

2.6.2 Islet Remodeling in Diabetes

Islet remodeling in diabetes distorts the normal paracrine relationships that mediate blood glucose regulation.[135] A reciprocal increase in the proportion of islet α-cells accompanies progressive β-cell dysfunction (de-differentiation, degranulation) in T2D, as observed in T1D.[136] Fractional δ-cell number and area, and SST content per islet are reportedly reduced or unchanged in T2D animals and humans with mild to moderate hyperglycemia and normo- or hyper-insulinemia (i.e., pre-diabetes and early-stage T2D).[90,137,138]. Alternatively, hypertrophy and hyperplasia of islet δ-cells have been documented in T1D rodent islets,[126] which may increase the contact density with neighbouring α-cells [139]. However, it remains unclear whether the δ-to-α-cell ratio is altered in T1D.[116,139,140] Regardless, remodeling of islet architecture may increase α-cell exposure to paracrine SST signaling in T1D, providing an anatomical basis for the impaired glucagon response to hypoglycemia. Few studies have examined δ-cell fate in more advanced (i.e., hypoinsulinemic) forms of T2D. It is important to remember,

however, that islet hormone secretion is glucose-dependent, and therefore, hormone content is not predictive of secretory behaviour under all glycemic conditions.

2.6.3 Hepatic Glucose Metabolism in Diabetes

Several studies have shown that in conditions of hyperglucagonemic, hyperglycemic diabetes, hepatic glucose production (HGP) is increased in the post-absorptive (fasted) and post-prandial (fed) states, owing to a hyperactivation of gluconeogenesis and reduced or absent glycogenolytic flux.[141] This alteration in hepatic glucose metabolism may be owing to several factors: (1) increased availability of gluconeogenic substrates, leading to chronic gluconeogenesis in people with T2D[142] but not in healthy controls[143,144]. This may be explained by a diabetes-related failure of autoregulatory mechanisms to drive gluconeogenesis based on energy demand rather than availability;[144] (2) hyperglucagonemia, leading to chronic activation of HGP and resulting glycogen depletion;[67] and (3) impaired hepatic insulin signaling (resulting from insulin deficiency and/or resistance), leading to reduced glycogen synthesis and inadequate suppression of hepatic glucose output.[67] Accordingly, upregulated gluconeogenic signalling accounts for postabsorptive and postprandial hyperglycemia in diabetes.[141] In agreement, exercise and insulin-induced hypoglycemia each failed to stimulate glycogenolysis in people with intensively treated T1D; however, these conditions failed to stimulate HGP altogether, which may be explained by blunted counterregulatory hormone responses, marked reductions in basal and fasting hepatic glycogen content, even after overnight insulinization, and/or increased dependence on gluconeogenesis.[145–147] Alternatively, moderately controlled T1D was associated with increased rates of both HGP and gluconeogenic flux relative to non-diabetic controls

during rest and exercise.[145] These data suggest that exogenous hyperinsulinemia and/or glycemic normalization, secondary to intensive insulin treatment, may reverse diabetes-related elevations in HGP during periods of low and high demand, ultimately supressing the response below normal levels.[146]

2.6.4 Effects of Recurrent Hypoglycemia on Central Glucose Sensing

Some of the glucose counterregulatory dysfunction in diabetes may be related to adaptations in central (i.e., brain) glucose sensing and regulation of glucose counterregulatory hormones.[46] For example, recurrent hypoglycemia impairs the glucose sensitivity of glucose-inhibited (GI) and glucose-excited (GE) neurons in the ventromedial hypothalamus (VMH).[148] While the mechanism(s) are not fully resolved, alterations in energy uptake and metabolism, oxidative stress, neurotransmission, and fuel sources have been proposed.[149]

2.6.4.1 Increased Glucose Uptake and Metabolism

Recurrent hypoglycemia has been shown to upregulate the mRNA and protein expression of glucose transporter, GLUT1 in endothelial cells of the blood-brain barrier, leading to increased cerebral glucose uptake.[150] Furthermore, the rate of glucose phosphorylation (i.e., the first committed step in glycolysis) is markedly increased by antecedent hypoglycemia in the hypothalamus, but not in other brain regions, such as the brainstem or frontal cortex.[151] Together, these complementary adaptations may reflect a compensatory mechanism for preserving cerebral glucose metabolism in response to recent glucose deprivation, which masks hypoglycemic detection by glucose-sensing neurons.[46] However, it remains unclear whether these adaptations, observed in rodent models, are conserved in humans.[46]

2.6.4.2 Oxidative Stress

Acute hypoglycemia increases levels of hypothalamic reactive oxygen species (ROS), resulting in the S-nitrosylation of various proteins, including the nitric oxide (NO) receptor, soluble guanylyl cyclase, which reduces its activation.[152] Impaired NO signaling prevents GI neurons from sensing a glucose deficit, so this hypothesis is consistent with the observation that neuronal nitric oxide synthase (nNOS) activity decreases with recurrent hypoglycemia.[62] In non-diabetic rats, pre-treatment with N-acetyl cysteine (NAC), which enhances the redox capacity of the glutathione antioxidant system, prevented both hypoglycemia-induced ROS production in the VMH and counterregulatory impairment during subsequent hypoglycemia.[62] However, other studies suggest that the overexpression of both glutathione and thioredoxin antioxidant systems in the VMH may be necessary to preserve the redox state, and therefore, the glucose-sensitivity of GI neurons.[153]

2.6.4.3 Altered Hypothalamic Signaling

Animal studies have shown that the suppression of hypothalamic *γ*-aminobutyric acid (GABA) signaling is critical to the initiation of counterregulatory hormone responses.[154,155] Antecedent hypoglycemia increases GABAergic tone within the VMH of rats during subsequent hypoglycemia, which suppresses glucagon and sympathoadrenal counterregulation.[155,156] This effect was reproduced in humans via pharmacologic activation of GABA receptors.[157]

2.6.4.4 Use of Alternate Fuels

Glycogen is the major energy reserve in the brain. Though it is localized almost exclusively to astrocytes, glycogen can be metabolized to lactate via glycolysis and

exported to neurons for use as an energy source during hypoglycemia.[158] It has been proposed that supercompensation of brain glycogen content following an episode of hypoglycemia may provide the brain with additional fuel stores during subsequent hypoglycemia in rodents[159] and humans[160]. Lactate infusions have been shown to suppress counterregulation during systemic hypoglycemia in rodents[161,162], and lactate transport and metabolism by the brain are thought to be upregulated in both recurrently hypoglycemic rodents[163,164] and humans[164]. Though the mechanism is unclear, lactate may supress AMP-activated protein kinase (AMPK; required for GI neuronal activation)[165] and increase inhibitory GABAergic tone in the VMH[161], which is typically withdrawn during hypoglycemia to permit counterregulatory activation[155]. However, other studies suggest that lactate may be insufficient to support metabolism during hypoglycemia.[163,166] One group that did not detect an increase in brain glycogen deposition did report faster glycemic recovery after recurrent hypoglycemic exposure compared to before.[167]

2.7 Treatment with SSTR2a for Hypoglycemia Prevention in T1D

Figure 2-3 depicts α- and δ-cell responses to hypoglycemia in the non-diabetic and insulin-deficient diabetic states. In the non-diabetic state, SST secretion and the resultant activation of SSTR2 are low during hypoglycemia, allowing for the release of glucagon to prevent and correct hypoglycemia. In conditions of T1D and late-stage T2D, the excess release of SST secretion during hypoglycemia may supress the counterregulatory glucagon response, which is reversible with SSTR2a.

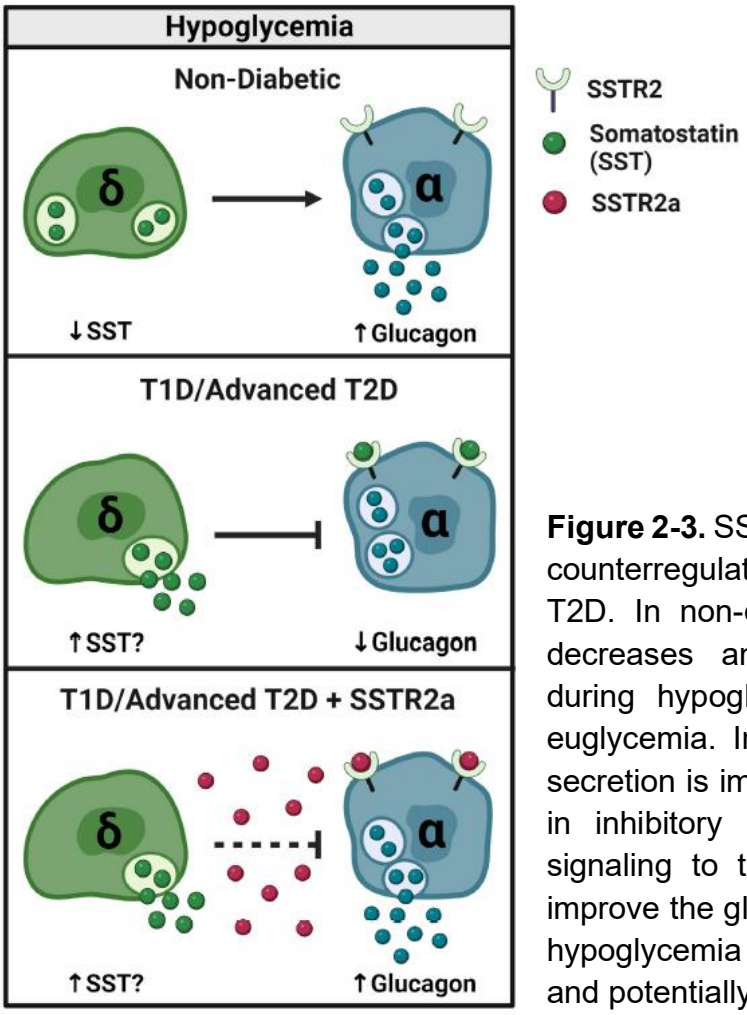

Figure 2-3. SSTR2 antagonism reverses glucagon counterregulatory failure in T1D and advanced T2D. In non-diabetic conditions, SST secretion decreases and glucagon secretion increases during hypoglycemia (≤3.9 mmol/L) to restore euglycemia. In T1D, counterregulatory glucagon secretion is impaired, possibly due to an increase in inhibitory SST signaling. By blocking SST signaling to the α-cell, pharmacologic SSTR2a improve the glucagon response to insulin-induced hypoglycemia in rodents and humans with T1D and potentially advanced (insulin-deficient) T2D.

2.7.1 Effects of SSTR2a in Rodent Models of T1D

As an adjunct to intensive glucose lowering treatment with insulin analogues and/or secretagogues (i.e., sulfonylureas and glinides), agents that block SSTR2 signaling in pancreatic α-cells may help alleviate the risk of treatment-induced hypoglycemia in T1D and late-stage T2D. The therapeutic effect of a selective SSTR2a, PRL-2903, on glucagon counterregulation was first demonstrated in streptozotocin (STZ), biobreeding, and non-obese diabetic (NOD) models of T1D during acute[1,3] and recurrent[2] hypoglycemia. Normalization of plasma glucagon levels with PRL-2903 fully alleviated the dependence on glucose infusion for the maintenance of clamped hypoglycemia in biobreeding rats[3] and reduced glucose dependence in STZ rats when hypoglycemia was induced with low- (5 U/kg) but not high- (10 U/kg) dose insulin[1]. No drug effect was observed under basal conditions in the STZ model of T1D.[1] In the same model, PRL-2903 prevented a 20-fold reduction in the plasma glucagon response to subsequent hypoglycemia after five antecedent episodes of hypoglycemia in three days.[2] SSTR2 blockade also raised the glucose nadir from level 2 (2.7 mM) to level 1 (3.7 mM) hypoglycemia and reduced the duration of subsequent hypoglycemia (≤3.9 mM) by ~50 min compared to vehicle treatment.[2] In adult T1D NOD mice, the SSTR2a, CYN154806, largely restored the 300% increase in plasma glucagon level observed in control mice during hypoglycemia.[5] Incomplete restoration of the response in these mice was attributed to markedly attenuated hepatic glycogen stores relative to contols.[5]

Aerobic forms of activity (e.g., walking, cycling, endurance exercise) often result in immediate and/or delayed episodes of hypoglycemia in people with T1D. This risk may stem from lower baseline hepatic glycogen stores (particularly if glycemic control is

suboptimal),[168] impaired or absent glucagon release during exercise,[5] and/or increased muscle glucose disposal without a corresponding reduction in circulating insulin levels (i.e., relative hyperinsulinemia)[169]. These metabolic disturbances collectively result in a blunting of hepatic glucose production during exercise. To help restore glucagon counterregulation and limit hypoglycemia during exercise, Leclair et al. evaluated the efficacy of a SSTR2a in STZ-T1D rodents during combined bolus insulin/exercise challenges.[170] STZ-T1D rodents were pre-treated with the SSTR2a, PRL-2903 (10 mg/kg, IP), or placebo, 90 minutes before performing a bout of treadmill exercise in a hyperinsulinemic state.[170] This hyperinsulinemic exercise protocol was repeated on two consecutive days, and again one week later with treatment crossover.[170] On all three occasions, plasma glucagon level increased 3-6-fold from baseline with PRL-2903 whereas no response was detected in the placebo group. PRL-2903 was without effect under basal glucose conditions.[170] These findings suggest that SSTR2 antagonism may help to improve glucagon counterregulation in animals during recurrent exercise-induced hypoglycemia.[170]

Since then, Farhat and others have demonstrated the superior efficacy of more potent SSTR2a peptide, ZT-01 (Zucara Therapeutics), compared to PRL-2903, in a setting of insulin-induced hypoglycemia.[171] ZT-01 raised the peak plasma glucagon response ~4-fold over PRL-2903 when compared head-to-head during clamped hypoglycemia in STZ-T1D rats.[171] ZT-01 was also more effective than PRL-2903 at raising the blood glucose nadir in free-living STZ-T1D rats after bolus insulin challenge, thereby reducing the incidence and duration of hypoglycemia (levels 1 and 2).[171] Additionally, the pharmacokinetic properties of ZT-01 were more favorable compared to PRL-2903 when

delivered by intraperitoneal (IP) or subcutaneous (SC) routes in STZ-T1D rats.[171] Namely, cumulative drug exposure (based on AUC of plasma drug concentration) was eleven times higher for ZT-01 than PRL-2903 over a 24-h period.[171] After establishing the superior therapeutic and pharmacokinetic properties of ZT-01 as compared to PRL-2903, the authors identified the minimum effective dose level of ZT-01 (0.3 mg/kg) that could increase glucagon secretion during clamped hypoglycemia (~2.5 mM) in poorly controlled, insulin-treated STZ-T1D rats.[171] Based on these and other safety and efficacy trials, ZT-01 advanced to phase 1 clinical trials, which assessed drug safety and pharmacodynamics in adults with and without T1D [19,172] In the phase 1b trial, the peak plasma glucagon response to ZT-01 (3 or 20 mg) was evaluated in subjects with T1D during clamped level 1 (3.5±0.3 mM) and level 2 (2.6± 0.2 mM) hypoglycemia (manuscript not yet peer-reviewed).[172] During level 1 hypoglycemia, ZT-01 raised peak plasma glucagon levels by up to 15 pg/mL from baseline, without an observable response in the placebo group.[172] During level 2 hypoglycemia, a maximal increase from baseline of 8 pg/mL in the placebo group was >3-fold larger with ZT-01 treatment.[172] A phase 2 study is currently recruiting participants with T1D to evaluate the efficacy of ZT-01 in reducing nocturnal hypoglycemia.[173]

2.8 Therapeutic Applications of other SSTR Antagonists (SSTR2a) for Diabetes Management

Beyond hypoglycemia prevention, SSTRa's may be capable of improving whole-body glucose metabolism through an incretin-dependent mechanism. Glucagon-like peptide-1 (GLP-1) is an incretin hormone secreted by enteroendocrine L-cells of the small intestine in response to nutrient stimulation. GLP-1 reduces postprandial glucose

excursions by stimulating insulin release from pancreatic β-cells, inhibiting gastric motility, and promoting satiety.[174] Incretin release[175,176] and/or function[177,178] are impaired in people with obesity and T2D, and GLP-1-targeted therapies offer effective management of body weight and blood glucose levels, with underlying improvements to β-cell function and peripheral insulin sensitivity.[179,180] It is well established that gut-derived SST inhibits GLP-1 secretion by paracrine effect, mediated primarily by SSTR5 in intestinal L-cells.[181] Several SSTR5 antagonists (SSTR5a) have been developed in the past decade with the ability to improve glucose tolerance by increasing circulating levels of active GLP-1 and insulin in healthy mice,[182,183] Zucker diabetic fatty (ZDF) rats,[184,185] diet-induced obese (DIO) mice,[183–185] and KK-Ay/Ta Jcl (KK-Ay) mice, a model of obese T2D with severe insulin resistance[186]. Further, oral administration of an SSTR5a for two weeks has been shown to significantly improve insulin sensitivity in KK-Ay mice, independent of weight loss and changes in food intake.[186] While the mechanism is unclear, SST may inhibit hepatic insulin signaling by blocking the insulin-induced phosphorylation of Akt, an effect that may be reversible with SSTR5a in mice.[186] Since hepatic insulin resistance can impair glucagon signaling and gluconeogenesis, restoring hepatic insulin sensitivity may have indirect benefits for glucose counterregulation.[186] The risk of hypoglycemia associated with SSTR5 antagonism was determined to be low in four-hour fasted lean C57BL/6N mice, since a supra-efficacious dose (30 mg/kg) of SSTR5a did not reduce basal glucose levels for five hours after administration.[182] In summary, SSTR2a and/or SSTR5a may lower blood glucose levels by mechanisms that are partially (via SSTR2a) or entirely (via SSTR5a) dependent on the stimulation of intestinally-derived GLP-1.[183] Since SSTR2a also stimulates insulin secretion by a direct effect on human β-cells,

engaging these complementary pathways through combined SSTR2 and SSTR5 antagonism may have additive effects on insulin release and whole-body glucose metabolism.

2.9 GIP and the α-Cell

Glucose-dependent insulinotropic polypeptide (GIP) is an intestinally derived incretin hormone that is secreted in the post-prandial state.[174] The GIP receptor (GIPR) is expressed in many cell types involved in the regulation of metabolism, including α-, β-, and δ-cells of the pancreatic islets.[174]. Like its sister hormone, GLP-1, GIP stimulates post-prandial insulin secretion by activating its receptor in β-cells; however, GLP-1, has received considerably more attention over the past thirty years owing to its clinical success in treating obesity and T2D.[187] One distinction between the two hormones is that GLP-1 inhibits glucagon secretion in the post-prandial state, which helps improve glucose tolerance.[188] Less is known about GIPR activity in α-cells or the control of glucagon secretion.[189]

Functionally, the activation of GIPRs in rodent α-cells has been shown to increase calcium influx and depolarization-evoked glucagon exocytosis by a PKA-dependent mechanism.[190] Accordingly, GIP likely potentiates glucagon secretion from activated α-cells in a setting of low-glucose stimulation by engaging cAMP/PKA signaling pathways.[190] While the available evidence points to a direct effect of GIP on islet α-cells, experiments involving α-cell GIPR knockouts are lacking.[189] Studies conducted in live humans and the perfused rat pancreas suggest that GIP infusion stimulates glucagon secretion in a dose- and glucose-dependent manner, with maximal effect during hypoglycemia.[191,192] That said, the stimulation of glucagon secretion by GIP, infused

during an acute episode of hypoglycemia, was relatively modest (+~3 pM) in non-diabetic adults compared to the effect of hypoglycemia alone (+~30 pM).[191]

2.1.0 The Artificial Pancreas

The artificial pancreas, also called a closed loop or automated insulin delivery (AID) system is composed of a glucose sensor, insulin infusion pump, and insulin dosing algorithm.[193] A real-time continuous glucose monitor inserted into the subcutaneous (SC) space measures glucose concentrations in the interstitial fluid every ~5 minutes and sends this data to a control algorithm device (i.e. an application hosted by a smartphone or the pump itself) that calculates the correct dose of insulin and automatically adjusts the infusion rate based on current and predicted glucose levels.[193]

Current AID systems are capable of temporarily suspending insulin delivery to reduce the severity and duration of existing or impending hypoglycemia compared with standard pump therapy.[194] However, this strategy does not prevent hypoglycemia altogether, since even the most rapid-acting insulins used in algorithmically advanced AID systems will remain active in circulation for three to five hours once delivery is withheld.[195] Therefore, this approach has limited efficacy in settings of rapidly declining glucose, such as after a meal-related insulin bolus or during continuous aerobic exercise.[194] Glucagon has a pharmacokinetic advantage over insulin suspension for preventing hypoglycemia since its subcutaneous absorption and onset/duration of action are faster than the most rapid-acting insulins.[196]

Two different approaches have been described for dosing glucagon in a dual-hormone artificial pancreas.[197] The first prioritizes hypoglycemia prevention by delivering a minimum daily dose of glucagon without increasing insulin administration.[197] The

second targets a lower mean glucose concentration by intensifying insulin treatment and ultimately balancing the increased risk of hypoglycemia with a higher daily dose of glucagon than the previous method.[197]

Eight studies to date have directly compared single- and dual-hormone artificial pancreas systems.[198] In each of these studies, the dual-hormone system used a glucagon-dosing algorithm aimed at reducing hypoglycemia rather than permitting more aggressive insulin treatment.[198] The dual-hormone artificial pancreas was shown to reduce the frequency and duration of hypoglycemia during daytime hours and exercise compared to the single hormone system in short-term studies but results for the late post-prandial period and nocturnal hypoglycemia are inconsistent.[199] It is also unclear whether the addition of glucagon to AID systems can further reduce HbA1C levels and the risk of severe hypoglycemia as compared to more conventional insulin-only systems.[199] The timing of glucagon delivery in relation to insulin is important to consider since the efficacy of glucagon in preventing hypoglycemia is dictated by circulating insulin levels.[200] Clinical studies show that micro-boluses of glucagon can correct hypoglycemia in a dose-dependent manner when insulin levels (and thus, the insulin-to-glucagon ratio in the hepatic portal vein) are low, but fail to influence hepatic glucose output when insulin levels are high, irrespective of glucose concentration.[200,201] Therefore, it is unlikely that the addition of glucagon to the closed-loop artificial pancreas will reproduce physiologic glucose control or completely eliminate the risk of hypoglycemia.[198]

Emergency glucagon, delivered at a dose of 1 mg in injectable and intranasal formats, is the standard of care for treating acute episodes of severe hypoglycemia, which require the assistance of another person for recovery.[202] The total daily dose of glucagon

tested in dual-hormone systems has ranged from 0.145 to 0.82 mg/day, for a maximum of eight days, however, the long-term safety and efficacy of chronic, low-dose delivery must still be assessed.[196]

Notably, glucagon is known to exert biological effects on multiple organ systems, including cardiovascular, renal, GI, and CNS.[203] For example, glucagon may reduce food intake by slowing GI motility and activating satiety centres in the CNS, and may increase energy expenditure by inducing thermogenesis of brown adipose tissue.[203] Acute side-effects of the standard rescue dose of glucagon (1 mg) commonly include nausea, vomiting, and headaches, and while available data suggest that these symptoms are rare at doses below 1 mg, longer-term studies are required.[203] Other concerns related to chronic glucagon delivery, including hepatic glycogen depletion, glucagon resistance, and/or tachyphylaxis, must also be addressed.[196] Finally, current glucagon formulations are not liquid stable, so glucagon cartridges must be replaced every 8-24 hours with freshly reconstituted glucagon.[196] While pump-compatible formulations are currently in development,[204] they have yet to undergo safety and efficacy testing.[197] In summary, the increased cost and complexity of a dual- versus single-hormone system must be weighed against its clinical benefit, which is focused on minimizing hypoglycemia during sleep, exercise, and the post-prandial period.[199]

Chapter 3: Research Objectives

The first objective of this dissertation was to investigate the therapeutic potential of pharmacologic somatostatin receptor 2 antagonism in restoring glucagon counterregulation and resisting the onset of insulin-induced hypoglycemia in rat models of recurrent hypoglycemia (Chapter 4) and pre-diabetes (Chapter 5). The second objective was to investigate the therapeutic potential of a somatostatin receptor 2 antagonist (SSTR2a) as an alternative to exogenous glucagon in rapidly reversing insulin-induced hypoglycemia without causing peripheral hyperglucagonemia (Chapter 6). We further investigated whether co-treatment with incretin hormone, glucose-dependent insulinotropic polypeptide (GIP) could augment glycemic rescue with SSTR2a (Chapter 6). Next, we extended our findings from rats to humans by characterizing glucagon and insulin responses to SSTR2 antagonism in isolated islets from T2D donors and from non-diabetic donors exposed to T2D culture conditions (Chapter 7.1). We also characterized the glucose dependence of somatostatin (SST) secretion in these islet preparations to elucidate the disease process that is reversible with SSTR2a, since islet-derived SST cannot be measured *in vivo* (Chapter 7.1). Finally, we examined the effects of advanced T2D, with and without basal insulin replacement, on islet hormone composition in rats and humans as a potential mechanism driving the inhibition of counterregulatory glucagon secretion by islet-derived SST (Chapter 7.2).

Chapter 4: Somatostatin Receptor Antagonism Reverses Glucagon Counterregulatory Failure in Recurrently Hypoglycemic Male Rats

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4.1 Abstract

Recent antecedent hypoglycemia is a known source of defective glucose counter-regulation in diabetes; the mechanisms perpetuating the cycle of progressive α-cell failure and recurrent hypoglycemia remain unknown. Somatostatin has been shown to suppress the glucagon response to acute hypoglycemia in rodent models of type 1 diabetes. We hypothesized that somatostatin receptor 2 antagonism (SSTR2a) would restore glucagon counterregulation and delay the onset of insulin-induced hypoglycemia in recurrently hypoglycemic, non-diabetic male rats. Healthy, male, Sprague-Dawley rats (n = 39) received bolus injections of insulin (10 U/kg, 8 U/kg, 5 U/kg) on 3 consecutive days to induce hypoglycemia. On day 4, animals were then treated with SSTR2a (10 mg/kg; n = 17) or vehicle (n = 12) 1 hour prior to the induction of hypoglycemia using insulin (5 U/kg). Plasma glucagon level during hypoglycemia was ~30% lower on day 3 (150 ± 75 pg/mL; $P < .01$), and 68% lower on day 4 in the vehicle group (70 ± 52 pg/mL; $P < .001$) compared with day 1 (219 ± 99 pg/mL). On day 4, SSTR2a prolonged euglycemia by 25 ± 5 minutes ($P < .05$) and restored the plasma glucagon response to hypoglycemia. Hepatic glycogen content of SSTR2a-treated rats was 35% lower than vehicle controls after hypoglycemia induction on day 4 (vehicle: 20 ± 7.0 vs SSTR2a: 13 ± 4.4 μmol/g; $P < .01$). SSTR2a treatment reverses the cumulative glucagon deficit resulting from 3 days of antecedent hypoglycemia in healthy rats. This reversal is associated with decreased hepatic glycogen content and delayed time to hypoglycemic onset. We conclude that recurrent hypoglycemia produces glucagon counterregulatory deficiency in healthy male rats, which can be improved by SSTR2a.

4.2 Introduction

Recurrent hypoglycemia is a pervasive clinical complication of insulin therapy in type 1 diabetes (T1D), owing to imperfect (nonphysiological) insulin replacement in a setting of defective glucose counterregulation.[18] The α-cell, which secretes the chief counter-regulatory hormone, glucagon, becomes unresponsive to hyperinsulinemic hypoglycemia with progressive β-cell failure and endogenous insulin deficiency.[18] Within about 5 years of T1D diagnosis, the counterregulatory burden has shifted to sympathoadrenal and other autonomic mechanisms.[18] However, these pathways are easily overwhelmed by intensive insulin therapy, commonly leading to recurrent hypoglycemia and the clinical syndrome of hypoglycemia-associated autonomic failure (HAAF).[18] In short, recent antecedent hypoglycemia lowers glycemic thresholds for sympathoadrenal activation to subsequent hypoglycemia, resulting in diminished counterregulatory and neuroglycopenic symptom responses.[18]

It is important to note that the literature models HAAF as the mechanism perpetuating recurrent hypoglycemia.[205] This model assumes a state in which the α-cell is already completely unresponsive to hypoglycemia; however, at least a partial glucagon response is typically present in cases of newly diagnosed T1D (≤5 years) and late stage type 2 diabetes even before HAAF develops.[123,206] In these and healthy animal models, it is well established that glucagon counterregulation is attenuated by acute, insulin-induced hypoglycemia [207,208] and diminishes further with repeat hypoglycemia exposure. [209,210] Unlike epinephrine, glucagon secretion in diabetic rats is not corrected by short-term (3-4 weeks) hypoglycemia avoidance.[211,212] Moreover, the glucagon response to hypoglycemia is preserved where neural inputs have been severed in vivo (cervical spinal

cord transection, partial or complete pharmacological adrenergic blockade, pancreas transplant)[213–215] and in vitro (perfused rodent pancreas and perifused rodent and human islets).[216,217] Conversely, a sustained rise in intra-islet insulin levels during hypoglycemia induced with insulin secretagogue, tolbutamide, silences the glucagon response to hypoglycemia despite intact autonomic signalling.[218] Collectively, these data suggest the involvement of non-neural mechanisms, potentially operating in parallel to HAAF, that underpin defective glucagon counterregulation to recurrent hypoglycemia.

Somatostatin-14 is an endocrine hormone secreted by δ-cells of the endocrine pancreas.[6] As the gatekeeper of the pancreatic islets, somatostatin exerts tonic inhibitory control over neighbouring α- and β-cells by activating somatostatin receptor type 2 (SSTR2) and types 3/5 (SSTR3/5), localized to α- and β-cells, respectively.[219] Reported elevations of pancreatic and plasma somatostatin in diabetic humans,[220] dogs,[221] and rodents[222–224] have long implicated δ-cell dysfunction in the failed α-cell response to insulin-induced hypoglycemia in T1D.[6] More recently, Rorsman's group demonstrated that a therapeutic dose of insulin inhibited hypoglycemic glucagon secretion by an indirect, paracrine mechanism mediated by somatostatin - a feature that was reversible with somatostatin type 2 receptor antagonism (SSTR2a).[225]

Pharmacological SSTR2a recently advanced to phase 1 clinical trials as a therapeutic tool for hypoglycemia prevention in T1D.[226] In pre-clinical trials, SSTR2a was shown to restore glucagon counterregulation in response to acute[1,3] and recurrent[2] episodes of clamped hypoglycemia in rodent models of streptozotocin- and biobreeding-T1D, and in healthy, human pancreas slices.[6]

In this study, we used a non-diabetic rat model of hypoglycemia-induced counterregulatory failure to determine whether somatostatin signalling mediates the suppressive effect of recent, antecedent hypoglycemia on subsequent glucagon counterregulation. To this end, we evaluated the restorative potential of pharmacological SSTR2 antagonism on hormonal (glucagon and C-peptide) and glycemic responses to hypoglycemia after three prior episodes in non-diabetic rodents. The absence of pre-existing α-cell dysfunction in our model enabled us to selectively target and reverse the glucagon secretory defect secondary to recurrent hyperinsulinemic-hypoglycemia per se. We hypothesized that SSTR2 blockade would rescue the glucagon secretory response and its catabolic effects on hepatic glycogen stores to resist subsequent hypoglycemia in recurrently hypoglycemic, non-diabetic, male Sprague-Dawley rats.

4.3 Materials and Methods

This study was conducted in accordance with the recommendations of the Canadian Council for Animal Care guidelines and has been approved by the York University Animal Care Committee (Protocol # 2017-7).

Rodent Treatment and Experimental Design Forty-five (n=45) healthy, non-diabetic, male Sprague-Dawley (SD) rats (Charles River Laboratories, ~250g body mass post weaned, age ~ 9-10 weeks old) were habituated in the York University Vivaria for one-week before handling. Rats were housed in a light controlled (12-hour light/dark cycle) room with a humidity of 50-60% and temperature of 22-23^{o}C. Rats had ad-libitum access to standard rodent chow (Purina Labdiet 5012, St. Louis, Missouri) and water. Following the 7-day habituation period, body weight, blood glucose, and food intake were monitored daily. Fig. 4-1A provides an outline of the study design, with an itemized

breakdown of animal sub-groupings, animal exclusions, and analytes measured for each condition.

Baseline Controls (Day 1) Following habituation, a subset of rats (n=6) was randomly selected for baseline analysis and excluded from all hypoglycemia protocols. A blood sample was collected via saphenous vein bleed for baseline (basal/fed) measurement of plasma glucagon and C-peptide concentrations. These rats (and all others in this study at their respective endpoints) were then anaesthetized using isoflurane gas for portal blood and tissue collection (see below).

Hypoglycemia Conditioning (Days 1-3) Hypoglycemia has been clinically defined by the American Diabetes Association Workgroup on Hypoglycemia as a whole blood glucose concentration of ≤3.9 mmol/l.[227] In this study, we undertook a three day hypoglycemia conditioning protocol to induce defective glucagon counterregulation in healthy rats. The experimental procedure for hypoglycemic conditioning is outlined in Fig. 4-1B**.** Thirty-nine rats underwent recurrent insulin-induced hypoglycemia, comprised of one, 120-min hypoglycemic event per day, on three consecutive days (food was removed ~60-min prior to each event). A stepwise reduction in the insulin dose was used to induce a similar level of hypoglycemia on each day (day 1: 10 U/kg, day 2: 8 U/kg, day 3: 5 U/kg, IP Humulin-R, Lilly, Canada). This insulin dose reduction over days 1-3 was designed in anticipation of diminished counterregulatory function over the 3-day conditioning period. [228] Blood glucose was measured in duplicate via tail prick using a hand-held glucometer (ATRAK, Abbott) before insulin administration (t=0 min) and every 10-min thereafter until each animal had remained between 1.7-2.5 mmol/l for 120 consecutive minutes. This is an established protocol for reproducing overt and progressive glucagon failure in healthy

rodents. [161,229,230] Animals that failed to reach the glycemic target by 60-min of their initial insulin injection received a second dose of insulin. If blood glucose levels dropped below range at any time, 0.1-1.0 ml of 35% D-glucose was administered, as necessary, to restore target hypoglycemia. If physically capable, animals drank the dextrose solution from a syringe; otherwise, it was delivered via oral gavage. Any animal that reached severe hypoglycemia (i.e., seizure and/or loss of consciousness) was immediately withdrawn from the study and excluded from all datasets (see Fig. 4-1A for one such exclusion).

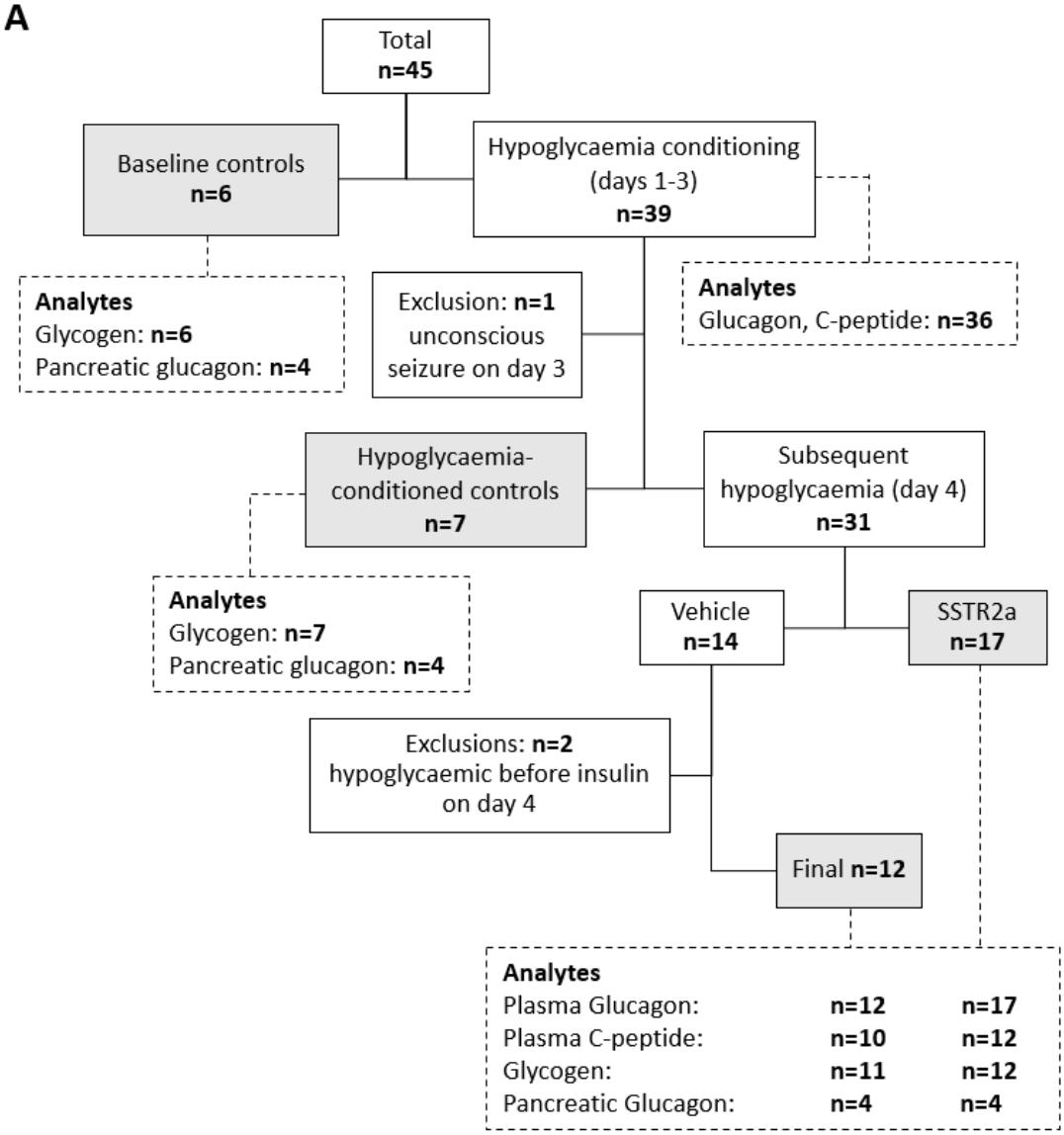
Saphenous vein blood samples were collected for subsequent glucagon and C-peptide analysis prior to insulin injection (t=0 min) and at the first blood glucose measurement ≤3.5 mmol/l (in duplicate samples). This glycemic target was chosen to ensure that the glucagon response was captured beyond its glycemic activation threshold of ~3.6-3.9 mmol/l,[231] especially since this threshold can shift to lower blood glucose concentrations with recurrent hypoglycemia.[232]

Hypoglycemia-Conditioned Controls Following three days of recurrent hypoglycemia, seven (n=7) rats were randomly selected for terminal basal analysis ~24 hours after their third hypoglycemic event (i.e., on the morning of day 4). These rats were anaesthetized for tissue and portal blood collection in the euglycemic state for comparison against baseline controls naive to hypoglycemia. One animal suffered a seizure after hypoglycemia conditioning on day 3 and was euthanized without advancing to day 4 (Fig. 4-1A). Their data were excluded from all analyses.

Subsequent Hypoglycemia (day 4) The experimental protocol for day 4 is provided in Fig. 4-1C. To assess the effects of recurrent hypoglycemia on glucagon counterregulation, with and without SSTR2a, a subset of rats (n=31) were subjected to the same insulin induction protocol as day 3, but without any oral glucose provided. More rats were allocated to the drug-treated group (n=17) than the vehicle group (n=14), based on our previous findings that the glucagon response to SSTR2a is more variable than to vehicle in both healthy and diabetic rodents.[3] Two rats in the vehicle group developed baseline hypoglycemia prior to insulin bolus on day 4 and were excluded from all data analyses (Fig. 4-1A; see below for possible limitation of αTOS as drug vehicle).

Duplicate blood glucose measurements were obtained via tail prick using a hand-held glucometer at t=-60 min (baseline), t=-30 min, t=0 min (before insulin administration) and every 10-min thereafter until a glycemic endpoint of ≤3.5 mmol/l. At this time, a terminal blood sample was collected via saphenous venipuncture for subsequent hormone analysis and rats were anaesthetized for portal blood and tissue extraction. Experiments were conducted in batches and technicians were blinded to the treatment condition.

A


Total
n=45
Baseline controls
n=6
Hypoglycaemia conditioning
(days 1-3)
n=39
Analytes
Glycogen: n=6
Pancreatic glucagon: n=4
Exclusion: n=1
unconscious
seizure on day 3
Analytes
Glucagon, C-peptide: n=36
Hypoglycaemia-
conditioned controls
n=7
Subsequent
hypoglycaemia (day 4)
n=31
Analytes
Glycogen: n=7
Pancreatic glucagon: n=4
Vehicle
n=14
SSTR2a
n=17
Exclusions: n=2
hypoglycaemic before insulin
on day 4
Final n=12
Analytes
Plasma Glucagon: n=12 n=17
Plasma C-peptide: n=10 n=12
Glycogen: n=11 n=12
Pancreatic Glucagon: n=4 n=4

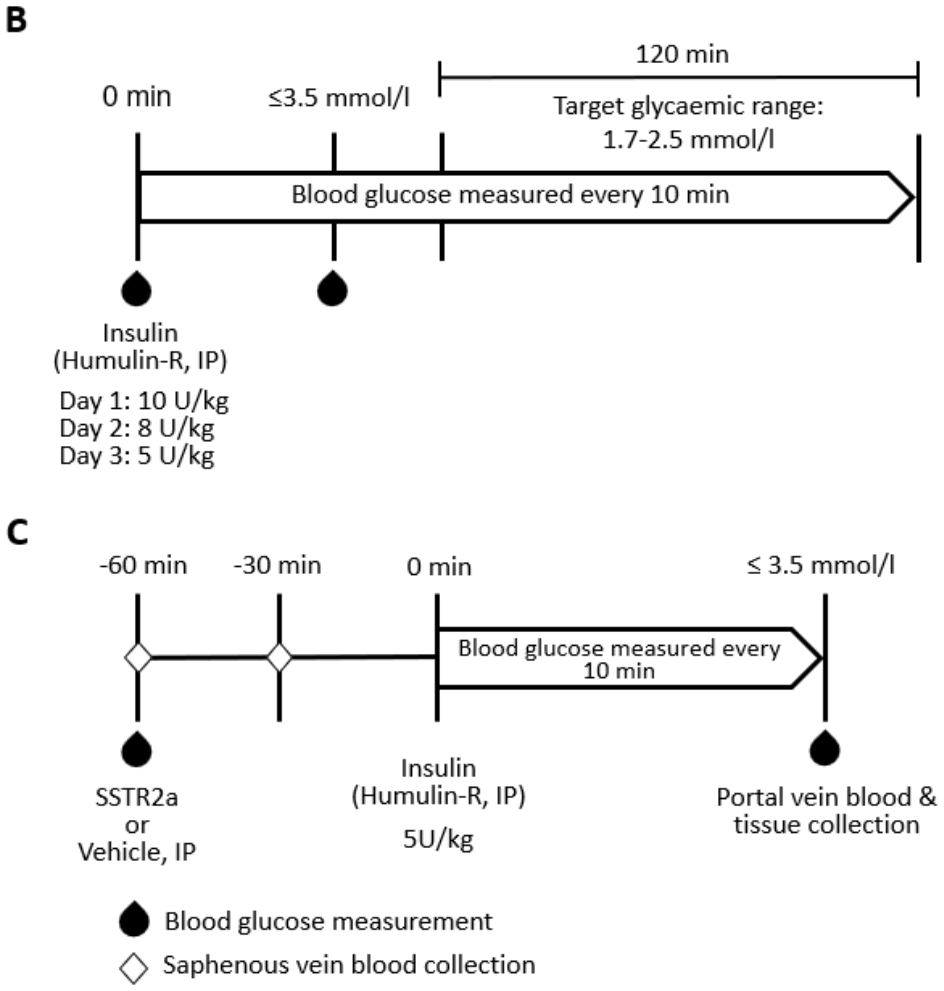


Figure 4-1. Study flow diagram and experimental protocols. **(A)** Flow diagram. Shaded boxes denote terminal conditions. **(B)** Hypoglycemia conditioning protocol (days 1-3). Thirty-nine (n=39) healthy rats received a daily bolus injection of Humulin-R insulin to induce similar hypoglycemia on 3 consecutive days using oral D-glucose as necessary. Blood glucose was measured before insulin administration (t=0 min) and every 10-min thereafter for the duration of the protocol. A venous blood sample was collected at t=0 min and again during hypoglycemia (i.e., first blood glucose measurement ≤3.5 mmol/l) for subsequent glucagon and insulin analysis. **(C)** Subsequent hypoglycemia (day 4) with or without SSTR2a treatment. Hypoglycemia-conditioned rats were administered SSTR2a (PRL-2903, 10mg/kg; n=17; IP) or vehicle (n=12) 1 h (t=-60 min) before a bolus injection of insulin (Humulin-R insulin, 5 U/kg, IP) at t=0 min. A terminal blood sample was collected at blood glucose ≤3.5 mmol/l and animals were anaesthetised for tissue and portal vein blood extraction prior to euthanisation.

SSTR2a and Vehicle The SSTR2a used (PRL-2903) has a half-maximal inhibitory concentration of 2.5 nmol/L and binds to SSTR2 with a Ki of 26 nmol/l. [233] This peptide is selective for SSTR2 over SSTR3 and SSTR5 by 10- and 20-fold, respectively, and has negligible binding affinity for SSTR1 and SSTR4. [233] Rats received an intraperitoneal injection of SSTR2a (10mg/kg PRL-2903, IP; formulated by CPC Scientific, Sunnyvale, CA, USA; supplied by CDRD, BC, Canada) with vehicle (0.5% α-tocopheryl succinate (αTOS) in pH 7 phosphate buffered saline, IP) or vehicle alone (controls) one-hour (t=-60 min) prior to a single intraperitoneal injection of 5 U/kg Humulin-R insulin. The vitamin E pro-drug, αTOS, was chosen over acetic acid, the more commonly used vehicle for PRL-2903 delivery [1–3] based on observations that αTOS offers: 1) improved drug solubility; 2) less pain on administration, which could elicit a confounding stress response, and 3) increased stability and predictability of drug absorption. Unlike its active tocopherol (vitamin E), αTOS does not have a redox potential and does not act as an antioxidant. [234]

Plasma Analysis Blood samples from saphenous vein bleed (or portal vein at endpoint on day 4) were collected in potassium-EDTA coated, microvette capillary tubes (Sarstedt, Cat # 16.444.100, Canada) and centrifuged at 12000 rpm for 5 min. Plasma was removed and stored in polyethylene tubes at -80°C for subsequent quantification of glucagon (Mercodia Cat# 10-1271-01, RRID:AB_2737304) and C-peptide (Crystal Chem Cat# 90055, RRID:AB_2893130) levels using ELISA.

Tissue Analysis Skeletal muscle (tibialis anterior and extensor digitorum longus) and liver glycogen content were quantified using a method modified from Carr and Neff. [235] Briefly, frozen tissue was digested in 0.5 ml of 1M KOH at 70°C for 1 hr, and the pH of the digest was titrated to 4.8 prior to the addition of acetate buffer and 0.5 mg/ml

amyloglucosidase. Glycogen was subsequently hydrolysed at 40°C for 2 h. Glucose was detected enzymatically, and the absorbance was read at 340 nm in a spectrophotometer (Ultraspec 2100 pro; Biochrom Ltd., Cambridge, UK).

Statistical Analysis All data are expressed as means ± SD. Statistical tests were conducted against a significance criterion of $P<0.05$ using Prism software (GraphPad Software, San Diego, CA). Because treatment was administered on day 4 only, we elected to use a one-way, independent samples ANOVA (factor: study group; levels: day 1, day 3, vehicle, SSTR2a) rather than a two-way, mixed-effects ANOVA (factors: day x treatment) to analyze the following outcomes: basal or hypoglycemic plasma hormone levels, time to hypoglycemia onset, and tissue glycogen content. Multiple comparisons were performed using Tukey's HSD post-hoc test. Day 4 glycemic responses were analyzed using a 2-way mixed-design ANOVA (factors: treatment x time), followed by a Sidak post-hoc test. A two-tailed, unpaired t-test was used to compare portal vein hormone levels between day 4 treatment groups. Survival curve analysis was performed using a log-rank (Mantel-Cox) test.

4.4 Results

Body Weight and Blood Glucose Body weight increased steadily over the experimental period, from 321±67 g at baseline on day 1 to 334±66 g on day 4 ($P<0.001$) but did not vary significantly between day 4 treatment groups (vehicle: 354±59 g; SSTR2a: 340±64 g). Blood glucose concentrations from the hypoglycemia conditioning phase of the protocol (days 1-3; n=36) are presented in Fig. 4-2A, while Fig. 4-2B shows the glycemic responses to subsequent insulin challenge (day 4) with or without SSTR2a. Basal blood glucose levels at t=-60 min were not significantly different across

experimental days or conditions (Fig. 4-2A, 2B). Glucose levels during hypoglycemia conditioning on days 1-3 were similar, with all animals remaining in target range for 120 min. As described in the methods, plasma and tissue samples were collected at the first blood glucose reading ≤3.5 mmol/l. As a result, hypoglycemic outcome variables corresponded to a mean blood glucose concentration of 3.2±0.4 mmol/l on day 1, 3.1±0.4 mmol/l on day 3, 3.0±0.4 mmol/l in vehicle (day 4), and 3.1± 0.4 mmol/l in SSTR2a (day 4) (not significantly different).

In Fig. 4-2B, each animal's terminal blood glucose concentration as measured on day 4 (at ≤3.5 mmol/l) was carried forward to all subsequent timepoints following their removal from the challenge. This data extrapolation procedure was done to illustrate glycemic trends over time, despite diminishing sample sizes, which would otherwise misrepresent group means. There was a significant treatment by time effect on glycemic responses to subsequent insulin challenge (day 4; $P<0.001$) plotted in Fig. 4-2B, with elevations in the SSTR2a group at 10- and 20-min post-insulin administration ($P<0.05$ and $P=0.06$, respectively).

Time to Hypoglycemia Onset Time from insulin administration to the onset of clinical hypoglycemia (blood glucose ≤3.9 mmol/l) did not differ significantly during the hypoglycemic conditioning days, averaging 50±39 min (days 1-3 combined) (Fig. 4-2C). On day 4, the drug-treated group reached hypoglycemia by an average of 58±40 min as compared to 33±34 min in the vehicle group ($P<0.05$). A comparison of survival curves in Fig. 4-2D revealed a significant overall reduction in the proportion of hypoglycemic animals in the treatment vs vehicle group ($P<0.05$). Three of twelve rats (25%) in the

vehicle group were euglycemic at 30 mins as compared with nine of seventeen rats (53%) in the treatment group.

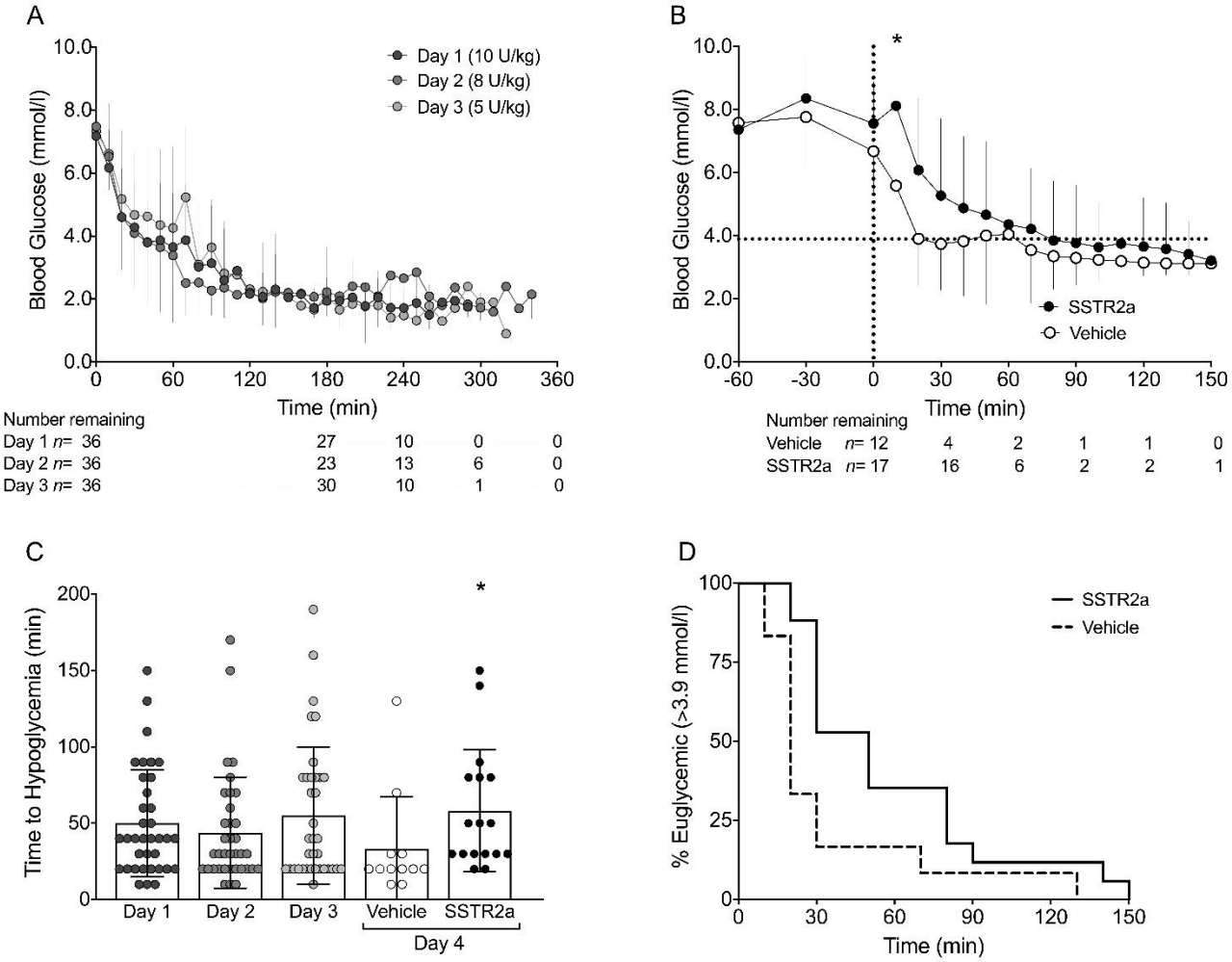


Figure 4-2. Blood glucose responses to hypoglycemic challenge on hypoglycemia conditioning days **(A)**. On day 4 **(B)**, hypoglycemia-conditioned rats were given SSTR2a (10 mg/kg; n=17) or vehicle (n=12) at t=-60 min, followed by a 5 U/kg IP bolus injection of insulin 1 h later (t=0min, indicated by vertical dotted line). Terminal blood glucose values from each animal have been extended out to the x-axis limit. Horizontal dotted line indicates the threshold for clinical hypoglycemia (3.9 mmol/l). Treatment by time interaction: *P*<0.001; *P*=0.06 at t=10, 20 min. **(C)** Time from insulin administration to hypoglycemia onset on days 1-3 (hypoglycemia conditioning) and day 4 (subsequent hypoglycemia) with or without SSTR2a pre-treatment. **P*<0.05 vehicle vs SSTR2a. **(D)** Survival curve comparing the percentage of animals in each group that remained euglycemic (>3.9 mmol/l) post-insulin exposure on day 4. **P*<0.05. All data are means ±

SD. Data in the tables below **(A)** and **(B)** indicate the number of animals remaining at each major interval time point.

Glucagon Mean plasma glucagon concentrations on experimental days 1, 3, and 4 are shown in Fig. 4-3A. Basal glucagon levels did not change significantly throughout the experimental period. The stimulated glucagon response to blood glucose ≤3.5 mmol/l diminished by 32% across 3 days of conditioning (day 1: 219±99 pg/ml vs day 3: 150±75 pg/ml; $P<0.01$) and by an additional 53% on day 4 in the vehicle group (day 4 vehicle: 70±52 pg/ml; $P<0.001$ vs day 1; $P<0.05$ vs day 3). Plasma glucagon was ~2.4-fold higher in SSTR2a-treated animals (171±122 pg/ml) than vehicle controls *(*$P<0.05$) and comparable to levels observed on days 1 and 3. Portal vein glucagon levels did not vary significantly between groups on day 4 (Fig. 4-3B).

C-peptide Basal concentrations of plasma C-peptide were comparable across all 4 days (Fig. 4-3C). Hypoglycemic levels (measured at blood glucose ≤3.5 mmol/l) of C-peptide were also comparable between days 1 and 3 but increased significantly on day 4 (vehicle: 0.59±0.23 nmol/l; SSTR2a: 0.36±0.20 nmol/l) relative to day 1 (0.15±0.09 nmol/l; $P<0.001$ vs vehicle or SSTR2a) and day 3 (0.14±0.11 nmol/l; $P<0.001$ vs vehicle or SSTR2a) (Fig. 4-3B). This marked elevation in the vehicle group was partially, yet significantly offset by SSTR2a pre-treatment, and was mirrored by a comparable, 1.6-fold reduction in portal vein C-peptide levels of SSTR2a-treated animals (vehicle: 0.32±0.11 nmol/l vs SSTR2a: 0.19±0.13 nmol/l; $P<0.01$) (Fig. 4-3D).

Portal vein glucagon-to-insulin ratio The calculated ratio of glucagon to C-peptide in the portal vein during hypoglycemia on day 4 was ~2.6-fold higher in the SSTR2a (2014±1698) vs vehicle group (776±406; $P<0.05$) (Fig. 4-3E).

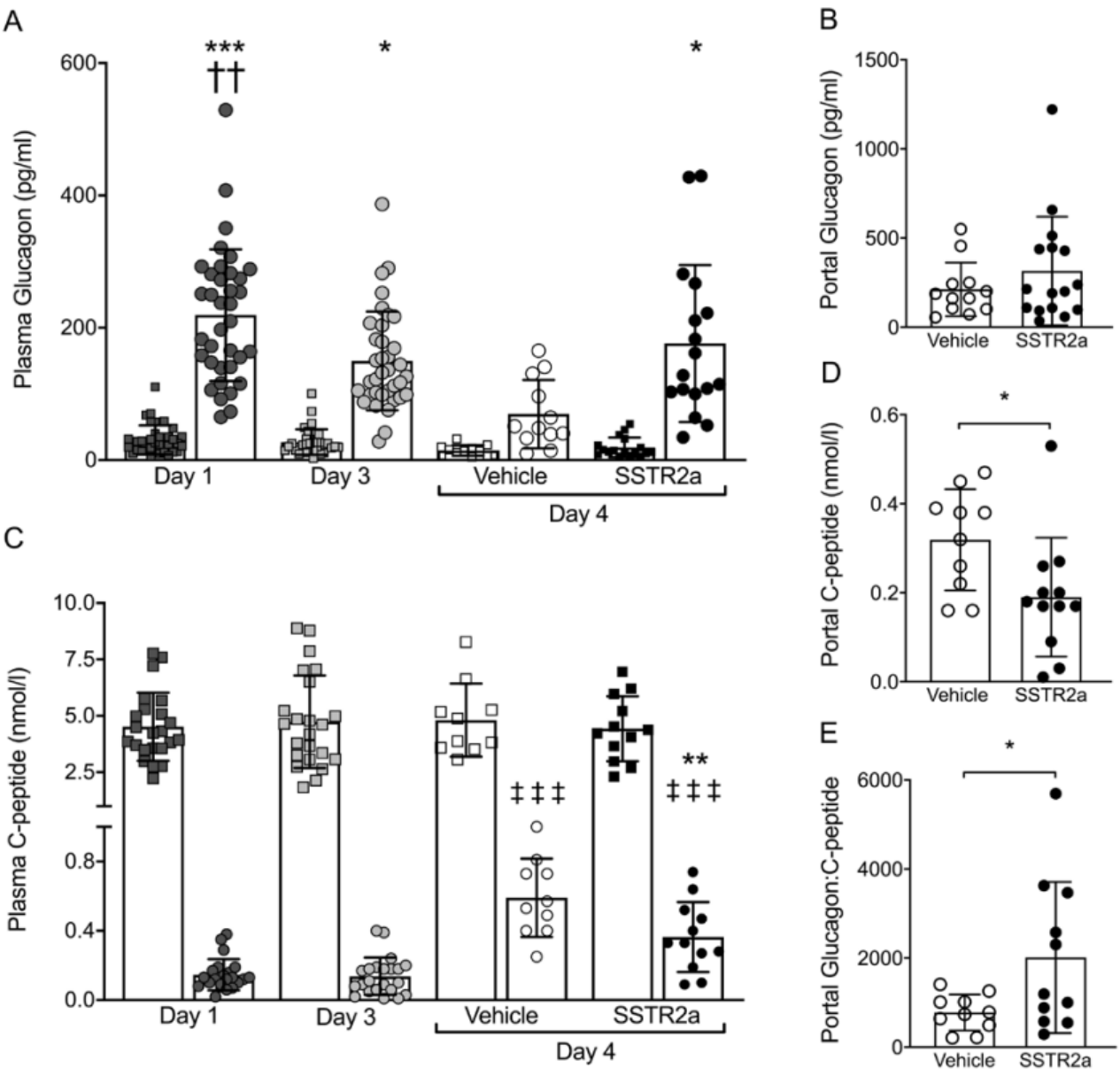


Figure 4-3. Basal and hypoglycemic plasma hormone levels. Baseline (squares) and endpoint (circles) plasma concentrations of **(A)** systemic glucagon; (**B)** portal vein glucagon (day 4 only); **(C)** systemic C-peptide; (**D)** portal vein C-peptide (day 4 only); and **(E)** the ratio of portal glucagon to portal C-peptide. Note: hypoglycemic measurements were obtained at the first blood glucose reading ≤3.5 mmol/l. **P*<0.05, ***P*<0.01, ****P*<0.001 vs vehicle; ††*P*<0.01 vs day 3; ‡ ‡ ‡*P*<0.001 vs day 1 and day 3. All data are means ± SD.

Hepatic and skeletal muscle glycogen content The hepatic glycogen content of euglycemic, baseline controls was not significantly different after 3 days of recurrent hypoglycemia (i.e., measured 24-hours after the 3rd hypoglycemic exposure) (Table 4-1). On day 4, hepatic glycogen stores were significantly lower in hypoglycemic animals as compared to their euglycemic counterparts (i.e., hypoglycemia-conditioned controls: $P<0.05$ vs vehicle; $P<0.001$ vs SSTR2a). Notably, glycogen levels were reduced by 35% in the SSTR2a vs vehicle group after hypoglycemia ($P<0.01$). No difference was detected in the glycogen content of the tibialis anterior or extensor digitorum longus muscle across days or treatment conditions (Table 4-1).

Table 4-1. Tissue glycogen content of control animals in the basal (euglycemic) state and of hypoglycemic animals at target blood glucose ≤3.5 mmol/L.

	Day 1	Day 4		
	Baseline controls	Hypoglycemia-conditioned controls	Subsequent hypoglycemia	
			Vehicle	SSTR2a
Hepatic Glycogen (µmol)	27 ± 0.4	26 ± 1.9	20 ± 7.0*	13 ± 4.4***††
TA Glycogen (µmol)	12 ± 1.9	12 ± 2.5	13 ± 3.2	11 ±1.9
EDL Glycogen (µmol)	11 ± 1.5	11 ± 2.0	11 ± 3.4	11 ± 1.7

Data are means ± SD.
Abbreviations: TA, tibialis anterior; EDL, extensor digitorum longus.
*P < .05, ***P < .001 vs. baseline controls or hypoglycemia-conditioned controls; ††P < .01 vs. vehicle.

4.5 Discussion

The goal of this study was to examine whether somatostatin signalling plays a role in the pathophysiology of hypoglycemia-induced counterregulatory failure. As hypothesized, SSTR2a restored glucagon secretion to subsequent hypoglycemia, which in turn, increased hepatic glycogen utilization and delayed time to hypoglycemia onset.

Antecedent hypoglycemia is known to blunt the glucagon response to hypoglycemia in healthy[207–209,236] and diabetic subjects.[237] Here, our non-diabetic rat model showed a 32% attenuation in counterregulatory glucagon secretion after three days of hypoglycemia conditioning. This response echoed the ~40-60% impairment reported by Chan et al. across a similar three-day protocol.[156] By reducing the insulin dose on each successive day of conditioning, we achieved a consistent rate of glycemic decline despite failing counterregulation. Without this compensatory dosing measure, vehicle-treated rats showed a further 53% impairment to subsequent hypoglycemia on day 4.

We demonstrated that delivery of the SSTR2a (10 mg/kg PRL-2903) one-hour before hypoglycemia induction normalized plasma glucagon levels and delayed the onset of clinical hypoglycemia (blood glucose ≤3.9 mmol/l) by 25 minutes. The absence of pre-existing α-cell dysfunction in our non-diabetic rodent model allowed us to selectively target and reverse glucagon counterregulatory failure induced by recurrent hyperinsulinemic-hypoglycemia per se. The lack of an observable drug effect in acutely hypoglycemic healthy rodents further supports the conclusion that somatostatin signalling in the pancreatic islets may increase with repeated hypoglycemia exposure.[1] Abnormal elevations in plasma and/or pancreatic somatostatin have traditionally been considered a diabetes-specific phenomenon.[1,6] The rescue of glucagon counterregulation by SSTR2a in various diabetic rodent models during acute[1,3] and recurrent[2] hypoglycemia suggests that somatostatin signalling may attenuate glucagon counterregulation.[2,6] We propose that in addition to diabetes, antecedent hypoglycemia per se may elevate somatostatin signalling to further suppress glucagon counterregulation.

Proinsulin connecting peptide (C-peptide) is a by-product of insulin biosynthesis, secreted with insulin in an equimolar ratio. It therefore serves as a reliable metric for measuring β-cell secretory activity in the presence of exogenous insulin. We unexpectedly observed significantly higher levels of systemic C-peptide during hypoglycemia on day 4, as compared to days 1 and 3, which was more pronounced in the vehicle-only group. This suggests that the vehicle, αTOS, may have acted as an insulin secretagogue prior to and/or during hypoglycemia. However, because the vehicle was given to both groups, any potential paracrine or glycemic interference would not modify relative treatment outcomes. Insulin secretagogues are known to suppress hypoglycemic glucagon secretion under healthy conditions,[218] and we propose that the SSTR2a used here may have inadvertently opposed this insult by reducing pharmacologically-stimulated insulin secretion. Any non-specific binding of the antagonist to SSTR3 and/or SSTR5 on the β-cell would be expected to raise C-peptide levels by blocking somatostatin's inhibitory input.[238] We instead propose that by occupying somatostatin binding sites on the α-cell, the antagonist may have promoted the somatostatin-mediated activation of its receptors on the β-cell, thereby inhibiting (and partially normalizing) hypoglycemic insulin release. Confirmed elevations in pancreatic and plasma somatostatin during PRL-2903 exposure, based on in vitro studies of the perfused isolated rat pancreas[238] and hypoglycemia-clamp experiments in healthy rats,[3] may lend further support to this theory.

Glucagon-stimulated glycogenolysis and gluconeogenesis by the liver are the predominant counterregulatory pathways that defend against hypoglycemic stressors.[239] T1D reduces liver glycogen stores,[240] even in those under good glycemic control.[241] The

reduction in liver glycogen is associated with impaired rates of glycogenolysis during hypoglycemia in humans with T1D[241] and in dog models of altered hepatic glycogen content.[242] Interestingly, we did not observe any change in basal hepatic glycogen stores following three days of hypoglycemic conditioning in these non-diabetic rodents. Diminished glycogenolytic capacity in recurrently hypoglycemic rats is supported by Shum et al., who reported impairments in both absolute and incremental glucose production during the first forty-five minutes of insulin-induced hypoglycemia, despite an intact neuroendocrine response.[243] Previous studies proposed that SSTR2a promotes homeostatic recovery from hypoglycemia by augmenting the rate of hepatic glycogenolysis,[2] though none had directly tested this hypothesis. We observed a 45% depletion of hepatic glycogen in rats treated with SSTR2a, indicating its ability to protect against hypoglycemia following a period of untreated, recurrent hypoglycemia. While hepatocytes have been shown to express SSTR1 and SSTR3,[244] the SSTR2 antagonist used in this study (PRL-2903) is incompatible with the former and binds to the latter with 10-fold lower affinity than SSTR2.[233] This supports the conclusion that somatostatin modulates hepatic glycogen stores by an indirect mechanism - likely, via suppression of glucagon counterregulation.[2]

Insulin antagonizes glucagon's catabolic effects on liver glycogen, so the ratio of glucagon to insulin in the portal vein (which serves as a direct conduit from the pancreas to the liver) is considered the primary mediator of hepatic glycogen breakdown during mild hypoglycemia and exercise.[245] Accordingly, the elevated portal vein ratio observed in SSTR2a-treated vs vehicle-control animals may account for the significant reduction in hepatic glycogen observed in this group. However, to conclude whether glycogenolytic

capacity was truly attenuated by antecedent hypoglycemia in this model, future studies must establish the glycogen response to acute hypoglycemia for comparison against each day of subsequent exposure.

We found that the circulating, but not portal concentration of plasma glucagon was significantly higher in drug- vs- vehicle-treated animals at terminal hypoglycemia on day 4. This finding may invite speculation of a SSTR2a-mediated reduction in hepatic glucagon clearance; however, no such effects have been described. Instead, studies in isolated rat islets and pancreas slices have consistently demonstrated the potent and direct α-cell glucagonotropic effects of selective SSTR2 antagonists.[3,246] We suspect that our sampling timepoint, which took place 60 minutes after insulin administration in the SSTR2a group (vs 30 minutes in the vehicle group), may have captured a time-dependent decline in glucagon secretion, shown to occur between ~30-40 minutes of hypoglycemic induction with or without SSTR2a.[1,2,247]

This study has several limitations to acknowledge. First, we elected to use αTOS as the vehicle for SSTR2a delivery. Long-term supplementation of vitamin E in its active form, α-tocopherol, has been shown to compensate for insulin resistance by increasing glucose-stimulated insulin release and peripheral insulin sensitivity in hyperglycemic, type 2 diabetic rats.[248,249] Evidence of a single-dose effect, especially in healthy subjects, is less convincing.[248,250,251] It is also worth clarifying that αTOS is a prodrug, which shows slow and continuous hydrolysis (activation) by a tissue esterase in pharmacokinetic studies of IV-infused rats.[252] A peak in serum tocopherol levels 7-8 hrs post-delivery and minimal non-hepatic tissue exposure overall[252] further reduces the likelihood of vehicle interference. Yet, we cannot discount the possibility that αTOS promoted insulin secretion

and/or peripheral insulin sensitivity in both treatment groups, owing to the premature glycemic decline following SSTR2a/vehicle administration (Fig. 4-2B). The use of α-tocopheryl succinate should thus be avoided in future anti-hypoglycemic drug formulations. As a second limitation, plasma glucagon and C-peptide levels were measured at a single hypoglycemic timepoint, which did not allow us to profile these responses over time. Finally, the majority of glucagon's counterregulatory response lies beyond the glycemic threshold for clinical hypoglycemia onset (3.9 mmol/l),[231] reflected in our glycemic sampling target of 3.5 mmol/l, which questions a role for glucagon in resisting hypoglycemia onset (versus its progression) under normal conditions. Thus, it remains to be determined how the elevation in plasma glucagon levels as measured at 3.5 mmol/l relates to a delay in hypoglycemia onset at 3.9 mmol/l. Notably, SSTR2a infusion prevents glucose-mediated glucagon suppression between 3.5-5 mmol/l in the isolated rat pancreas, suggesting that SSTR2a may promote earlier glucagon recruitment and glycemic recovery.[238] Future studies are required to elucidate how SSTR2a may modify the glycemic threshold for glucagon counterregulation in this and other models of health and disease.

In summary, we demonstrated that the SSTR2a, PRL-2903, restored glucagon counterregulation and significantly delayed the onset of subsequent hypoglycemia in recurrently hypoglycemic, non-diabetic rats. Hypoglycemia resistance with SSTR2a treatment is associated with accelerated hepatic glycogenolysis. Our findings suggest a role for somatostatin in the glucagon counterregulatory defect that develops in parallel with HAAF. Further investigation into these findings stands to advance our understanding

of intra-islet paracrine dynamics in health and disease, as well as the therapeutic applications of SSTR2a in diabetic patients with advanced counterregulatory failure.

Chapter 5: Effects of Somatostatin Receptor Type 2 Antagonism During Insulin-Induced Hypoglycemia in Male Rats with Pre-Diabetes.

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Editing the manuscript: EGH, RL, MCR

5.1 Abstract

Aims: Somatostatin receptor 2 antagonists (SSTR2a) block somatostatin's inhibitory input to pancreatic α-cells and enhance glucagon counterregulation during hypoglycemia in type 1 diabetes. Here, we examine if glucagon counterregulatory defects exist in a rat model of pre-diabetes (pre-T2D) and assess if a selective SSTR2a (ZT-01) enhances the glucagon response to insulin-induced hypoglycemia. **Materials/Methods:** Hyperglycemia was induced in 8-9-week-old, male, Sprague-Dawley rats via seven-weeks of high-fat feeding followed by a single, low-dose injection of streptozotocin (30 mg/kg; IP). After two-weeks of basal insulin therapy (0-4 U/d insulin glargine; SC) to facilitate partial glycemic recovery and a pre-T2D phenotype, n=17 pre-T2D and n=10 normal chow-fed control rats underwent the first of two hypoglycemic treatment-crossover experiments, separated by a one-week washout period. On each experimental day, SSTR2a (3 mg/kg ZT-01; SC) or vehicle was administered 1 h prior to insulin-induced hypoglycemia (insulin aspart, 6 U/kg; SC). **Results:** Glucagon counterregulation was marginally reduced with the induction of pre-T2D. Treatment with SSTR2a raised peak plasma glucagon levels and glucagon area under the curve (AUC) before and after insulin overdose in both control and pre-T2D rats. Blood glucose concentration was elevated by 30 min after SSTR2a treatment in pre-T2D rats and hypoglycemia onset (≤3.9 mmol/L) was delayed by 15±12 min compared to vehicle (P<0.001), despite similar glucose nadirs between treatment groups (1.4±0.3 mmol/L). SSTR2a treatment had no effect on blood glucose levels in the control group or on the hypoglycemia-induced decline in plasma C-peptide levels in either group. **Conclusions:** SSTR2a treatment increases glucagon

responsiveness and delays the onset of insulin-induced hypoglycemia in this rat model of pre-T2D where only a modest deficiency in glucagon counterregulation exists.

5.2 Introduction

Glucagon, secreted by pancreatic α-cells prevents and/or attenuates the development of hypoglycemia by mobilising glucose from the liver.[253] In type 1 diabetes (T1D), the counterregulatory glucagon response is typically absent within a few years of diagnosis, predisposing affected individuals to insulin-induced hypoglycemia.[21] While the natural history of this acquired α-cell defect is not well characterised in type 2 diabetes (T2D), owing, at least in part, to heterogeneity in disease phenotype and progression, it is well established that hypoglycemia, secondary to the intensive use of insulin analogues and/or secretagogues, becomes limiting to glycemic control in the late or advanced stages of disease.[21,27,35,36,43,254] Each episode of hypoglycemia impairs the glucagon and sympathoadrenal (epinephrine) responses to subsequent hypoglycemia, often perpetuating a cycle of recurrence.[121]

After more than 40 years of research into the mechanisms of glucagon counterregulatory failure in diabetes, evidence points to the involvement of lesser known islet hormone, somatostatin (SST).[6,255] SST is secreted by δ-cells of the pancreatic islets, which comprise 5-10% of the total islet cell mass.[256] As the putative islet gatekeeper, SST inhibits glucagon and insulin secretion via SST receptor 2 (SSTR2) and receptor 5 (SSTR5), localised to rodent α- and β-cells, respectively.[102,257] Pharmacological antagonism of SSTR2 augments the plasma glucagon response to both acute[1,3,171] and recurrent[2] hypoglycemia in rodent models of streptozotocin (STZ) and biobreeding T1D. This restorative effect was replicated in non-diabetic rodents with overt glucagon counterregulatory failure following recurrent episodes of insulin-induced hypoglycemia.[258] Currently in clinical trials,[19] the highly selective SSTR2 antagonist (SSTR2a), ZT-01

(Zucara Therapeutics), is emerging as a promising therapeutic for the prevention of insulin-induced hypoglycemia in people with T1D.

Owing to relative disease prevalence, T2D, rather than T1D, now accounts for the majority of severe diabetes-related hypoglycemic events (i.e., requiring hospitalisation).[29] Hypersecretion of SST, observed in T2D mouse and human islets at low glucose, offers a potential mechanism of impaired glucagon output (65-75%) that is reversible with SSTR2a.[4] Since δ-cells are electrically silenced by β-cells via gap junction coupling at low glucose, mathematical modelling of δ-cells predicts increasing hyperactivity and SST release under conditions of progressive β-cell death.[99] Yet, the setting in which this defect first arises remain unclear. Conflicting *in vivo* observations suggest that glucagon counterregulation is increased,[131] decreased,[259] or unchanged[260] in chronically high-fat fed (HFF) mice. However, the commonly used rodent model of HFF-induced pre-diabetes (pre-T2D) shows sufficient β-cell compensation to maintain normoglycemia,[131,260] and therefore, the effect of mild hyperglycemia on glucagon counterregulation in a setting of insulin resistance is unclear. Mild hyperglycemia in pre-T2D is predictive of T2D risk, and β-cell exposure to mild hyperglycemia in the transition to diabetes impairs glucose-stimulated insulin secretion,[261,262] which is most readily reversible in this early state.[8,263] This led us to question whether stimulated (i.e., counterregulatory) glucagon secretion is similarly impacted by mild hyperglycemia during the progression to diabetes and, if so, whether SSTR2 antagonism may provide a method of early correction.

We induced mild hyperglycemia in HFF rats with a single, low-dose injection of STZ followed by a period of glycemic recovery, yielding a stable pre-T2D phenotype. We took this approach since low-dose STZ (i.e., 30-40 mg/kg) induces acute hyperglycemia

and normo- or hypoinsulinemia reminiscent of late-stage T2D in HFF rats, whereas doses below this range fail to elicit significant hyperglycemia in normal and HFF male, Sprague-Dawley rats.[264] The goal of this study was to characterise the counterregulatory responses of islet hormones to insulin-induced hypoglycemia (i.e., decreased insulin secretion coupled with increased glucagon secretion) in this novel pre-T2D model, with and without SSTR2a pre-treatment. We hypothesized that this model would exhibit a mild defect in counterregulatory glucagon secretion that is reversible with a highly selective SSTR2a, ZT-01, to reduce hypoglycemia exposure without modifying endogenous insulin levels or basal glycemia.

5.3 Materials and Methods

This study was conducted in accordance with the recommendations of the Canadian Council for Animal Care guidelines and has been approved by the York University Animal Care Committee (Protocol # 2017-7).

Animals and T2D Induction

The experimental protocol is outlined in Supplementary Figure 5-1. Thirty (n=30) male Sprague Dawley rats (initial weight 200-250 g) were purchased from Envigo RMS Inc. (Indianapolis, IN, USA) at an age of 8-9 weeks. Rats were individually housed in the York University vivarium in a 12-hour light-dark cycle with *ad libitum* access to food and water. Following a one-week habituation period, twenty (n=20) rats were randomly selected to begin a high fat diet (5.21 kcal/g of food), containing 60% fat, 20% carbohydrate, and 20% protein (Research Diets, Inc., Cat# D12492, New Brunswick NJ, USA) for a period of ~7 weeks to induce obesity and insulin resistance (Fig. S5-1). The remaining ten rats served as healthy normal chow-fed (NC) controls (LabDiet, Cat# 5012,

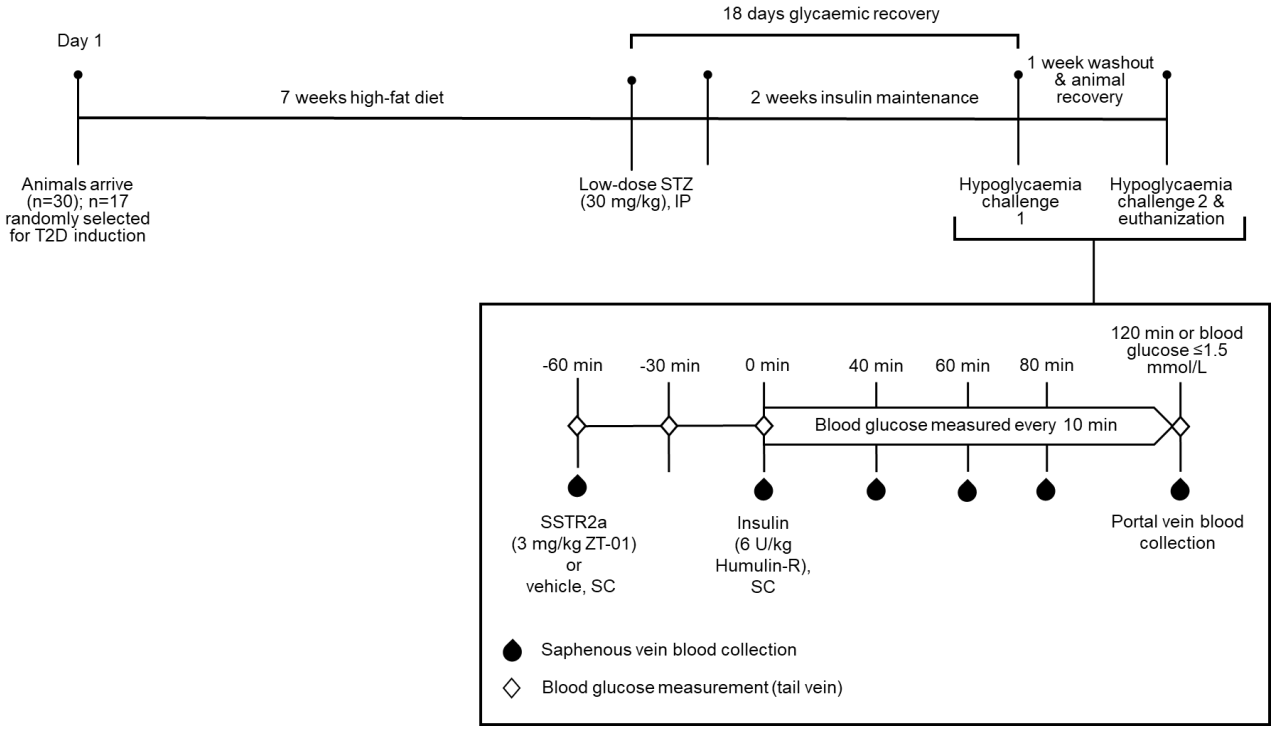
St. Louis, MO, USA; 13% fat, 58% carbohydrates, 29% protein; 3.5 kcal/g). After seven weeks of high-fat feeding (HFF), rats (n=20) received a single, low-dose injection of streptozotocin (STZ) (30 mg/kg, IP; Sigma). This HFF/STZ model results in acute hyperglycemia followed by a glucose recovery period from β-cell expansion that results in mild hyperglycemia or pre-T2D.[265]

Following STZ, regular drinking water was replaced with sugar water (10% w/v sucrose solution) for two days as a precautionary measure to prevent hypoglycemic episodes within the first 48 h. Hyperglycemia was confirmed in all STZ-treated rats based on a post-absorptive whole blood glucose measurement ≥11.1 mmol/L two days after STZ administration. Rats that met this criterion (n=20) remained on high-fat chow for the remainder of the study period. From this point onward, body weight, blood glucose (Contour Next glucose meter and test strips, Ascensia Diabetes Care, Mississauga, Canada), and food consumption were measured daily. Initiated five days after STZ treatment, basal insulin (Lantus SoloSTAR insulin glargine, Sanofi-Aventis, Bridgewater, NJ, USA) was administered each evening (~18:00h), as needed, for two weeks, to help promote glycemic recovery to the status of pre-T2D.[263,266] Therapeutic insulin was dosed based on evening (i.e., pre-feeding) blood glucose measurements according to the following sliding scale: <12 mmol/L: 0 U; 12-15 mmol/L: 1 U; >15 mmol/L: 4 U.

Hypoglycemia Challenges

After two weeks of basal insulin therapy, animals were randomly assigned to the SSTR2a or vehicle group for the first of two hypoglycemic challenges, conducted one week apart in a treatment crossover design (Fig. S5-1). Baseline glucose and hormone measurements were taken from all animals at 09:00h on hypoglycemia challenge days

following a controlled overnight feed to standardise food intake and preserve liver glycogen stores.[2] SSTR2a (3 mg/kg ZT-01; formulated by AdMare BioInnovations, Vancouver, BC, Canada.; supplied by Zucara Therapeutics Inc., Toronto, ON, Canada) with vehicle (2.1% v/v glycerol in 10 mM acetate buffer pH 4.2), or vehicle alone, were then administered by subcutaneous (SC) injection one hour (t=-60 min) before a SC bolus injection of insulin aspart (6 U/kg NovoRapid insulin, Novo Nordisk, Bagsværd, Denmark) at t=0 min (Fig. S5-1). This dose of ZT-01 was selected since it reflects the upper limit of the dosing range tested previously in T1D rodents under hypoglycemic clamp conditions.[171] Blood glucose was measured in duplicate via tail prick using the hand-held glucometer (described above) at t=-60, -30, and 0 min, and every 10 min thereafter until t=60 and t=120 min in healthy and pre-T2D rats, respectively (Fig. S5-1). Saphenous vein blood samples were collected at t=-60, 0, 40, and 60 min from healthy and pre-T2D rats, and at t=80 and 120 min, from pre-T2D rats only for subsequent hormone analysis (Fig. S5-1). Most healthy rats (regardless of treatment group) reached the humane endpoint of the study (i.e., signs of distress, extreme lethargy, and/or blood glucose ≤1.5 mmol/) by 80 min post-insulin injection, so data collected beyond t=60 min were excluded from analysis due to low N remaining. Following the second crossover challenge, all animals were anaesthetised for portal vein blood collection and then euthanised via exsanguination. Data were pooled from both hypoglycemia challenges for analysis (Fig. S5-1).



Supplementary Figure 5-1. Experimental protocol. After 7 weeks of high-fat diet (HFD)-feeding, n = 17 rats received a low-dose, intraperitoneal (IP) injection of β-cell toxin, streptozotocin (STZ; 30 mg/kg). With continued HFD-feeding (for the duration of the protocol), rats underwent ~2.5 weeks (18 days) of glycemic recovery, including 2 weeks (14 days) of basal insulin maintenance, to yield a model of pre-diabetes (pre-T2D). Pre-T2D (n = 17) and normal chow-fed, non-diabetic control (healthy; n = 10) rats then underwent two hypoglycemia challenges conducted in a SSTR2a/vehicle crossover design and separated by a one-week washout period. SSTR2a (3 mg/kg ZT-01) or vehicle was administered subcutaneously (SC) 1 hour (t= -60 min) prior to a bolus injection of NovoRapid insulin (6 U/kg, SC) at t= 0 min. Whole blood glucose concentration was measured via tail prick at t= -60, -30, and 0 min, and every 10 minutes thereafter for 120 minutes, unless an animal reached the glycemic threshold for premature removal (≤1.5 mmol/L). Saphenous vein blood samples were collected at t= -60, 0, 40, 60 80, and 120 minutes for the subsequent quantification of glucagon and C-peptide concentrations in circulating plasma. Upon completion of the second hypoglycemia challenge, rats were anaesthetized for portal vein blood collection prior to euthanization.

Plasma Analysis

Blood samples were collected from saphenous or portal vein bleed (terminal time point only) in a potassium-EDTA coated, microvette capillary tubes (Sarstedt, Cat # 16.444.100, Canada) and centrifuged at 12000 rpm for 5 min. Plasma was removed and stored in polyethylene tubes at -80°C for the subsequent quantification of glucagon (Mercodia Cat# 10-1271-01, RRID:AB_2737304) and C-peptide (Crystal Chem Cat# 90055, RRID:AB_2893130) levels using ELISA.

Statistical Analysis

Data are expressed as means ± SD, unless otherwise stated. Statistical tests were conducted against a significance criterion of $P<0.05$ using Prism 8 software (GraphPad, San Diego, CA, USA). Daily body weight and blood glucose levels measured throughout the study period (within-subjects factor: time x between-subjects factor: diabetes status); baseline (i.e., pre-hypoglycemia challenge) body weight, blood glucose, and plasma hormone levels; peak and nadir plasma hormone and nadir blood glucose levels; plasma hormone AUC; percent change in C-peptide levels from baseline; and portal vein glucagon levels (within-subjects factor: treatment x between-subjects factor: diabetes status) were analyzed by two-way mixed-model ANOVAs followed by Sidak post-hoc tests. Blood glucose and plasma hormone concentrations measured during hypoglycemia challenges were analyzed by two-way repeated measures ANOVAs (factors: treatment x time), followed by Sidak post-hoc testing. Paired t-tests were used to compare time to hypoglycemia onset and blood glucose AUC between treatment groups. Probability of euglycemia was evaluated using a log-rank (Mantel-Cox) test. The linear relationship between whole blood glucose and plasma glucagon concentration was modelled using

simple linear regression and regression line slopes were analyzed by two-way mixed-model ANOVA (within-subjects factor: treatment x between-subjects factor: diabetes status) and Sidak post-hoc tests.

5.4 Results

Baseline Rat Model Characteristics

Body weight and whole blood glucose levels measured from the time of STZ administration until study completion, as well as body weight, whole blood glucose and plasma hormone levels measured prior to drug/vehicle dosing on the day of experimental hypoglycemia challenges are shown in Figure 5-1. Rats in the STZ/HFF group were severely hyperglycemic after STZ treatment, reaching a peak on study day 3 (22.5±6.8 mmol/L vs. 5.1±0.4 mmol/L in healthy rats), and gradually reverting towards pre-T2D over the glycemic recovery period (Fig. 5-1A). Mean daily basal insulin requirement diminished throughout the study period, from 3.3±1.6 U/day on day one of insulin maintenance (study day 6) to 0.2±1.0 U/day ~three weeks later (study day 27) (data not shown). At the time of hypoglycemia challenges, only one of seventeen HFF/STZ rats required basal insulin therapy (data not shown). Body weight remained stable in the normal-chow-fed healthy controls throughout the four-week study period, whereas HFF rats exhibited rapid weight loss after STZ treatment, reaching a nadir by day three and recovering one-week later (Fig. 5-1B). Body weight plateaued with continued high-fat feeding for the remainder of the study (Fig. 5-1B).

Mean baseline measurements in pre-T2D rats did not differ significantly between experimental days (i.e., hypoglycemia challenges) one and two, separated by a one-week washout period [i.e., body weight (415±26 g vs. 417±28 g), whole blood glucose (7.4±1.1

mmol/L vs. 7.3±0.6 mmol/), plasma glucagon (23±15 pg/mL vs. 24±19 pg/mL) and plasma C-peptide (1038±230 pmol/L vs. 1111±281 pmol/L) levels], so data from both experiments were pooled for analysis. Collectively, three animals met the diagnostic criteria for overt T2D by a random glucose measurement in humans (≥11.1 mmol/L)[202] on challenge day one (24.4 mmol/L) and day two (13.0 and 11.9 mmol/L, respectively), so their data were excluded from all analyses, reducing the sample size of the pre-T2D group to n=17.

At the time of hypoglycemia challenges (i.e., two and three weeks after STZ treatment), a mild hyperglycemic phenotype was observed in our pre-T2D rat model, as measured in the post-absorptive state after a controlled overnight feed and without basal insulin therapy (pre-T2D: 7.4±1.2 mmol/L vs. healthy: 5.3±0.5 mmol/L; $P<0.001$) (Fig. 5-1C). In combination with mild baseline hyperglycemia, plasma C-peptide levels were also increased relative to healthy controls (pre-T2D: 1061±249 pmol/L vs. healthy: 836±211 pmol/L; $P<0.01$) (Fig. 5-1D), while plasma glucagon concentration was unchanged (pre-T2D: 24±16 pg/mL vs. healthy: 33±19 pg/mL) (Fig. 5-1E). Finally, body weight was modestly yet significantly higher ($P<0.05$) in pre-T2D (416±26 g) vs. healthy control (393±39 g) rats (Fig. 5-1F).

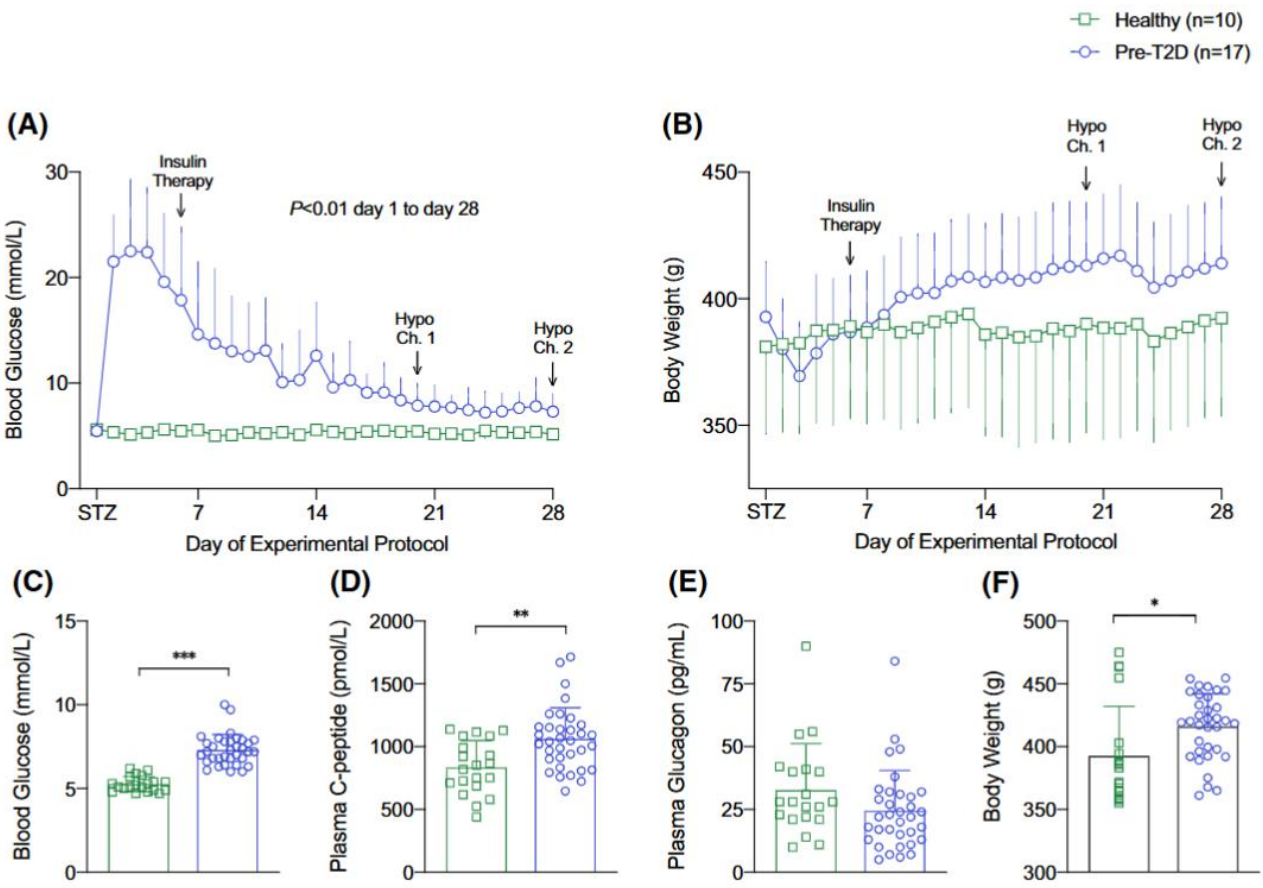


Figure 5-1. Pre-diabetes (pre-T2D) model characteristics. **(A)** Whole blood glucose concentration and **(B)** body weight measured from the time of STZ administration (day 1) to the second hypoglycemia challenge (study completion; day 28). The initiation of daily (blood glucose-dependent) insulin therapy is indicated on day 6. Pooled baseline measurements of **(C)** whole blood glucose concentration, **(D)** plasma C-peptide concentration, **(E)** plasma glucagon concentration, and **(F)** body weight on hypoglycemia challenge days 1 and 2. *P<0.05, **P<0.01, ***P<0.001. All data are means ± SD. Hypo Ch., hypoglycemia challenge; STZ, streptozotocin.

Hypoglycemia Challenges

Blood Glucose In the healthy rats, blood glucose levels were not significantly different between vehicle and SSTR2a groups at baseline or any subsequent sampling time point (Fig. 5-2A). Blood glucose levels remained unchanged from basal values for 1 h after SSTR2a or vehicle treatment (i.e., t=0 min) and fell uniformly in both groups after insulin bolus to reach similar hypoglycemic nadirs by t=60 min (healthy-vehicle:1.6±0.3

mmol/L vs. healthy-SSTR2a: 1.4±0.3 mmol/) (Fig. 5-2A). Time to hypoglycemia onset (healthy-vehicle: 27±8 min vs. healthy-SSTR2a: 24±7 min) (Fig. 5-2B) and probability of euglycemia (≥4.0 mmol/L) over time (Fig. 5-2C) were unaffected by SSTR2a treatment in healthy rats.

In the pre-T2D rats, blood glucose levels were identical between treatment groups at baseline (vehicle: 7.3±0.9 mmol/L vs. SSTR2a: 7.3±0.9 mmol/L) (Fig. 5-2D). However, glycemia rose by 1.5 mmol/L from baseline (7.3±0.9 mmol/L to 8.8±2.1 mmol/L; N.S.) in the vehicle group and by 4.3 mmol/L (7.3±0.9 mmol/L to 11.6±3.3 mmol/L; P<0.001) in the SSTR2a group by 30 min (t=-30 min) after treatment (Fig. 5-2D). Blood glucose levels were significantly higher in the SSTR2a vs. vehicle group from 30 min post-SSTR2a/vehicle administration (t=-30min) until 30 min post-insulin administration (t=30 min) (P<0.001 for t=-30, 0, 10, and 20 min; P<0.05 for t=30 min) (Fig. 5-2D). Blood glucose nadir during hypoglycemia was unaffected by treatment (pre-T2D-vehicle: 1.4±0.3 mmol/L vs. T2D-SSTR2a: 1.3±0.4 mmol/L) (Fig. 5-2D). Blood glucose AUC after t=0 min was 1.3-fold higher (P<0.001) in the pre-T2D-SSTR2a group (498±203 mmol*min/L) vs. the pre-T2D-vehicle group (375±133 mmol*min/L) (Fig. 5-2D').

The onset of hypoglycemia, defined by the ADA Workgroup on Hypoglycemia as blood glucose ≤3.9 mmol/L in humans with either T1D or T2D,[227] was delayed by 15±12 min (P<0.001) in the T2D-SSTR2a (44±13 min) compared to the pre-T2D-vehicle (28±7 min) group (Fig. 5-2E). A comparison of survival curves in Figure 5-2F revealed a lower proportion of hypoglycemic pre-T2D animals treated with SSTR2a as compared to vehicle (P<0.001) throughout the challenge period. Notably, 18% (n=3/17) of vehicle-treated vs. 65% (n=1/17) of SSTR2a-treated rats remained euglycemic (≥4.0 mmol/L) 30 min after

insulin administration, and 100% of rats in each group reached hypoglycemia by 40- and 70 min, respectively (Fig. 5-2F).

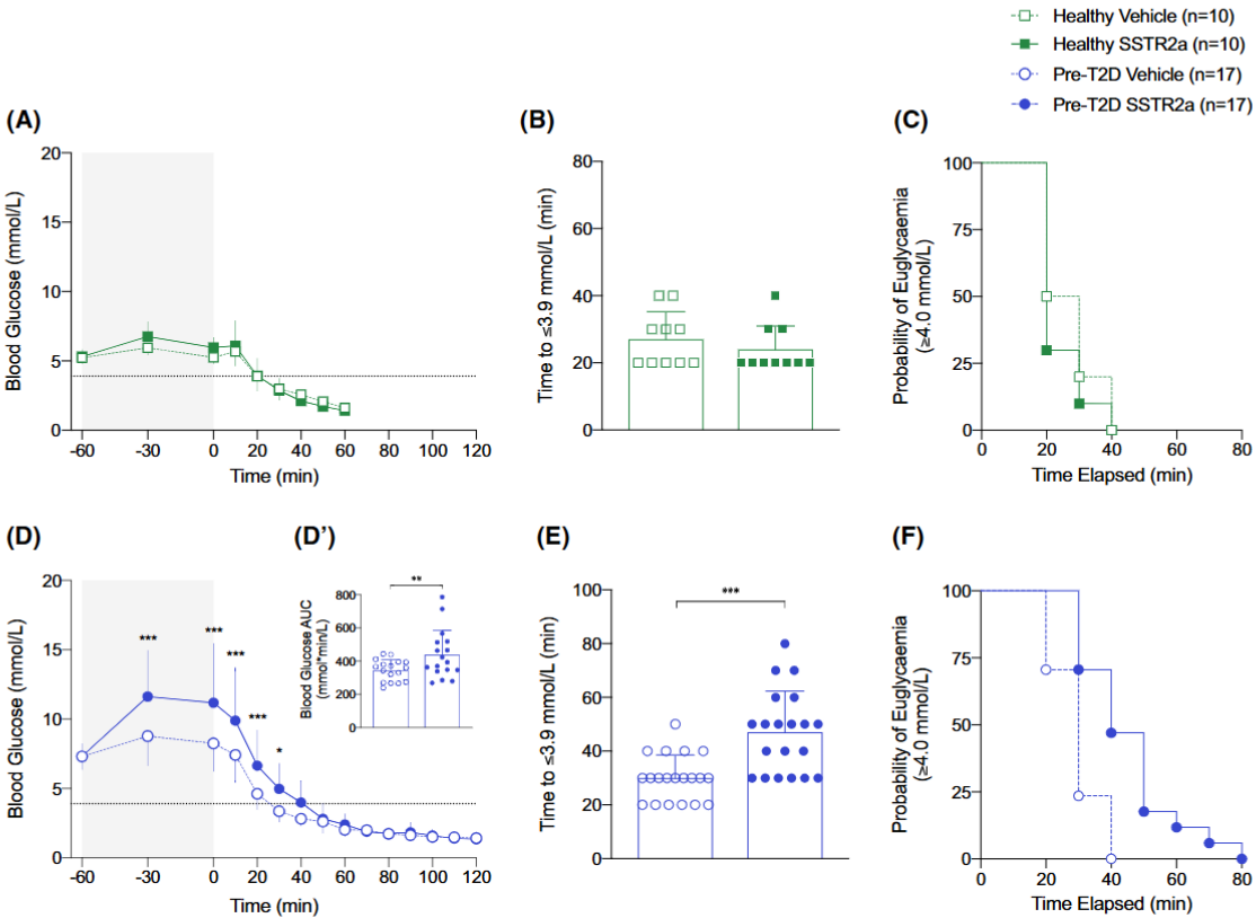


Figure 5-2. Whole blood glucose responses to insulin-induced hypoglycemia in **(A)** healthy and **(B)** pre-T2D rats with or without SSTR2a. Grey shaded region indicates the period of basal monitoring before hypoglycemia induction at t=0 minutes. Time from insulin administration to the onset of clinical hypoglycemia (≤3.9 mmol/L) in **(C)** healthy and **(D)** pre-T2D rats. **(D')** Blood glucose AUC from t=0 to t=120 minutes in pre-T2D rats. Survival curve comparing the proportion of **(E)** healthy and **(F)** pre-T2D rats (P<0.001 SSTR2a vs. vehicle) remaining euglycemic (≥4.0 mmol/L) after hypoglycemia induction. *P<0.05, **P<0.01, ***P<0.001 between treatment groups. All data are means ± SD.

Figure 5-3 illustrates the effects of SSTR2a treatment on depth of hypoglycemia (Fig. 5-3A) and hyperglycemia (Fig. 5-3B) in pre-T2D rats. Blood glucose AUC ≤3.9 mmol/L was 25% lower (*P*<0.01) with SSTR2a (128±44 mmol*min/L) vs. vehicle (171±43 mmol*min/L) treatment (Fig. 5-3A). Blood glucose AUC ≥11.1 mmol/L was not significantly higher in the SSTR2a group (vehicle: 4 ±10 mmol*min/L vs SSTR2a: 77±160 mmol*min/L (Fig. 5-3B); however, 30% (n=3/10) vs. 63% (n=9/17) of pre-T2D rats reached a blood glucose concentration of ≥11.1 mmol/L after treatment with vehicle vs SSTR2a, respectively (chi square *P*=0.25). Hyperglycemia was defined by a lower limit of 11.1 mmol/L for this outcome, since it reflects the clinical threshold for T2D diagnosis from a random blood glucose measurement.[202]

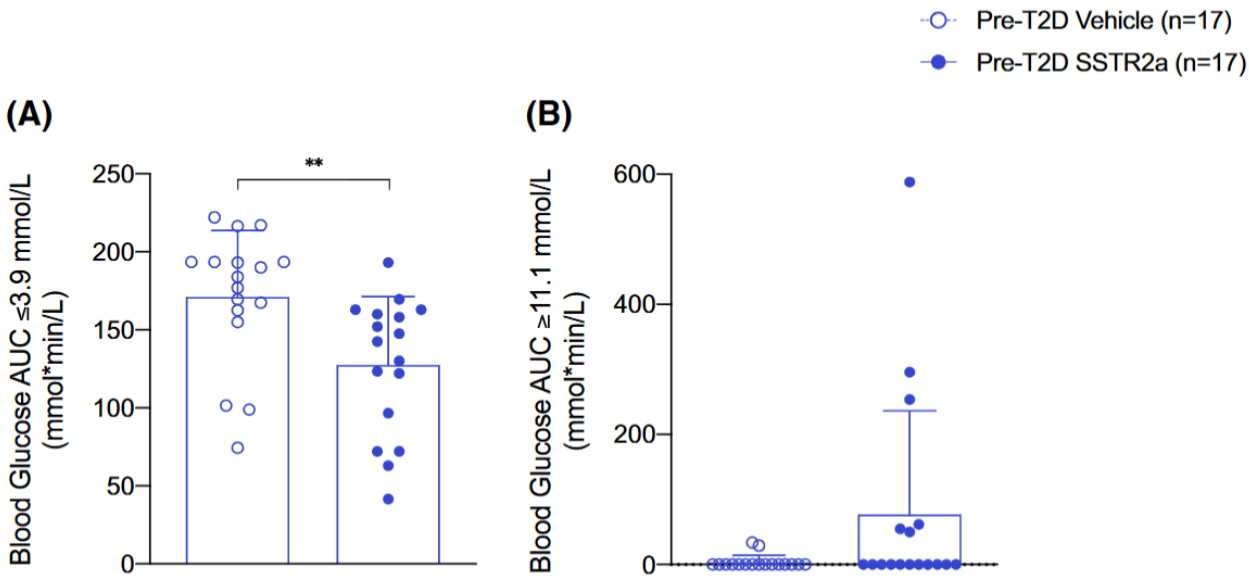


Figure 5-3. Whole blood glucose area under the curve (AUC) of pre-diabetes (pre-T2D) rats **(A)** ≤3.9 mmol/L and **(B)** ≥11.1 mmol/L with or without a somatostatin receptor 2 antagonist (SSTR2a). ***P*< 0.01. All data are means ± SD.

Plasma Hormones Plasma glucagon and C-peptide concentrations measured during iatrogenic hypoglycemia challenge are shown in Figure 5-4 Plasma glucagon levels did not differ significantly between the two treatment groups of healthy (i.e., control)

rats at baseline (vehicle: 30±13 pg/mL vs. SSTR2a: 35±24 pg/mL) (Fig 5-4A). In healthy rats, glucagon levels rose 4.6-fold from baseline 1 h after SSTR2a treatment (t=0 min: 158±76 pg/mL vs. t=-60 min: 35±24 pg/mL; $P<0.05$) (Fig. 5-4A). This increase was not significant relative to vehicle-control levels (t=0 min: 27±15 pg/mL), which remained stable from baseline (t=-60 min: 29 ±12 pg/mL) (Fig. 5-4A). In response to hypoglycemia induction by insulin aspart bolus, plasma glucagon levels were significantly elevated for 60 min in the healthy-SSTR2a versus healthy-vehicle group (time x treatment: $P<0.01$) (Fig. 5-4A). In the pre-T2D rats, plasma glucagon levels did not differ significantly between treatment groups at baseline (vehicle: 25±20 pg/mL vs. SSTR2a: 24±12 pg/mL) (Fig. 5-4B). Plasma glucagon levels were ~12-fold higher ($P<0.001$) within 1 h of SSTR2a (vehicle: 12±5 pg/mL vs. SSTR2a: 141±62 pg/mL) and remained significantly elevated for 60 min post-insulin administration ($P<0.05$ for t=40 and 60 min) (Fig. 5-4B).

A comparison of plasma glucagon responses across all four groups within 60 min of insulin administration is shown in Figure 5-4C. The plasma glucagon response to hypoglycemia (as measured by glucagon AUC) was 1.4-fold lower in pre-T2D (394±198 pg*min/mL) vs. healthy (540±291 pg*min/mL) rats, independent of treatment (main effect of diabetes status: $P<0.01$), and ~2-fold higher with STSR2a (595±237 pg*min/mL) vs. vehicle (302±137 pg*min/m) treatment, independent of diabetes status (main effect of treatment: $P<0.001$) (Fig. 5-4C). Peak plasma glucagon concentration was ~1.5-fold higher with SSTR2a (477±124 pg/mL) vs. vehicle (321±134 pg/mL) treatment, independent of diabetes status (main effect of treatment: $P<0.001$) but was not significantly different between healthy and pre-T2D groups (data not shown).

Baseline circulating C-peptide levels were higher in pre-T2D (1061±249 pmol/L) as compared to healthy (836±212 pmol/L) rats (main effect of diabetes status: $P<0.01$) (Fig. 5-4D). SSTR2a had no significant effect on circulating C-peptide levels under basal or hypoglycemic conditions in healthy (Fig. 5-4D) or pre-T2D (Fig. 5-4E) rodents. However, peak C-peptide levels and plasma C-peptide AUC between t=-60 and 0 min were both ~1.3-fold higher in pre-T2D (peak: 1380±309 pmol/L; AUC: 72935±14441 pmol*min/L) vs. healthy (peak: 1048±209 pmol/L; AUC: 54151±9814 pmol*min/L) rats, independent of treatment (main effect of diabetes status: $P<0.001$ for both), and 1.1-1.2-fold higher with SSTR2a (peak: 1383±272 pmol/L; AUC: 68600±11983 pmol*min/L) vs. vehicle (peak: 1131±315 pmol/L; AUC: 61617±18285 pmol*min/L), independent of diabetes status (main effect of treatment: $P<0.01$ and $P<0.05$ for peak and AUC, respectively) (data not shown). Bolus insulin administration at t=0 min triggered a decline in plasma C-peptide levels over 40 min in all groups, yielding a comparable reduction from baseline across all four groups (healthy-vehicle: 87±6%; healthy-SSTR2a: 85±8%; pre-T2D-vehicle: 79±9%; pre-T2D-SSTR2a: 81±8%) (Fig. 5-4D,E).

Portal vein glucagon concentration, measured at the terminal time point in hypoglycemia, did not differ significantly across the four conditions (healthy-vehicle:142±211 pg/mL; healthy-SSTR2a: 116±162 pg/mL; pre-T2D-vehicle: 89±85 pg/mL; pre-T2D SSTR2a: 62±63 pg/mL) (data not shown), while C-peptide was undetectable in the portal vein across conditions at terminal hypoglycemia.

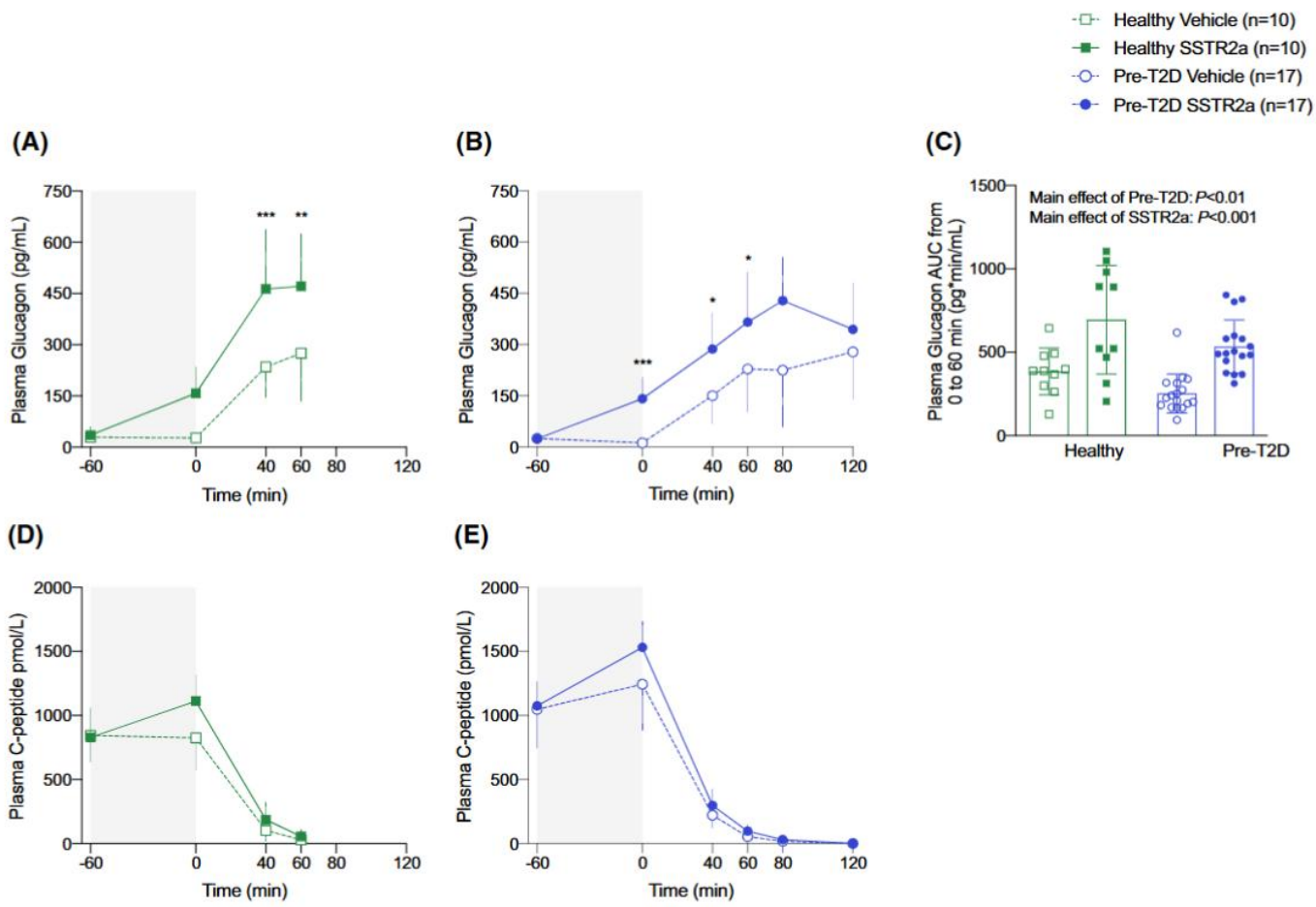


Figure 5-4. Plasma glucagon responses to insulin-induced hypoglycemia in **(A)** healthy, and **(B)** pre-T2D rats, with or without SSTR2a. **(C)** Plasma glucagon AUC from t=0 minutes to t=60 minutes in all conditions. Plasma C-peptide concentration in **(D)** healthy and **(E)** pre-T2D rats. Grey shaded region indicates period between SSTR2a/vehicle dosing and bolus insulin administration. **P*<0.05, ***P*<0.01, ****P*<0.001 between treatment groups. All data are means ± SD.

The effects of pre-T2D and SSTR2a treatment on the relationship between whole blood glucose and plasma glucagon levels after insulin overdose are illustrated in Figure 5-5. For each group, average blood glucose level and glucagon level at baseline and each sampling timepoint after insulin bolus (t=-60, 40, 60, or 80 min) were linearly correlated (R^2>0.94 for each condition) (Fig. 5-5A). Regression line slopes, representing the increase in plasma glucagon concentration (pg/mL) per 1 mmol/L drop in blood

glucose concentration, were significant (slope > 0) for all conditions (Fig. 5-5A). A comparison of slopes for each rat in each group (Fig. 5-5A') showed increased glucagon responsiveness to insulin-induced hypoglycemia with SSTR2a vs. vehicle (glucagon increase of 88±43 vs. 52±35 pg/mL per 1 mmol/L drop in glucose) treatment, independent of diabetes status (main effect of treatment: $P<0.001$), and attenuated glucagon responsiveness in pre-T2D vs. healthy (glucagon increase of 56±30 vs. 97±50 pg/mL per mmol/L decline in glucose) rats, independent of treatment (main effect of diabetes status: $P<0.001$) (Fig. 5-5A').

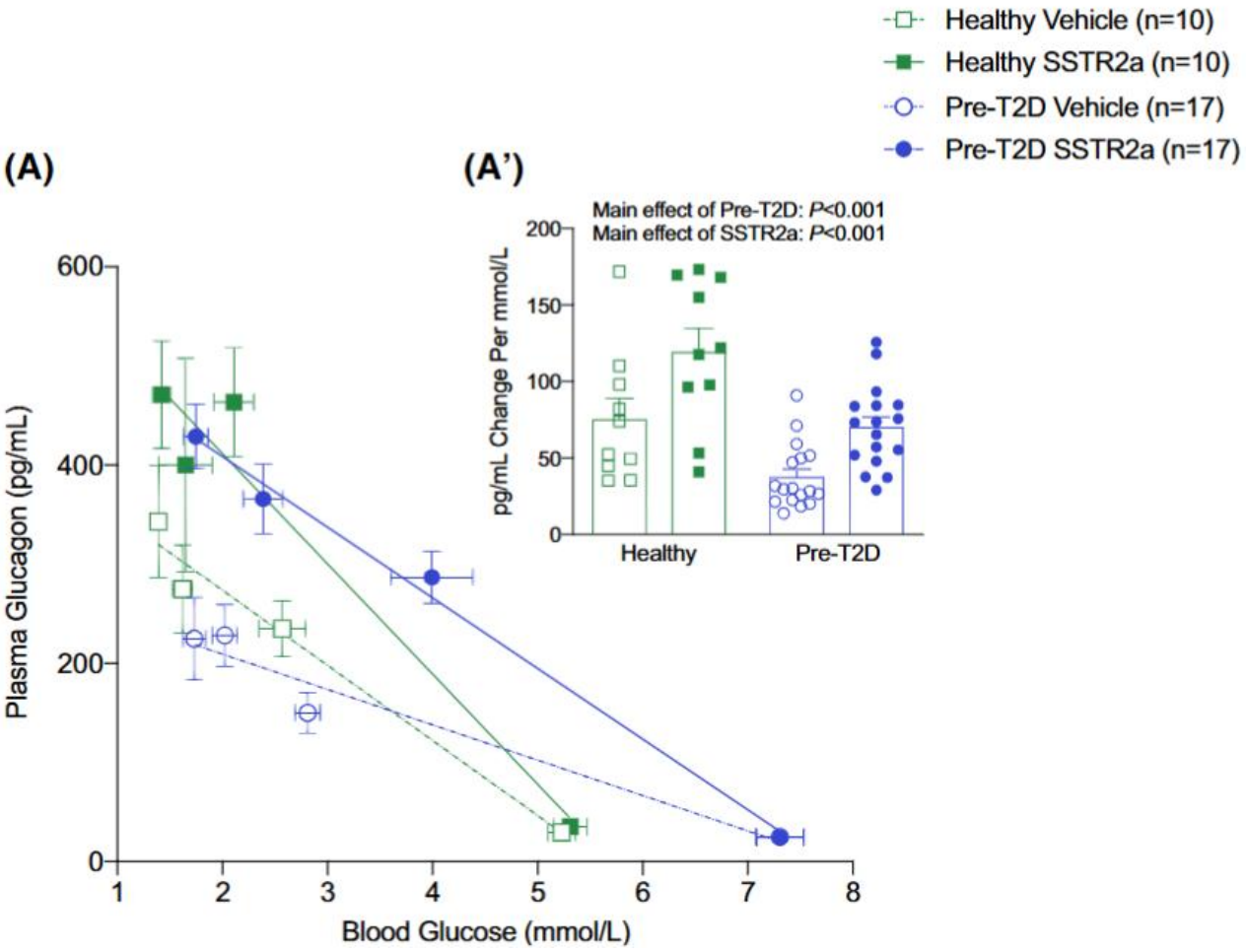


Figure 5-5. Relationship between plasma glucagon and whole blood glucose levels in healthy and pre-T2D rats after insulin overdose with or without SSTR2a. **(A)** Regression line fit to grouped timepoint data (t= -60, 40, 60, and 80 minutes) for each condition. **(A')** Comparison of regression line slopes across conditions. All data are means ± SEM.

5.5 Discussion

In this study, we developed a novel rat model of pre-T2D, that exhibited mild hyperglycemia and moderate insulin resistance, to better understand the combined effects of these pre-T2D hallmarks on glucagon counterregulation to hypoglycemia, with and without SSTR2 antagonism. We report a mild attenuation in the magnitude of the glucagon response, as well as glucagon responsiveness (i.e., α-cell sensitivity) to hypoglycemia development following insulin bolus challenge in our novel pre-T2D model. Pre-treatment with SSTR2a increased the plasma glucagon response during hypoglycemia by all measures (peak concentration, AUC, responsiveness) in both healthy and pre-T2D rats. This glucagon response was associated with a ~30 minute elevation in blood glucose levels over vehicle controls and a 15 minute delay in the onset of hypoglycemia that were not observed in healthy rats. Consistent with evidence from somatostatin knockout islets,[255] SSTR2a treatment in the present study did not affect the C-peptide "switch-off" response to insulin-induced hypoglycemia in healthy or pre-T2D animals. This finding confirms antagonist selectivity for SSTR2 (expressed by α-cells) over SSTR5 (expressed by rodent β-cells).[267] Collectively, these data suggest that SSTR2a treatment may have therapeutic applications for hypoglycemia prevention in more advanced stages of insulin-deficient T2D.

Recent findings suggest that the suppression of SST secretion from islet δ-cells may act as a permissive signal for counterregulatory glucagon release, which is

compromised in T2D.[4] In one study, islets from hyperglycemic Fh1βKO T2D mice showed a 6-fold increase in SST secretion at 1 mmol/L glucose relative to healthy control islets, which correlated with a >75% reduction in glucagon secretion.[4] Consistent with elevated SST tone in T2D islets, application of a SSTR2a at 1 mmol/L glucose raised glucagon secretion by 143±11% in Fh1βKO islets compared with 13±14% in control islets.[4] Alternatively, reports of a decrease in SST secretion[4,110] and acquired α-cell resistance to SST[110] at higher glucose concentrations may help to explain the hyperglucagonemia in T2D. These findings were replicated in the perfused pancreas and isolated islets of HFF mice across a wider glucose range (1, 6, and 15 mmol/L glucose).[131] Accordingly, SSTR2 antagonism had little to no effect on glucagon secretion in HFF islets, or in T2D islets at high glucose, unlike the marked stimulatory effect observed in control islets.[4,131] However, it is important to note that these *in situ* and *in vitro* responses were observed under conditions of low glucose alone – that is, without the inhibitory effect of exogenous insulin on counterregulatory glucagon secretion.[268] When measured in HFF mice following bolus insulin challenge *in vivo*, the counterregulatory glucagon response was only increased relative to controls when insulin was dosed according to lean and not total body mass.[131] In the present study, the stimulatory effect of SSTR2 antagonism on plasma glucagon levels was similar between pre-T2D and healthy rats during basal glycemia and insulin-induced hypoglycemia, suggesting that SST signalling may be relatively normal in this model compared to more advanced stages of T2D.[4] This outcome further highlights the potential for SSTR2a to induce transient hyperglucagonemia in the absence of hypoglycemia at the present dose in T2D.

Islet β-cells undergo morphological (i.e., β-cell expansion) and functional adaptations to maintain normoglycemia in a setting of insulin resistance.[269] Over time, increased insulin demand leads to gradual β-cell de-differentiation, and the loss of adequate compensation precipitates the onset of pre-T2D, characterised by impaired glucose tolerance and impaired fasting glucose.[269,270] Hyperinsulinemia, which persists from pre-T2D through early-stage T2D, is eventually normalised (relative to controls) with advancing β-cell failure, resulting in the onset of late-stage T2D.[271] Despite phenotypic heterogeneity within and between clinical T2D study samples (i.e., mean disease and treatment duration, treatment intensity and modality, etc.), basal hypoinsulinemia relative to non-diabetic control subjects (i.e., late-stage T2D) has emerged as a clinical surrogate of defective glucose counterregulation in T2D.[121,123,124] Here, we report a mild defect in counterregulatory glucagon secretion during insulin-induced hypoglycemia in pre-T2D rats, despite basal hyperinsulinemia. These observations suggest that this progressive α-cell defect may begin to develop in a state of relative insulin deficiency (relative to ambient blood glucose concentration), before the onset of overt or absolute insulin deficiency (relative to healthy controls), as previously speculated.

A robust SSTR2a-induced increase in plasma glucagon levels, observed before and after hypoglycemic induction in healthy and pre-T2D rats, only amounted to a detectable increase in blood glucose levels in the pre-T2D group. We propose that the basal hyperglycemic insult posed by SSTR2 antagonism in the healthy group was offset by endogenous insulin action (based on increases in peak plasma C-peptide levels and plasma C-peptide AUC before bolus insulin challenge) to preserve euglycemia. This increase in endogenous insulin levels, observed after SSTR2a treatment but before

insulin bolus in both healthy and pre-T2D rats, may reflect paracrine stimulation by increased levels of intra-islet glucagon.[272] After insulin bolus, SSTR2a afforded no protection against hypoglycemia in healthy rats, perhaps because the glucagon response was outmatched by the dose of exogenous insulin used. This outcome in healthy rats was similar to that of a previous study, in which SSTR2a treatment in STZ-T1D rats reduced the dependence on glucose infusion for the maintenance of clamped hypoglycemia in the presence of low- but not high-dose insulin, despite a complete normalisation of glucagon levels at both doses.[1] We suspect that in a setting of peripheral insulin resistance and relative insulin deficiency, the hyperglycemic effect of SSTR2a could not be offset by the actions of endogenous or exogenous insulin, delaying hypoglycemia onset in our pre-T2D model.

This study contained several limitations to acknowledge. First, this study tested a single low dose of ZT-01, so future studies are necessary to determine the minimum effective dose of this particular SSTR2a that does not aggravate basal hyperglycemia in this or other models of pre-T2D and T2D. Second, portal vein hormones were measured only at hypoglycemic challenge completion; however, a measurable effect of SSTR2a on portal hormone levels may have dissipated by this time. Thus, regular portal vein sampling from pre-implanted catheters may offer more descriptive insights into islet hormone responses *in vivo* (including response rates and glycemic thresholds). Finally, non-normalised blood glucose levels in our pre-T2D rats at baseline, compounded by insulin resistance in this model, extended the time to terminal hypoglycemia by 60 minutes relative to healthy rats. This limited our comparison of total hormone responses to hypoglycemia (i.e., AUC analyses) between healthy and pre-T2D rats.

In summary, the glucagon counterregulatory response to insulin-induced hypoglycemia was mildly impaired in this novel rat model of pre-T2D that exhibits insulin resistance and mild hyperglycemia. Nonetheless, SSTR2a treatment augmented plasma glucagon levels after bolus insulin overdose and delayed the onset of hypoglycemia in pre-diabetic rodents without affecting the plasma C-peptide response in this rodent model. However, promising, these treatment outcomes were not low-glucose-dependent, suggesting that somatostatin may be important for regulating glucagon secretion under basal (post-prandial) and hypoglycemic conditions *in vivo*. Consequently, SSTR2a may aggravate basal hyperglycemia at the current dose in a setting of relative insulin deficiency and peripheral insulin resistance. Collectively, these discoveries stand to advance our understanding of the paracrine mechanisms governing α-cell behaviour in health and disease, which may be vital to improving therapeutic options for individuals living with early- and late-stageT2D.

Chapter 6: Comparing a Somatostatin Receptor 2 Antagonist to Exogenous Glucagon for the Treatment of Severe Insulin-Induced Hypoglycemia in Rats.

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Author Contributions:

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Data acquisition: EGH, NCD, NA, NN, SF, SN

Data analysis and interpretation: EGH, OC, RL, MCR

Drafting the manuscript: EGH

Editing the manuscript: EGH, OC, RL, MCR

Target Journal: Diabetes

6.1 Abstract

Background: Exogenous glucagon is effective at treating severe hypoglycemia but causes supraphysiologic hyperglucagonemia and transient glycemic recovery, which is often complicated by gastrointestinal side effects. Somatostatin receptor 2 antagonists (SSTR2a) augment endogenous glucagon release and reduce the frequency and duration of hypoglycemia when administered before insulin overdose but have not been evaluated as a rescue therapy. We compared the effects of the selective SSTR2a, ZT-01, with or without gastric inhibitory polypeptide (GIP), against exogenous glucagon on glucagon counterregulation and glycemic recovery from recurrent insulin-induced hypoglycemia. **Materials/Methods:** Male Sprague–Dawley rats (n=39) underwent three days of insulin-induced hypoglycemia to impair counterregulatory glucagon secretion. On day 4, rats received a bolus insulin overdose followed by a rescue injection of vehicle, exogenous glucagon (0.15 mg, SC), ZT-01 (3 or 10 mg/kg, SC), or ZT-01 (10 mg/kg, SC) + GIP (10 nmol/kg, IP) once blood glucose levels reached level 2 (L2) hypoglycemia (<3.0 mM glucose) and were monitored for 2 hours before portal vein plasma and tissue collection. **Results:** Vehicle-treated rats remained in L2 hypoglycemia for 2 hours whereas EG restored euglycemia in all rats for at least 30 min. Mean blood glucose level in the 10 mg/kg ZT-01 condition stabilized in level 1 hypoglycemia for hour 1 and in euglycemia for hour 2. Glycemic recovery was improved by GIP co-treatment in the first 15 min but impaired thereafter. Total peripheral glucagon exposure was ~70-fold higher with exogenous glucagon but only ~2–4-fold higher with ZT-01 compared to vehicle. In the high-dose conditions, portal vein glucagon concentration and the activities of hepatic enzymes, glycogen phosphorylase and phosphoenolpyruvate carboxykinase (PEPCK) were elevated, whereas hepatic glycogen content was reduced compared to vehicle-

and/or glucagon-treated controls. **Conclusions:** SSTR2 antagonism restored physiological glucagon counterregulation and provided gradual, sustained recovery from severe hypoglycemia without inducing peripheral hyperglucagonemia. Unlike exogenous glucagon, maximal glycemic recovery occurred in the second hour after administration of high-dose ZT-01, highlighting its potential as a novel strategy for treating severe hypoglycemia.

6.2 Introduction

In people with type 1 diabetes (T1D) and advanced type 2 diabetes (T2D), the use of insulin therapy to achieve strict glycemic control is undermined by the associated risk of severe hypoglycemia.[23] Defined as cognitive and/or physical dysfunction requiring external assistance for recovery, severe hypoglycemia commonly involves a loss of consciousness, seizures, and/or inability to swallow.[23] This complication is treated outside of healthcare settings with emergency glucagon, available in an injectable (intramuscular or subcutaneous) and intranasal formulation.[23,273]

Since the hepatic portal vein acts as a direct conduit for hormone transit from the pancreas to the liver, plasma glucagon levels are 1.5x higher in hepatic portal versus peripheral venous circulation.[274] Whether delivered by intramuscular, subcutaneous, or intranasal routes, the dose of exogenous glucagon must provide adequate portal vein exposure despite systemic distribution and enzymatic degradation in circulating plasma.[273] Accordingly, peripheral venous glucagon levels are 5.5x higher after treatment with the efficacious rescue dose (1 mg for injectable, 3 mg for intranasal) than during maximal metabolic demand, such as fasted exercise.[275]

Peripheral hyperglucagonemia, resulting from the standard rescue dose of exogenous glucagon, relaxes smooth muscle throughout the gastrointestinal (GI) tract, slowing motility and causing full visceral distention.[276] Consequently, nausea (22-42%) and vomiting (9-17%) are the most common side-effects of treatment independent of delivery route.[277] Another factor limiting the clinical utility of exogenous glucagon is its short duration of action ranging from 12-27 minutes,[278] which may allow for a recurrence of hypoglycemia depending on the type, dose, and timing of insulin administration. Therefore, the goal of treatment is to (at least) temporarily restore blood glucose to a level

that enables treatment with fast-acting oral carbohydrate while minimizing dose-dependent side-effects.[278] However, if GI distress deters the oral intake of carbohydrate, recovery may be transient, especially without replenishing hepatic glycogen stores.[23]

The risk of hypoglycemia in individuals with T1D and advanced T2D stems from therapeutic hyperinsulinemia (owing to imperfect insulin dosing) and inadequate release of glucagon - the body's chief counterregulatory hormone.[279] Once pre-disposed to hypoglycemia by these factors, each exposure further attenuates the glucagon and epinephrine responses to future episodes, inviting a vicious cycle of recurrent hypoglycemia.[258,280,281]

Somatostatin-14, secreted by pancreatic δ-cells, acts as a paracrine hormone that inhibits glucagon secretion from pancreatic α-cells via somatostatin receptor 2 (SSTR2) in rodents and humans.[6,257] Treatment with somatostatin receptor 2 antagonists (SSTR2a) has been shown to reduce the depth, duration, and/or frequency of insulin-induced hypoglycemia by augmenting counterregulatory glucagon secretion in rat models of T1D[1–3], T2D,[282,283] T1D/exercise,[170] and recurrent hypoglycemia.[258] Having recently advanced to phase 2 clinical trials, the highly selective SSTR2a, ZT-01 (Zucara Therapeutics, Toronto, ON, Canada), will be evaluated as a therapeutic strategy for reducing the frequency of nocturnal hypoglycemia in adults with T1D.[20] To date, this novel class of therapeutic has only been investigated as a preventative measure against hypoglycemia (i.e., when administered one hour before insulin overdose);[22] however, its ability to reverse existing hypoglycemia remains unexplored.

Like GLP-1, GIP is an incretin hormone secreted by the small intestine in the post-prandial state.[284] However, unlike GLP-1, pharmacologic dosing of GIP has been shown to stimulate, rather than inhibit, glucagon secretion during hypoglycemia in animals and humans, both with and without diabetes.[113,188,191,285,286] Because these studies were performed under hyperinsulinemic-hypoglycemic clamp conditions,[191,285,286] it is unclear whether exogenous GIP can promote glycemic recovery from hypoglycemia. Like GLP-1, GIP is an attractive treatment candidate for diabetes since it regulates islet hormone release in a glucose-dependent fashion, and therefore, does not carry a risk of iatrogenic hyperglycemia or hypoglycemia.[191,285,287]

The purpose of this study was to determine whether SSTR2 antagonism, with or without co-administration of GIP, can effectively treat severe hypoglycemia without causing peripheral hyperglucagonemia.

6.3 Materials and Methods

This study was conducted in accordance with the recommendations of the Canadian Council for Animal Care guidelines and has been approved by the York University Animal Care Committee (Protocol # 2017-7).

Animals

Thirty-nine (n=39) male Sprague-Dawley rats were obtained at age of 8-10 weeks and body weight of ~250 g. Due to availability from suppliers, thirty-three (n=33) rats were supplied by Charles River Laboratories (Montreal, QC, Canada) and five (n=5) by Inotiv (West Lafayette, IN, USA). Rats were housed in the York University vivarium in a light-controlled (12-hour light/dark cycle) room at 22-23°C and 50-60% relative humidity with ad libitum access to standard rodent chow (Purina Labdiet, 5012, St. Louis, MO, USA)

and water. Following a 7-day habituation period, all rats underwent 3 consecutive days of hypoglycemia conditioning.

Hypoglycemia Conditioning (Days 1-3)

Food was limited to 20 g per rat overnight and removed at 9 am the morning of conditioning. Hypoglycemia was induced using a descending daily dose of Humulin-R insulin (Eli Lilly, Canada): 10, 8, and 5 U/kg on days 1, 2 and 3, respectively, to achieve a similar level of hypoglycemia throughout the conditioning period as counterregulatory function declined[151,258]. After hypoglycemia induction, blood glucose levels were maintained within a target range of 1.7-2.5 mM for ~120 min using 50% dextrose solution (via oral gavage and/or intraperitoneal injection) as necessary. Animals that failed to reach the glycemic target range within 60 minutes of their initial insulin dose received a second full or partial dose.[258] After 120 min in the target hypoglycemic range, blood glucose levels were recovered with a 1-2 mL oral gavage of 50% dextrose solution.

Whole blood glucose levels were measured in duplicate from the saphenous vein using a hand-held glucometer (ATRAK, Abbott) every 1-5 min until a concentration of <3.0 mM was reached, and every 10 min thereafter. Blood samples were collected by saphenous venipuncture at baseline (t=0 min; before insulin administration), at blood glucose concentrations of ≤3.6 mM and <3.0 mM, and 15 and 30 min after reaching <3.0 mM, for subsequent hormone analysis.

Pharmacological Rescue from Recurrent Hypoglycemia (Day 4)

Following a controlled overnight feed of 20 g, chow was removed at 9 am and hypoglycemia was induced using 2 U/kg Humulin-R insulin. Rats were randomly assigned to one of the following treatment groups: vehicle (veh; 2.1% glycerol in 10 mM acetate

buffer, pH 4.5, formulated by AdMare BioInnovations, Vancouver, BC, Canada; supplied by Zucara Therapeutics; n=8), exogenous glucagon (EG; 0.15 mg Glucagon for Injection, rDNA Origin, DIN 02243297, Eli Lilly; n=7), low dose SSTR2a (3ZT; 3 mg/kg SSTR2a in vehicle, adMare BioInnovations; n=8) high dose SSTR2a (10ZT; 10 mg/kg ZT-01 in vehicle, adMare BioInnovations; n=8) and 10 mg/kg ZT-01+rat gastric inhibitory polypeptide (10ZT+GIP; 10 nmol/kg GIP, Phoenix Pharmaceuticals, 027-14, Burlingame, CA, USA; n=8). All treatments were injected subcutaneously, excluding GIP, which was administered immediately after 10ZT by intraperitoneal injection. The dose of exogenous glucagon (0.15 mg) was selected based on evidence that 0.1 mg could restore euglycemia from a baseline glucose concentration of ~3.3 mM in hypoglycemia naïve rats without causing reactive hyperglycemia.[288] Since 1) we administered rescue treatment at a lower baseline glucose level (<3.0 mM), 2) our rat model had diminished counterregulatory function, and 3) a positive dose response had been modeled beyond 0.1 mg[289], we opted for a dose of 0.15 mg.

Whole blood glucose was measured from the saphenous vein of either leg using a handheld glucometer every 1-5 min until a blood glucose of <3.0 mM was reached, and then every 10 min thereafter for 120 min. At the first blood glucose measurement <3.0 mM, thirty-nine (n=39) rats were treated according to their group assignments. Saphenous blood was collected using the same method and at the same time points evaluated during hypoglycemia conditioning on days 1-3, with four additional samples collected for hormone analysis at 30, 60, 90, and 120 min after the administration of rescue treatment. If blood glucose reached ≤1.5 mM (i.e., the humane endpoint), a blood sample was obtained, and the observation period was terminated to avoid severe

symptoms of hypoglycemia. At challenge completion (i.e., 120 min after rescue dosing or upon early termination), rats were anesthetized with isoflurane gas for portal vein blood collection and then euthanized via exsanguination prior to tissue collection. Liver samples were washed in PBS, flash-frozen in liquid nitrogen, and stored at -80°C for future analysis.

Glucagon and C-peptide Analysis

Blood samples were collected from saphenous or portal vein bleed (terminal time point only) in potassium-EDTA coated, microvette capillary tubes (16.444.100, Sarstedt, Canada) and centrifuged at 12000 RPM at 4°C for 5 minutes. Plasma was removed and stored in polyethylene tubes at -80°C for the subsequent quantification of glucagon (Mercodia,10-1271-01, RRID: AB 2737304) and C-peptide (Crystal Chem, 90055, RRID: AB 2893130) levels using ELISA.

Liver Glycogen and Enzyme Activity Analysis

Liver Glycogen content (Abcam, ab65620) and the enzyme activities of glycogen phosphorylase (GP; Abcam, ab273271) and phosphoenolpyruvate carboxykinase (PEPCK; Abcam, ab239714) were measured using colorimetric assay kits according to kit instructions. For measurement of GP activity, supernatant collected from homogenized tissue was diluted 10x with PBS before assaying. Glycogen concentration and enzyme activities were normalized to total protein content, as determined using a BCA protein assay kit (Thermo Fisher, 23227).

Statistical Analysis

Data are expressed as means ± standard deviation. Statistical tests were conducted against a significance criterion of $P<0.05$ using Prism software (GraphPad

Software, San Diego, CA). Effects between groups were evaluated by ANOVA with time-based analyses using repeated measures ANOVA. Significant differences were further evaluated with Tukey post-hoc tests as appropriate. Glycemic trends between t=0-15 min, t=15-60 min, and t=60-120 min analyzed by simple linear regression. 10% of C-peptide concentration values in five rats could not be obtained at all scheduled time points and were interpolated as the mean of neighboring time point values. Plasma glucagon concentration, C_{max}, and AUC analyses were conducted on log-transformed data.

6.4 Results

Hypoglycemia Conditioning (Days 1-4)

Body weight, whole blood glucose, and plasma glucagon measurements obtained on days 1-3, and before rescue treatment on day 4, are provided in table 6-1. Body weight was consistent across study days whereas basal blood glucose concentration (i.e., prior to hypoglycemia induction) was 1.4-1.5 mM higher on day 1 vs. days 3 and 4 ($P \leq 0.012$ for days 3 and 4 vs. day 1). Time from insulin dosing to the onset of level 2 hypoglycemia (blood glucose <3.0 mM) was ≤18 min shorter on days 2-4 vs. day 1 ($P<0.006$) and became less variable throughout the study period. (Table 6-1). Basal and peak plasma glucagon levels, measured before hypoglycemia induction and at a blood glucose concentration of ≤3.6 mM, respectively, were ~30% lower on day 2 vs. day 1 (both $P \leq 0.025$), but did not change on subsequent days (Table 6-1).

Table 6-1. Hypoglycemia Conditioning Characteristics.

	Day 1	Day 2	Day 3	Day 4
Body Weight (g)	325±33	321±34	320±34	321±34
Whole Blood Glucose				
Baseline (mM)	7.0±0.9	7.2±0.8	7.4±0.8*	7.5±0.7**
Time to BG <3.0 mM (min)	41±34	23±12**	18±8**	20±9**
Plasma Glucagon				
Baseline (pg/mL)	18±9	12±9*	14±6	15±8
Peak at BG ≤3.6 mM (pg/ml)	121±58	85±30*	94±46	97±51

*P<0.05, **P<0.01 vs. day 1, †††P<0.001 vs. day 2. BG: blood glucose. Data are means ± SD.

Pharmacological Rescue from Recurrent Hypoglycemia (Day 4)

Whole blood glucose

Basal blood glucose concentration (i.e., before insulin administration; P=0.470), time from insulin administration to the hypoglycemic dosing target of <3.0 mM (P=0.059), and blood glucose concentration at the hypoglycemia dosing target (P≥0.620) were comparable between treatment groups (Table 6-2).

Whole blood glucose responses to rescue treatment are shown in figure 6-1A. Compared to vehicle, mean blood glucose concentration was higher at t=15 min in all other treatment groups, as well as at t=30 min in the 10ZT+GIP group, t=30-120 min in the EG group, and t=100-120 min in the 10ZT group (all P<0.05; Fig. 6-1A).

The glycemic trends described below were analyzed using simple linear regression (Fig. 6-1A). In the control group, mean blood glucose level did not change significantly from 2.7±0.3 mM over two hours (P ≥0.071; Fig. 6-1A). Three vehicle-treated rats were euthanized upon reaching severe hypoglycemia (≤1.5 mM) at t=80, 90, and 100 min post-rescue, respectively. In the EG group, glucose concentration increased from 2.8±0.2 mM to 5.6±0.5 mM within 15 min of dosing (P<0.001) and then decreased between t=15-60

min (*P*=0.002), without further change between t=70-120 min *(P*=0.496; Fig. 6-1A). In the 10ZT group, glucose blood concentration increased from 2.9±0.1 mM to 3.6±0.6 mM within 15 min (*P*=0.005) and stabilized until 60 min (*P*=0.782) before resuming an upward trajectory to a terminal concentration of 5.5±2.2 mM at t=120 min (*P*=0.037) (Fig. 6-1A). In the 3ZT and 10ZT+GIP groups, blood glucose concentration increased from 2.9±0.1 mM to 3.9±0.5 mM within 15 min of treatment (*P*<0.001) before decreasing to 2.4±0.6 mM and 2.8±0.5 mM, respectively, by t=60 min (both *P*≤0.002) (Fig. 6-1A). Subsequent recovery between t=60-120 min resulted in a terminal glucose concentration of 3.2±1.8 mM with 3ZT (*P*=0.041) and 4.0±2.1 mM with 10ZT+GIP (*P*=0.002) (Fig. 6-1A). Two rats in the 3ZT group and one rat in the 10ZT+GIP group were terminated upon reaching severe hypoglycemia of ≤1.5 mM glucose (3ZT: 50 min and 100 min, 10ZT+GIP: 80 min).

Glucose peak and nadir, and euglycemia recovery rates post rescue treatment

Table 6-2 summarizes various metrics of glycemic recovery after hypoglycemia treatment on day 4. Peak glucose concentration was higher in all groups compared to vehicle (all *P*≤0.025) and in the 10ZT vs. 3ZT group (*P*=0.042). All rats treated with EG reached peak glycemia in the first hour after dosing, compared to 75% with 3ZT, 25% with 10ZT, and 38% with 10ZT+GIP (data not shown). These differences did not reach statistical significance due to high within group variability (*P*=0.079; Table 6-2). Nadir blood glucose concentration was higher in the EG (*P*=0.002) and 10ZT (*P*=0.005) groups compared to vehicle but time to reach nadir blood glucose concentration did not differ between groups (*P*=0.159; Table 6-2).

GIP co-treatment resulted in a higher incidence of euglycemia at t=15 min compared to 10ZT alone, but this effect subsided by t=30 min and euglycemia became

less common in the 10ZT+GIP vs. 10ZT group from t=60-120 min (Table 6-2). Between t=30 and t=120 min, the incidence of euglycemia increased in the 10ZT group and decreased in the EG and 3ZT groups (Table 6-2).

Figure 6-1B compares net blood glucose AUC across treatment conditions for up to 120 min after dosing, calculated as the sum of the area above and below baseline for each group. Vehicle was the only treatment associated with a net negative glucose AUC (Fig. 6-1B). Net glucose exposure was higher in the EG (P=0.003) and 10ZT (P=0.002) groups but not the 3ZT (P=0.672) or 10ZT+GIP (P=0.215) groups compared to vehicle (Fig. 6-1B).

All rescue treatments reduced time spent in L2 hypoglycemia by at least ~50 min compared to vehicle (veh: 117±6 min; EG: 60±23 min, P<0.001; 3ZT: 66±51 min, P=0.049; 10ZT: 44±41 min, P=0.002; 10ZT+GIP: 46±37 min, P=0.003) (data not shown). However, only treatment with EG and 10ZT reduced total exposure to hypoglycemia (L1 or L2) as compared to vehicle (veh: 120±0 min, EG: 60±23 min, P=0.002; 3ZT: 96±36 min, P=0.441; 10ZT: 79±38 min, P=0.041; 10ZT+GIP: 89±24 min, 75±20%, P=0.202; data not shown).

Table 6.2. Blood Glucose Response Profile to Hypoglycemia Rescue Treatment.

	Vehicle	EG	3ZT	10ZT	10ZT+GIP
Baseline (mM)	7.4±0.9	7.7±0.6	7.3±0.8	7.6±0.7	7.5±0.4
Time to BG <3.0 mM (min)	22±5	30±7	19±6	18±8	22±13
Peak (mM)	2.6±0.3	5.9±0.5***†††	4.4±1.2**	6.5±2.5***†††§§§	4.8±1.3***†††
Time to Peak (min)	34±31	32±18	49±45	86±46	67±50
Nadir (mM)	1.7±0.3	2.9±0.6††	2.2±0.7	2.7±0.6††	2.3±0.4
Time to Nadir (min)	83±37	91±16	68±24	57±34	68±26
% in Euglycemia					
t=15 min	0	100	38	13	50
t=30 min	0	100	38	13	13
t=100min	0	38	13	63	25
t=120min	0	13	13	75	50

**P<0.1, ***P<0.001 vs. baseline; †P<0.05, ††P<0.01, †††P<0.001 vs. vehicle; §§§P<0.001 vs. 3ZT. EG: exogenous glucagon; 3ZT: 3 mg/kg ZT-01; 10ZT: 10 mg/kg ZT-01; 10ZT+GIP: 10 mg/kg ZT-01 + glucose-dependent insulinotropic polypeptide. Euglycemia is defined as blood glucose ≥4.0 mM. Data are means ± SD.

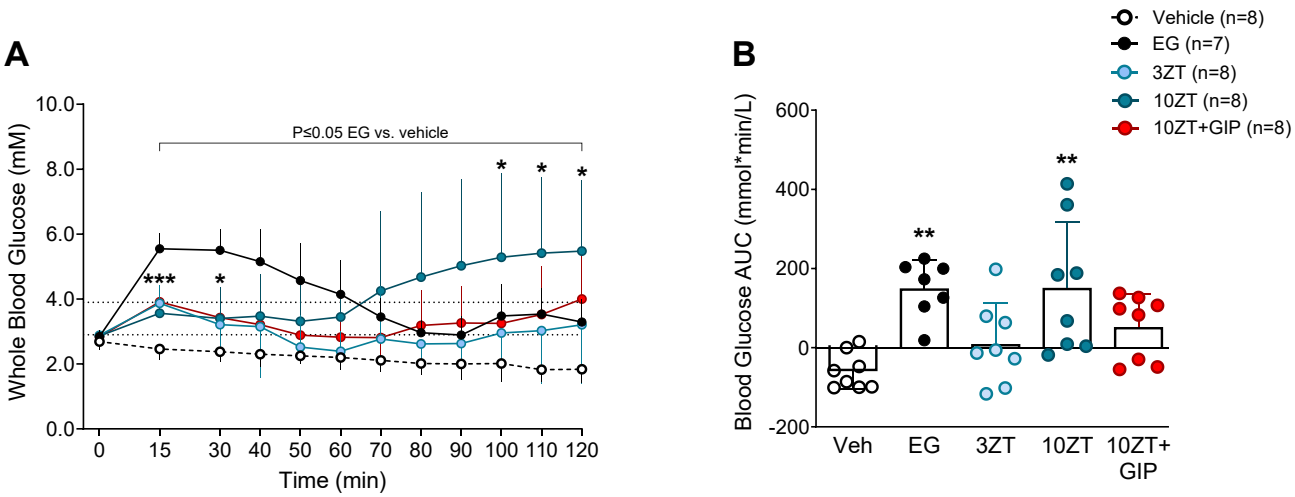

Figure 6-1. Whole blood glucose concentration (**A**) and AUC (**B**) for 120 min following administration of treatment at the onset of level 2 hypoglycemia (≤2.9 mM, t=0 min). Horizontal dashed lines indicate glycemic thresholds for level 1 (2.9 mM) and level 2 (3.9 mM) hypoglycemia. *P<0.05, **P<0.01, ***P<0.001 vs. vehicle. EG: exogenous glucagon, 3ZT: 3 mg/kg ZT-01, 10ZT: 10 mg/kg ZT-01, 10ZT+GIP: 10 mg/kg ZT-01 + glucose-dependent insulinotropic polypeptide. Data are means ± SD.

Plasma Hormones

Plasma glucagon and C-peptide responses to hypoglycemia rescue treatment are shown in figure 6-2. Peripheral plasma glucagon concentration was comparable between treatment groups at baseline (i.e., before hypoglycemia induction; *P*=0.208) and at the

hypoglycemia dosing target of <3.0 mM; *P*=0.366) (Fig. 6-2A). Mean plasma glucagon level increased significantly from baseline in all groups besides the vehicle group, in which we 55% reduction occurred between t=60 and t=120 min (*P*=0.041). Compared to vehicle, plasma glucagon level was elevated for 30 min after treatment with 10ZT, 60 min with 3ZT or 10ZT+GIP, and 120 min with EG (Fig. 6-2A). Peak plasma glucagon level (Fig. 6-2A) and plasma glucagon AUC (Fig. 6-2B) for 120 min after rescue dosing, can be arranged by group in order of increasing response size: vehicle < 10ZT < 3ZT or 10ZT+GIP < EG. Compared to vehicle, peak plasma glucagon and AUC responses were collectively 1.6-2-fold higher with 10ZT (*P*=0.043), 3-4.5 fold higher with 3ZT or 10ZT+GIP (*P*<0.001), and 70-180 fold higher with EG (*P*<0.001) (Fig. 6-2B). Time to reach peak glucagon concentration was at least 26 min shorter with EG (*P*=0.049) and 3ZT (*P*=0.021) compared to vehicle (Table 6-2).

Figure 6-2C compares plasma glucagon levels in peripheral and portal vein circulation at terminal sampling. Mean peripheral vein concentration was similar across treatments (*P*=0.107), whereas mean portal vein concentration was ~14-fold higher in the 10ZT group and 5.6-fold higher in the 10ZT+GIP group compared to the vehicle group and when compared within-groups to peripheral glucagon levels (10ZT: *P*≤0.008 vs. both; 10ZT+GIP: *P*≤0.007 vs. both) .

Peripheral plasma C-peptide concentration did not differ between groups at baseline (before hypoglycemia induction; *P*=0.064), or before rescue dosing at the onset of L2 hypoglycemia (blood glucose <3.0 mM; *P*=0.201) (Fig. 6-2D). In response to rescue treatment, peripheral C-peptide concentration fell to 25-50% of pre-dosing levels within 15 min (*P*<0.001 for 0 vs. 15 min in all groups) and was nearly undetectable (<7% of

baseline) after 60 min (all *P*<0.001 for 0 vs. 60 min) in all groups. Plasma C-peptide concentration (Fig. 6-2D) and AUC (Fig. 6-2E), representing total peripheral C-peptide exposure for up to 120 min after rescue dosing, were unaffected by treatment (*P*=0.170).

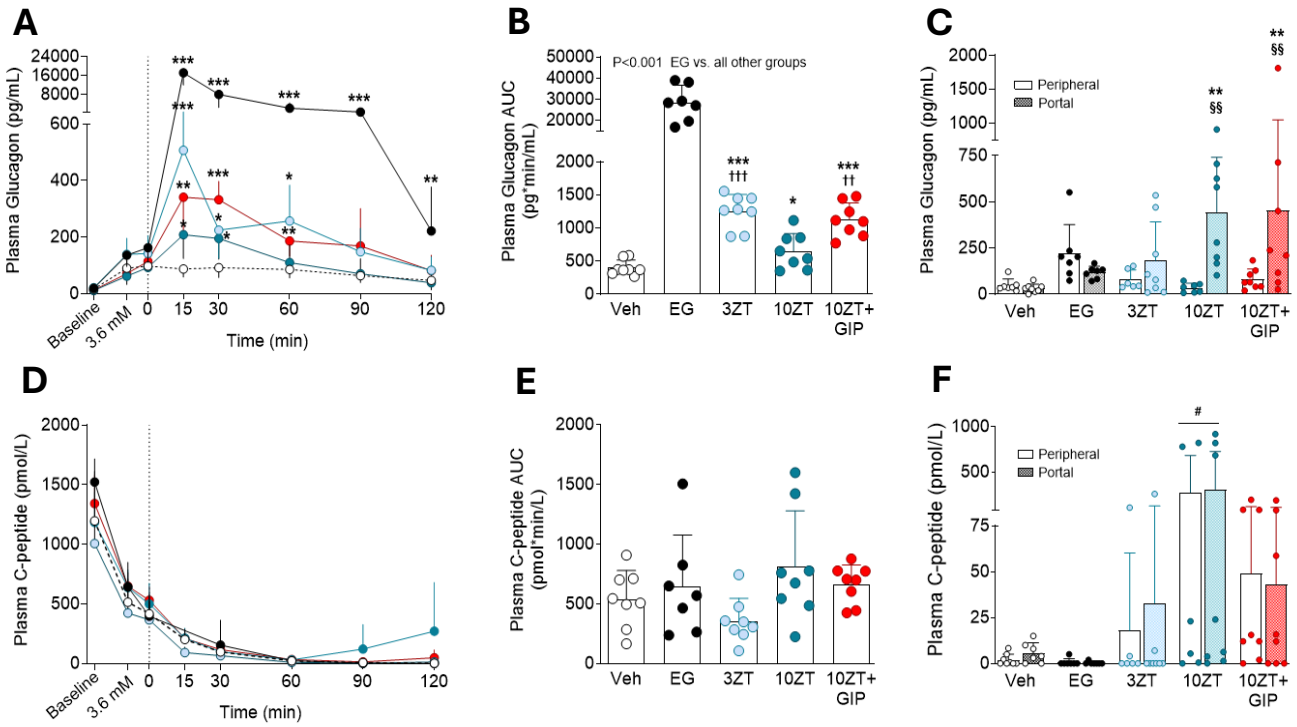

Figure 6-2. Plasma (A-C) glucagon and (D-F) C-peptide responses to rescue treatment administered at the onset of level 2 hypoglycemia (<3.0 mM, t=0 min). (A) Peripheral plasma glucagon concentration. (B) AUC of peripheral plasma glucagon concentration between t=0 and t=120 min. (C) Comparison of peripheral and hepatic portal vein glucagon concentrations at t=120 min. (D) Peripheral plasma C-peptide concentration. (E) AUC of peripheral plasma C-peptide concentration between t=0 and t=120 min. (F) Comparison of peripheral and hepatic portal vein C-peptide concentrations at t=120 min. (F') Linear regression modelling the relationship between hepatic portal and peripheral C-peptide concentration (top) and hepatic portal C-peptide and whole blood glucose concentration (bottom) in the 10ZT and 10ZT+GIP groups at t=120 min. Vertical dotted lines in (A) and (D) indicate the time of treatment. **P*<0.05, ***P*<0.01, ****P*<0.001 vs. vehicle. ††P<0.01 †††P<0.001 vs. 10ZT. §§*P*<0.01 vs. within-group peripheral. #*P*<0.05 vs. vehicle, EG, and 3ZT. EG: exogenous glucagon; 3ZT: 3 mg/kg ZT-01; 10ZT: 10 mg/kg ZT-01; 10ZT+GIP: 10 mg/kg ZT-01 + glucose-dependent insulinotropic polypeptide. Data are means ± SD.

Liver glycogen content and glycogenolytic/gluconeogenic enzyme activity

Liver glycogen content, and the activities of GP and PEPCK, measured 120-min after rescue treatment, are shown in figure 6-3. Liver glycogen content was lower in all treatment groups compared to vehicle (EG: -43%, 3ZT or 10ZT: ~-63%, 10ZT+GIP: -82%; *P*<0.001) and in the 10ZT+GIP group compared to the EG group (-68%; *P*=0.027; Fig. 6-3A). In agreement, GP activity was higher in all groups compared to vehicle (EG: +116%; 3ZT/10ZT/10ZT+GIP: ~+200%; *P*<0.001) and in the 10ZT and 10ZT+GIP groups compared to EG (~+40%; *P*≤0.012; Fig. 6-3B). PEPCK activity was higher in the 10ZT+GIP group compared to all other treatments (by 26-50%; *P*≤0.012), except for 10ZT (*P*=0.131), which showed higher enzyme activity compared to vehicle (~30% *P*=0.014; Fig. 6-3C).

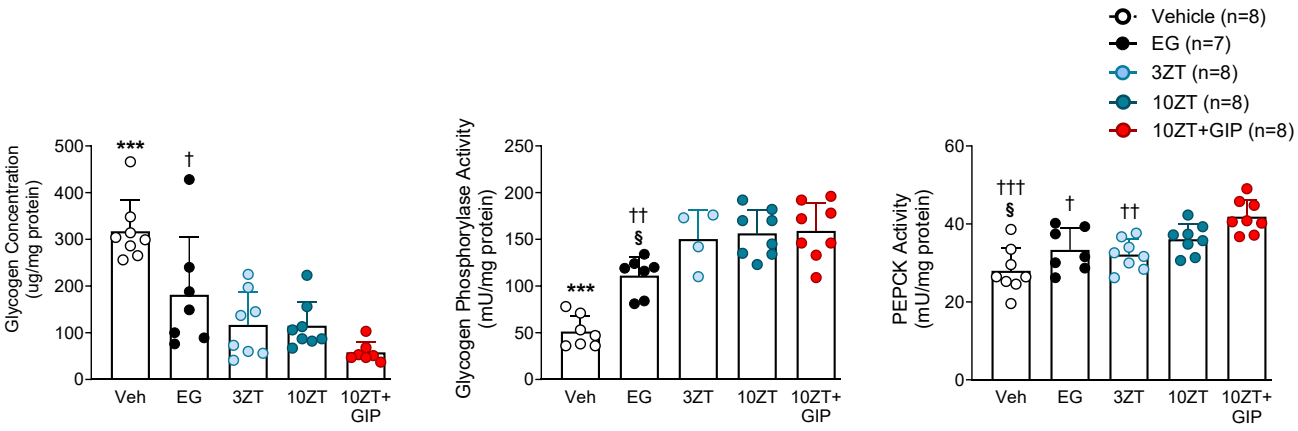

Figure 6-3. Hepatic (**A**) glycogen content and activity of (**B**) glycogen phosphorylase and (**C**) PEPCK enzymes measured 120-min after hypoglycemia treatment was administered at a glycemic target of <3.0 mM. ***P<0.001 vs. all other groups; †P<0.05, ††P<0.01, †††P<0.001 vs. 10ZT+GIP; §P<0.05 vs. 10ZT. EG: exogenous glucagon; 3ZT: 3 mg/kg ZT-01; 10ZT: 10 mg/kg ZT-01; 10ZT+GIP: 10 mg/kg ZT-01 + glucose-dependent insulinotropic polypeptide; PEPCK: phosphoenolpyruvate carboxykinase. Data are means ± SD.

6.5 Discussion

This is the first study to examine whether an acute dose of the highly selective SSTR2 antagonist, ZT-01, either alone or in combination with GIP, could serve as a rescue treatment for level two (L2) hypoglycemia (<3.0 mM glucose) in rats with established counterregulatory failure. At both a low (3 mg/kg; 3ZT) and high (10 mg/kg; 10ZT) dose level, ZT-01 restored a physiological rise in plasma glucagon concentration that was absent in vehicle-treated controls. This counterregulatory response reduced the mean depth of insulin-induced hypoglycemia from level 2 (L2; severe) to level 1 (L1; alert range) within 15 minutes of treatment with either dose; however, only 10ZT alone (i.e., without GIP) resisted a recurrence of L2 in the first hour. In the second hour, 10ZT restored euglycemia in a majority of rats owing to sustained or biphasic secretion of pancreatic glucagon into the hepatic portal vein. For the first time, our findings demonstrate a contribution of both gluconeogenesis and glycogenolysis to the maintenance of hepatic glucose output by SSTR2a. In support of our previous findings, these results suggest that somatostatin signaling may impair the glucagon counterregulatory response to recurrent hypoglycemia.[258] Beyond its established use for hypoglycemia prevention,[19] ZT-01 may have therapeutic potential as a hypoglycemia rescue agent by restoring physiological glucagon secretion into the hepatic portal vein during insulin-induced hypoglycemia.

In previous studies, SSTR2as have been shown to reduce the depth, duration, and/or frequency of hypoglycemia when dosed one hour before insulin overdose by augmenting glucagon counterregulation in animal models of T1D, T1D/exercise, pre-T2D, T2D, and recurrent hypoglycemia.[1–3,22,170,171,258,282,283] As the first study to compare the 3 mg/kg and 10 mg/kg doses of ZT-01, our data suggest an inverse dose effect on peak

and total glucagon exposure over two hours. In support, Farhat et al. reported a bell-shaped dose-response in the 0.0001-3 mg/kg dose range with a peak at 0.3 mg/kg.[171] All ZT-01 treatment conditions in the present study reduced exposure to L2 hypoglycemia over two hours; however, only 10ZT eliminated the incidence of severe hypoglycemia (i.e., blood glucose ≤1.5 mM) and attenuated hypoglycemic (L1 and L2) exposure altogether.[33] 10ZT ultimately outperformed EG in the second hour after dosing, despite a persistence of peripheral hyperglucagonemia in EG-treated animals. Accordingly, a maximal plasma concentration of 10ZT was previously achieved two hours after subcutaneous injection in rat,[171] which, in the present study, coincides with maximal glycemic recovery and active (possibly even increasing) glucagon release into the portal vein. The duration of glycemic recovery provided by exogenous glucagon is limited by its short half-life (8-18 minutes) in circulation;[290] however, duration of action is an important metric of treatment success in the case of hypoglycemia given the risk of recurrence, especially in a setting of defective glucagon counterregulation.[23]

GIP is an intestinally-derived incretin hormone that potentiates insulin secretion in the post-prandial state.[284] Yet, pharmacological dosing of exogenous GIP under euglycemic- and hypoglycemic-clamp conditions has been shown to stimulate glucagon secretion in a dose-dependent manner without altering insulin release.[191,192] Here, GIP augmented peak and total glucagon exposure in ZT-01-treated rats by 75%, and while co-treatment was more effective at restoring euglycemia (i.e., in 50% versus 13% of rats) within fifteen minutes of dosing than 10ZT alone, GIP became deleterious to ZT-01-mediated recovery during the second hour after treatment. Hepatocytes do not express GIP receptors and GIP is unlikely to act directly on the liver;[174] however, the additive effect

of GIP on hepatic glucagon exposure (versus 10ZT alone) may have increased hepatocyte sensitivity to insulin-mediated glucose disposal in a setting of peripheral insulin overdose.[291,292] Alternatively, GIP can directly sensitize adipocytes to insulin signaling by potentiating insulin-stimulated GLUT4 translocation and glucose uptake in a dose- and time-dependent manner.[293] In cultured adipocytes, this synergy between GIP and insulin was activated after fifteen minutes of combined hormone exposure and doubled in potency between thirty and sixty minutes.[293] In agreement, a one-hour infusion of GIP during clamped hypoglycemia in adults with T1D improved glucose kinetics (i.e., increased the rate of endogenous glucose production and decreased the rate of exogenous glucose infusion) fifteen minutes after hypoglycemia induction but not thereafter.[285] As a result, dual treatment with ZT-01 and GIP may provide more immediate but temporary recovery from severe hypoglycemia than ZT-01 alone.

Plasma C-peptide concentration was supressed by exogenous insulin overdose at a similar rate across treatment groups, reaching nearly undetectable levels within one hour of treatment. That said, we observed a subsequent elevation in both peripheral and portal vein C-peptide levels two hours after treatment compared to vehicle-treated controls. Because this response coincided with the recovery of euglycemia in affected animals, the increase in plasma C-peptide may have resulted from glycemic normalization rather than a direct stimulatory action of ZT-01 on the pancreatic β-cell. Since plasma glucagon concentration was elevated in hepatic portal versus peripheral venous circulation two hours after injection of high-dose ZT-01, with and without GIP, it is also possible that the diminishing inhibitory effect of therapeutic insulin on islet α-cells invited a second phase of glucagon release, which in turn, stimulated C-peptide secretion via

intra-islet paracrine effect.[294] It is important to note that our rat model of glucagon counterregulatory failure displays intact C-peptide secretion, unlike the majority of people with T1D, who represent the target demographic for hypoglycemia rescue treatments. It will be important to repeat these experiments in an animal model of T1D to better understand how residual β-cell function influences SSTR2a-mediated recovery from iatrogenic hypoglycemia.

Hepatic glycogenolysis accounts for the majority of glucagon-mediated glucose output during hypoglycemia; however, gluconeogenesis is recruited in settings of severe and/or prolonged exposure.[295] We previously showed that when administered one hour before bolus insulin challenge, the SSTR2a, PRL-2903, delayed hypoglycemia onset by increasing hepatic glycogen utilization in recurrently hypoglycemic rats.[258] In agreement, the high-dose of ZT-01 tested here reduced hepatic glycogen stores and increased the activity of rate-limiting glycogenolytic enzyme, glycogen phosphorylase, compared to vehicle- and EG-treated controls, which were hypoglycemic at the time of measurement. Notably, a gluconeogenic contribution to the maintenance of hepatic glucose output was observed with high-dose ZT-01, with and without GIP, but not with any other treatment. Therefore, prolonged glycemic recovery in the high-dose group was associated with increased portal vein glucagon levels and downstream stimulation of hepatic glucose production via glycogenolytic and gluconeogenic pathways.

This study has several limitations to address. First, we did not have a GIP-only condition to control for the stress associated with IP injection. Instead, all animals were given 15 minutes to recover from treatment before any measurements were obtained. However, the effect of GIP alone in this setting remains unclear. Second, due to supplier

issues, six rats, allocated to the vehicle and 3ZT groups, were ordered from a different vendor than the other 33. These six rats were ~50 g smaller, on average, with a lower blood volume that could not satisfy our pre-established sampling protocol. As a result, no C-peptide data was collected from this subset of rats at the 15-minute timepoint after dosing, necessitating the exclusion of this timepoint from the C-peptide dataset (Fig. 6-2D). Third, portal vein plasma and liver tissue were only sampled ~2-hours after test agents were administered, and therefore, these endpoints (i.e., portal vein hormone levels, hepatic enzyme activity) did not coincide with peak drug action. Fourth, the dose of exogenous glucagon used as a positive control caused a >100-fold rise in systemic glucagon levels, (consistent with a previous report in rat)[8], which is double the rise typically seen in humans treated with the standard dose of emergency glucagon.[275] Therefore, the dose selected may overestimate the efficacy of our positive control, especially without the ability to assess dose-dependent side effects, like nausea and vomiting, in rat.[296]

For the first time, we showed that the SSTR2a, ZT-01 (10 mg/kg), provides gradual and sustained recovery from level 2 hypoglycemia by restoring physiological glucagon counterregulation in recurrently hypoglycemic rats. By reversing the therapeutic time course of EG, high-dose ZT-01 may target a period of glycemic instability that arises once the effect of exogenous glucagon has diminished. Future studies may wish to explore the combination of high-dose ZT-01 with a sub-therapeutic or “mini-dose” of glucagon to temper the dose-dependent side-effects of the exogenous hormone while harnessing the sustained action and enhanced tolerability of ZT-01 to prevent hypoglycemia recurrence in the late recovery period. SSTR2 antagonism has emerged as a promising therapeutic

strategy for preventing the onset of hypoglycemia in people with T1D. Here, we extend this body of evidence to support a potential role for ZT-01 in the treatment and reversal of existing hypoglycemia without inducing peripheral hyperglucagonemia over two hours.

Chapter 7.1: Counterregulatory Glucagon Secretion is Supressed by Hypersecreted Somatostatin and Recovered by Somatostatin Receptor 2 Antagonism in Type 2 Diabetic Human Islets.

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Data analysis and interpretation: EGH, LGG, EI, RL

Drafting the manuscript: EGH

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Target Journal: American Journal of Physiology-Cell Physiology

7.1.1 Abstract

Background: Glucagon counterregulation is impaired in type 2 diabetes (T2D), contributing to increased risk of hypoglycemia. We have previously proposed that in somatostatin (SST), secreted by islet δ-cells, inhibits glucagon release from neighbouring α-cells via somatostatin receptor 2 (SSTR2) in diabetic rodents. Here we investigated whether SST secretion is elevated under low glucose conditions in human T2D and GLTX-treated islets and whether pharmacologic SSTR2 antagonism can restore the counterregulatory glucagon response. **Materials/Methods:** Human islets were obtained from one T2D donor and five non-diabetic (ND) donors. ND islets were also cultured in glucolipotoxic (GLTX) conditions for four days to model T2D. Static incubation experiments measured insulin, glucagon, and SST secretion at varying glucose concentrations (1–16.7 mM), with or without the selective SSTR2 antagonist, PRL-2915. **Results:** In T2D and GLTX islets incubated at 1- or 2.8-mM glucose, glucagon secretion was 3-5-fold lower, whereas SST secretion was 1.5-3-fold higher compared to ND islets. Application of the SSTR2a at 1 mM glucose restored a 3.5-fold increase in glucagon secretion from basal glucose conditions (4 mM) in GLTX islets (vs. +1.6-fold in ND islets) and potentiated insulin release at 16.7 mM glucose in ND (+2.2-fold) but not GLTX islets. **Conclusions:** We demonstrate, for the first time, that SST is hypersecreted under low glucose conditions in isolated islets from humans with long-standing T2D. In turn, elevated SST tone appears to inhibit glucagon secretion from pancreatic α-cells in persons with long-standing T2D by paracrine effect, which is reversible with SSTR2 antagonism.

7.1.2 Introduction

Somatostatin-14 (SST) is a paracrine hormone secreted by δ-cells of the pancreatic islets that provides inhibitory feedback signaling to both glucagon-secreting α-cells and insulin-secreting β-cells. In individuals with type 1 diabetes (T1D) or advanced type 2 diabetes (T2D), glucagon secretion is inappropriately elevated under basal and hyperglycemic conditions but paradoxically suppressed under hypoglycemic conditions.[9] Basal and postprandial hyperglucagonemia (6-20 mM glucose) have been linked to deficient SST secretion and/or signaling via acquired α-cell resistance in islets isolated from mouse models of pre-diabetes[131] and T1D[4], and human donors with T2D[4,110]. Emerging evidence also suggests that SST may be hypersecreted under hypoglycemic conditions in humans and mice with T1D.[4,5] Accordingly, pharmacological antagonism of SST receptor 2 (SSTR2), the functionally dominant somatostatin receptor in pancreatic α-cells, rescues defective glucagon counterregulation in vitro[4,5,297] and in vivo[1–3,170,283]. Despite a limited number of experiments performed in human islets from donors with T2D (i.e., three donor preparations)[4] versus T1D[4], the data collectively suggest that SST secretion decreases under low glucose conditions in non-diabetic islets to "release the brake" on counterregulatory glucagon secretion, and that this permissive paracrine signal may be compromised in insulin-deficient diabetes.[22]

Based on observations in non-diabetic islets from human donors, SST secretion is simulated by glucose beyond a threshold of ~3 mM and increases linearly to a peak at ~20 mM glucose, suggesting tonic inhibition of islet α-cells throughout the physiologic glucose range. However, pharmacological antagonism of SSTR2 in human islets from non-diabetic donors has been shown to increase glucagon secretion under

hyperglycemic[106,110,298] but not euglycemic or hypoglycemic[96,106,298–300] conditions. These findings suggest that SST may contribute to the inhibition of glucagon secretion by glucose, but only after counterregulatory (i.e., stimulated) glucagon secretion has been maximally supressed (i.e., ~at 4-5 mM glucose). Conversely, immunoneutralization of SST[113] and pharmacological antagonism of SSTR2 in non-diabetic rodent islets[95,98,113,115,183,301] have been shown to stimulate glucagon secretion across the physiologic glucose range. Further, the effects of pharmacological SSTR2 antagonism on insulin secretion in human islets with or without T2D have not been thoroughly investigated. Accordingly, the glucose-dependent regulation of insulin and glucagon secretion by SST in humans with and without T2D remains unclear.

Prolonged exposure (i.e., 4 days) of isolated islets to glucolipotoxic (GLTX) culture conditions induces a dysfunctional β-cell phenotype, characteristic of T2D.[302] This *in vitro* model of T2D has important experimental utility due to the scarcity and phenotypic heterogeneity of T2D donor islets. However, no studies, to our knowledge, have investigated the effects of glucolipotoxicity on glucagon and SST secretion from isolated human islets.

The purpose of this study was to characterize the glucose and SSTR-dependent regulation of insulin, glucagon, and SST secretion in islets from human donors with and without T2D. We also sought to determine whether the GLTX model of T2D recapitulates the altered secretory phenotypes and paracrine signaling relationship between α- and δ-cells in T2D islets.

7.1.3 Materials and Methods

Human Islets

Human islets, isolated from one T2D donor and five non-diabetic (ND) donors, were purchased from Prodo Laboratories Inc. Islets were cultured for at least 48 hours in CMRL media (Thermo Fisher, 11530037) containing 5.6 mM glucose, 1% L-glutamine (GIBCO, 35050-038), 1% penicillin/streptomycin, and 10% FBS at 37°C and 5% CO_2. To model islet conditions in T2D, ND islets were cultured in glucolipotoxic (GLTX) media for four days prior to experimentation. GLTX media had a glucose concentration of 16.7 mM, and was supplemented with 100 µM palmitate (Sigma, P9767) and 200 µM sodium oleate (Sigma, O3880).

Static Islet Incubation Experiments

Batches of 15 size-matched islets (in triplicates or quadruplicates) were preincubated in KRBH (VWR, AMPQ99730.1000) containing 2% 0.1 g/mL HSA stock solution (Sigma, A9511), 1% L-glutamine (Gibco, 35050-038), 2.8% $NaHCO_3$ (Gibco, 25080), and 2.8, 4 or 16.7 mM glucose (as indicated) for 1 h at 37°C. This was followed by a 1-h test incubation in KRBH containing 1 or 16.7 mM glucose, with or without 1 µM of the SSTR2 antagonist, PRL-2915 (MedChemExpress, HY-P4452). The hormone content of the supernatant was determined using ELISA (Mercodia, 10-1132-01, 10-1271-01; Phoenix Pharmaceuticals Inc., EK-060-03) and DNA content in cell lysates was determined using an assay kit (Invitrogen, P7589). In a separate set of experiments, GLTX islets were pre-incubated in KRBH with 4 mM glucose for one hour, followed by a one-hour test incubation in 1, 2.8, 4, 7, 10, or 16.7 mM glucose as indicated. In pilot experiments, the glucose transitions selected for this study were shown to maximally

stimulate or inhibit the secretion of targeted hormones in nondiabetic islets while minimizing secretory fatigue and prioritizing physiological relevance. For example, 2.8 mM glucose was selected as physiologically relevant stimulus of glucagon secretion and an inhibitory basal glucose condition,[96] which maximized the subsequent stimulation of insulin and SST secretion by glucose. Alternatively, a glycemic transition from 4 to 1 mM was chosen to represent the development of hypoglycemia in diabetes, which typically occurs in the post-absorptive state secondary to strict glycemic control (i.e., the maintenance of blood glucose concentration in the euglycemic range). Further, a basal condition of 4 mM glucose is unlikely to exhaust SST secretion and may aid in the detection of pathologically elevated secretion during subsequent hypoglycemia in T2D and/or GLTX islets. Following exposure to 4 mM glucose, a deeper hypoglycemic stimulus of 1 mM glucose was required to induce a detectable glucagon response in nondiabetic islets.

Statistical Analysis

Data are expressed as means of batches of 15 donor islets (not donor replicates), connected by dashed lines between incubation conditions. Statistical tests were conducted against a significance criterion of $P<0.05$ using Prism 10 software (GraphPad, San Diego, CA, USA). Hormone secretion was analyzed by two-way mixed-effects ANOVA (factors: culture condition i.e., with or without GLTX x static incubation condition, i.e., glucose concentration with or without SSTR2) followed by Tukey post-hoc testing.

7.1.4 Results

Glucagon

The first set of experiments, shown in figures 7-1A, 1B, were performed in isolated islets from one non-diabetic (ND) donor and one donor with T2D. Lowering the glucose concentration from 4 to 1 mM increased glucagon secretion 4.8-fold in the control islets but only 2.8-fold in the T2D islets (Fig 7-1A). The stimulated glucagon response was larger when glucose concentration was lowered from 16.7 to 1 mM (vs. 4 to 1 mM) in islets from control (+9.5-fold) but not T2D (+3.2-fold; Fig. 7-1A) donors. Similarly, the inhibition of glucagon secretion resulting from a rise in glucose concentration from 2.8 to 16.7 mM, was blunted in T2D versus control islets, owing to markedly lower (8.5-fold) secretion at 2.8 mM glucose in the T2D islets and comparable secretion between groups at 16.7 mM glucose (Fig. 7-1B).

In GLTX-treated islets, lowering the glucose concentration from 4 to 1 mM failed to stimulate a glucagon response (P=0.537) as it did in ND islets (1.6-fold, P=0.040; Fig. 7-1C). Application of a SSTR2 antagonist at 1 mM glucose stimulated glucagon secretion compared to 1 mM glucose alone in both groups (CTL: 11.5-fold, GLTX: ~8-fold; P<0.001 for both), restoring a robust counterregulatory response in the GLTX group relative to basal secretion at 4 mM glucose (3.5-fold; P<0.001; Fig. 7-1C).

Next, we found that raising the glucose concentration from 2.8 to 16.7 mM failed to inhibit glucagon secretion in GLTX islets (P=0.078) as it did in ND islets (P=0.008; Fig. 7-1D). Application of a SSTR2 antagonist at 16.7 mM glucose stimulated glucagon secretion in both groups compared to 16.7 mM glucose alone (CTL: 18.4-fold, GLTX: 8.3-fold; P<0.001 for both; Fig. 7-1D).

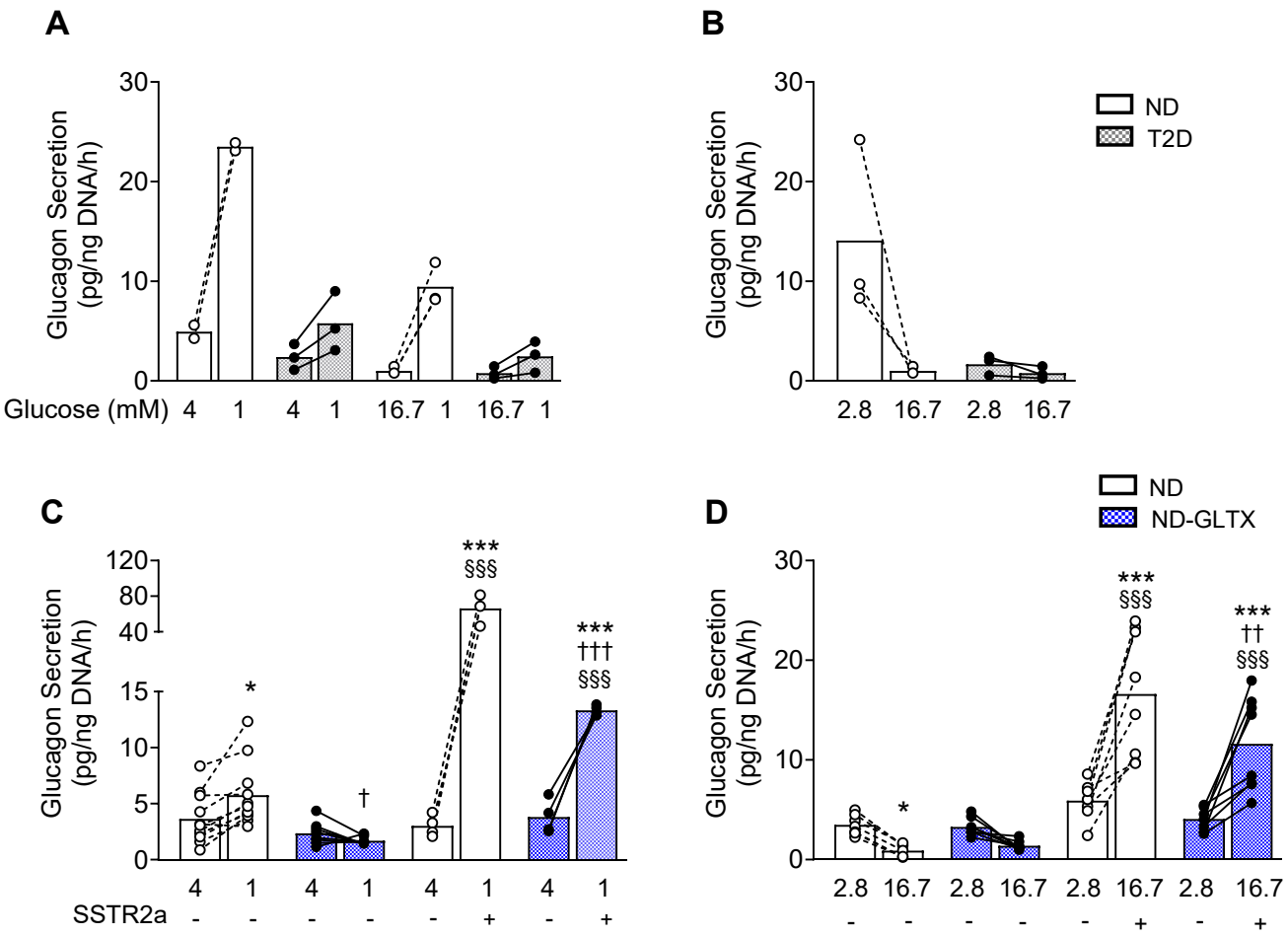
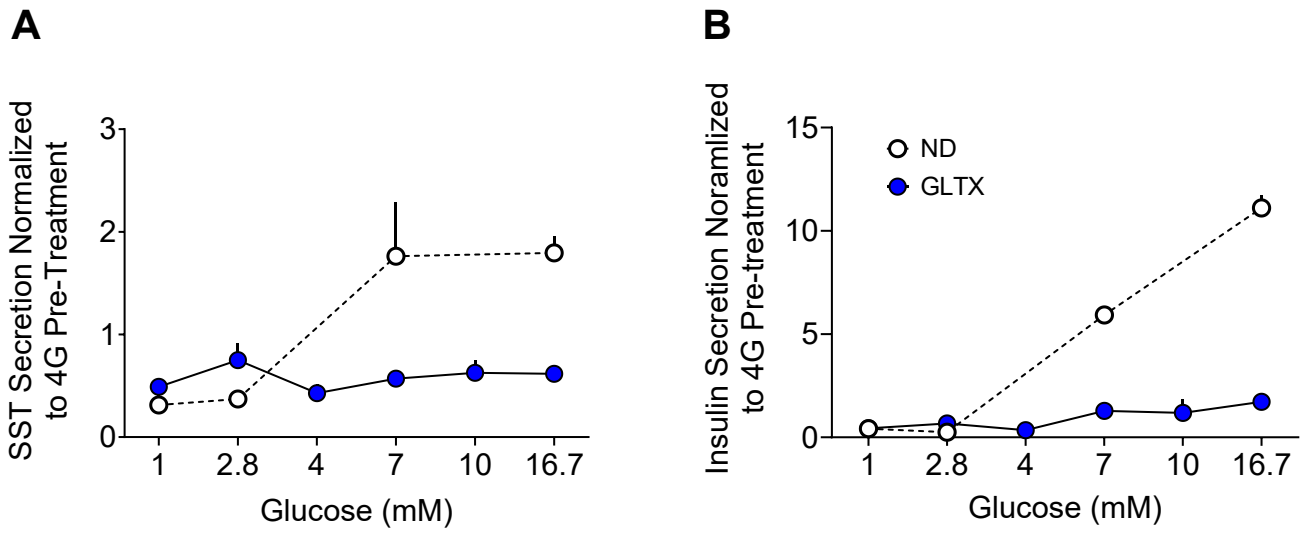


Figure 7-1. Glucagon secretion from T2D islets (A,B) and GLTX islets with or without SSTR2a (C,D) in response to a decrease (A,C) or increase (B,D) in glucose exposure. Experiments in: (A,B) triplicate from 2 donors (1 ND, 1 T2D), (C) quadruplicate from 1-3 ND donors, or (D) quadruplicate from 2 ND donors. *P<0.05, ***P<0.001 vs. baseline glucose condition (same group); †P<0.05, ††P<0.01, †††P<0.001 vs. ND under the same glucose condition; §§§P<0.001 vs. the same group and glucose condition without SSTR2a. ND: non-diabetic, GLTX: glucolipotoxic, SSTR2a: somatostatin receptor 2 antagonist. Data are means.

Somatostatin

In the T2D donor experiments, lowering the glucose concentration from 4 to 1 mM modestly reduced SST secretion in the ND islets and modestly increased secretion in the T2D islets. Next, lowering the glucose concentration from 16.7 to 1 mM markedly inhibited SST secretion in the ND (18.6-fold) but not the T2D (1.5-fold) donor islets. (Fig. 7-2A). Basal SST secretion at 2.8 mM glucose was 3-fold higher, whereas stimulated secretion at 16.7 mM glucose was 20-fold lower in the T2D vs. the ND donor islets (Fig. 7-2B).

In the first set of GLTX experiments, glucose-stimulated SST secretion was first detected at 7 mM glucose and was not further stimulated at 16.7 mM glucose, amounting to a maximal 5-fold increase from basal secretion at 4 mM glucose (Fig. S7-1A). In donor-matched GLTX islets, SST secretion was higher than ND islets at 2.8 mM glucose and unresponsive to further increments in glucose concentration (Fig. S7-1A). In agreement, lowering the glucose concentration from 4 to 1 mM inhibited SST secretion non-significantly in both ND and GLTX islets (P=0.287 for both; Fig. 7-2C). SST secretion was ~2-fold higher in GLTX vs. control islets at 2.8 mM glucose (P=0.005), and raising the glucose concentration to 16.7 mM failed to stimulate SST secretion in GLTX islets (P=0.136) as it did in ND islets (P<0.001; Fig. 7-2D).



Supplementary Figure 7-1. Glucose-dependent secretion of (A) SST and (B) insulin from ND and GLTX islets. Data normalized to secretion during 1 h pre-treatment in 4 mM glucose. Experiments in triplicate from 1 ND donor per hormone. SST: somatostatin, 4G: 4 mM glucose, ND: non-diabetic, GLTX: glucolipotoxic. Data are means ± SD.

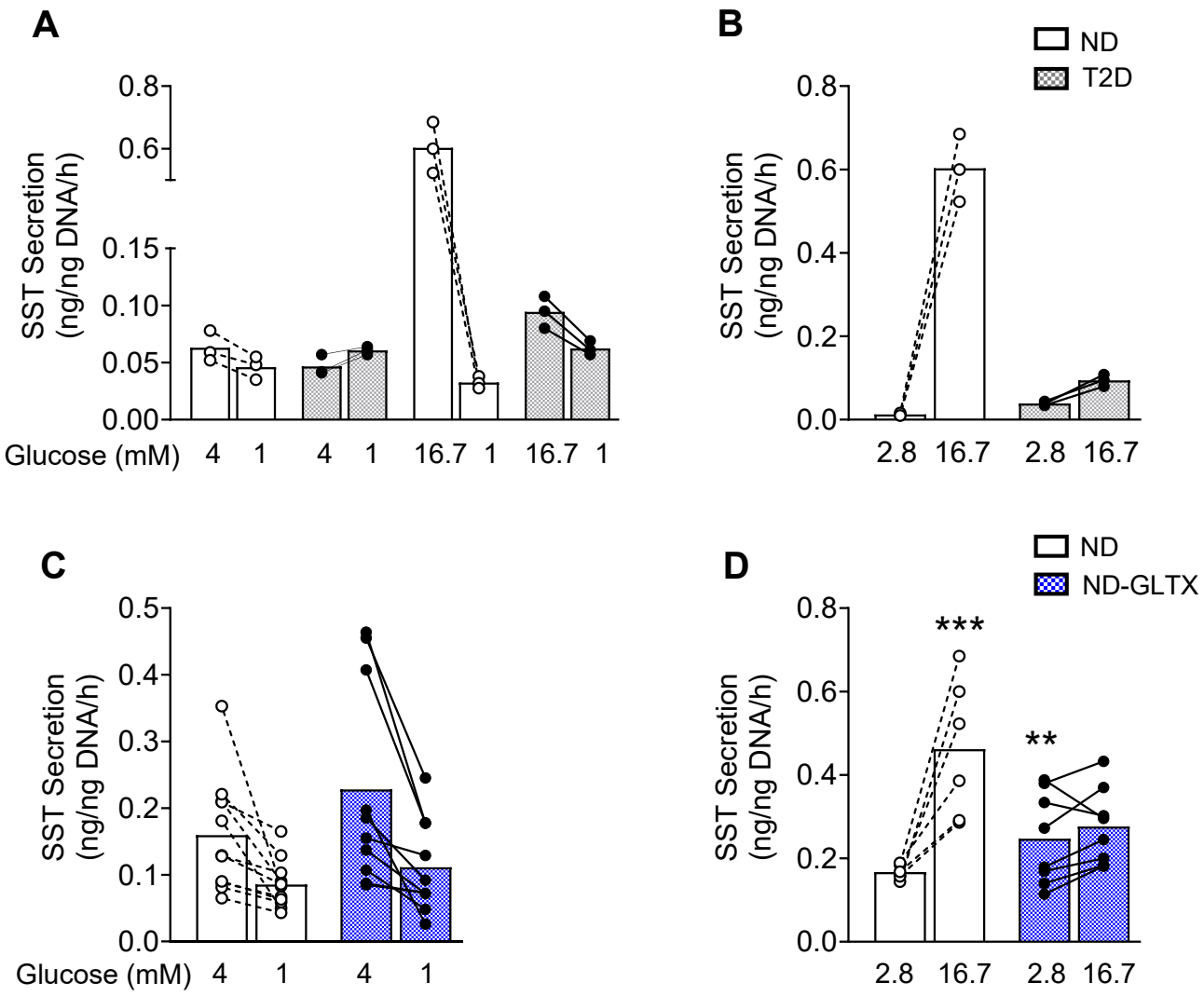


Figure 7-2. SST secretion from (A,B) T2D and (C,D) GLTX islets in response to a (A,C) decrease or (B,D) increase in glucose exposure. Experiments in: (A,B) triplicate from 2 donors (1 ND, 1 T2D), (C) triplicate from 3 ND donors, or (D) quadruplicate from 2 ND donors. **P<0.01, ***P<0.001 vs. ND at 2.8 mM glucose. SST: somatostatin, ND: non-diabetic, GLTX: glucolipotoxic. Data are means.

Insulin

In the T2D donor experiments, raising the glucose concentration from 2.8 to 16.7 mM increased insulin secretion ~20-fold in ND islets and ~4-fold in T2D islets (Fig. 7-3A). In the GLTX experiments, glucose-stimulated insulin secretion was first detected at 7 mM glucose in ND and GLTX islets; however, the response was 4.6-fold lower in the GLTX islets and did not respond to further increments in glucose concentration (Fig. 7-S1B). In agreement, raising the glucose concentration from 2.8 to 16.7 triggered a markedly blunted (i.e., 9-fold) insulin response in GLTX versus ND islets (P<0.006; Fig. 7-2B). Application of a SSTR2 antagonist at 16.7 mM glucose increased glucose-stimulated insulin secretion 2.2-fold compared to 16.7 mM glucose alone (P<0.001) in ND islets but was without effect in GLTX islets (P=0.389; Fig. 7-3B).

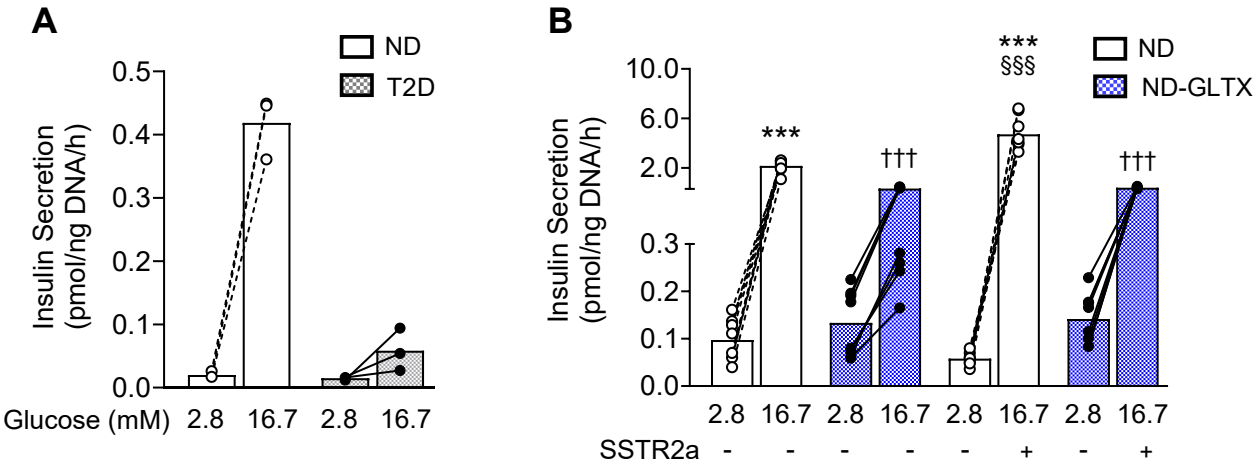


Figure 7-3. Insulin secretion from T2D islets (A) and GLTX islets with or without SSTR2a (B) in response to an increase in glucose exposure. Experiments in: (A) triplicate from 2 donors (1 ND, 1 T2D) or (B) triplicate from 2-3 ND donors. ***P<0.001 vs. 2.8 mM glucose (same group); †††P<0.001 vs. ND under the same incubation conditions; §§§P<0.001 vs. ND at 16.7 mM glucose without SSTR2a. ND: non-diabetic, GLTX: glucolipotoxic, SSTR2a: somatostatin receptor 2 antagonist. Data are means.

7.1.5 Discussion

For the first time, we demonstrated that somatostatin (SST) secretion is pathologically elevated under low glucose conditions in islets from human donors with T2D.[4] We extended these findings to a GLTX model of T2D and linked excess paracrine inhibition by SST to defective glucagon counterregulation by the restorative effect of a selective SSTR2 antagonist (SSTR2a). We also showed, for the first time, that SSTR2 antagonism potentiates glucose-stimulated insulin secretion in non-diabetic human islets, consistent with reports that SSTR2 is the functionally dominant SST receptor in both human α- and β-cells.[100,101] Finally, we propose that chronic (glucolipotoxic) GLTX exposure may recapitulate the aberrant secretory phenotypes of α- and δ-cells in T2D donor islets and may serve an important role in modelling these distorted paracrine relationships *in vitro*.

In agreement with previous reports, we found that SSTR2 antagonism increased glucagon secretion in non-diabetic human islets under high glucose (i.e., non-stimulated) conditions, suggesting that SST contributes to the inhibition of glucagon secretion by glucose.[106,110,298] Consistent with studies in rodent[95,113,115,183,301] but not human[96,106,298–300] islets, we also found that SSTR2 antagonism potentiated counterregulatory (i.e., stimulated) glucagon release at 1 mM glucose, suggesting tonic inhibition of islet α-cells by SST. Since glucose-stimulated SST secretion was first detected above a glycemic threshold of ~3 mM, our data may suggest that basal concentrations of the hormone exert functional inhibition over glucagon release. As expected, the glucagonotropic effect of SSTR2 antagonism was greater in ND islets at 16.7 vs. 1 mM glucose, consistent with the glucose-stimulated secretion of SST. Alternatively, the glucagonotropic effect of

SSTR2 antagonism was comparable at high and low glucose concentrations in GLTX islets, reflecting the absence of glucose-stimulated SST secretion in this setting.

Next, we report that the SSTR2a, PRL-2915, markedly augmented counterregulatory glucagon secretion at 1 mM glucose in islets from non-diabetic human donors. This finding conflicts with existing evidence demonstrating that the SSTR2a, CYN154806, more commonly used in research, does not affect glucagon release under low glucose conditions.[5,96,106,298–300] This discrepancy may be owing, at least in part, to the binding affinities of the two antagonist compounds. PRL-2915 is ~70x more selective for SSTR2 than SSTR5, whereas CYN154806 binds both receptors with similar affinity.[233,303] Since SSTR5 plays a secondary role to SSTR2 in mediating the inhibition of insulin secretion by SST in human islets,[101] we suspect that CYN154806 may have stimulated insulin output at 1 mM glucose, which, in turn, suppressed glucagon release. It will be necessary to confirm our findings in a larger sample of human islets using multiple dose levels of PRL-2915.

As previously observed in T1D mouse islets,[5] the inhibition of SST secretion at low glucose and stimulation at high glucose were markedly blunted in T2D and GLTX-treated islets compared to controls. Accordingly, the observed hypersecretion of SST under low glucose conditions in these islet preparations seemed to reflect failed inhibition rather than stimulated secretion *per se.* Therefore*,* T2D and *in vitro* GLTX exposure may render the pancreatic δ-cell resistant to both stimulatory and inhibitory fluctuations in glucose concentration.

In support of preliminary findings from a small sample of T2D donor islets,[4] we show that SSTR2 antagonism can restore counterregulatory glucagon secretion at 1 mM

glucose in GLTX-treated human islets, which was undetectable without treatment. Additionally, as the first study to investigate whether SSTR2 antagonism impacts glucose-stimulated insulin secretion (GSIS) in human islets, we report a potentiating effect in non-diabetic but not in GLTX preparations. While the reason is unclear, this finding may undermine the potential dual function of SSTR2a as an insulin secretagogue in settings of residual β-cell mass. We suspect that insulin secretion from GLTX islets, which was markedly diminished compared to controls, may have been maximally stimulated by 16.7 mM glucose alone. This finding may also reflect a loss of autoregulation by the β-cell, as previously reported in T2D donor islets.[10] In response to increasing glucose concentration, Urocortin 3 (UCN3) is co-secreted with insulin from islet β-cells, which in turn, triggers the release of SST from neighbouring δ-cells.[10] SST then feeds back on a delay to inhibit insulin secretion via SSTR2.[10] Since UCN3 is reportedly depleted in β-cells from T2D donor islets, SST's contribution to the autoregulation of GSIS may be lost in T2D.[10]

In summary, our data suggest that SST may regulate counterregulatory glucagon secretion from islet α-cells under non-diabetic conditions and that this inhibitory paracrine tone may be increased in T2D and GLTX-exposed islets from human donors. As a result, SST may account for the characteristic deficiency in counterregulatory glucagon secretion in these conditions, which was reversible with SSTR2 antagonism. Lastly, we provide preliminary evidence supporting the physiological relevance of glucolipotoxic culture conditions for modelling the effects of T2D on glucagon and SST secretion *in vitro*.

Chapter 7.2: Relative Somatostatin Expression is Increased in Pancreatic Islets from Rats and Humans with Insulin-Deficient Type 2 Diabetes.

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Data analysis and interpretation: EGH, RL, MCR

Drafting the manuscript: EGH

Editing the manuscript: EGH, RL, MCR

Target Journal: American Journal of Physiology-Cell Physiology

7.2.1 Abstract

Background: Pancreatic islet remodeling in diabetes disrupts the paracrine relationships that govern blood glucose regulation. The relative expression of somatostatin (SST) is known to increase in type 1 diabetes (T1D) islets, but it remains unclear how SST expression changes in rodents and humans with advanced (i.e., insulin-deficient) stages of type 2 diabetes (T2D). **Materials/Methods:** Insulin resistance and insulin deficiency were induced in male Sprague–Dawley rats via high-fat feeding (HFF) and low-dose streptozotocin (STZ), respectively, with or without two weeks of basal insulin therapy. Pancreas tissue was harvested for immunohistochemical staining of insulin, glucagon, and SST, and hormone expression was quantified as a percent of islet area. Findings were validated in human pancreas sections from donors with T1D and T2D. **Results:** In untreated T2D rats, insulin-positive area per islet was ~60% lower compared to non-diabetic rats, whereas glucagon and SST expression had approximately doubled. Basal insulin therapy attenuated the decrease in insulin (−26%) and increase in SST (+~60%) expression, without affecting glucagon expression. In human islets from T2D donors, insulin expression per islet was 32% lower and SST expression was 70% higher compared to non-diabetic controls. **Conclusions:** The relative expression of SST per islet was markedly higher in HFF/STZ-T2D rats and human donors with T2D compared to non-diabetic controls, which could be partially normalized by basal insulin therapy in T2D rats. Accordingly, islet remodeling in insulin-deficient T2D may increase the available pool of secretable SST, as observed in T1D.

7.2.2 Introduction

Pancreatic islet remodeling in type 1 (T1D) and type 2 diabetes (T2D) distorts the paracrine relationships that coordinate blood glucose regulation.[135] Accordingly, islet α-cells, which secrete glucagon to mitigate the onset and progression of hypoglycemia, become increasingly "blind" (i.e., less responsive) to hypoglycemia with the progression of insulin deficiency.[5] Based on numerous reports of elevated somatostatin expression in the islets of T1D humans[116,126], dogs[304], and rodents[305–307] and a higher contact density between α- and δ-cells,[138,139] islet remodelling in diabetes likely increases α-cell exposure to inhibitory SST signaling[139].

Due to the influence of treatment and disease stage on islet architecture, the fate of the δ-cell is less clear in T2D compared to T1D. In hyperinsulinemic forms of T2D, characterized by increased or unchanged β-cell mass, δ-cell and SST expression per islet are reportedly reduced or unchanged.[127,308,309] We hypothesize that the reverse may be true in settings of insulinopenic T2D, whereby SST expression increases reciprocally with decreasing β-cell mass, as observed in T1D.[5,126] For the first time, we characterized islet hormone composition in an established rodent model of insulin-deficient and insulin-resistant T2D and validated these findings in human islets from donors with T2D. Finally, we investigated the effects of early glycemic intervention, using basal insulin therapy, on islet SST expression in rodents with advanced T2D.

7.2.3. Materials and Methods

Rats and T2D induction

Thirteen (n=13) male, Sprague-Dawley rats were purchased from Charles River Laboratories at an age of 8-10 weeks (post weaned, initial weight 200-250 g). Rats were

individually housed in the York University vivarium in a 12-hour light-dark cycle with ad libitum access to food and water. Eight rats were randomly selected for T2D induction via three weeks of high fat feeding (HFF; 5.21 kcal/g: 60% fat, 20% carbohydrate and 20% protein; Research Diets, D12492) before a single injection of low-dose streptozotocin (STZ; 35 mg/kg, IP) to model mid-to-late stage T2D.[310] Following STZ treatment, regular drinking water was replaced with 10% sucrose solution for 48 hours as a precautionary measure to prevent hypoglycemic episodes in this timeframe Hyperglycemia was confirmed in all STZ-treated rats based on a post-absorptive whole blood glucose measurement ≥11.1 mM 48 hours after STZ administration. Five rats served as non-diabetic (ND) normal-chow-fed (3.5 kcal/g: 13% fat, 58% carbohydrate, 29% protein; LabDiet, 5012) controls.

A subset of four HFF/STZ-T2D rats were randomly selected to receive basal insulin treatment (T-T2D), while the remaining four rats were left untreated (UT-T2D). Insulin glargine (Lantus SoloSTAR, Sanofi-Aventis) was administered once daily in the evening (~6:00 pm) for 14 days based on post-absorptive (pre-feeding) blood glucose measurements according to the following sliding scale: <12 mmol/L: 0 U; 12 to 15 mM: 1 U; >15 mM: 4 U. On day 15, food was removed at 9 am, and body weight, blood glucose, and plasma hormone levels were measured in the evening after a ~9 hour fast to provide a sufficient washout period for insulin administered the day prior[311]. Animals were then euthanized via exsanguination and pancreases were extracted, rinsed in PBS, fixed in 10% buffered formalin solution for 24 hours, and finally before transferred to 70% ethanol for storage.

Human Tissue

Pancreas slices were obtained from seven non-diabetic (n=6-7; ND), five (n=5) T1D, and six (n=6) T2D donors with consent from ADI IsletCore. T2D subjects were selected based on the following criteria: dependence on insulin and/or treatment non-compliance, disease duration >10 years, and/or HBA1C >8%. T2D donors were age, BMI, and sex matched (where possible) to ND controls.

Immunohistochemical Staining

Serial sections of paraffin embedded tissue were mounted onto glass slides by Princess Margaret hospital and returned to York University for analysis. Samples were pre-incubated in 3% hydrogen peroxide for 15 min to block endogenous peroxidases, and heat-mediated antigen retrieval was performed using a pressure cooker microwaved at high power for 7-12 min. Samples were then incubated with a primary anti-insulin (Abcam, ab108546; 1:500), anti-SST (Abcam, ab63820; 1:200), or anti-glucagon (Abcam, ab108426, 1:7500) antibody for two hours at room temperature and hormones were detected using the avidin-biotin complex (ABC) method according to kit instructions (Vector Laboratories, PK-401). DAB peroxidase (Vector Laboratories, SK-4105) was applied for 1.5-2 min before counterstaining with hematoxylin (Sigma-Aldrich, HHS16) for 15-30 seconds.

A similar protocol was used to stain and analyze human tissue with a few notable exceptions. First, antigen retrieval was performed in a hot water bath at 95°C for 5 min. Serial sections were incubated with a primary anti-insulin antibody (1:500) overnight at 4°C and with a primary anti-glucagon or anti-SST antibody for one hour at room temperature.

Hormone Quantification

Hormone expression was quantified by a blinded technician using Aperio Image Analysis software (Leica Biosystems) for rat islets and ImageJ software (open source) for human islets. The following number of islets were analyzed in rats: 102-127 ND, 52-55 UT-T2D, and 102-108 T-T2D and humans: 100-148 ND, 100-158 T1D, and 120-158 T2D.

Studies commonly report the effects of diabetes and glycemic normalization on pancreatic hormone content; [304,309,312] however, intercellular communication is localized to each islet, discounting total pancreas values as biologically relevant measures of cell-to-cell interactions. Further, we chose not to conflate cell area with hormone area, due to evidence that these variables are differentially affected by diabetes induction and glycemic intervention.[140]

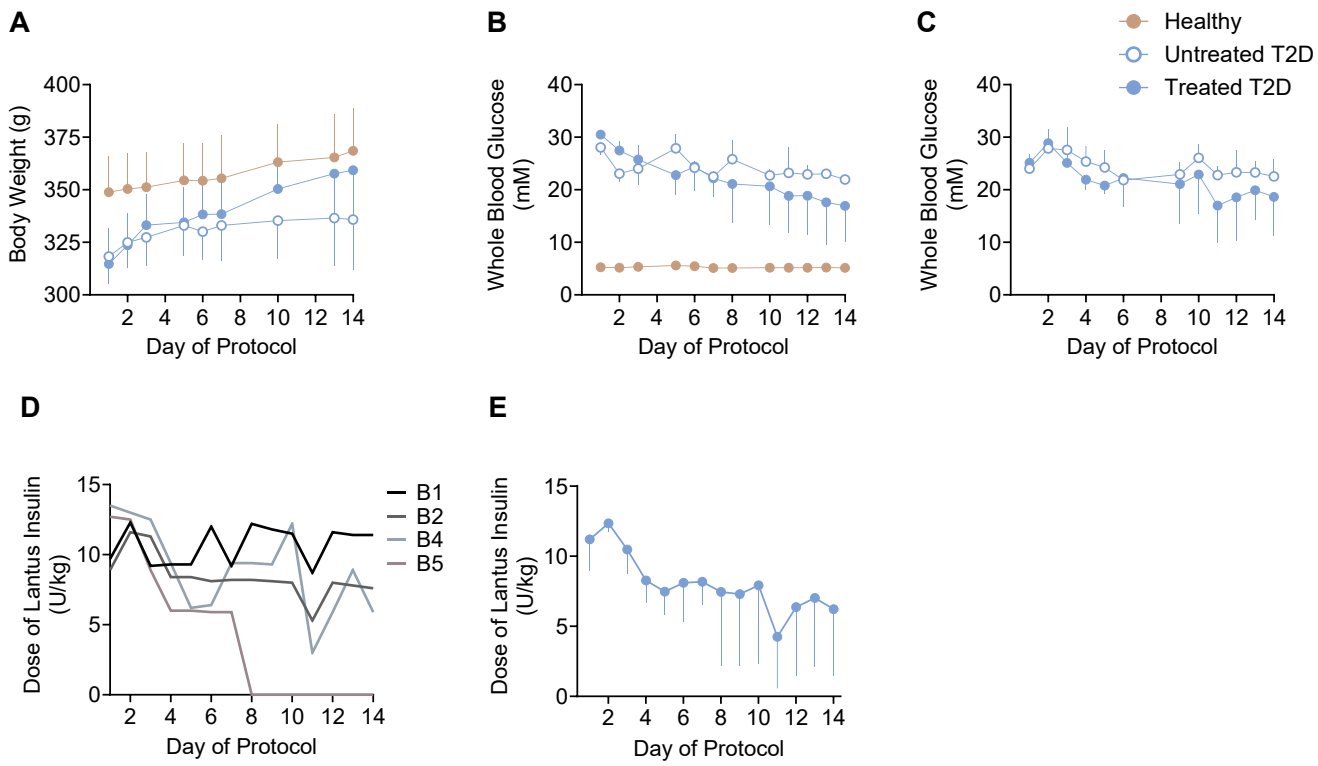
Statistical Analysis

Statistical tests were conducted against a significance criterion of $P < 0.05$ using GraphPad Prism Software. In vivo measurements taken immediately prior to sacrifice, islet area, and percent hormone expression were analyzed by ordinary one-way ANVOA and Tukey post-hoc testing (factor: condition). Average daily glucose concentration during the glycemic maintenance period (within-subject factor: day, between-subject factor: condition) and SST expression by islet size (within-subject factor: islet size, between-islet factor: condition) were analyzed by two-way mixed model ANOVA and Tukey post-hoc testing. The linear relationships between expression levels of each hormone were modelled using simple linear regression and analyzed using the Pearson correlation coefficient.

7.2.4 Results

HFF/STZ-T2D Model Characteristics

Daily body weight, morning and evening blood glucose levels, and basal insulin dosing for the T-T2D group throughout the 14-day glycemic maintenance period are shown in S7-2A-D, respectively. At the time of sacrifice, both groups of T2D rats were severely hyperglycemic on average ($P \leq 0.008$ vs. ND for both), however, one T-T2D rat (B5) had near-normal (6.8 mM) blood glucose levels and no longer required insulin treatment after day 8 (Fig. S7-2D), leading to high glycemic variability in the T-T2D group (Table 7-1). Plasma C-peptide level was ~45% lower in UT-T2D vs. ND rats ($P<0.024$) but comparable between T-T2D and ND rats ($P=0.959$; Table 7-1). Despite a trend towards hyperglucagonemia in the UT-T2D group, plasma glucagon levels were highly variable in all three conditions and no significant effect of T2D or insulin treatment was detected ($P=0.445$; Table 7-1).



Supplementary Figure 7-2. Maintenance data from HFF/STZ-T2D rats during 2 weeks with or without basal insulin replacement. **(A)** Body weight **(B)** morning blood glucose **(C)** evening blood glucose **(D)** dose of Lantus insulin per rat, and **(E)** average dose of Lantus insulin for pooled T-T2D rats. ND: non-diabetic, UT-T2D: untreated T2D, T-T2D: treated T2D, HFF: high-fat feeding, STZ: streptozotocin.

Table 7-1. STZ/HFF-T2D Model Characteristics.

	Non-diabetic	Untreated T2D	Insulin-Treated T2D
Body weight (g)	369±20	336±24	359±24
Whole blood glucose (mM)	5.6±0.3	24.8±3.3***	17.2±7.4**
Peripheral plasma C-peptide (pmol/L)	1107±121	624±64*	1064±397
Hepatic portal C-peptide (pmol/L)	831±338	247±102*	449±202
Peripheral plasma glucagon (pg/mL)	60±70	131±87	78±90

Measurements were obtained immediately prior to sacrifice and pancreas removal. *P<0.05, **P<0.01, ***P<0.001 vs. ND. STZ: streptozotocin, HFF: high-fat fed. Data are means ± SD.

Human Donor Characteristics

Donor ID, age, sex, BMI, HbA1C%, disease duration, and treatment modality (where available) are provided in Table 7-2. All ADI-registered donors with insulin-treated T2D (n=4) were included in the study. Of the two remaining donors selected for the T2D cohort, one satisfied the inclusion criteria for advanced disease duration (>10 years) whereas the other qualified based on treatment non-compliance. All T2D donors and four of five T1D donors had HbA1C levels in the diabetic range (≥6.5%). Males and females were equally represented in the T2D group and T2D donors were age-, BMI-, and sex-matched (where possible) to ND donors.

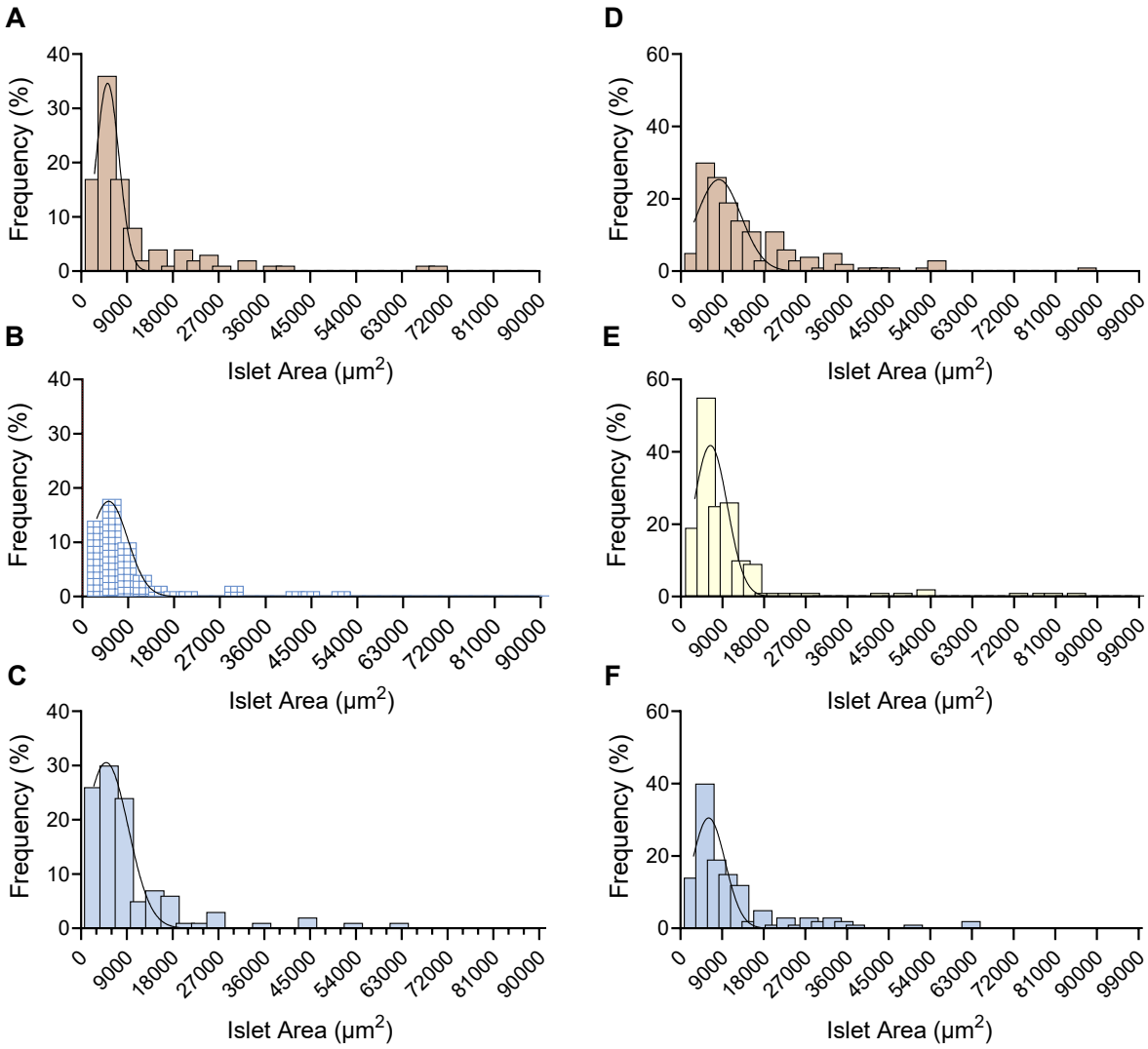
Table 7-2. Human Donor Characteristics.

Donor Condition	Donor ID	Age	Sex	BMI (kg/m²)	HbA1C (%)	Years Since Diagnosis	Treatment
Non diabetic	R026	68	F	28.5	5.3		
	R145	55	F	24.1	5.5		
	R186	68	M	28.1	5.6		
	R195	54	M	35.0	5.5		
	R273	57	M	33.9	5.8		
	R490	39	M	28.2	5.3		
	R492	68	M	21.2	5.2		
T1D	R095	44	M	23.7	14.6	24	
	R132	39	F	24.6	5.9	31	
	R339	30	M	25.7	9.2	16	
	R429	23	F	23.9	11.8	4	
	R437	38	F	38.6	10.0	20	
T2D	R057	53	F	35.5	10.3	20	Metformin
	R064	36	M	28.1	10.9	1.5	Insulin
	R083	71	F	27.5	6.6	20	Metformin, insulin
	R236	51	M	35.3	8.6	N/A	Non-compliant
	R241	65	M	21.8	9.9	25	Humalog Mix 25
	R276	54	F	24.4	7.2	10	Insulin (1.5 years)

Islet Area and Hormone Expression in ND, UT-T2D, and T-T2D Rats

Average islet area was similar among the three groups (*P*=0.664; Fig. 7-4A). Within each group, the frequency distribution of islet size was positively skewed, with a majority of islets concentrated in the smallest size categories (Fig. S7-3A-C). Notably, the islet size distribution for the UT-T2D group (n=55; Fig. S7-3B) contained approximately half the number of observations (reflecting half the number of islets detected and analyzed) compared to the ND (n=102; Fig. S7-3A) and T-T2D groups (n=108; Fig. S7-3C). Insulin-positive staining, expressed as a percentage of total islet area, was 63% lower in UT-T2D islets (27±16% vs. 73±16%; *P*<0.001) but only 26% lower in T-T2D islets (54±23% vs. 73±16%; *P*<0.001 vs. ND and UT-T2D) compared to ND islets (Fig. 7-4B). Percent glucagon-positive staining was 70-110% higher in UT-TD (29±17%; P<0.001) and T-T2D islets (24 ±16%) compared to ND islets (14±9%) (Fig 7-4C). Similarly, percent SST-expression was 2-fold higher in UT-T2D islets (14± 7% vs. 7±5%; *P*<0.001) but only 1.6-fold higher in T-T2D islets (11±8% in T-T2D; *P*<0.001 vs. ND and *P*=0.020 vs. UT-T2D; Fig. 7-4D) compared to ND controls. Percent SST expression was inversely related to islet size, such that smaller islets contained a higher proportion of SST than large islets in ND, T-T2D, and pooled T2D (Fig. 7-4E'), but not UT-T2D rats (Fig. 7-4E). When compared across treatment groups, relative SST expression was higher in small- and medium-sized islets from UT-T2D (sm: *P*=0.006, med: *P*=0.003) and T-T2D (sm: *P*<0.001, med: *P*=0.004) compared to ND rats, whereas in large islets, SST expression was only higher than control levels in the absence of treatment (*P*=0.007 vs. ND and T-T2D; Fig. 7-4E). Correlation analysis of relative hormone expression revealed a positive association

between glucagon and SST (*P*=0.022; Fig S7-4A), and a negative association between both hormones and insulin (*P*≤0.006 for both; Fig. S7-4B,C).



Supplementary Figure 7-3. Frequency distribution of islet size in **(A)** non-diabetic, **(B)** untreated-T2D**,** and **(C)** treated-T2D rats (left) and **(D)** non-diabetic, **(E)** T1D, and **(F)** T2D human donors (right). T2D: type 2 diabetes.

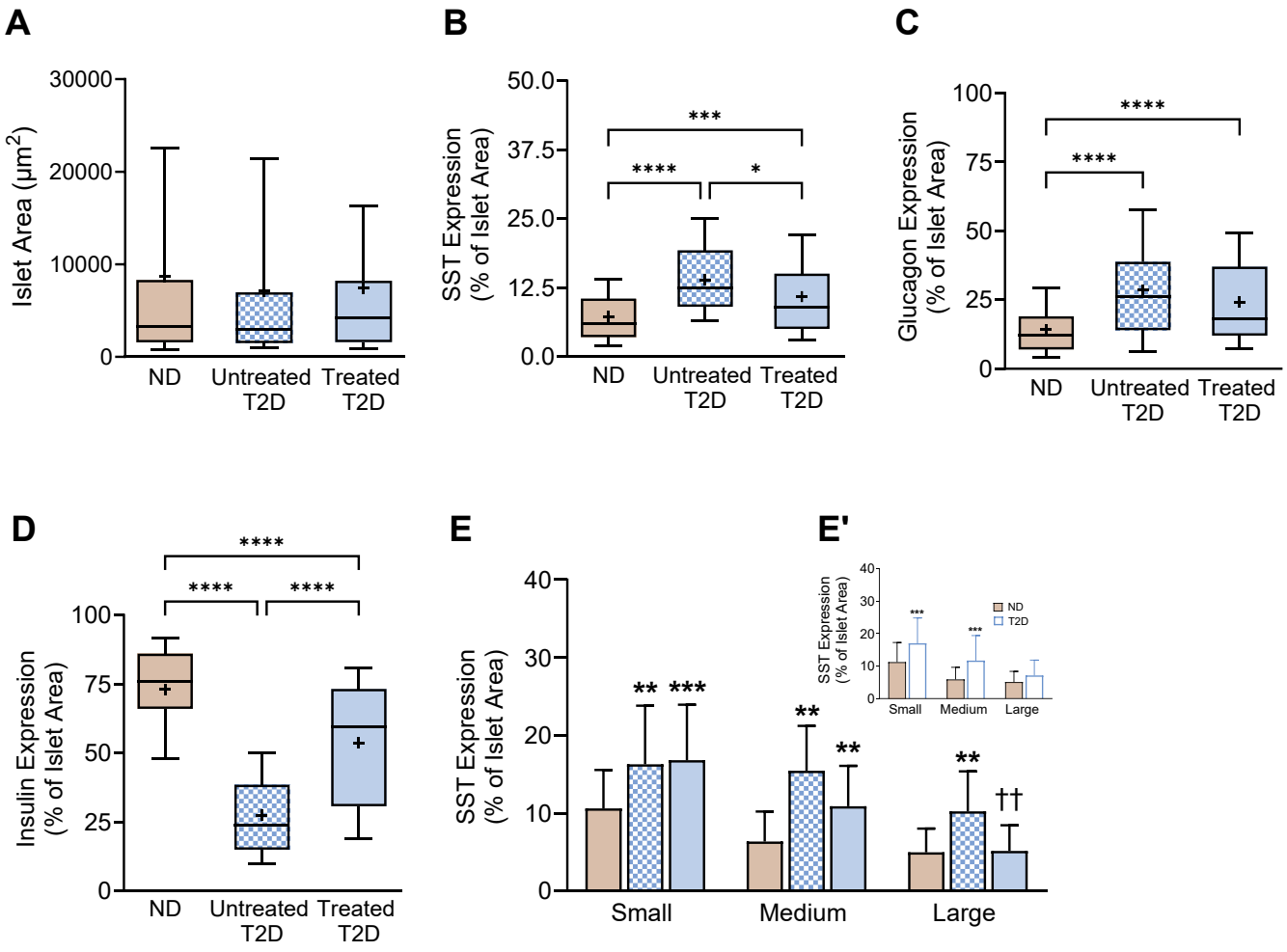
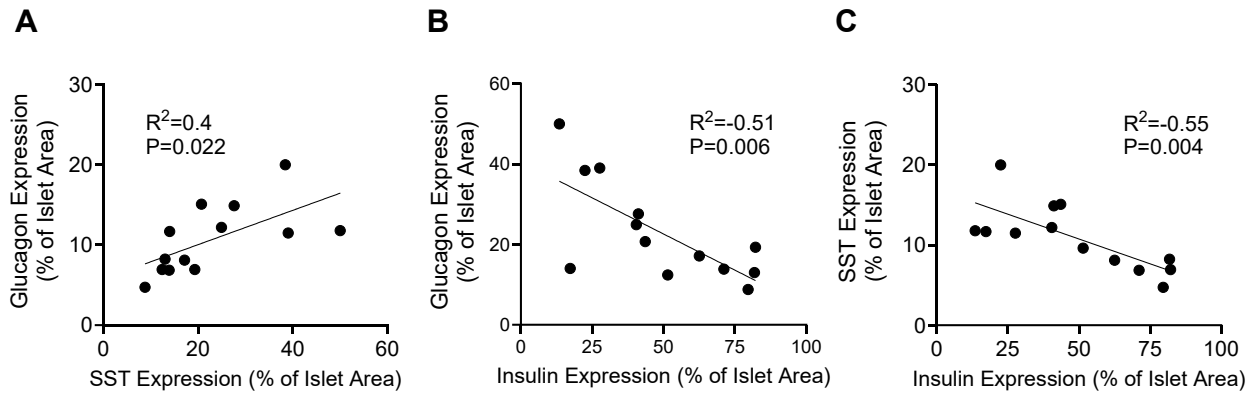


Figure 7-4. Islet hormone composition in HFF/STZ-T2D rats, with and without basal insulin replacement. Islet **(A)** area and proportions of **(B)** insulin **(C)** glucagon and **(D)** SST staining expressed as a percentage of total islet area. *P<0.05, ***P<0.001, ****P<0.0001. **(E)** Percent SST expression stratified by islet size in separate and **(E')** pooled T2D conditions. **P<0.01, ***P<0.001 vs. ND; ††P<0.01 vs. UT-T2D. Whiskers indicate 10th and 90th percentiles, "+" indicates the mean.



Supplementary Figure 7-4. Correlation analysis of relative hormone area in HFF/STZ-T2D rat islets. **(A)** glucagon vs. SST **(B)** glucagon vs. insulin, and **(C)** SST vs. insulin. SST: Somatostatin.

Representative images of rat islets stained for insulin, glucagon, and SST are shown in Figure 7-5. Insulin staining was ubiquitous in ND islets (Fig. 7-5A) and fragmented in UT-T2D (Fig 7-5B) and T-T2D (Fig. 7-5C) islets. Glucagon staining, wich was localized to the outer mantle of ND islets (Fig. 7-5D), increased in area but retained its peripheral distribution in T-T2D (Fig. 7-5E) and UT-T2D islets (Fig 7-5F). SST-positive staining was relatively sparse but similarly localized to the outer mantle region of ND islets (Fig. 7-5G) and migrated inward to occupy the core of T2D islets (Fig. 7-5H,I). δ-cells appeared elongated and contained filopodia-like-extensions, as previously described (Fig. 7-5H,I).[313]

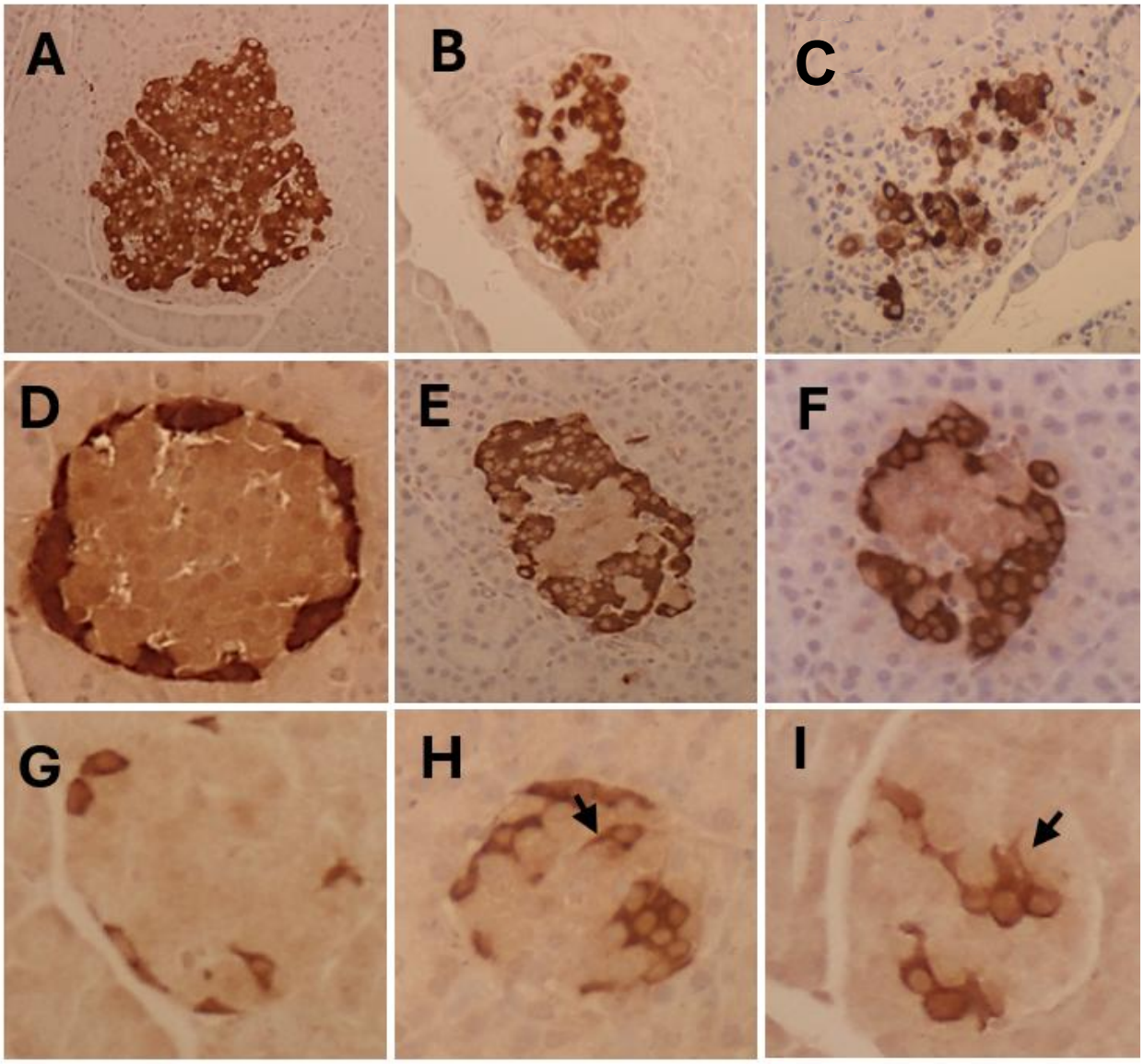


Figure 7-5. Representative images of ND (left), UT-T2D (middle), and T-T2D (right) rat islets stained for insulin **(A-C)**, glucagon **(D-F)**, and SST **(G-I)**. Arrows indicate δ-cell filopodia. ND: non-diabetic, SST: somatostatin, UT-T2D: untreated T2D, T-T2D: treated T2D.

Islet Area and Hormone Expression in Human Donors with ND, T1D, or T2D

Average islet area was ~30% lower in T1D (*P*=0.044) and T2D (*P*=0.093) vs. ND donor islets but the difference was only significant in the T1D group (Fig 7-6A). The frequency distribution of islet size was less positively skewed in the T1D donor cohort compared to the ND (Fig. S7-3D) and T2D (Fig. S7-3F) cohorts, since the majority of islets were concentrated in the smallest size categories rather than the tail of the distribution, despite the presence of fewer but more extreme outliers in the T1D cohort (Fig. S7-3E). Insulin expression accounted for 71±15% of islet area in the ND group, 48±15% in the T2D group (*P*<0.001 vs. ND), and was undetectable in the T1D group (Fig. 7-6B). SST expression accounted for 6±4% of islet area in ND islets, compared to 14±14% inT1D islets (*P*<0.001) and 10±6% in T2D islets (P<0.001 vs. ND and T1D) (Fig. 7-6C). Percent SST expression was inversely proportional to islet size within each condition, such that smaller islets contained higher relative expression of SST (Fig 7-6D). Between conditions, percent SST expression was higher in both small- and medium-sized islets from T1D vs. ND (sm: *P*=0.001, med: *P*<0.001) and T2D donors (sm: *P*=0.005, med: *P*=0.005), whereas in large islets, expression was lower in T1D vs. ND (*P*=0.020) and T2D (*P*=0.017) donors (Fig. 7-6D).

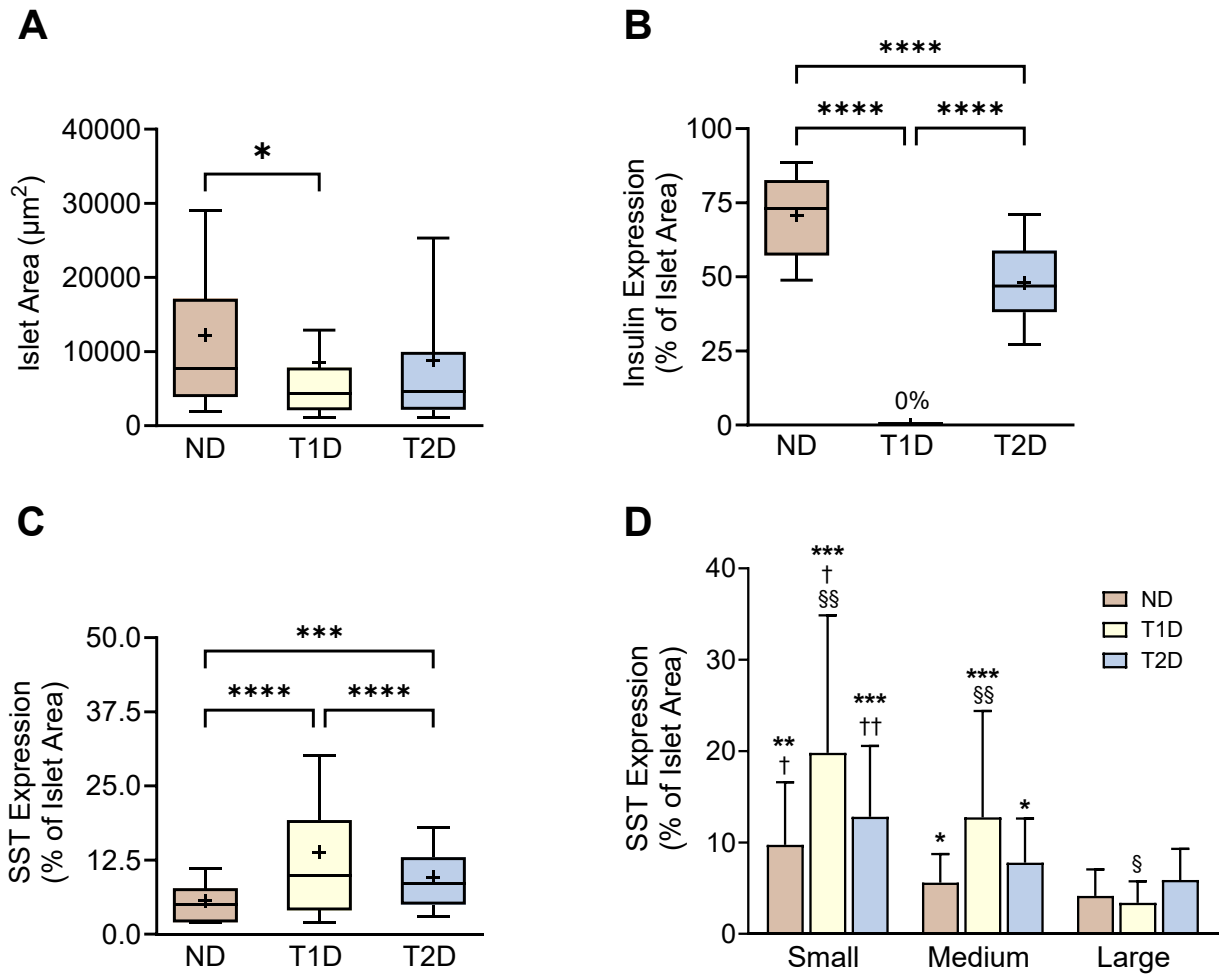


Figure 7-6. Islet hormone composition in human donors with T1D and T2D. Islet **(A)** area and proportions of **(B)** insulin **(C)** glucagon and **(D)** SST staining, expressed as a percentage of total islet area. *P>0.05; ***P<0.001, ****P<0.0001. **(E)** Percent SST expression stratified by islet size. *P<0.05, **P<0.01, ***P<0.001 vs. large islets; †P<0.05, ††P<0.01 vs. medium islets; §P<0.05, §§P<0.01 vs. ND and T2D islets. Whiskers represent 10th and 90th percentiles, "+" denotes the mean.

Representative images of human islets stained for insulin, glucagon, and SST are shown in Figure 7-7. Of note, some T2D islets retained a normal appearance, while others had irregular/ill defined borders and were fragmented into lobules by fibrous tissue and amorphous amyloid deposits (Fig. 7-7C,F). Interior sections of necrotic cells were observed in both T2D (Fig. 7-7C) and T1D (Fig. 7-7E) islets. Insulin staining was

ubiquitous in ND donor islets (Fig. 7-7A), undetectable in T1D donor islets (Fig. 7-7B), and fragmented in T2D donor islets (Fig.7-7C). Glucagon staining was localized to the outer mantle of ND islets (Fig. 7-7D) but was ubiquitously expressed among regions of hormone-positive cells in T1D (Fig. 7-7E) and T2D (Fig. 7-7F) donor islets. Unlike rodent islets, SST staining was scattered throughout ND donor islets (Fig. 7-7G) but tended to form internal clusters in T1D (Fig. 7-7H) and T2D (Fig. 7-7I) donor islets.

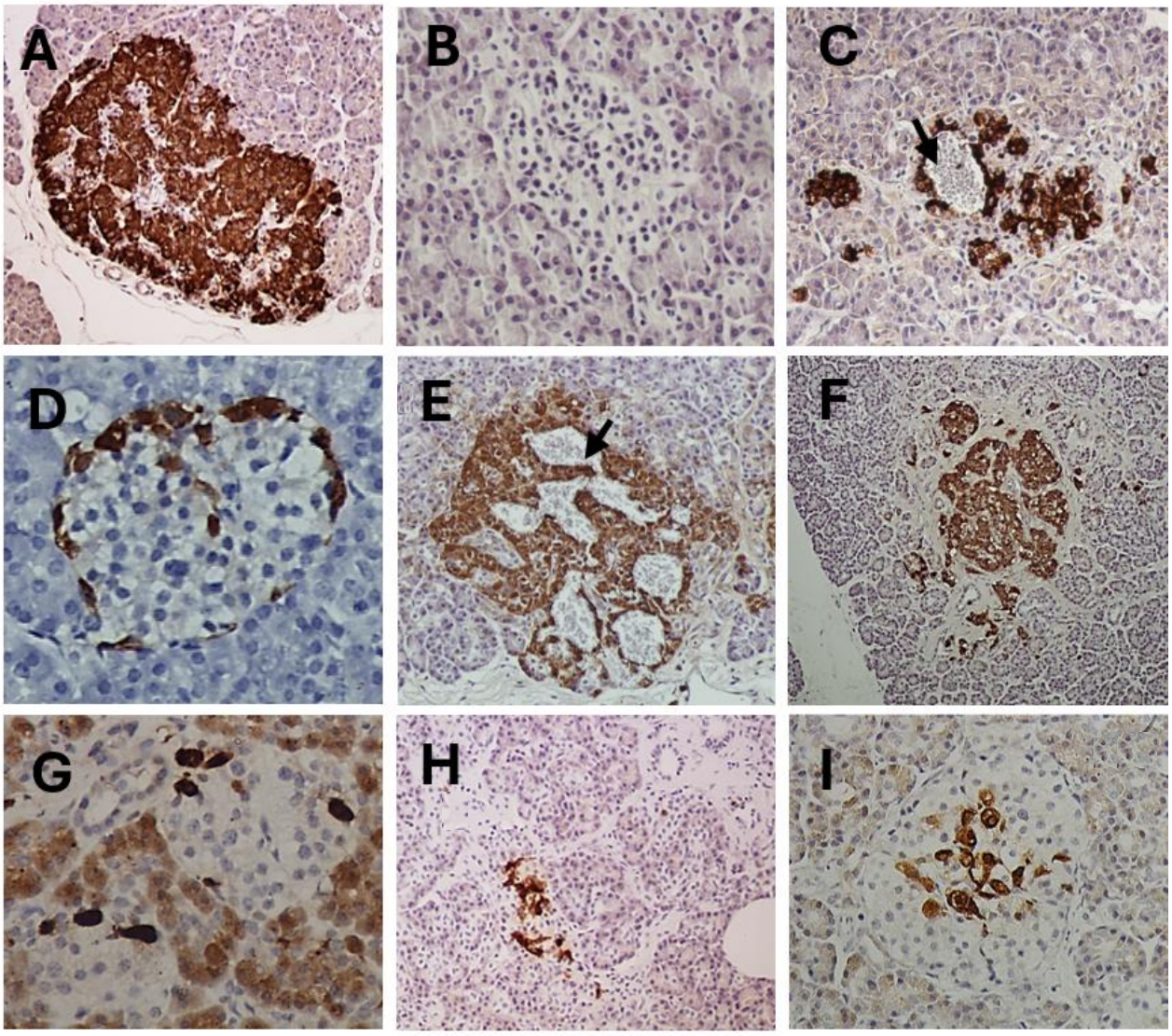


Figure 7-7. Representative images of ND (left), T1D (middle), and T2D (right) human islets immunostained for insulin **(A-C),** glucagon **(D-F)**, and SST **(G-I).** Arrow indicates mass of necrotic cells. ND: non-diabetic, SST: somatostatin.

7.2.5 Discussion

For the first time, we demonstrate an increase in the relative expression of somatostatin (SST)-positive staining per islet in humans and rats with advanced (i.e., insulin-deficient) T2D, as we (in the present study) and others have observed in islets from humans and animals with T1D[126]. Our findings suggest that despite their etiological differences, T2D may reflect an intermediate islet phenotype in the progression of insulin deficiency from non-diabetes to overt T1D, characterized by milder deviations in hormone composition from the ND state. We also showed that early glycemic intervention using basal insulin therapy in a rat model of insulin-deficient and insulin-resistant T2D could markedly preserve islet insulin expression while partially offsetting the relative expansion of SST-positive area.

The hallmark features of advanced T2D – insulin resistance and relative or absolute insulin deficiency – are modelled in rodents via high fat feeding (HFF) and low-dose streptozotocin (STZ), respectively. However, the effects of these interventions on islet architecture have only been examined independently.[260] Here, we show that percent insulin expression per islet was reduced by ~65% (from 73% to 27%) in HFF/STZ-T2D versus ND rats and this depletion of insulin could be attenuated (to 54%) by basal insulin therapy, consistent with previous observations in other murine models of T2D[314]. Alternatively, islet proportions of glucagon and SST increased by ~100% following T2D induction, and like insulin, physiologic SST expression could be partially preserved with basal insulin therapy (i.e., 7% in ND vs 11% with treatment and 14% without). As a result of early glycemic intervention in HFF/STZ-T2D rats, islet hormone composition was consistent with that observed in T2D islets from human donors selected for advanced

disease stage (i.e., 54% insulin, 24% glucagon, and 11% SST in rats vs. 48% insulin, and 10% SST in humans). Therefore, a once-daily injection of basal insulin dosed according to evening (post-absorptive) blood glucose concentration, may reproduce a modest level of glycemic control that is representative of our human donor cohort. Owing to the cost and relative scarcity of diabetic donor islets, those derived from HFF/STZ-T2D rats may serve as a useful tool for studying islet pathology in diabetes *ex vivo*.

Evidence from mouse models of autoimmune T1D suggests that SST expression may increase with advancing insulin deficiency.[5,315] Here, we extended these findings to HFF/STZ-T2D rats by modeling the progression of insulin deficiency using early glycemic intervention to partially preserve islet insulin content after STZ treatment in a subset of insulin-resistant rats. Despite this discovery, a proportional increase in glucagon expression per islet conserved the ratio of SST to glucagon across disease stages. In previous studies, this hormone ratio was elevated in islets from animals[140,304,316] but not humans[116] with T1D. Even so, islet remodeling may still increase α-cell exposure to SST signaling as an anatomical basis for the inhibition of counterregulatory glucagon secretion in rats with insulin-deficient and resistant T2D.

In summary, the relative expression of SST was markedly higher in islets from HFF/STZ-T2D rats and human donors with T2D compared to controls and could be partially normalized by basal insulin therapy in T2D rats. Accordingly, islet remodeling in insulin-deficient T2D may increase the available pool of secretable SST, as observed in T1D. Future studies should explore the effect of more intensive glycemic management on islet hormone composition in both T1D and T2D. It will also be important to determine how islet remodeling in advanced T2D influences SST and glucagon secretion during

episodes of hyperglycemia and hypoglycemia stemming from imperfect glucose management.

Chapter 8: Integrative Summary and Future Directions

Compared to insulin and glucagon, the role of somatostatin (SST) in blood glucose regulation is relatively understudied. Before its contribution to normal islet function could be fully elucidated, evidence linking SST to the breakdown of islet crosstalk in diabetes began to emerge.[317] The discovery that pharmacologic SSTR2 antagonism could restore the plasma glucagon response to insulin-induced hypoglycemia in T1D rodents suggested that δ-cell signaling may contribute to the pathogenesis of glucagon counterregulatory failure in diabetes.[1–3] In the thesis experiments presented in chapters 4-6, we extended these findings to rat models of pre-diabetes (Chapter 5) and recurrent insulin-induced hypoglycemia (Chapters 4 and 6).

Chapter 4 was the first study to evaluate the effects of an SSTR2 antagonist (SSTR2a) on glucagon counterregulation and glycemic recovery from hypoglycemia outside of a clamped environment. We showed that intraperitoneal injection of the SSTR2a, PRL-2903, could delay the onset of a fourth recurrent episode of insulin-induced hypoglycemia by twenty-five minutes.[258] The absence of pre-existing (i.e., diabetes-related) α-cell dysfunction in these rats allowed us to selectively target and reverse the cumulative attenuation (30%) in plasma glucagon levels during hypoglycemia resulting from three prior episodes of hyperinsulinemic hypoglycemia *per se*.[258] Notably, pre-treatment with the SSTR2a offset a further ~40% reduction in the plasma glucagon response during a fourth episode of hypoglycemia compared to the third.[258] While these results may imply that inhibitory SST signaling increases during recurrent hypoglycemia, no studies, to our knowledge have proven this effect. Since islet-derived SST cannot be measured in circulating plasma, future studies may wish to model the effects of recurrent

low-glucose exposure on SST secretion and signaling in isolated islets. If SST does in fact mediate the inhibition of counterregulatory glucagon secretion by recurrent hypoglycemia, it can next be determined whether this δ-cell dysfunction originates at the islet level or whether it is mediated by neural mechanisms.

Proof of concept studies, establishing a restorative effect of pharmacologic SSTR2a on glucagon counterregulation during insulin-induced hypoglycemia, were previously conducted in pre-clinical (i.e., rodent) models of T1D.[1–3,170] Unlike these models, the rat models of defective glucagon counterregulation studied in chapters 4-6 retained significant insulin-secretory function, characteristic of humans and other mammals with T2D. This disease phenotype introduced the potential for off-target effects of pharmacologic SSTR2 antagonism on islet β-cells, which, like α-cells, express SSTR2 and are primary targets of intra-islet SST signaling.[102] That said, SSTR5, rather than SSTR2, is the functionally dominant SST receptor in rodent β-cells[105], and since the antagonists used were >20x more selective for SSTR2 than SSTR5[233], it was unlikely that they would disinhibit insulin secretion by binding non-specifically to SSTR5. In chapter 4, plasma C-peptide level was unexpectedly lower during a fourth episode of recurrent hypoglycemia in rats treated with the SSTR2a, PRL-2903, versus vehicle.[258] Since SSTR2 antagonism has been shown to increase intra-islet and plasma levels of SST in rodents,[3,115] this effect may have increased the interaction between SST and SSTR5 in islet β-cells, inhibiting insulin release. That said, plasma C-peptide levels were higher in the vehicle group on day four of recurrent hypoglycemia compared to previous days, ;leading to speculation that the SSTR2a was suppressing inadvertent stimulation of endogenous insulin secretion caused by α-tocopheryl succinate (αTOS), a component of

the vehicle formulation. Evidence from rodent SST knockout islets lends support to this theory, suggesting that insulin secretion is not inhibited by SST below insulin's stimulatory threshold of ~7 mM glucose.[255] In chapter 5, pre-treatment of non-diabetic and pre-diabetic rats with the SSTR2a, ZT-01, did not significantly alter basal C-peptide levels (i.e., before hypoglycemia induction) or modify the suppression of plasma C-peptide by exogenous insulin overdose. However, when administered as a rescue therapy during severe hypoglycemia in chapter 6, ZT-01 (high dose, 10 mg/kg) increased portal vein C-peptide concentration relative to control levels two hours after dosing in rats exhibiting robust glycemic recovery (i.e., blood glucose >6.0 mM). The observed stimulation of endogenous insulin secretion was likely owing to a corresponding elevation in portal vein glucagon concentration, as occurs in the fed state.[294] Therefore, SSTR2a may engage (rather than override) physiologic feedback mechanisms that prevent post-prandial (and in this case, SSR2a-induced) hyperglycemia by limiting further glycemic recovery once euglycemia has been restored.

Unlike in rats, SSTR2 is the functionally dominant SST receptor in *both* human α- and β-cells.[100] Consistent with the dual regulation of insulin and glucagon secretion by SSTR2 in human islets,[100] chapter 7.1 demonstrated, for the first time, that SSTR2 antagonism may augment glucose-stimulated insulin secretion in non-diabetic but not GLTX-treated islets from human donors. These findings confirm that SST inhibits glucose-stimulated insulin secretion under physiologic conditions in human islets; however, it remains unclear whether SSTR2 antagonism stands to improve glucose tolerance in rodents or humans with T2D. Collectively, the data in this dissertation, obtained from rodent models of glucagon counterregulatory failure *in vivo* (Chapters 4-6) and isolated

human islets *in vitro* (Chapter 7.1), support continued interest into the development of pharmacologic SSTR2a for hypoglycemia prevention in T2D. These findings also highlight interspecies differences in intra-islet signalling pathways that may limit the translation of therapeutic targets from rodents to humans.

A comparison of findings in chapters 4-6 reveals that endogenous glucagon may have a longer duration of action on the liver than the exogenous hormone, and this action may be extended by the administration of SSTR2a during hypoglycemia versus euglycemia. These characteristics, as well as the avoidance of peripheral hyperglucagonemia, may highlight the utility of SSTR2a for use in a bi-hormonal artificial pancreas system. However, there is a limit to the pharmacological stimulation of endogenous glucagon release, which falls within physiological bounds, and may not be adequate to provide rapid and robust recovery from severe insulin-induced hypoglycemia, especially in settings of high metabolic demand, like exercise. Future studies may wish to investigate whether combined treatment with other SST receptor agonists/antagonists in human islets may help to maximize drug potency. For example, SSTR1 plays a secondary role to SSTR2 in mediating the inhibition of glucagon secretion by SST in humans,[100,101] and therefore, co-treatment with an SSTR1 antagonist may alleviate more of SST's inhibitory tone, especially if it is elevated in diabetes.

It should also be noted that aside from inhibiting the counterregulatory actions of glucagon (i.e., hepatic glucose production),[318] therapeutic insulin also inhibits the section of endogenous glucagon in diabetic animals.[268] Therefore, an SSTR2 antagonist may be competing against the inhibitory effect of therapeutic insulin to liberate glucagon from the α-cell, whereas levels of exogenous glucagon in circulation are not dictated by insulin

dosing. Exogenous insulin can also directly stimulate SST secretion in rodent islets,[297] but it remains unclear what portion of insulin's inhibitory effect on glucagon secretion during hyperinsulinemic hypoglycemia is mediated by SST and reversible with an SSTR2a. When it comes to a dual-hormone artificial pancreas system, the timing of glucagon delivery in relation to insulin is important to consider since the efficacy of glucagon in preventing hypoglycemia depends on circulating insulin levels.[196] Accordingly, micro-boluses of glucagon delivered by a pump system have been shown to correct hypoglycemia in a dose-dependent manner when insulin levels are low, but fail to influence hepatic glucose output when insulin levels are high, irrespective of glucose concentration.[200,201] Therefore, pharmacologic stimulation of endogenous glucagon release may be subject to the same shortcomings as exogenous glucagon, as observed in chapter 5. Specifically, pre-treatment of non-diabetic and prediabetic rats with an SSTR2a one hour before hypoglycemia induction increased glucagon secretion in both groups as compared to their respective vehicle-treated controls but only augmented glycemic recovery (i.e., increased blood glucose levels and delayed the onset of hypoglycemia) in the prediabetic rats. We reasoned that in non-diabetic rats, hepatic glucagon signaling was supressed by antagonistic inputs from therapeutic insulin at the dose used. Alternatively, the presence of insulin resistance in the T2D group may have reduced the potency of insulin signaling in this model, allowing for pharmacologically stimulated glucagon to influence blood glucose levels. Future studies are required to evaluate the benefits and limitations of exogenous glucagon versus a SSTR2a in a dual-hormone pump system, particularly in settings that confer the highest risk of hypoglycemia, such the post-prandial period, sleep, and exercise.[198]

The study presented in chapter 4 of this dissertation was the first in the literature to establish a mechanism of hepatic glucose production driving glycemic recovery by pharmacologic SSTR2 antagonism. Whether SSTR2a was dosed before or during hypoglycemia, hepatic glycogen content was lower than control levels after a fourth recurrent episode of insulin-induced hypoglycemia, suggestive of increased glycogen utilization. We further probed the enzymatic contribution to hepatic glucose production in chapter 6, demonstrating elevated activity of glycogenolytic enzyme, glycogen phosphorylase and glycogenotic enzyme, phosphoenolpyruvate carboxykinase (PEPCK) in SSTR2a- versus vehicle-treated rats. Since gluconeogenesis is typically recruited in settings of prolonged hypoglycemia, consistent with glycogen depletion, we suspect that gluconeogenesis may at least partially underscore the delay in maximal glycemic recovery observed at the high dose of SSTR2a (10 mg/kg) when administered as a hypoglycemia rescue agent (Chapter 6). That said, gluconeogenesis is largely regulated by transcriptional programs, and therefore, gluconeogenic enzyme expression may be a more descriptive measure of gluconeogenic activity for future studies to explore.

The goal of the second study in this thesis (Chapter 5) was to evaluate the therapeutic potential of SSTR2a in a T2D model of insulin resistance and relative insulin deficiency, induced by high-fat feeding and low-dose streptozotocin, respectively[264,319] However, COVID-19-related delays allowed time for the model to recover from streptozotocin-induced insulin deficiency and severe hyperglycemia to a state of basal hyperinsulinemia. Even though this rat model did not exhibit advanced counterregulatory failure as intended, it did exhibit mild hyperglycemia – a metabolic hallmark of pre-diabetes that is not recapitulated by the traditional use of high-fat feeding alone to model

the condition in rodents.[260,319] Since the time course for the development of glucagon counterregulatory failure in T2D remained unclear,[123] we used this model to investigate the combined effects of mild hyperglycemia and moderate hyperinsulinemia, secondary to insulin resistance, on glucagon counterregulatory function in pre-diabetes. To that end, we detected a mild deficiency in the plasma glucagon response to insulin-induced hypoglycemia in rats with pre-T2D, which was reversible with SSTR2a. Previous studies have demonstrated this glucagon secretory defect in settings of absolute insulin deficiency (i.e., T1D and advanced T2D), characterized by basal hypoinsulinemia relative to healthy controls;[121] however, our results suggest that α-cell impairment, potentially mediated by SST signalling, may begin to develop in a state of relative insulin deficiency (i.e., relative to blood glucose level) and basal hyperinsulinemia, once the compensatory capacity of the β-cell has been reached and β-cell function begins to decline. This is consistent with decades of research, demonstrating a dependence of physiologic glucagon secretion on intact β-cell signalling[320] and more recent evidence suggesting that SST may act as a paracrine messenger, delivering glucose-mediated feedback from β- to α-cells.[5,6,297,321] Supported by our analysis of islet remodelling in chapter 7.2, these data also suggest that preservation of β-cell mass with early glycemic intervention may be capable of offsetting diabetes-induced impairments in glucagon counterregulation, as long as hypoglycemia can be avoided.[119]

In chapter 5, we developed a novel rat model of pre-T2D exhibiting moderate hyperinsulinemia and mild hyperglycemia. This phenotype was established after a period of glycemic recovery from STZ treatment facilitated by exogenous insulin therapy. However, this method of pre-T2D induction reflects the reverse sequence of disease

progression in humans. With the alleviation of metabolic stress (typically through weight loss), human β-cells can recover from a dedifferentiated or quiescent state in the early stages of decompensation; however, recovery from degranulation and β-cell death, as observed in our STZ-treated animals, may be less representative of the natural disease history.[322] It also remains unclear whether our model of pre-T2D is phenotypically stable with continued high fat feeding after the period of experimentation in the present study (i.e., 4 weeks post STZ injection). Additionally, future studies are necessary to confirm whether compensatory hyperinsulinemia in this model relies on the expansion of β-cell mass relative to healthy controls, as is observed in humans.[269] Therefore, our model must be thoroughly characterized to determine its clinical relevance. As a potential alternative, the addition of nicotinamide to the HFF/STZ-T2D induction protocol may improve the physiological relevance of the pre-T2D model by protecting pancreatic β-cells from the cytotoxicity of STZ while preserving key T2D features, such as β-cell fatigue and impaired insulin secretion[323]

The established model of HFF/STZ-T2D used in chapter 7.2 also presents limitations that must be considered when translating findings to humans. First, there are no standardized glycemic thresholds used to confirm the induction of diabetes in animal models. That said, fasting glucose levels >8.3 mM and non-fasting glucose levels >11 mM are deemed confirmatory of diabetes in STZ-treated animals.[324] Other tests, such as oral glucose tolerance and insulin tolerance tests are useful for detecting insulin resistance and impaired glucose disposal.[324] Second, β-cells are resistant to diet-induced apoptosis and do not form cytotoxic amyloid plaques, necessitating the use of STZ to induce β-cell destruction.[264] However, the rapid onset of chemically-induced β-cell death

is contrasted by the gradual progression of T2D and its multifactorial basis in humans (i.e., owing to a complex interplay of genetic, metabolic, and age-related factors). This limitation may be addressed with the use of multiple low-dose injections, which has been shown to elicit more gradual β-cell death.[325326] Third, the sequential loss of first and second phase insulin secretion in humans with T2D is not reproduced in this model.[327] Fourth, the age of the rats used to model diabetes in this thesis (8-10 weeks upon initiation of high-fat feeding; classified as “young”) may not accurately reflect the older age of T2D onset in humans.[328] Accordingly, older rats (>1 year), like adults >30 years, lack the capacity for β-cell regeneration and may be better suited for this diabetic model [328] Fourth, acute and transient weight loss resulting from STZ treatment is characteristic of overt T1D onset but not T2D.[329] Fifth, obesity is a central driver of peripheral insulin resistance in humans, but not in rodents, as HFF-induced insulin resistance precedes the onset of significant weight gain by 2-3 months.[330] As a result, obesity was absent from our rodent models owing to time and financial constraints.[329] A high fat and high sucrose diet is not only more reflective of the Western diet but is likely to confer more rapid and severe weight gain than HFF alone.[327] That said, a standardized definition of obesity in rodent models of T2D is lacking.[324]

Chronic exposure of isolated islets to glucolipotoxic (GLTX) culture conditions is a common approach to modeling β-cell dysfunction in T2D;[302] however, to our knowledge, the secretory phenotypes of islet α- and δ-cells had not been characterized in this model. In chapter 7.2, we demonstrated in a limited sample of healthy and diabetic human islets, that glucagon secretion was reduced under low glucose conditions and unresponsive to elevations in glucose concentration, consistent with the dysregulation observed in islet

preparations from one T2D donor. These findings are also supported by previous observations in islets from humans and mice with T1D[5] and a subset of human islets from donors with T2D[301]. Alternatively, we observed that the inhibition of SST secretion at low glucose and stimulation at high glucose were both blunted in T2D and GLTX-treated islets compared to controls. Since mounting evidence suggests that SST is hypersecreted from T1D and advanced T2D islets under low glucose conditions, we[22] and others[24] have speculated (owing to a lack of data) as to whether this outcome reflects failed inhibition (i.e., secretion fails to decrease with decreasing glucose level) or stimulation *per se* (i.e., secretion increases with decreasing glucose level, mimicking physiologic glucagon secretion) (Fig. 2-2). Preliminary findings from chapter 7.2 of this dissertation may help to resolve some of this uncertainty by demonstrating that T2D and *in vitro* GLTX exposure reduced δ-cell sensitivity to both stimulatory and inhibitory fluctuations in glucose concentration. Drawing on findings from chapter 7.2, which indicate that islet SST expression may increase with the progression of insulin deficiency in diabetic rats and humans, future work may wish to assess whether SST secretion during hypoglycemia also increases along this time course to account for the progressive impairment in glucagon counterregulation.[5]

In chapter 7.1, we discovered a potential role for SST, via SSTR2, in regulating counterregulatory glucagon secretion in non-diabetic human islets. Previous studies performed in healthy human islets, all using a common SSTR2a (CYN154806), observed no effect of treatment on glucagon secretion below 6 mM glucose, [5,96,106,298–300] unlike the marked stimulation observed in T2D islets[4,301]. Since SST secretion increases linearly with increasing glucose concentration above 3 mM,[96] SST's inhibitory tone was

considered negligible below this glycemic threshold. However, chapter 7.1 of this dissertation showed that SSTR2 antagonism markedly increased counterregulatory glucagon secretion in non-diabetic human islets incubated at low glucose, suggesting that SST exerts tonic inhibition over the α-cell throughout the physiologic glucose range, as observed in rodent islets.[115] Accordingly, SSTR2a may relieve both pathological and physiological inhibition of glucagon secretion by SST but whether the magnitude of recovery is proportional to SST tone is a topic of future study.

In chapter 7.2, we demonstrated that remodelling of islet architecture in humans and rats with insulin-deficient T2D increases the proportion of SST-positive area per islet. This may reflect a compensatory adaption to preserve paracrine signaling as glucose-stimulated SST secretion becomes blunted, while consequently, increasing inhibitory SST tone. It will be important to determine whether other factors, such as glucose-dependent changes in SSTR2 expression, also sensitize the α-cell to SST in diabetic states. In support, α-cell resistance to SST signaling was observed at higher glucose concentrations in T2D islets from human donors, owing to dynamic changes in receptor expression, which was proposed to underscore post-prandial hyperglucagonemia in T2D.[110] Since we did not quantify the number or distribution of each cell type per islet, we cannot determine whether hypertrophy and/or hyperplasia of α- and δ-cells contributed to respective increases in glucagon- and SST-positive staining. It also unclear how relative insulin deficiency in T2D may impact the contact density between α- and δ-cells, which was recently reported to double in T1D islets from human donors.[139] Without further insights into cellular distribution, we can simply conclude that islet remodeling in advanced T2D increased the relative pool of secretable SST, which may be necessary to

sustain the higher rate of secretion observed under low-glucose conditions in chapter 7.1. Histological analysis of diabetic islets is complicated by the presence of islet atrophy, immune cell infiltration, and amyloid deposits, which produce vast, diffuse, and/ or segmented islets with ill-defined borders. Establishing a standard protocol for defining islet borders (and other qualitative parameters) obscured by diabetes-related pathology may help resolve some of the uncertainty surrounding the fate of islet δ-cells in the progression of T2D.

When administered either *in vivo* (pre-T2D rats; Chapter 5) or *in vitro* (non-diabetic and GLTX-treated human islets; Chapter 7.1), SSTR2a-dependent increases in glucagon secretion were no glucose dependent. Specifically, we found that SSTR2a increased plasma glucagon and whole blood glucose concentrations during euglycemia in pre-T2D rats. Additionally, isolated human islets secreted more glucagon at both 1 and 16.7 mM glucose, independent of GLTX treatment, when incubated with SSTR2a versus without. These data suggest that SSTR2a may promote hyperglycemia unless there is a corresponding rise in plasma insulin levels. Accordingly, the administration of ZT-01 after the onset of severe insulin-induced hypoglycemia in recurrently hypoglycemic rodents (Chapter 6), produced a corresponding increase in plasma C-peptide level that managed to offset the development of hyperglycemia once euglycemia had been restored. This result may also reflect a sensitizing effect of glucagon on hepatic insulin signaling, which may develop with prolonged glucagon exposure.[291,292] Glycemic outcomes of SSTR2a treatment remain unclear in humans; however, co-stimulation of C-peptide secretion, owing to its secretory regulation by SSTR2 in human but not rodent β-cells,[101,102] may similarly act to limit glucagon's hyperglycemic actions. Further, the secretion and

inhibitory paracrine actions of SST are reportedly diminished within and above the euglycemic range in T2D, which could reduce its inhibition of the α-cell and any resulting hyperglycemic effect of SSTR2a treatment.[110]

In summary, this dissertation established a common therapeutic target for treating diabetes- and hypoglycemia-associated defects in the counterregulatory glucagon response to insulin-induced hypoglycemia. As an adjunct to intensive glucose-lowering therapies, pharmacological SSTR2 antagonism may be a promising tool for managing acute and recurrent hypoglycemia in T2D without compromising metabolic control. Accordingly, we propose a paracrine mechanism of glucagon counterregulatory failure in T2D driven by elevations in SST expression and low-glucose-dependent secretion. Collectively, these data extend the body of pre-clinical evidence supporting the development of pharmacological SSTR2a and identify potential off-label indications for its use. Additionally, this work establishes a novel rodent model of chemically-induced pre-diabetes that recapitulates mild hyperglycemia in the early stages of β-cell decompensation. Lastly, we provide preliminary evidence supporting the physiological relevance of glucolipotoxic culture conditions for modelling the effects of T2D on glucagon and SST secretion *in vitro*. These contributions stand to advance both our understanding and the treatment of glucagon counterregulatory failure in T1D and advanced T2D.

References

1. Yue JTY, Burdett E, Coy DH, Giacca A, Efendic S, Vranic M. Somatostatin receptor type 2 antagonism improves glucagon and corticosterone counterregulatory responses to hypoglycemia in streptozotocin-induced diabetic rats. *Diabetes*. 2012;61(1):197-207.

2. Yue JTY, Riddell MC, Burdett E, Coy DH, Efendic S, Vranic M. Amelioration of hypoglycemia via somatostatin receptor type 2 antagonism in recurrently hypoglycemic diabetic rats. *Diabetes*. 2013;62(7):2215-2222.

3. Karimian N, Qin T, Liang T, et al. Somatostatin receptor type 2 antagonism improves glucagon counterregulation in biobreeding diabetic rats. *Diabetes*. 2013;62(8):2968-2977.

4. Vergari E, Denwood G, Salehi A, et al. Somatostatin secretion by Na+-dependent Ca2+-induced Ca2+ release in pancreatic delta cells. *Nat Metab*. 2020;2(1):32-40.

5. Hill TG, Gao R, Benrick A, et al. Loss of electrical β-cell to δ-cell coupling underlies impaired hypoglycaemia-induced glucagon secretion in type-1 diabetes. *Nat Metab*. 2024;6(11):2070-2081.

6. Rorsman P, Huising MO. The somatostatin-secreting pancreatic δ-cell in health and disease. *Nat Rev Endocrinol*. 2018;14(7):404-414.

7. Siafarikas A, Johnston RJ, Bulsara MK, O'Leary P, Jones TW, Davis EA. Early loss of the glucagon response to hypoglycemia in adolescents with type 1 diabetes. *Diabetes Care*. 2012;35(8):1757-1762.

8. Weir GC, Laybutt DR, Kaneto H, Bonner-Weir S, Sharma A. Beta-cell adaptation and decompensation during the progression of diabetes. *Diabetes*. 2001;50 Suppl 1(suppl_1):S154-9.

9. Gaisano HY, MacDonald PE, Vranic M. Glucagon secretion and signaling in the development of diabetes. *Front Physiol*. 2012;3:349.

10. van der Meulen T, Donaldson CJ, Cáceres E, et al. Urocortin3 mediates somatostatin-dependent negative feedback control of insulin secretion. *Nat Med*. 2015;21(7):769-776.

11. Hermansen K. Characterisation of the Abnormal Pancreatic D and A Cell Function in Streptozotocin Diabetic Dogs: Studies with D-Glyceraldehyde, Dihydroxyacetone, D-Mannoheptulose, D-Glucose, and L-Arginine. *Diabetologia*. 1981;21:94.

12. Abdel-Halim SM, Guenifi A, Efendić S, Ostenson CG. Both somatostatin and insulin responses to glucose are impaired in the perfused pancreas of the spontaneously noninsulin-dependent diabetic GK (Goto-Kakizaki) rats. *Acta Physiol Scand*. 1993;148(2):219-226.

13. Wysham C, Bhargava A, Chaykin L, et al. Effect of insulin degludec vs insulin glargine U100 on hypoglycemia in patients with type 2 diabetes. *JAMA*. 2017;318(1):45.

14. Lane W, Bailey TS, Gerety G, et al. Effect of insulin degludec vs insulin glargine U100 on hypoglycemia in patients with type 1 diabetes: the SWITCH 1 randomized clinical trial. *JAMA*. 2017;318(1):33-44.

15. Heinemann L, Freckmann G, Ehrmann D, et al. Real-time continuous glucose monitoring in adults with type 1 diabetes and impaired hypoglycaemia awareness or severe hypoglycaemia treated with multiple daily insulin injections (HypoDE): a multicentre, randomised controlled trial. *The Lancet*. 2018;391(10128):1367-1377.

16. Lin YK, Hung M, Sharma A, et al. Impaired awareness of hypoglycemia continues to be a risk factor for severe hypoglycemia despite the use of continuous glucose monitoring system in type 1 diabetes. *Endocrine Practice*. 2019;25(6):517-525.

17. Rickels MR. Hypoglycemia-associated autonomic failure, counterregulatory responses, and therapeutic options in type 1 diabetes. *Ann N Y Acad Sci*. 2019;1454(1):68-79.

18. Cryer PE. Mechanisms of hypoglycemia-associated autonomic failure and its component syndromes in diabetes. *Diabetes*. 2005;54(12):3592-3601.

19. Effect of ZT-01 on glucagon during hypoglycemia in type 1 diabetes mellitus. Identifier: NCT05007977. https://www.clinicaltrials.gov/ct2/show/NCT05007977.

20. A Study of the Effect of ZT-01 on Night-time Hypoglycemia in Type 1 Diabetes. Identifier: NCT05762107. https://clinicaltrials.gov/study/NCT05762107

21. Cryer P. Hypoglycaemia: the limiting factor in the glycaemic management of type I and type II diabetes. *Diabetologia*. 2002;45(7):937-948.

22. Hoffman EG, D'Souza NC, Liggins RT, Riddell MC. Pharmacologic inhibition of somatostatin receptor 2 to restore glucagon counterregulation in diabetes. *Front Pharmacol*. 2023;14:1295639.

23. American Diabetes Association Professional Practice Committee. 6. Glycemic Goals and Hypoglycemia: Standards of Care in Diabetes-2024. *Diabetes Care*. 2024;47(Suppl 1):S111-S125.

24. Noguchi GM, Huising MO. Integrating the inputs that shape pancreatic islet hormone release. *Nat Metab*. 2019;1(12):1189-1201.

25. McCoy RG, Van Houten HK, Ziegenfuss JY, Shah ND, Wermers RA, Smith SA. Increased mortality of patients with diabetes reporting severe hypoglycemia. *Diabetes Care*. 2012;35(9):1897-1901.

26. Aronson R, Goldenberg R, Boras D, Skovgaard R, Bajaj H. The Canadian hypoglycemia assessment tool program: insights into rates and implications of hypoglycemia from an observational study. *Can J Diabetes*. 2018;42(1):11-17.

27. Heller SR, Choudhary P, Davies C, et al. Risk of hypoglycaemia in types 1 and 2 diabetes: effects of treatment modalities and their duration. *Diabetologia*. 2007;50(6):1140-1147.

28. Ratner RE. Hypoglycemia: new definitions and regulatory implications. *Diabetes Technol Ther*. 2018;20(S2):S2-50-S2-53.

29. Zhong VW, Juhaeri J, Cole SR, et al. Incidence and trends in hypoglycemia hospitalization in adults with type 1 and type 2 diabetes in England, 1998–2013: a retrospective cohort study. *Diabetes Care*. 2017;40(12):1651-1660.

30. Lipska KJ, Ross JS, Wang Y, et al. National trends in US hospital admissions for hyperglycemia and hypoglycemia among Medicare beneficiaries, 1999 to 2011. *JAMA Intern Med*. 2014;174(7):1116-1124.

31. Lipska KJ, Yao X, Herrin J, et al. Trends in drug utilization, glycemic control, and rates of severe hypoglycemia, 2006-2013. *Diabetes Care*. 2017;40(4):468-475.

32. Gregg EW. The changing tides of the type 2 diabetes epidemic-smooth sailing or troubled waters ahead? Kelly West Award Lecture 2016. *Diabetes Care*. 2017;40(10):1289-1297.

33. Weinstock RS, Xing D, Maahs DM, et al. Severe hypoglycemia and diabetic ketoacidosis in adults with type 1 diabetes: results from the T1D exchange clinic registry. *J Clin Endocrinol Metab*. 2013;98(8):3411-3419.

34. Berlin I, Sachon C, Grimaldi A. Identification of factors associated with impaired hypoglycaemia awareness in patients with type 1 and type 2 diabetes mellitus. *Diabetes Metab*. 2005;31(3):246-251.

35. Duckworth W, Abraira C, Moritz T, et al. Glucose control and vascular complications in veterans with type 2 diabetes. *N Engl J Med*. 2009;360(2):129-139.

36. Patel A, Chalmers J, Poulter N. ADVANCE: Action in Diabetes and Vascular Disease. *J Hum Hypertens*. 2005;19(S1):S27-S32.

37. Patel A, MacMahon S, Chalmers J, Neal B. Intensive blood glucose control and vascular outcomes in patients with type 2 diabetes. *N Engl J Med*. Published online 2008.

38. Chow E, Bernjak A, Williams S, et al. Risk of cardiac arrhythmias during hypoglycemia in patients with type 2 diabetes and cardiovascular risk. *Diabetes*. 2014;63(5):1738-1747.

39. Whitmer RA. Hypoglycemic episodes and risk of dementia in older patients with type 2 diabetes mellitus. *JAMA*. 2009;301(15):1565.

40. Amiel SA, Aschner P, Childs B, et al. Hypoglycaemia, cardiovascular disease, and mortality in diabetes: epidemiology, pathogenesis, and management. *Lancet Diabetes Endocrinol*. 2019;7(5):385-396.

41. Beck RW, Bergenstal RM, Riddlesworth TD, Kollman C. The association of biochemical hypoglycemia with the subsequent risk of a Severe hypoglycemic event: analysis of the DCCT data set. *Diabetes Technol Ther*. 2019;21(1):1-5.

42. Zammitt NN, Frier BM. Hypoglycemia in type 2 diabetes: pathophysiology, frequency, and effects of different treatment modalities. *Diabetes Care*. 2005;28(12):2948-2961.

43. Peacey SR, Robinson R, Bedford C, et al. Does the choice of treatment for type 2 diabetes affect the physiological response to hypoglycemia? *Diabetes Care*. 2000;23(7):1022-1023.

44. Mitrakou A, Ryan C, Veneman T, et al. Hierarchy of glycemic thresholds for counterregulatory hormone secretion, symptoms, and cerebral dysfunction. *Am J Physiol Endocrinol Metab*. 1991;260(1):E67-E74.

45. Cryer PE, Tse TF, Clutter WE, Shah SD. Roles of glucagon and epinephrine in hypoglycemic and nonhypoglycemic glucose counterregulation in humans. *Am J Physiol*. 1984;247(2 Pt 1):E198-205.

46. Stanley S, Moheet A, Seaquist ER. Central mechanisms of glucose sensing and counterregulation in defense of hypoglycemia. *Endocr Rev*. 2019;40(3):768-788.

47. Thorens B. Brain glucose sensing and neural regulation of insulin and glucagon secretion. *Diabetes Obes Metab*. 2011;13(s1):82-88.

48. Stanley SA, Kelly L, Latcha KN, et al. Bidirectional electromagnetic control of the hypothalamus regulates feeding and metabolism. *Nature*. 2016;531(7596):647-650.

49. Tong Q, Ye C, McCrimmon RJ, et al. Synaptic glutamate release by ventromedial hypothalamic neurons is part of the neurocircuitry that prevents hypoglycemia. *Cell Metab*. 2007;5(5):383-393.

50. McCrimmon R. The mechanisms that underlie glucose sensing during hypoglycaemia in diabetes. *Diabet Med*. 2008;25(5):513-522.

51. Niimi M, Sato M, Tamaki M, Wada Y, Takahara J, Kawanishi K. Induction of Fos protein in the rat hypothalamus elicited by insulin-induced hypoglycemia. *Neurosci Res*. 1995;23(4):361-364.

52. Dunn-Meynell AA, Govek E, Levin BE. Intracarotid glucose selectively increases Fos-like immunoreactivity in paraventricular, ventromedial and dorsomedial nuclei neurons. *Brain Res*. 1997;748(1-2):100-106.

53. Kang L, Routh VH, Kuzhikandathil E V, Gaspers LD, Levin BE. Physiological and molecular characteristics of rat hypothalamic ventromedial nucleus glucosensing neurons. *Diabetes*. 2004;53(3):549-559.

54. Kang L, Dunn-Meynell AA, Routh VH, et al. Glucokinase Is a Critical Regulator of Ventromedial Hypothalamic Neuronal Glucosensing. *Diabetes*. 2006;55(2):412-420.

55. Bachelard HS. Glucose-phosphorylating enzyme with high Km in human brain. *Nature*. 1967;215(5104):959-960.

56. Ashford ML, Boden PR, Treherne JM. Glucose-induced excitation of hypothalamic neurones is mediated by ATP-sensitive K+ channels. *Pflugers Arch*. 1990;415(4):479-483.

57. O'Malley D, Reimann F, Simpson AK, Gribble FM. Sodium-Coupled Glucose Cotransporters Contribute to Hypothalamic Glucose Sensing. *Diabetes*. 2006;55(12):3381-3386.

58. Hallows KR, Raghuram V, Kemp BE, Witters LA, Foskett JK. Inhibition of cystic fibrosis transmembrane conductance regulator by novel interaction with the metabolic sensor AMP-activated protein kinase. *J Clin Invest*. 2000;105(12):1711.

59. Fioramonti X, Marsollier N, Song Z, et al. Ventromedial hypothalamic nitric oxide production is necessary for hypoglycemia detection and counterregulation. *Diabetes*. 2010;59(2):519-528.

60. Murphy MP. How mitochondria produce reactive oxygen species. *Biochem J*. 2009;417(1):1-13.

61. Murphy BA, Fakira KA, Song Z, Beuve A, Routh VH. AMP-activated protein kinase and nitric oxide regulate the glucose sensitivity of ventromedial hypothalamic glucose-inhibited neurons. *Am J Physiol Cell Physiol*. 2009;297(3):C750-C758.

62. Fioramonti X, Deak A, Deshpande S, et al. Hypothalamic S-Nitrosylation Contributes to the Counter-Regulatory Response Impairment following Recurrent Hypoglycemia. Gaetano C, ed. *PLoS One*. 2013;8(7):e68709.

63. Iversen J. Adrenergic Receptors and the Secretion of Glucagon and Insulin from the Isolated, Perfused Canine Pancreas. *J Clin Invest*. 1973;52(9):2102.

64. Borg WP, During MJ, Sherwin RS, Borg MA, Brines ML, Shulman GI. Ventromedial hypothalamic lesions in rats suppress counterregulatory responses to hypoglycemia. *J Clin Invest*. 1994;93(4):1677.

65. Ramnanan CJ, Edgerton DS, Kraft G, Cherrington AD. Physiologic action of glucagon on liver glucose metabolism. *Diabetes Obes Metab*. 2011;13(1):118-125.

66. Svoboda M, Tastenoy M, Vertongen P, Robberecht P. Relative quantitative analysis of glucagon receptor mRNA in rat tissues. *Mol Cell Endocrinol*. 1994;105(2):131-137.

67. Sharabi K, Tavares CDJ, Rines AK, Puigserver P. Molecular pathophysiology of hepatic glucose production. *Mol Aspects Med*. 2015;46:21-33.

68. Lecavalier L, Bolli G, Cryer P, Gerich J. Contributions of gluconeogenesis and glycogenolysis during glucose counterregulation in normal humans. *Am J Physiol*. 1989;256(6 Pt 1):E844-51.

69. Cherrington AD, Williams PE, Shulman GI, Lacy WW. Differential time course of glucagon's effect on glycogenolysis and gluconeogenesis in the conscious dog. *Diabetes*. 1981;30(3):180-187.

70. Kajani S, Laker RC, Ratkova E, Will S, Rhodes CJ. Hepatic glucagon action: beyond glucose mobilization. *Physiol Rev*. 2024;104(3):1021-1060.

71. Cullen KS, Al-Oanzi ZH, O'Harte FPM, Agius L, Arden C. Glucagon induces translocation of glucokinase from the cytoplasm to the nucleus of hepatocytes by transfer between 6-phosphofructo 2-kinase/fructose 2,6-bisphosphatase-2 and the glucokinase regulatory protein. *Biochim Biophys Acta*. 2014;1843(6):1123-1134.

72. Castaño JG, Nieto A, Felíu JE. Inactivation of phosphofructokinase by glucagon in rat hepatocytes. *J Biol Chem*. 1979;254(13):5576-5579.

73. Pilkis SJ, El-Maghrabi MR, McGrane M, Pilkis J, Claus TH. Regulation by glucagon of hepatic pyruvate kinase, 6-phosphofructo 1-kinase, and fructose-1,6-bisphosphatase. *Fed Proc*. 1982;41(10):2623-2628.

74. Veneziale CM, Deering NG, Thompson HJ. Gluconeogenesis in isolated rat hepatic parenchymal cells. IX. Differential effects of glucagon and epinephrine on phosphofructokinase and pyruvate kinase. *Mayo Clin Proc*. 1976;51(10):624-631.

75. Petersen MC, Vatner DF, Shulman GI. Regulation of hepatic glucose metabolism in health and disease. *Nat Rev Endocrinol*. 2017;13(10):572-587.

76. Altarejos JY, Montminy M. CREB and the CRTC co-activators: sensors for hormonal and metabolic signals. *Nature Reviews Molecular Cell Biology 2011 12:3*. 2011;12(3):141-151.

77. Koo SH, Flechner L, Qi L, et al. The CREB coactivator TORC2 is a key regulator of fasting glucose metabolism. *Nature*. 2005;437(7062):1109-1111.

78. Le Lay J, Tuteja G, White P, Dhir R, Ahima R, Kaestner KH. CRTC2 (TORC2) contributes to the transcriptional response to fasting in the liver but is not required for the maintenance of glucose homeostasis. *Cell Metab*. 2009;10(1):55-62.

79. Stark R, Guebre-Egziabher F, Zhao X, et al. A Role for Mitochondrial Phosphoenolpyruvate Carboxykinase (PEPCK-M) in the Regulation of Hepatic Gluconeogenesis. *J Biol Chem*. 2014;289(11):7257-7263.

80. Kimball SR, Siegfried BA, Jefferson LS. Glucagon represses signaling through the mammalian target of rapamycin in rat liver by activating AMP-activated protein kinase. *J Biol Chem*. 2004;279(52):54103-54109.

81. Baum JI, Kimball SR, Jefferson LS. Glucagon acts in a dominant manner to repress insulin-induced mammalian target of rapamycin complex 1 signaling in perfused rat liver. *Am J Physiol Endocrinol Metab*. 2009;297(2):E410-5.

82. Ezaki J, Matsumoto N, Takeda-Ezaki M, et al. Liver autophagy contributes to the maintenance of blood glucose and amino acid levels. *Autophagy*. 2011;7(7):727-736.

83. Kilberg MS, Barber EF, Handlogten ME. Characteristics and Hormonal Regulation of Amino Acid Transport System A in Isolated Rat Hepatocytes. *Curr Top Cell Regul*. 1985;25(C):133-163.

84. Charlton MR, Adey DB, Nair KS. Evidence for a catabolic role of glucagon during an amino acid load. *J Clin Invest*. 1996;98(1):90-99.

85. Swenson TL, Porter JW. Mechanism of glucagon inhibition of liver acetyl-CoA carboxylase. Interrelationship of the effects of phosphorylation, polymer-protomer transition, and citrate on enzyme activity. *J Biol Chem*. 1985;260(6):3791-3797.

86. Zhang X, Yang S, Chen J, Su Z. Unraveling the regulation of hepatic gluconeogenesis. *Front Endocrinol (Lausanne)*. 2019;10(JAN):431525.

87. Mcgarry JD, Brown NF. The mitochondrial carnitine palmitoyltransferase system From concept to molecular analysis. *Eur J Biochem*. 1997;244:1-14.

88. Gastaldelli A, Toschi E, Pettiti M, et al. Effect of Physiological Hyperinsulinemia on Gluconeogenesis in Nondiabetic Subjects and in Type 2 Diabetic Patients. *Diabetes*. 2001;50(8):1807-1812.

89. Saltiel AR, Kahn CR. Insulin signalling and the regulation of glucose and lipid metabolism. *Nature*. 2001;414(6865):799-806.

90. Folli F, La Rosa S, Finzi G, et al. Pancreatic islet of Langerhans' cytoarchitecture and ultrastructure in normal glucose tolerance and in type 2 diabetes mellitus. *Diabetes Obes Metab*. 2018;20 Suppl 2:137-144.

91. Marre M, Miller J, Helman AM, Assan R. Reciprocal gastropancreatic modulations for the release of somatostatin-like immunoreactivity, glucagon, and insulin in the rat. *Diabetes*. 1983;32(8):768-773.

92. Taborsky GJ, Ensinck JW. Contribution of the pancreas to circulating somatostatin-like immunoreactivity in the normal dog. *J Clin Invest*. 1984;73(1):216-223.

93. Gutniak M, Grill V, Wiechel KL, Efendić S. Basal and meal-induced somatostatin-like immunoreactivity in healthy subjects and in IDDM and totally pancreatectomized patients. Effects of acute blood glucose normalization. *Diabetes*. 1987;36(7):802-807.

94. Francis BH, Baskin DG, Saunders DR, Ensinck JW. Distribution of somatostatin-14 and somatostatin-28 gastrointestinal-pancreatic cells of rats and humans. *Gastroenterology*. 1990;99(5):1283-1291.

95. Vieira E, Salehi A, Gylfe E. Glucose inhibits glucagon secretion by a direct effect on mouse pancreatic alpha cells. *Diabetologia*. 2007;50(2):370-379.

96. Walker JN, Ramracheya R, Zhang Q, Johnson PR V., Braun M, Rorsman P. Regulation of glucagon secretion by glucose: paracrine, intrinsic or both? *Diabetes Obes Metab*. 2011;13(s1):95-105.

97. Briant LJB, Reinbothe TM, Spiliotis I, Miranda C, Rodriguez B, Rorsman P. δ-cells and β-cells are electrically coupled and regulate α-cell activity via somatostatin. *J Physiol*. 2018;596(2):197-215.

98. Svendsen B, Holst JJ. Paracrine regulation of somatostatin secretion by insulin and glucagon in mouse pancreatic islets. *Diabetologia*. 2021;64(1):142-151.

99. Miranda C, Begum M, Vergari E, Briant LJB. Gap junction coupling and islet delta-cell function in health and disease. *Peptides (NY)*. 2022;147:170704.

100. Kailey B, van de Bunt M, Cheley S, et al. SSTR2 is the functionally dominant somatostatin receptor in human pancreatic β- and α-cells. *Am J Physiol Endocrinol Metab*. 2012;303(9):E1107-E1116.

101. Singh V, Brendel MD, Zacharias S, et al. Characterization of somatostatin receptor subtype-specific regulation of insulin and glucagon secretion: an in vitro study on isolated human pancreatic islets. *J Clin Endocrinol Metab*. 2007;92(2):673-680.

102. Strowski MZ, Parmar RM, Blake AD, Schaeffer JM. Somatostatin inhibits insulin and glucagon secretion via two receptor subtypes: an in vitro study of pancreatic islets from somatostatin receptor 2 knockout Mice. *Endocrinology*. 2000;141(1):111-117.

103. Rorsman P, Ashcroft FM. Pancreatic β-Cell electrical activity and insulin secretion: of mice and men. *Physiol Rev*. 2018;98(1):117-214.

104. Briant L, Salehi A, Vergari E, Zhang Q, Rorsman P. Glucagon secretion from pancreatic α-cells. *Ups J Med Sci*. 2016;121(2):113-119.

105. Braun M. The somatostatin receptor in human pancreatic β-Cells. In: *Vitamins and Hormones*. Vol 95. Academic Press; 2014:165-193.

106. Elliott AD, Ustione A, Piston DW. Somatostatin and insulin mediate glucose-inhibited glucagon secretion in the pancreatic α-cell by lowering cAMP. *Am J Physiol Endocrinol Metab*. 2015;308(2):E130-E143.

107. Dickerson MT, Dadi PK, Zaborska KE, et al. Gi/o protein-coupled receptor inhibition of beta-cell electrical excitability and insulin secretion depends on Na+/K+ ATPase activation. *Nat Commun*. 2022;13(1):6461.

108. Hartig SM, Cox AR. Paracrine signaling in islet function and survival. *J Mol Med*. 2020;98(4):451-467.

109. Hsu WH, Xiang HD, Rajan AS, Kunze DL, Boyd AE. Somatostatin inhibits insulin secretion by a G-protein-mediated decrease in Ca2+ entry through voltage-dependent Ca2+ channels in the beta cell. *J Biol Chem*. 1991;266(2):837-843.

110. Omar-Hmeadi M, Lund PE, Gandasi NR, Tengholm A, Barg S. Paracrine control of α-cell glucagon exocytosis is compromised in human type-2 diabetes. *Nat Commun*. 2020;11(1):1896.

111. Gilon P. The role of α-cells in islet function and glucose homeostasis in health and type 2 diabetes. *J Mol Biol*. 2020;432(5):1367-1394.

112. MacDonald PE, De Marinis YZ, Ramracheya R, et al. A K ATP channel-dependent pathway within alpha cells regulates glucagon release from both rodent and human islets of Langerhans. Vidal-Puig AJ, ed. *PLoS Biol*. 2007;5(6):e143.

113. de Heer J, Rasmussen C, Coy DH, Holst JJ. Glucagon-like peptide-1, but not glucose-dependent insulinotropic peptide, inhibits glucagon secretion via somatostatin (receptor subtype 2) in the perfused rat pancreas. *Diabetologia*. 2008;51(12):2263-2270.

114. Brunicardi FC, Kleinman R, Moldovan S, et al. Immunoneutralization of somatostatin, insulin, and glucagon causes alterations in islet cell secretion in the isolated perfused human pancreas. *Pancreas*. Published online 2001.

115. Xu SFS, Andersen DB, Izarzugaza JMG, Kuhre RE, Holst JJ. In the rat pancreas, somatostatin tonically inhibits glucagon secretion and is required for glucose-induced inhibition of glucagon secretion. *Acta Physiologica*. 2020;229(3):e13464.

116. Rahier J, Goebbels RM, Henquin JC. Cellular composition of the human diabetic pancreas. *Diabetologia*. 1983;24(5).

117. Zenz S, Mader JK, Regittnig W, et al. Impact of C-Peptide status on the response of glucagon and endogenous glucose production to induced hypoglycemia in T1DM. *J Clin Endocrinol Metab*. 2018;103(4):1408-1417.

118. Madsbad S, Hilsted J, Krarup T, et al. Hormonal, metabolic and cardiovascular responses to hypoglycaemia in type 1 (insulin-dependent) diabetes with and without residual B cell function. *Diabetologia*. 1982;23:499-503.

119. Liu D, McManus RM, Ryan EA. Improved counter-regulatory hormonal and symptomatic responses to hypoglycemia in patients with insulin-dependent diabetes mellitus after 3 months of less strict glycemic control. *Clin Invest Med*. 1996;19(2):71-82.

120. Flatt AJ, Peleckis AJ, Dalton-Bakes C, et al. Automated insulin delivery for hypoglycemia avoidance and glucose counterregulation in long-standing type 1 diabetes with hypoglycemia unawareness. *Diabetes Technol Ther*. 2023;25(5):302-314.

121. Segel SA, Paramore DS, Cryer PE. Hypoglycemia-associated autonomic failure in advanced type 2 diabetes. *Diabetes*. 2002;51(3):724-733.

122. Israelian Z, Gosmanov NR, Szoke E, et al. Increasing the decrement in insulin secretion improves glucagon responses to hypoglycemia in advanced type 2 diabetes. *Diabetes Care*. 2005;28(11):2691-2696.

123. Israelian Z, Szoke E, Woerle J, et al. Multiple defects in counterregulation of hypoglycemia in modestly advanced type 2 diabetes mellitus. *Metabolism*. 2006;55(5):593-598.

124. Bolli GB, Tsalikian E, Haymond MW, Cryer PE, Gerich JE. Defective glucose counterregulation after subcutaneous insulin in noninsulin-dependent diabetes mellitus. *J Clin Invest*. 1984;73(6):1532-1541.

125. Raju B, Cryer PE. Loss of the decrement in intraislet insulin plausibly explains loss of the glucagon response to hypoglycemia in insulin-deficient diabetes: documentation of the intraislet insulin hypothesis in humans. *Diabetes*. 2005;54(3):757-764.

126. Orci L, Baetens D, Rufener C, et al. Hypertrophy and hyperplasia of somatostatin-containing D-cells in diabetes. *Proc Natl Acad Sci U S A*. 1976;73(4):1338-1342.

127. Baetens D, Stefan Y, Ravazzola M, Malaisse-Lagae F, Coleman DL, Orci L. Alteration of islet cell populations in spontaneously diabetic mice. *Diabetes*. 1978;27(1):1-7.

128. Hermansen K, Orskov H, Christensen SE. Streptozotocin diabetes: a glucoreceptor dysfunction affecting D cells as well as B and A cells. *Diabetologia*. 1979;17(6):385-389.

129. Weir GC, Clore ET, Zmachinski CJ, Bonner-Weir S. Islet secretion in a new experimental model for non-insulin-dependent diabetes. *Diabetes*. 1981;30(7):590-595.

130. Adam J, Ramracheya R, Chibalina M V., et al. Fumarate hydratase deletion in pancreatic β cells leads to progressive diabetes. *Cell Rep*. 2017;20(13):3135-3148.

131. Kellard JA, Rorsman NJG, Hill TG, et al. Reduced somatostatin signalling leads to hypersecretion of glucagon in mice fed a high-fat diet. *Mol Metab*. 2020;40:101021.

132. Singh B, Khattab F, Gilon P. Glucose inhibits glucagon secretion by decreasing [Ca2+]c and by reducing the efficacy of Ca2+ on exocytosis via somatostatin-dependent and independent mechanisms. *Mol Metab*. 2022;61:101495.

133. Berts A, Ball A, Dryselius S, Gylfe E, Hellman B. Glucose stimulation of somatostatin-producing islet cells involves oscillatory Ca2+ signaling. *Endocrinology*. 1996;137(2):693-697.

134. Weir GC, Bonner-Weir S. Conflicting views about interactions between pancreatic α-cells and β-cells. *Diabetes*. 2023;72(12):1741-1747.

135. Brereton MF, Vergari E, Zhang Q, Clark A. Alpha-, delta- and PP-cells. *J Histochem Cytochem*. 2015;63(8):575-591.

136. Amo-Shiinoki K, Tanabe K, Hoshii Y, et al. Islet cell dedifferentiation is a pathologic mechanism of long-standing progression of type 2 diabetes. *JCI Insight*. 2021;6(1):e143791.

137. Deng S, Vatamaniuk M, Huang X, et al. Structural and functional abnormalities in the islets isolated from type 2 diabetic subjects. *Diabetes*. 2004;53(3):624-632.

138. Kilimnik G, Zhao B, Jo J, et al. Altered islet composition and disproportionate loss of large islets in patients with type 2 diabetes. Chung S, ed. *PLoS One*. 2011;6(11):e27445.

139. Tegehall A, Korsgren O, Ingvast S, Gasparyan G, Granlund L, Lundberg M. Islet delta-cell architecture is remodelled in the human pancreas during type 1 diabetes. *Sci Rep*. 2025;15(1):19776.

140. Rastogi KS, Brubaker PL, Kawasaki A, Efendic S, Vranic M. Increase in somatostatin to glucagon ratio in islets of alloxan-diabetic dogs: effect of insulin-induced euglycemia. *Can J Physiol Pharmacol*. 1993;71(7):512-517.

141. Jiang S, Young JL, Wang K, Qian Y, Cai L. Diabetic-induced alterations in hepatic glucose and lipid metabolism: The role of type 1 and type 2 diabetes mellitus. *Mol Med Rep*. 2020;22(2):603.

142. Exton JH, Park CR. Control of Gluconeogenesis in Liver: General features of gluconeogenesis in the perfused livers of rats. *J Biol Chem*. 1967;242(11):2622-2636.

143. Jahoor F, Peters EJ, Wolfe RR. The relationship between gluconeogenic substrate supply and glucose production in humans. *Am J Physiol Endocrinol Metab*. 1990;258(2 21-2).

144. Jenssen T, Nurjhan N, Consoli A, Gerich JE. Failure of substrate-induced gluconeogenesis to increase overall glucose appearance in normal humans: Demonstration of hepatic autoregulation without a change in plasma glucose concentration. *J Clin Invest*. 1990;86(2):489-497.

145. Petersen KF, Price TB, Bergeron R. Regulation of Net Hepatic Glycogenolysis and Gluconeogenesis during Exercise: Impact of Type 1 Diabetes. *J Clin Endocrinol Metab*. 2004;89(9):4656-4664.

146. Kishore P, Gabriely I, Cui MH, et al. Role of hepatic glycogen breakdown in defective counterregulation of hypoglycemia in intensively treated type 1 diabetes. *Diabetes*. 2006;55(3):659-666.

147. Orskov L, Alberti KG, Mengel A, et al. Decreased hepatic glucagon responses in type 1 (insulin-dependent) diabetes mellitus. *Diabetologia*. 1991;34(7):521-526.

148. Song Z, Routh VH. Recurrent hypoglycemia reduces the glucose sensitivity of glucose-inhibited neurons in the ventromedial hypothalamus nucleus. *Am J Physiol Regul Integr Comp Physiol*. 2006;291(5):R1283-7.

149. Tesfaye N, Seaquist ER. Neuroendocrine Responses to Hypoglycemia. *Ann N Y Acad Sci*. 2010;1212:12.

150. Simpson IA, Appel NM, Hokari M, et al. Blood-brain barrier glucose transporter: Effects of hypo- and hyperglycemia revisited. *J Neurochem*. 1999;72(1):238-247.

151. Osundiji MA, Hurst P, Moore SP, et al. Recurrent hypoglycemia increases hypothalamic glucose phosphorylation activity in rats. *Metabolism*. 2011;60(4):550-556.

152. Sayed N, Baskaran P, Ma X, van den Akker F, Beuve A. Desensitization of soluble guanylyl cyclase, the NO receptor, by S-nitrosylation. *Proc Natl Acad Sci U S A*. 2007;104(30):12312-12317.

153. Mastrocola R, Restivo F, Vercellinatto I, et al. Oxidative and nitrosative stress in brain mitochondria of diabetic rats. *J Endocrinol*. 2005;187(1):37-44.

154. Chan O, Zhu W, Ding Y, McCrimmon RJ, Sherwin RS. Blockade of GABA(A) receptors in the ventromedial hypothalamus further stimulates glucagon and sympathoadrenal but not the hypothalamo-pituitary-adrenal response to hypoglycemia. *Diabetes*. 2006;55(4):1080-1087.

155. Zhu W, Czyzyk D, Paranjape SA, et al. Glucose prevents the fall in ventromedial hypothalamic GABA that is required for full activation of glucose counterregulatory responses during hypoglycemia. *Am J Physiol Endocrinol Metab*. 2010;298(5):E971-7.

156. Chan O, Cheng H, Herzog R, et al. Increased GABAergic tone in the ventromedial hypothalamus contributes to suppression of counterregulatory responses after antecedent hypoglycemia. *Diabetes*. 2008;57(5):1363-1370.

157. Hedrington MS, Farmerie S, Ertl AC, Wang Z, Tate DB, Davis SN. Effects of antecedent GABAA activation with alprazolam on counterregulatory responses to hypoglycemia in healthy humans. *Diabetes*. 2010;59(4):1074-1081.

158. Dienel GA, Cruz NF. Astrocyte activation in working brain: Energy supplied by minor substrates. *Neurochem Int*. 2006;48(6-7):586-595.

159. Choi IY, Seaquist ER, Gruetter R. Effect of Hypoglycemia on Brain glycogen metabolism in vivo. *J Neurosci Res*. 2003;72(1):25.

160. Öz G, DiNuzzo M, Kumar A, et al. Cerebral glycogen in humans following acute and recurrent hypoglycemia: Implications on a role in hypoglycemia unawareness. *J Cereb Blood Flow Metab*. 2016;37(8):2883.

161. Chan O, Paranjape SA, Horblitt A, Zhu W, Sherwin RS. Lactate-induced release of GABA in the ventromedial hypothalamus contributes to counterregulatory failure in recurrent hypoglycemia and diabetes. *Diabetes*. 2013;62(12):4239-4246.

162. Borg MA, Tamborlane W V., Shulman GI, Sherwin RS. Local lactate perfusion of the ventromedial hypothalamus suppresses hypoglycemic counterregulation. *Diabetes*. 2003;52(3):663-666.

163. Herzog RI, Jiang L, Herman P, et al. Lactate preserves neuronal metabolism and function following antecedent recurrent hypoglycemia. *J Clin Invest*. 2013;123(5):1988-1998.

164. Shah M, Addison A, Wang P, Zhu W, Chan O. Recurrent glucose deprivation leads to the preferential use of lactate by neurons in the ventromedial hypothalamus. *Am J Physiol Endocrinol Metab*. 2019;316(5):E948.

165. Alquier T, Kawashima J, Tsuji Y, Kahn BB. Role of Hypothalamic Adenosine 5′-Monophosphate-Activated Protein Kinase in the Impaired Counterregulatory Response Induced by Repetitive Neuroglucopenia. *Endocrinology*. 2007;148(3):1367-1375.

166. Lubow JM, Piñón IG, Avogaro A, et al. Brain oxygen utilization is unchanged by hypoglycemia in normal humans: Lactate, alanine, and leucine uptake are not sufficient to offset energy deficit. *Am J Physiol Endocrinol Metab*. 2006;290(1).

167. Herzog RI, Chan O, Yu S, Dziura J, McNay EC, Sherwin RS. Effect of Acute and Recurrent Hypoglycemia on Changes in Brain Glycogen Concentration. *Endocrinology*. 2008;149(4):1499-1504.

168. Bischof MG, Bernroider E, Krssak M, et al. Hepatic glycogen metabolism in type 1 diabetes after long-term near normoglycemia. *Diabetes*. 2002;51(1):49-54.

169. Mallad A, Hinshaw L, Schiavon M, et al. Exercise effects on postprandial glucose metabolism in type 1 diabetes: a triple-tracer approach. *Am J Physiol Endocrinol Metab*. 2015;308(12):E1106-E1115.

170. Leclair E, Liggins RT, Peckett AJ, et al. Glucagon responses to exercise-induced hypoglycaemia are improved by somatostatin receptor type 2 antagonism in a rat model of diabetes. *Diabetologia*. 2016;59(8):1724-1731.

171. Farhat R, Aiken J, D'Souza NC, et al. ZT-01: A novel somatostatin receptor 2 antagonist for restoring the glucagon response to hypoglycaemia in type 1 diabetes. *Diabetes Obes Metab*. 2022;24(5):908-917.

172. Abitbol A, Peers S, Simonson E, Midmer M, Riddell M, Liggins R. 219-OR: Glucagon counterregulation in a hypoglycemic clamp in type 1 diabetes Is increased by ZT-01, a novel somatostatin receptor 2 antagonist—a phase 1b study. *Diabetes*. 2023;72(Supplement_1).

173. A study of the effect of ZT-01 on night-time hypoglycemia in type 1 diabetes (ZONE). Identifier: NCT05762107. https://clinicaltrials.gov/study/NCT05762107.

174. Baggio LL, Drucker DJ. Biology of incretins: GLP-1 and GIP. *Gastroenterology*. 2007;132(6):2131-2157.

175. Toft-Nielsen MB, Damholt MB, Madsbad S, et al. Determinants of the impaired secretion of glucagon-like peptide-1 in type 2 diabetic Patients. *J Clin Endocrinol Metab*. 2001;86(8):3717-3723.

176. Vilsbøll T, Krarup T, Deacon CF, Madsbad S, Holst JJ. Reduced postprandial concentrations of intact biologically active glucagon-like peptide 1 in type 2 diabetic patients. *Diabetes*. 2001;50(3):609-613.

177. Nauck M, Stöckmann F, Ebert R, Creutzfeldt W. Reduced incretin effect in type 2 (non-insulin-dependent) diabetes. *Diabetologia*. 1986;29(1):46-52.

178. Muscelli E, Mari A, Casolaro A, et al. Separate impact of obesity and glucose tolerance on the incretin effect in normal subjects and type 2 diabetic patients. *Diabetes*. 2008;57(5):1340-1348.

179. Zander M, Madsbad S, Madsen JL, Holst JJ. Effect of 6-week course of glucagon-like peptide 1 on glycaemic control, insulin sensitivity, and β-cell function in type 2 diabetes: a parallel-group study. *The Lancet*. 2002;359(9309):824-830.

180. Hinnen D. Glucagon-like peptide 1 receptor agonists for type 2 diabetes. *Diabetes Spectrum*. 2017;30(3):202-210.

181. Jepsen SL, Grunddal K V., Wewer Albrechtsen NJ, et al. Paracrine crosstalk between intestinal L- and D-cells controls secretion of glucagon-like peptide-1 in mice. *Am J Physiol Endocrinol Metab*. 2019;317(6):E1081-E1093.

182. Liu W, Shao PP, Liang GB, et al. Discovery and pharmacology of a novel somatostatin subtype 5 (SSTR5) antagonist: synergy with DPP-4 inhibition. *ACS Med Chem Lett*. 2018;9(11):1082-1087.

183. Jepsen SL, Albrechtsen NJW, Windeløv JA, et al. Antagonizing somatostatin receptor subtype 2 and 5 reduces blood glucose in a gut- and GLP-1R-dependent manner. *JCI Insight*. 2021;6(4).

184. Farb TB, Adeva M, Beauchamp TJ, et al. Regulation of endogenous (male) rodent GLP-1 secretion and human islet insulin secretion by antagonism of somatostatin receptor 5. *Endocrinology*. 2017;158(11):3859-3873.

185. Sprecher U, Mohr P, Martin RE, et al. Novel, non-peptidic somatostatin receptor subtype 5 antagonists improve glucose tolerance in rodents. *Regul Pept*. 2010;159(1-3):19-27.

186. Tamura YO, Sugama J, Abe S, Shimizu Y, Hirose H, Watanabe M. Selective somatostatin receptor 5 inhibition improves hepatic insulin sensitivity. *Pharmacol Res Perspect*. 2023;11(1).

187. Drucker DJ, Nauck MA. The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *Lancet*. Preprint posted online 2006.

188. El K, Gray SM, Capozzi ME, et al. GIP mediates the incretin effect and glucose tolerance by dual actions on α cells and β cells. *Sci Adv*. 2021;7(11).

189. El K, Campbell JE. The role of GIP in α-cells and glucagon secretion. *Peptides (NY)*. 2020;125:170213.

190. Ding WG, Renström E, Rorsman P, Buschard K, Gromada J. Glucagon-like peptide I and glucose-dependent insulinotropic polypeptide stimulate Ca2+-induced secretion in rat α-cells by a protein kinase A–mediated mechanism. *Diabetes*. 1997;46(5):792-800.

191. Christensen M, Vedtofte L, Holst JJ, Vilsbøll T, Knop FK. Glucose-dependent insulinotropic polypeptide: a bifunctional glucose-dependent regulator of glucagon and insulin secretion in humans. *Diabetes*. 2011;60(12):3103-3109.

192. Meier JJ, Gallwitz B, Siepmann N, et al. Gastric inhibitory polypeptide (GIP) dose-dependently stimulates glucagon secretion in healthy human subjects at euglycaemia. *Diabetologia*. 2003;46(6):798-801.

193. Nwokolo M, Hovorka R. The Artificial Pancreas and Type 1 Diabetes. *J Clin Endocrinol Metab*. 2023;108(7):1614-1623.

194. Bergenstal RM, Klonoff DC, Garg SK, et al. Threshold-Based Insulin-Pump Interruption for Reduction of Hypoglycemia. *N Engl J Med*. 2013;369(3):224-232.

195. Wong EY, Kroon L. Ultra-Rapid-Acting Insulins: How Fast Is Really Needed? *Clinical Diabetes*. 2021;39(4):415-423.

196. Infante M, Baidal DA, Rickels MR, et al. Dual-hormone artificial pancreas for management of type 1 diabetes: Recent progress and future directions. *Artif Organs*. 2021;45(9):968.

197. Haidar A, Smaoui MR, Legault L, Rabasa-Lhoret R. The role of glucagon in the artificial pancreas. *Lancet Diabetes Endocrinol*. 2016;4(6):476-479.

198. Peters TM, Haidar A. Dual-hormone artificial pancreas: benefits and limitations compared with single-hormone systems. *Diabetic Medicine*. 2018;35(4):450-459.

199. Haidar A. Insulin-and-Glucagon Artificial Pancreas Versus Insulin-Alone Artificial Pancreas: A Short Review. *Diabetes Spectr*. 2019;32(3):215.

200. Russell SJ, El-Khatib FH, Nathan DM, Damiano ER. Efficacy determinants of subcutaneous microdose glucagon during closed-loop control. *J Diabetes Sci Technol*. 2010;4(6):1288-1304.

201. El Youssef J, Castle JR, Bakhtiani PA, et al. Quantification of the Glycemic Response to Microdoses of Subcutaneous Glucagon at Varying Insulin Levels. *Diabetes Care*. 2014;37(11):3054-3060.

202. American Diabetes Association Professional Practice Committee. Classification and diagnosis of diabetes: standards of medical care in diabetes—2022. *Diabetes Care*. 2022;45(Supplement_1):S17-S38.

203. Taleb N, Haidar A, Messier Msc V, Gingras Msc V, Legault L, Rabasa-Lhoret R. Glucagon in artificial pancreas systems: Potential benefits and safety profile of future chronic use. Published online 2016.

204. Hövelmann U, Olsen MB, Mouritzen U, Lamers D, Kronshage B, Heise T. Low doses of dasiglucagon consistently increase plasma glucose levels from hypoglycaemia and euglycaemia in people with type 1 diabetes mellitus. *Diabetes Obes Metab*. 2019;21(3):601-610.

205. Cryer PE. Mechanisms of hypoglycemia-associated autonomic failure and its component syndromes in diabetes. *Diabetes*. 2005;54(12):3592-3601.

206. Mokan M, Mitrakou A, Veneman T, et al. Hypoglycemia unawareness in IDDM. *Diabetes Care*. 1994;17(12):1397-1403.

207. Heller SR, Cryer PE. Reduced neuroendocrine and symptomatic responses to subsequent hypoglycemia after 1 episode of hypoglycemia in nondiabetic humans. *Diabetes*. 1991;40(2):223-226.

208. Davis SN. Effects of morning hypoglycemia on neuroendocrine and metabolic responses to subsequent afternoon hypoglycemia in normal man. *J Clinc Endocrinol Metab*. 2001;86(5):2043-2050.

209. Davis MR, Shamoon H. Counterregulatory adaptation to recurrent hypoglycemia in normal humans. *J Clin Endocrinol Metab*. 1991;73(5):995-1001.

210. Davis SN, Shavers C, Mosqueda-Garcia R, Costa F. Effects of differing antecedent hypoglycemia on subsequent counterregulation in normal humans. *Diabetes*. 1997;46(8):1328-1335.

211. Fanelli CG, Epifano L, Rambotti AM, et al. Meticulous prevention of hypoglycemia normalizes the glycemic thresholds and magnitude of most of neuroendocrine responses to, symptoms of, and cognitive function during hypoglycemia in intensively treated patients with short-term IDDM. *Diabetes*. 1993;42(11):1683-1689.

212. Dagogo-Jack S, Rattarasarn C, Cryer PE. Reversal of hypoglycemia unawareness, but not defective glucose counterregulation, in IDDM. *Diabetes*. 1994;43(12):1426-1434.

213. Gupta V, Wahoff DC, Rooney DP, et al. The defective glucagon response from transplanted intrahepatic pancreatic islets during hypoglycemia is transplantation site-determined. *Diabetes*. 1997;46(1):28-33.

214. Palmer JP, Henry DP, Benson JW, Johnson DG, Ensinck JW. Glucagon response to hypoglycemia in sympathectomized man. *J Clin Invest*. 1976;57(2):522-525.

215. Hilsted J, Frandsen H, Holst JJ, Christensen NJ, Nielsen SL. Plasma glucagon and glucose recovery after hypoglycemia: the effect of total autonomic blockade. *Acta Endocrinol (Copenh)*. 1991;125(4):466-469.

216. Gerich JE, Charles MA, Grodsky GM. Characterization of the effects of arginine and glucose on glucagon and insulin release from the perfused rat pancreas. *J Clin Invest*. 1974;54(4):833-841.

217. Walker JN, Ramracheya R, Zhang Q, Johnson PR V., Braun M, Rorsman P. Regulation of glucagon secretion by glucose: paracrine, intrinsic or both? *Diabetes Obes Metab*. 2011;13(s1):95-105.

218. Banarer S, McGregor VP, Cryer PE. Intraislet hyperinsulinemia prevents the glucagon response to hypoglycemia despite an intact autonomic response. *Diabetes*. 2002;51(4):958-965.

219. Cejvan K, Coy DH, Efendic S. Intra-islet somatostatin regulates glucagon release via type 2 somatostatin receptors in rats. *Diabetes*. 2003;52(5):1176-1181.

220. Orci L, Baetens D, Rufener C, et al. Hypertrophy and hyperplasia of somatostatin-containing D-cells in diabetes. *Proceedings of the National Academy of Sciences*. 1976;73(4):1338-1342.

221. Rastogi KS, Lickley L, Jokay M, Efendic S, Vranic M. Paradoxical reduction in pancreatic glucagon with normalization of somatostatin and decrease in insulin in normoglycemic alloxan-diabetic dogs: a putative mechanism of glucagon irresponsiveness to hypoglycemia. *Endocrinology*. 1990;126(2):1096-1104.

222. Patel YC, Cameron DP, Bankier A, et al. Changes in somatostatin concentration in pancreas and other tissues of streptozotocin diabetic rats. *Endocrinology*. 1978;103(3):917-923.

223. Inouye K, Shum K, Chan O, Mathoo J, Matthews SG, Vranic M. Effects of recurrent hyperinsulinemia with and without hypoglycemia on counterregulation in diabetic rats. *Am J Physiol Endocrinol Metab*. 2002;282(6):E1369-E1379.

224. Inouye KE, Yue JTY, Chan O, et al. Effects of insulin treatment without and with recurrent hypoglycemia on hypoglycemic counterregulation and adrenal catecholamine-synthesizing enzymes in diabetic rats. *Endocrinology*. 2006;147(4):1860-1870.

225. Vergari E, Knudsen JG, Ramracheya R, et al. Insulin inhibits glucagon release by SGLT2-induced stimulation of somatostatin secretion. *Nat Commun*. 2019;10(1):139.

226. Effect of ZT-01 on Glucagon During Hypoglycemia in Type 1 Diabetes Mellitus. Identifier: NCT05007977. Accessed August 26, 2021. https://www.clinicaltrials.gov/ct2/show/NCT05007977

227. Seaquist ER, Anderson J, Childs B, et al. Hypoglycemia and diabetes: a report of a workgroup of the American Diabetes Association and the Endocrine Society. *Diabetes Care*. 2013;36(5):1384-1395.

228. Osundiji MA, Hurst P, Moore SP, et al. Recurrent hypoglycemia increases hypothalamic glucose phosphorylation activity in rats. *Metabolism*. 2011;60(4):550-556.

229. Chowdhury GM, Wang P, Ciardi A, et al. Impaired glutamatergic neurotransmission in the ventromedial hypothalamus may contribute to defective counterregulation in recurrently hypoglycemic rats. *Diabetes*. 2017;66(7):1979-1989.

230. McCrimmon RJ, Evans ML, Fan X, et al. Activation of ATP-sensitive K+ channels in the ventromedial hypothalamus amplifies counterregulatory hormone responses to hypoglycemia in normal and recurrently hypoglycemic rats. *Diabetes*. 2005;54(11):3169-3174.

231. Schwartz NS, Clutter WE, Shah SD, Cryer PE. Glycemic thresholds for activation of glucose counterregulatory systems are higher than the threshold for symptoms. *J Clin Invest*. 1987;79(3):777-781.

232. Widom B, Simonson DC. Intermittent hypoglycemia impairs glucose counterregulation. *Diabetes*. 1992;41(12):1597-1602.

233. Hocart SJ, Jain R, Murphy WA, Taylor JE, Coy DH. Highly potent cyclic disulfide antagonists of somatostatin. *J Med Chem*. 1999;42(11):1863-1871.

234. Traber MG, Atkinson J. Vitamin E, antioxidant and nothing more. *Free Radic Biol Med*. 2007;43(1):4-15.

235. Carr RS, Neff JM. Quantitative semi-automated enzymatic assay for tissue glycogen. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*. 1984;77(3):447-449.

236. Moheet A, Kumar A, Eberly LE, Kim J, Roberts R, Seaquist ER. Hypoglycemia-associated autonomic failure in healthy humans: comparison of two vs three periods of hypoglycemia on hypoglycemia-induced counterregulatory and symptom response 5 days later. *J Clin Endocrinol Metab*. 2014;99(2):664-670.

237. Powell AM, Sherwin RS, Shulman GI. Impaired hormonal responses to hypoglycemia in spontaneously diabetic and recurrently hypoglycemic rats. Reversibility and stimulus specificity of the deficits. *J Clin Invest*. 1993;92(6):2667-2674.

238. Xu SFS, Andersen DB, Izarzugaza JMG, Kuhre RE, Holst JJ. In the rat pancreas, somatostatin tonically inhibits glucagon secretion and is required for glucose-induced inhibition of glucagon secretion. *Acta Physiologica*. 2020;229(3):e13464.

239. Ramnanan CJ, Edgerton DS, Kraft G, Cherrington AD. Physiologic action of glucagon on liver glucose metabolism. *Diabetes Obes Metab*. 2011;13(1):118-125.

240. Hwang JH, Perseghin G, Rothman DL, et al. Impaired net hepatic glycogen synthesis in insulin-dependent diabetic subjects during mixed meal ingestion. A

13C nuclear magnetic resonance spectroscopy study. *J Clin Invest*. 1995;95(2):783-787.

241. Kishore P, Gabriely I, Cui MH, et al. Role of hepatic glycogen breakdown in defective counterregulation of hypoglycemia in intensively treated type 1 diabetes. *Diabetes*. 2006;55(3):659-666.

242. Winnick JJ, Kraft G, Gregory JM, et al. Hepatic glycogen can regulate hypoglycemic counterregulation via a liver-brain axis. *J Clin Invest*. 2016;126(6):2236-2248.

243. Shum K, Inouye K, Chan O, et al. Effects of antecedent hypoglycemia, hyperinsulinemia, and excess corticosterone on hypoglycemic counterregulation. *Am. J. Physiol. Endocrinol. Metab*. 2001;281(3):E455-E465.

244. Patel YC. Somatostatin and its receptor family. *Front Neuroendocrinol*. 1999;20(3):157-198.

245. Sindelar DK, Chu CA, Venson P, Donahue EP, Neal DW, Cherrington AD. Basal hepatic glucose production is regulated by the portal vein insulin concentration. *Diabetes*. 1998;47(4):523-529.

246. de Heer J, Rasmussen C, Coy DH, Holst JJ. Glucagon-like peptide-1, but not glucose-dependent insulinotropic peptide, inhibits glucagon secretion via somatostatin (receptor subtype 2) in the perfused rat pancreas. *Diabetologia*. 2008;51(12):2263-2270.

248. Tajiri Y, Grill VE. Interactions between vitamin E and glucose on B-cell functions in the rat: an in vivo and in vitro study. *Pancreas*. 1999;18(3):274-281.

249. Minamiyama Y, Takemura S, Bito Y, et al. Supplementation of α-tocopherol improves cardiovascular risk factors via the insulin signalling pathway and reduction of mitochondrial reactive oxygen species in type II diabetic rats. *Free Radic Res*. 2008;42(3):261-271.

250. Facchini F, Coulston AM, Reaven GM. Relation between dietary vitamin intake and resistance to insulin-mediated glucose disposal in healthy volunteers. *Am J Clin Nutr*. 1996;63(6):946-949.

251. Al Shamsi MS, Amin A, Adeghate E. Beneficial effect of vitamin E on the metabolic parameters of diabetic rats. *Mol Cell Biochem*. 2004;261(1):35-42.

252. Teng XW, Davies NM, Fukuda C, Good RL, Fariss MW. Pharmacokinetics and tissue distribution of d-alpha-tocopheryl succinate formulations following intravenous administration in the rat. *Biopharm Drug Dispos*. 2005;26(5):195-203.

253. Rizza RA, Cryer PE, Gerich JE. Role of glucagon, catecholamines, and growth hormone in human glucose counterregulation. *J Clin Invest*. 1979;64(1):62-71.

254. Davis SN, Mann S, Briscoe VJ, Ertl AC, Tate DB. Effects of intensive therapy and antecedent hypoglycemia on counterregulatory responses to hypoglycemia in type 2 diabetes. *Diabetes*. 2009;58(3):701-709.

255. Hauge-Evans AC, King AJ, Carmignac D, et al. Somatostatin secreted by islet δ-cells fulfills multiple roles as a paracrine regulator of islet function. *Diabetes*. 2009;58(2):403-411.

256. Cabrera O, Berman DM, Kenyon NS, Ricordi C, Berggren PO, Caicedo A. The unique cytoarchitecture of human pancreatic islets has implications for islet cell function. *Proc Natl Acad Sci*. 2006;103(7):2334-2339.

257. Cejvan K, Coy DH, Efendic S. Intra-islet somatostatin regulates glucagon release via type 2 somatostatin receptors in rats. *Diabetes*. 2003;52(5):1176-1181.

258. Hoffman EG, Jahangiriesmaili M, Mandel ER, et al. Somatostatin receptor antagonism reverses glucagon counterregulatory failure in recurrently hypoglycemic male rats. *Endocrinology*. 2021;162(12).

259. Stern JH, Smith GI, Chen S, Unger RH, Klein S, Scherer PE. Obesity dysregulates fasting-induced changes in glucagon secretion. *J Endocrinol*. 2019;243(2):149-160.

260. Merino B, Alonso-Magdalena P, Lluesma M, et al. Pancreatic alpha-cells from female mice undergo morphofunctional changes during compensatory adaptations of the endocrine pancreas to diet-induced obesity. *Sci Rep*. 2015;5(1):11622.

261. Ebrahimi AG, Hollister-Lock J, Sullivan BA, Tsuchida R, Bonner-Weir S, Weir GC. Beta-cell identity changes with mild hyperglycemia: implications for function, growth, and vulnerability. *Mol Metab*. 2020;35:100959.

262. Leahy JL, Bonner-Weir S, Weir GC. Beta-cell dysfunction induced by chronic hyperglycemia. Current ideas on mechanism of impaired glucose-induced insulin secretion. *Diabetes Care*. 1992;15(3):442-455.

263. Wang Z, York NW, Nichols CG, Remedi MS. Pancreatic β-cell dedifferentiation in diabetes and redifferentiation following insulin therapy. *Cell Metab*. 2014;19(5):872-882.

264. Srinivasan K, Viswanad B, Asrat L, Kaul CL, Ramarao P. Combination of high-fat diet-fed and low-dose streptozotocin-treated rat: a model for type 2 diabetes and pharmacological screening. *Pharmacol Res*. 2005;52(4):313-320.

265. Tschen SI, Dhawan S, Gurlo T, Bhushan A. Age-dependent decline in beta-cell proliferation restricts the capacity of beta-cell regeneration in mice. *Diabetes*. 2009;58(6):1312-1320.

266. Grossman EJ, Lee DD, Tao J, et al. Glycemic control promotes pancreatic beta-cell regeneration in streptozotocin-induced diabetic mice. *PLoS One*. 2010;5(1):e8749.

267. Rossowski WJ, Coy DH. Specific inhibition of rat pancreatic insulin or glucagon release by receptor-selective somatostatin analogs. *Biochem Biophys Res Commun*. 1994;205(1):341-346.

268. Ito K, Maruyama H, Hirose H, et al. Exogenous insulin dose-dependently suppresses glucopenia-induced glucagon secretion from perfused rat pancreas. *Metabolism*. 1995;44(3):358-362.

269. Weir GC, Bonner-Weir S. Five stages of evolving beta-cell dysfunction during progression to diabetes. *Diabetes*. 2004;53(suppl_3):S16-S21.

270. Colagiuri S. Definition and classification of diabetes and prediabetes and emerging data on phenotypes. *Endocrinol Metab Clin North Am*. 2021;50(3):319-336.

271. Fonseca VA. Defining and characterizing the progression of type 2 diabetes. *Diabetes Care*. 2009;32(suppl_2):S151-S156.

272. Campbell JE, Newgard CB. Mechanisms controlling pancreatic islet cell function in insulin secretion. *Nat Rev Mol Cell Biol*. 2021;22(2):142-158.

273. Thieu VT, Mitchell BD, Varnado OJ, Frier BM. Treatment and prevention of severe hypoglycaemia in people with diabetes: Current and new formulations of glucagon. *Diabetes Obes Metab*. 2020;22(4):469.

274. Rodgers RL. Glucagon, cyclic AMP, and hepatic glucose mobilization: A half-century of uncertainty. *Physiol Rep*. 2022;10(9):e15263.

275. Blauw H, Wendl I, DeVries JH, Heise T, Jax T, PCDIAB consortium. Pharmacokinetics and pharmacodynamics of various glucagon dosages at different blood glucose levels. *Diabetes Obes Metab*. 2016;18(1):34-39.

276. Pacchioni M, Orena C, Panizza P, Cucchi E, Del Maschio A, Pontiroli AE. The hypotonic effect of intranasal and intravenous glucagon in gastrointestinal radiology. *Abdom Imaging*. 1995;20(1):44-46.

277. Christiansen MP, Cummins M, Prestrelski S, Close NC, Nguyen A, Junaidi K. Comparison of a ready-to-use liquid glucagon injection administered by autoinjector to glucagon emergency kit for the symptomatic relief of severe hypoglycemia: two

randomized crossover non-inferiority studies. *BMJ Open Diabetes Res Care*. 2021;9(1):e002137.

278. Amphastar Pharmaceuticals, Inc. Glucagon for Injection. Prescribing Information. https://amphastar.com/assets/glucagon-10-19.pdf

279. Cryer PE. The Barrier of Hypoglycemia in Diabetes. *Diabetes*. 2008;57(12):3169-3176.

280. Davis MR, Shamoon H. Counterregulatory adaptation to recurrent hypoglycemia in normal humans. *J Clin Endocrinol Metab*. 1991;73(5):995-1001.

281. Davis SN, Shavers C, Mosqueda-Garcia R, Costa F. Effects of differing antecedent hypoglycemia on subsequent counterregulation in normal humans. *Diabetes*. 1997;46(8):1328-1335.

282. Hoffman EG, D'Souza NC, Aiken J, Atherley S, Liggins R, Riddell MC. Effects of somatostatin receptor type 2 antagonism during insulin-induced hypoglycaemia in male rats with prediabetes. *Diabetes Obes Metab*. 2023;25(6):1547-1556.

283. D'Souza NC, Aiken JA, Hoffman EG, et al. Evaluating the effectiveness of a novel somatostatin receptor 2 antagonist, ZT-01, for hypoglycemia prevention in a rodent model of type 2 diabetes. *Front Pharmacol*. 2024;15:1302015.

284. Orskov C, Wettergren A, Holst JJ. Secretion of the incretin hormones glucagon-like peptide-1 and gastric inhibitory polypeptide correlates with insulin secretion in normal man throughout the day. *Scand J Gastroenterol*. 1996;31(7):665-670.

285. Christensen M, Calanna S, Sparre-Ulrich AH, et al. Glucose-dependent insulinotropic polypeptide augments glucagon responses to hypoglycemia in type 1 diabetes. *Diabetes*. 2015;64(1):72-78.

286. Christensen MB, Calanna S, Holst JJ, Vilsbøll T, Knop FK. Glucose-dependent insulinotropic polypeptide: blood glucose stabilizing effects in patients with type 2 diabetes. *J Clin Endocrinol Metab*. 2014;99(3):E418-26.

287. Pederson RA, Brown JC. Interaction of gastric inhibitory polypeptide, glucose, and arginine on insulin and glucagon secretion from the perfused rat pancreas. *Endocrinology*. 1978;103(2):610-615.

288. Young AA, Cooper GJ, Carlo P, Rink TJ, Wang MW. Response to intravenous injections of amylin and glucagon in fasted, fed, and hypoglycemic rats. *Am J Physiol*. 1993;264(6 Pt 1):E943-50.

289. Wang MW, Carlo P, Rink TJ, Young AA. Amylin is more potent and more effective than glucagon in raising plasma glucose concentration in fasted, anesthetized rats. *Biochem Biophys Res Commun*. 1991;181(3):1288-1293.

290. Eli Lily, Canada. Baqsimi. Product Monograph. https://pi.lilly.com/ca/baqsimi-ca-pm.pdf.

291. Kim T, Nason S, Antipenko J, et al. Hepatic mTORC2 signaling facilitates acute glucagon receptor enhancement of insulin-stimulated glucose homeostasis in mice. *Diabetes*. 2022;71(10):2123-2135.

292. Kim T, Holleman CL, Nason S, et al. Hepatic glucagon receptor signaling enhances insulin-stimulated glucose disposal in rodents. *Diabetes*. 2018;67(11):2157-2166.

293. Mohammad S, Ramos LS, Buck J, Levin LR, Rubino F, McGraw TE. Gastric inhibitory peptide controls adipose insulin sensitivity via activation of cAMP-response element-binding protein and p110β isoform of phosphatidylinositol 3-kinase. *J Biol Chem*. 2011;286(50):43062-43070.

294. Svendsen B, Larsen O, Gabe MBN, et al. Insulin secretion depends on intra-islet glucagon signaling. *Cell Rep*. 2018;25(5):1127-1134.e2.

295. Rivera N, Ramnanan CJ, An Z, et al. Insulin-induced hypoglycemia increases hepatic sensitivity to glucagon in dogs. *J Clin Invest*. 2010;120(12):4425-4435.

296. Horn CC, Kimball BA, Wang H, et al. Why can't rodents vomit? A comparative behavioral, anatomical, and physiological study. *PLoS One*. 2013;8(4):e60537.

297. Vergari E, Knudsen JG, Ramracheya R, et al. Insulin inhibits glucagon release by SGLT2-induced stimulation of somatostatin secretion. *Nat Commun*. 2019;10(1):139.

298. Yu Q, Shuai H, Ahooghalandari P, Gylfe E, Tengholm A. Glucose controls glucagon secretion by directly modulating cAMP in alpha cells. *Diabetologia*. 2019;62(7):1212-1224.

299. Ramracheya R, Chapman C, Chibalina M, et al. GLP-1 suppresses glucagon secretion in human pancreatic alpha-cells by inhibition of P/Q-type Ca2+ channels. *Physiol Rep*. 2018;6(17):e13852.

300. Ramracheya R, Ward C, Shigeto M, et al. Membrane potential-dependent inactivation of voltage-gated ion channels in alpha-cells inhibits glucagon secretion from human islets. *Diabetes*. 2010;59(9):2198-2208.

301. Zhang Q, Ramracheya R, Lahmann C, et al. Role of KATP channels in glucose-regulated glucagon secretion and impaired counterregulation in type 2 diabetes. *Cell Metab*. 2013;18(6):871-882.

302. Poitout V, Amyot J, Semache M, Zarrouki B, Hagman D, Fontés G. Glucolipotoxicity of the pancreatic beta cell. *Biochim Biophys Acta*. 2010;1801(3):289-298.

303. Hannon JP, Nunn C, Stolz B, et al. Drug Design at Peptide Receptors. *J Mol Neurosci*. 2002;18(1-2):15-28.

304. Rastogi KS, Lickley L, Jokay M, Efendic S, Vranic M. Paradoxical reduction in pancreatic glucagon with normalization of somatostatin and decrease in insulin in normoglycemic alloxan-diabetic dogs: a putative mechanism of glucagon irresponsiveness to hypoglycemia. *Endocrinology*. 1990;126(2):1096-1104.

305. Patel YC, Cameron DP, Bankier A, et al. Changes in somatostatin concentration in pancreas and other tissues of streptozotocin diabetic rats. *Endocrinology*. 1978;103(3):917-923.

306. Inouye K, Shum K, Chan O, Mathoo J, Matthews SG, Vranic M. Effects of recurrent hyperinsulinemia with and without hypoglycemia on counterregulation in diabetic rats. *Am J Physiol Endocrinol Metab*. 2002;282(6):E1369-79.

307. Inouye KE, Yue JTY, Chan O, et al. Effects of insulin treatment without and with recurrent hypoglycemia on hypoglycemic counterregulation and adrenal catecholamine-synthesizing enzymes in diabetic rats. *Endocrinology*. 2006;147(4):1860-1870.

308. Unger RH, Orci L. Possible roles of the pancreatic D-cell in the normal and diabetic states. *Diabetes*. 1977;26(3):241-244.

309. Henquin JC, Ibrahim MM, Rahier J. Insulin, glucagon and somatostatin stores in the pancreas of subjects with type-2 diabetes and their lean and obese non-diabetic controls. *Sci Rep*. 2017;7(1):11015.

310. Mansor LS, Gonzalez ER, Cole MA, et al. Cardiac metabolism in a new rat model of type 2 diabetes using high-fat diet with low dose streptozotocin. *Cardiovasc Diabetol*. 2013;12(1).

311. *Instructions for Use: Lantus Solostar (Insulin Glargine [RDNA Origin] Injection). Sanofi.*
https://www.accessdata.fda.gov/drugsatfda_docs/label/2005/21081s017lbl.pdf

312. Shi ZQ, Rastogi KS, Lekas M, Efendic S, Drucker DJ, Vranic M. Glucagon response to hypoglycemia is improved by insulin-independent restoration of normoglycemia in diabetic rats. *Endocrinology*. 1996;137(8):3193-3199.

313. Arrojo e Drigo R, Jacob S, García-Prieto CF, et al. Structural basis for delta cell paracrine regulation in pancreatic islets. *Nat Commun*. 2019;10(1):4218.

314. Brereton MF, Iberl M, Shimomura K, et al. Reversible changes in pancreatic islet structure and function produced by elevated blood glucose. *Nat Commun*. 2014;5(1):4639.

315. Plesner A, Ten Holder JT, Verchere CB. Islet remodeling in female mice with spontaneous autoimmune and streptozotocin-induced diabetes. Fiorina P, ed. *PLoS One*. 2014;9(8):e102843.

316. Patel YC, Weir GC. Increased somatostatin content of islets from streptozotocin-diabetic rats. *Clin Endocrinol (Oxf)*. 1976;5(2):191-194.

317. Kadowaki S, Taminato T, Chiba T, et al. Somatostatin release from the isolated, perfused diabetic rat pancreas: inverse relationship between pancreatic somatostatin and insulin. *Diabetes*. 1980;29(12):960-963.

318. Edgerton DS, Kraft G, Smith M, et al. Insulin's direct hepatic effect explains the inhibition of glucose production caused by insulin secretion. *JCI Insight*. 2017;2(6).

319. Reed MJ, Meszaros K, Entes LJ, et al. A new rat model of type 2 diabetes: The fat-fed, streptozotocin-treated rat. *Metabolism*. 2000;49(11):1390-1394.

320. McCulloch DK, Raghu PK, Koerker DJ, Palmer JP, Klaff LJ. Responses of the pancreatic A cell during hypoglycemia and hyperglycemia are dependent on the B cell. *Metabolism*. 1989;38(7):702-707.

321. Fukuda M, Tanaka A, Tahara Y, et al. Correlation between minimal secretory capacity of pancreatic beta-cells and stability of diabetic control. *Diabetes*. 1988;37(1):81-88.

322. White MG, Shaw JAM, Taylor R. Type 2 diabetes: the pathologic basis of reversible β-Cell dysfunction. *Diabetes Care*. 2016;39(11):2080-2088.

323. Ghasemi A, Khalifi S, Jedi S. Streptozotocin-nicotinamide-induced rat model of type 2 diabetes (review). *Acta Physiol Hung*. 2014;101(4):408-420.

324. Furman BL. Streptozotocin-Induced diabetic models in mice and rats. *Curr Protoc Pharmacol*. 2015;70(1):e78.

325. Zhang M, Lv XY, Li J, Xu ZG, Chen L. The characterization of high-fat diet and multiple low-dose streptozotocin induced type 2 diabetes rat model. Kern T, ed. *J Diabetes Res*. 2008;2008(1):704045.

326. Prentki M, Nolan CJ. Islet β cell failure in type 2 diabetes. *J Clin Invest*. 2006;116(7):1802-1812.

327. Skovsø S. Modeling type 2 diabetes in rats using high fat diet and streptozotocin. *J Diabetes Invest*. 2014;5:349-358.

328. Kushner JA. The role of aging upon β cell turnover. *J Clin Invest*. 2013;123(3):990.

329. Skovsø S. Modeling type 2 diabetes in rats using high fat diet and streptozotocin. *J Diabetes Investig*. 2014;5(4):349-358.

330. Barnard RJ, Roberts CK, Varon SM, Berger JJ. Diet-induced insulin resistance precedes other aspects of the metabolic syndrome. 1998;84(4):1311-1315.