Cholecystokinin Receptor Activation Promotes Pancreatic β-Cell Survival

By

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This work is dedicated to Mom, Dad, and the love of my life Soyoun: for all of your support and love.

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Abstract

The functional decline of β-cell and eventual reduction of islet mass by β-cell apoptosis is the hallmark of diabetes pathogenesis. A mechanism-based therapeutic approach to diabetes prevention is necessary to address this epidemic. Identifying drug targets that can promote pancreatic β-cell survival safely and effectively will add an important new approach to the current diabetes therapies. Therapies based on cholecystokinin receptor agonism have been previously considered for obesity and diabetes. However, concerns about the adverse effects of cholecystokinin on the exocrine pancreas, gastrointestinal system, and central nervous system largely prevented the translation of these drugs for clinical use. This thesis describes the pro-survival effect of cholecystokinin by activation of cholecystokinin receptors to attenuate the pancreatic β-cell death and apoptosis in mouse and human pancreatic β-cells. Chapter two provides evidence that CCK can protect rat β-cell lines, mouse pancreatic islets, and human β-cells from apoptosis, both in culture and transplanted in vivo. Chapter three delineates findings from knockout mouse models that the CCKA receptor, but not the CCKB receptor, is essential for β-cell protection in mouse islets. In the remainder of chapter three, I present current evidence from an ongoing study where CCKR mediated protection in human donor islets has a variable protective result that may depend on the preexisting conditions or receptor expression levels. I also present compelling evidence that both CCKAR and CCKBR agonists can promote survival of human islet grafts to reduce hyperglycemia in diabetic mice, and even short-term pre-treatment of islets with CCK may have sustained benefits.

My work provides evidence that CCK protects both mouse, rat, and human islets through CCKA receptor signaling. It also hints that CCKA receptor activation before introducing cytotoxic stressors may be sufficient to provide an extended cytoprotective effect in β-cells. Based on the evidence in this dissertation, I propose that cholecystokinin receptor signaling can protect human β-cells against diabetogenic stress.

Chapter 1: Introduction

1.0 Preface to Introductory Chapter

My first ever exposure to a biomedical laboratory, or any wet laboratory for that matter, was in a spinal cord surgery lab located deep within the K tower. Behind the musky hallways of the department of neurological surgery here at the University of Wisconsin Madison, the senior lab scientist introduced me to the overarching goal of the lab:

"We aim to stop stressed neurons from dying and hopefully make them grow back."

Now that sounded pretty simple to me at the time.

It turns out that discovering a way to prevent, reverse, or slow the progressive death of any quiescent cell is the holy grail of neuroscientists and endocrinologists alike.

After spending four years in that lab as an undergraduate student, I joined a neuropathic pain lab as a molecular pharmacology Ph.D. student. Their goal was again simple: *Stop the ERK1/2 signaling in maladaptive neurons*.

The specific goal was to find a DNA sequence that folds to mimic quercetin's MEK binding structure, a naturally occurring plant flavonoid compound found to inhibit cytosolic phosphorylation of mitogen-activated kinase signaling (MAPK) cascade. Quercetin binds to a region of MEK overlapping the ATP-binding pocket, effectively inhibiting damaged or chronically *stressed* peripheral neurons from transducing

maladaptive GPCR signaling that causes neuropathy. The end goal was first to find this unknown sequence that can bind like quercetin. Then, transduce an oligonucleotide expressing an episomal cassette using a viral vector to provide a permanent inhibitor to the neuron. The product of this research would give an anatomically confined inhibition of MEK1/2 of the dorsal root ganglia as a cure for neuropathy. This project sounded very complex but promising at the time.

Unfortunately, even before my project began, I discovered that someone else had achieved much of this a decade ago. A much simpler overexpression of the constitutively inactive form of MEK had reached the same end goal. Behind many technically challenging and extraordinary experiments, it felt like there was no real question to be answered. After a few publications and a sequence of unfortunate events, I departed from that lab, found a tumor in my esophageal sphincter, spent three weeks cycling, and was about to quit my Ph.D.

Then I met Dr. Dawn B. Davis.

This dissertation is a collection of my research under her supervision.

My dissertation focuses on cholecystokinin in the pancreatic β-cell. One might ask the purpose of bringing up what happened before I joined the Davis lab. The reason for the paragraphs above is because I wanted first to introduce the three ideas that made me want to work on cholecystokinin in β-cells:

I. Neurons and β-cells are pretty similar, and there may be something to learn from each other.

II. Often, the most pivotal problem in need of a solution in medical research is straightforward, and it should be asked in such a manner.

III. An approach to answering a scientific question should follow the *law of parsimony* until it fails you.

1.1 β-cell & Neuron

From the perspective of a student who just transitioned from a neuro lab to a β-cell lab during his second year of graduate school, there was something highly similar between neurons and β-cells. Speculated to be coined by James Whitcomb Riley and later popularized by Richard Cunningham Patterson Jr. during the height of the cold war, a "duck test" is a humorous heuristic that uses abductive reasoning to classify an observation. The mechanism of a duck test is simple. It goes: *"If it looks like a duck, swims like a duck, and quacks like a duck, then it probably is a duck."* Similarly, an excited second-year biology student may also thus falsely conclude that β-cell signals like a neuron, secretes like a neuron, and dies like a neuron, then it must be a neuron. Praise thee, O Sir William Hamilton, Occam, and his razor!

It turns out a β-cell is nothing like a neuron. Mentioning the similarity as an opening line for a casual evening conversation to socialize at an academic conference will undoubtedly anger many islet biologists and neuroscientists. Nevertheless, to

demonstrate what got me excited about this project, I think it is worth pointing out the number of similarities between the two cells that helped me study the effects of cholecystokinin in β-cell survival.

Over the past half-century, many others have documented the similarity between neurons and pancreatic β-cells. In summary, both cell types specialize in sensing the physiological environment external to the cell and accordingly synthesize and release the signaling macromolecules that convey feedback to another cell, distant or adjacent. In 1971, Pearse and Polak recognized that both cell types could synthesize and metabolize biogenic amines (1,2) and classified them as APUD cells (Amine Precursor Uptake and Decarboxylation) (3,4). Previous findings of substance P (5) and somatostatin (6) in both brain and the "*gastrointestinal endocrine-like cells*" and the discovery of cholecystokinin-like peptides (2) in the brain by several laboratories using an immunoassay (7) further augmented an exciting (but later disproved) idea that neurons and β-cells may share embryological and evolutionary origin (8). Many biologists thought that β-cells and other non-neuronal secretory cells were classified through a newly coined term: a *'Paraneuron'* (9).

1.2 Similar but Different Cells

The microenvironment around neurons and β-cells is heavily vascularized and surrounded by support cells such as glial or pancreatic δ-cells that augment or inhibit

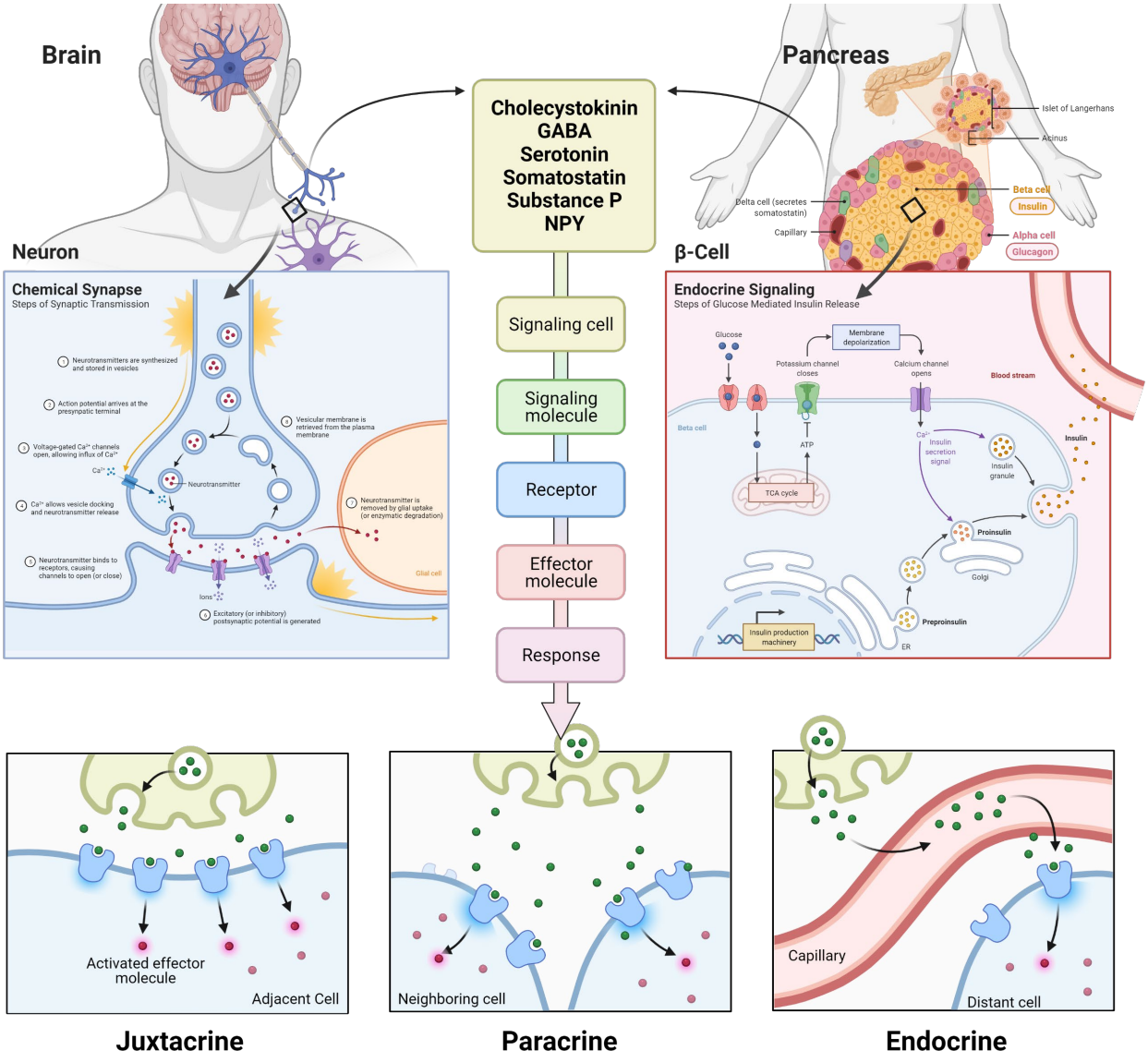

Figure 1. - Similar but Different Cells - Neurons and β-cells have many common chemical signaling messenger macromolecules and also some principles of chemical signaling in human body.

stimulation (10). They both synthesize, store, and release vesicles that contain common signaling molecules. Both β-cells and neurons produce GABA, Serotonin, NPY, Somatostatin, Substance P, and Cholecystokinin (Fig.1). Electrochemical stimulation can make neurons and β-cells release messengers; they share a similar vesicular release

mechanism, and they do not readily divide after the developmental phase has ended and when an organism has reached adulthood. Morphological studies of mammalian endocrine islets reveal the presence of tight and gap junctions between islet cells that range from 15-20 nM. The significance of this number can be better understood when it is compared to those of neuromuscular junctions (30 nm) or synaptic cleft (20 ~ 100 nm) as found in a typical nerve system. In neural synaptic transmission, a single presynaptic vesicle filled with neurotransmitters is defined as the elementary unit of signaling called "quantum"(11). A typical small synaptic vesicle is about 50 nm in diameter and a large one being 70~200 nm wide in diameter. A single insulin granule in β-cell is around 150 nm in diameter, comparable to a large synaptic vesicle, and a β-cell is estimated to have >10,000 granules per cell (12). In other words, β-cells can also quickly signal through intra-islet junction even with the low amount of macromolecule secretion to reach concentrations higher than other signaling neurotransmitters seen in synaptic transmission. β-cells, despite their endodermal origin, show a transcriptional and active chromatin signature that is most similar to ectoderm-derived neural tissues and share multiple mutual features. More recent genome-wide mRNA expression profile studies comparing expression profiles of isolated human and rodent pancreatic β-cells and islets cells to other cells with various developmental origins confirmed that β-cells share the highest gene activity profile to neural tissues, even more than to the tissues with a common endoderm origin, such as acinar or liver cells (13). More than 15% of β-cell specific gene markers are expressed in neurons but no other cell types (14). Furthermore, outside the mammalian kingdom, neurons often assume the responsibilities of β-cells for

systemic glucose homeostasis. For example, non-mammalian animals such as lobster, crabs, and worms share the insulin-secreting burden among neurons and β-cells, while insects like fruit fly rely solely on neuronal insulin secretion for glucose homeostasis (15).

Surprisingly, β-cells and neurons do not share an embryonic origin, and yes, this proves to be a major fallacy to the aforementioned "duck test." The β-cells are derived from the endoderm layer, whereas virtually all neurons originate from the neural crest formed from an ectodermal layer. Therefore, β-cells and neurons can only be seen as a product of convergent evolution. The idea of which cell type could have adopted the set of genes and mechanism of exocytotic communication is still largely elusive. A decade ago, Arntfield and van der Kooy suggested a possibility that mutations in random gut cells may have activated the neuronal transcription factors that gave rise to neuron-like traits and functional capacity to pancreatic β-cells in animals (15). A few years later, Daniel Eberhard further alluded that this may have been less of a random mutation by any gut cell but a much more controlled adoption, specifically by endocrine cells (16). How β-cells came to adopt neuronal traits is still unknown, and there have not been any extensive studies or reviews comparing β-cells and neurons since Arntfield, van der Kooy, and Eberhard.

1.3 Functional Plasticity

Differences aside, at least for me, the single most intriguing trait shared between these two cell types is that, unlike other cells, neurons and β-cells are both extremely

plastic. The fundamental concept behind memory, learning, and cognition lies in the neurons' ability to adapt to different environmental inputs and modulate their output (neurotransmitters) both in frequency and magnitude. In recent years, significant advancements in understanding adaptive islet cell response, namely β-cell de-differentiation and trans-differentiation, reveal that pancreatic islets cells and insulin-producing cells are capable of metabolic demand-driven β-cell-plasticity (17–19). Like the once-popular dogma overturned in neuroscience, research shows that islets and the β-cells themselves are also malleable and that *"you can teach an old islet new tricks."*

1.4 The Cost of Cellular Plasticity

Pancreatic β-cells and neurons share expectations for cellular and systemic homeostasis to keep our bodies in balance. Both cell types are master regulators and central signaling hubs for controlling energy consumption and expenditure. As a result, β-cells and neurons must be high-performing protein synthesizers among cells within our body. Neurons and β-cells are constantly under the pressure of making signaling macromolecules and a complex set of exocytotic machinery, making both of them high energy-consuming cells. The brain accounts for more than 20% of total oxygen metabolism in the human body (20) while β-cells heavily rely on mitochondrial oxidative phosphorylation (OXPHOS).

Unsurprisingly both β-cells and neurons can adapt to environmental changes such as heightened output demand, stress, and insult at a moment's notice. However, this kind

of cellular plasticity seems to come at a heavy price, similar to cancellation-free plane tickets.

Whether a temporal burst of transcriptional demand or sustained chronic high levels of protein synthesis, a heightened rate of macromolecule synthesis by a cell requires an adequately functioning unfolded protein response (UPR). High oxygen-dependent energy use also means that cells need to be equipped with extensive machinery to clear the free radicals generated via mitochondrial OXHPHOS and deal with reactive oxygen species (ROS) before they cause havoc on the cells. Failure to resolve either of these issues accumulates as cytotoxic stress and manifests as a functional failure or cell death that reduces brain or islet mass. Furthermore, when cells become unhealthy and start to show signs of malfunction, our immune systems are prone to flag these chronically stressed, overworked, and underperforming cells to be disposed of, causing inflammation and auto-immune-mediated destruction.

For both neurons and β-cells, demand-driven cellular adaptive processes have a tendency to fail towards the latter half of an organism's lifespan. Consequently, it is well known that aging increases the likelihood of diseases in the brain and pancreas, with increased susceptibility to progressive and chronic degenerative, inflammatory, or autoimmune diseases. Reflecting this, diabetes and neurodegenerative diseases tend to manifest more frequently in an older population.

1.5 Similar Stress & Shared Stress Response

So how do these cells manage their stress? From the perspective of evolutionary biology, life usually does not reinvent the wheel across different species, and such events occur even more infrequently for a single organism's cells. It is well observed that the cellular stress response is mediated through a conserved core set of homologous pathways in all cell types. Only a handful of organisms have been discovered to have a unique cellular stress response that does not overlap between different stress origins (21). As for the rest of us, most of the cellular responses to multiple stresses are synergistic, and pre-exposure to one form of stress induces transient stress-hardening or cross-tolerance to other forms of stress (22). Cells respond to all types of stress by activating a handful of mechanisms to stabilize the subcellular structure and functional integrity during adverse, abnormal, or pathological conditions while conserving metabolic energy for homeostatic adaptations (21–25). Therefore, I suspect that our not-so-related quiescent cells may, after all, have a shared mode of apoptosis or cell death, and as a result, are likely both equipped with similar if not shared mediators to counter some of these stressors.

Therefore, even if the exact mechanism of action for a drug is unknown, the therapeutic effect observed in one system is likely to have a similar benefit in another that shares comparable function-driven stress.

A prime example that supports this idea is the glucagon-like peptide-1 receptor (GLP-1R) agonist, liraglutide. Liraglutide, a pharmacological agonist that is a stable analog of the peptide hormone GLP-1, has recently proven effective in managing both

diabetic patients and patients with Parkinson's and Alzheimer's disease (26–30). Thus, it would not be surprising that for β-cells and neurons, the ability of the GLP-1R agonist to alter diabetic progression or protect from neurodegenerative diseases is due to a shared stress response mechanism in these different organs (26,31–34).

Cholecystokinin is a prime candidate as another peptide hormone that has the potential as a therapeutic agent that reduces cellular stress and promotes survival. Over the past few decades, an accumulation of evidence demonstrates that cholecystokinin can benefit cell survival, especially in β-cells (35–37). Hence this dissertation focuses on the protective effect of cholecystokinin in pancreatic β-cells.

However, before we explore cholecystokinin, it is time for us to push away from the parallel between β-cell and neurons and briefly review what is known to cause β-cell death. To set the stage for cholecystokinin, I should introduce the disease of interest, diabetes mellitus.

1.6 Diabetes Mellitus

Diabetes mellitus (DM) is, to put it as succinctly as possible, a diseased state of constant hyperglycemia. However, β-cell loss or β-cell failure contribute to hyperglycemia in most forms of DM. The development of DM involves characteristic early impairment of β-cell function followed by gradual loss of β-cell mass that occurs before the clinical manifestation of elevated glucose (38,39). The general mechanism

driving the pathogenesis of Type 1 DM (T1D) and Type 2 DM (T2D) have distinct pathways.

T1D results from autoimmune-mediated destruction of the pancreatic β-cells, resulting in insulin dependence. During the prediabetic stage for T1D, progressive loss of functional β-cells starts to manifest with signs of attenuated acute insulin response in response to intravenous glucose, paralleling the extent of the β-cell loss (40). Throughout the prediabetic phase of T1D and onwards, the immune system infiltrates the pancreatic islets and destroys the β-cells. At some point, a loss of glycemic control occurs when the remaining β-cell number or aggregate functionality falls below the critical threshold to produce enough insulin to maintain euglycemia. The clinical onset of T1D is often detected as acute ketosis or ketoacidosis (41).

T2D results from increased insulin resistance in peripheral tissues that fail to respond to normal insulin levels. Consequently, β-cells overwork to make more insulin to get the message across to the peripheral tissues to take up excess circulating glucose. As a result, sustained insulin resistance causes metabolic stress on β-cells beyond repair or expansion capacity. Eventually, the result is often β-cell failure, death, and reduced islet mass (42). Decreased total insulin production capacity due to metabolic stress further begets β-cell burden causing further loss of adaptive pancreatic β-cell proliferation and increased β-cell apoptosis (38,39,43–45).

Both forms of DM result from the collective loss of functional β-cells. At a single cell level, when damage is caused by various sources of stress on β-cell that the

subcellular stress response cannot repair, cells die through either necrosis or apoptosis. In either case of DM, a substantial decrease in β-cell mass characterized by increased β-cell apoptosis leads to insufficient net insulin production capacity and an inability to maintain euglycemia (44,46).

Unfortunately, except for surgical islet or pancreas transplantation, existing drugs used in the clinic cannot cure diabetes. I will discuss diabetes pharmacotherapy in the following subchapter.

1.7 Current Pharmacotherapy for Diabetes Mellitus

For patients with T1D, pharmacologic management options are still limited to lifelong dependence on an exogenous source of insulin. Research efforts to find better pharmacologic solutions to this cumbersome and often dangerous daily insulin therapy have been ongoing for over 50 years but were largely unsuccessful. Notably, in preventing T1D, experimental strategies to thwart auto-immune destruction of the β-cells have been ongoing since the 70s (47,48). Early approaches of antigen-specific antibody immunotherapy made from mouse antibody (mAb) designed to bind to target and deplete immune components or block the immunogenic cell receptor function were only partially successful. Such antibody-based therapies had severe side effects resulting from the patients developing a human antibody against mouse antibody (HAMA), leading to lethal renal failure. Today, improvement through humanized Abs has become available. A few examples include anti-TNFα (Golimumab), anti-IL-1β (Canakinumab), anti-IL-12

(Ustekinumab), and anti-CD4 (Zanolimumab) that are used as immunosuppressants but are not effective at prevention of T1D (48,49).

Ongoing efforts on developing anti-CD3 immunotherapeutic agents resulted in Teplizumab and Otelixizumab (50). Teplizumab had the most promising effect on delaying the onset of T1D and decreased the glycosylated hemoglobin (HbA1c) in phase I and II clinical trials (48) and stabilized β-cell function (51) providing long-term benefits in the prevention of T1D. As a result, on May 27^{th,} 2021, the FDA approved Teplizumab as the first preventive therapy for T1D. However, no drugs are known to reverse established T1D.

For T2D, Thiazolidinediones (TZDs) were once widely used from 1997 to 2007 in conjunction with metformin as T2D drugs. TZDs function by stimulating nuclear receptor peroxisome proliferator-activated receptor-γ (PPARγ) in the periphery, increasing muscle and adipose insulin sensitivity. Conflicting reports exist about TZDs ability to both increase or decrease β-cell mass. The changes of opposing proliferative or growth-inhibiting action by TZDs on β-cells through PPAR-γ receptors were attributed to varying depending on the glucose concentrations (52) and warrants a further study. PPAR-γ receptors are part of the steroid superfamily of nuclear receptors found in muscle, fat, and liver and modulate the gene expression that controls lipid and glucose metabolism, insulin signal transduction, and tissue differentiation (53). However, TZDs quickly retired from the first line of treatment due to concerns of weight gain and the possibility of increased myocardial infarction, cardiovascular toxicity, bladder cancer,

and increased bone fracture. Due to conflicting reports, the risk profiles of some of these conditions are still controversial. However, the drug has lost its support base in clinical use (54).

Sulfonylureas are the diabetes drug that was first available, approved in the US in the 50s. However, sulfonylureas are currently used as a second line of therapy after metformin failure in most clinical practice guidelines due to the dangers of hypoglycemia and over-stimulation of β-cells (55). A secondary β-cell failure from sulfonylurea use occurs due to their glucose-independent mechanism of action, which forces depolarization of β-cell and insulin release through the closing of the K_{ATP} channels (56). The use of sulfonylureas is limited to patients with remaining β-cells, but ironically, prolonged use in diabetic patients is known to cause β-cell burnout and aggravation of diabetes (57).

Metformin has now replaced sulfonylureas as the first line of drug for T2D patients. Unlike sulfonylureas, metformin does not elicit hypoglycemia and does not further stress the remaining β-cells to induce glucose homeostasis. The primary mechanism of action of metformin and other biguanides occurs by lowering blood glucose by restoring hepatic and additional peripheral insulin sensitivity and suppressing hepatic gluconeogenesis (58,59).

Research efforts focused on understanding the complex pathophysiology of T2D have led to the successful development of many new diabetes drugs over the past decade, resulting in breakthrough treatment options using incretin-based therapies such as

glucagon-like peptide 1 (GLP-1) analogs and dipeptidyl-peptidase-four inhibitors (DPP4i) (60–62). GLP-1R agonists were first approved for the treatment of T2D in 2005. Since then, GLP-1R agonists have grown as a widely used class of drugs for T2D due to their secondary impact on weight loss. DPP4i work by inhibiting the enzyme that inactivates the GLP-1 hormone. These drugs stimulate postprandial insulin secretion and augment insulin release only during high circulating blood glucose (63,64).

Sodium-glucose cotransporter 2 (SGLT2) inhibitors are a relatively newer drug class that lowers circulating glucose via blocking renal reabsorption, increasing the amount of glucose released in urine (65). Currently, a combination of the drugs above is frequently employed in clinics.

Interestingly, evidence shows that GLP-1R stimulation can promote mature β-cell proliferation in mice and rats (66–69). However, there seems to be a universal intrinsic regulatory mechanisms block and tightly controls cells proliferation signals such as cAMP, PKA, CREB, MAPK inducible by GLP-1R agonist (70). Recent research on $G_{\alpha}z$ activation through suppression of PGE2 receptor isoform, EP3, has been suggested as an emerging target for T2DM therapeutics (71). EP3 signaling is a potent inhibitor of the GLP-1R mediated β-cell response (72,73). Thus, inhibiting EP3 receptors on β-cells along with the use of GLP-1R agonists or other proliferative GPCR signaling may prove to be a novel and clinically translatable strategy to promote β-cell proliferation.

In summary, many advancements to expand the options for diabetes drugs have been made over the last few decades. However, it is essential to point out that there is

still an incomplete understanding of the direct contribution of most of these drugs to β-cell health. For the medicines mentioned above that have evidence that they improve the survival and function of β-cells, the subcellular signaling mechanisms often remain unclear. As a result, drugs designed to protect the β-cell mass and function through modulation of β-cell signaling have been absent. Therefore, identifying the factors that can directly protect β-cells is a critical therapeutic need to develop drugs that can prevent or reverse established DM.

1.8 Slow & Asynchronous β-Cell Death May Mean Wider Therapeutic Window

Similar to the slowly progressing nature of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease, β-cells do not die concurrently or in synchrony. In both cases, the disease progression occurs like a domino effect where each subsequent loss of a functioning neuron or β-cell burdens the rest of the population even further. It is clinically observed that in either type of DM, complete obliteration of all β-cells usually does not occur (38). It can thus be hypothesized that some β-cells are less susceptible to dysfunction or death than others. Even among β-cells within the same islet, differences in the microenvironment and islet architecture exist (74), which can result in different stress tolerance and thus varied susceptibility towards β-cell death or failure. Such examples can include the 1) relative location of the cell to the vasculature, 2) different spatial arrangement and resulting varied contact of other support cells within the

islet, 3) vicinity to vagal/spinal innervation, and 4) availability of intra-islet or extra-islet communication within an organ.

One of the ideal goals of therapy would be to augment the subcellular stress management in the more susceptible population of β-cells to prevent damage and β-cell loss from external stressors. In that case, protecting the susceptible β-cell population through pharmacotherapy could stop the next domino block from falling. Hypothetically, similar to the β-cell rest theory (75,76), this may allow the cells to reverse or slow early-stage diabetes from progressing further and ideally prevent it altogether.

DM is an unsolved clinical problem with ever-increasing prevalence in the global population. Ideally, a new mechanism-based therapeutic approach to diabetes prevention is necessary to address this epidemic. Identifying drug targets that can promote pancreatic β-cell survival safely and effectively will be transformative.

Thus, understanding the molecular mechanisms of β-cell adaptation to stress and identifying a chemical or biological agent that can provide a buffer or increased stress tolerance can lead to developing a new class of drugs to complement the currently available drug armamentarium.

1.9 Cholecystokinin & Pancreatic β-cells

Cholecystokinin (CCK) is one of the most abundant peptide hormones and a neuropeptide produced endogenously in the intestine and nervous system (77). CCK was first discovered in 1928 as a hormone described to induce gallbladder contraction (78). Since then, numerous studies have found that endogenous CCK has various

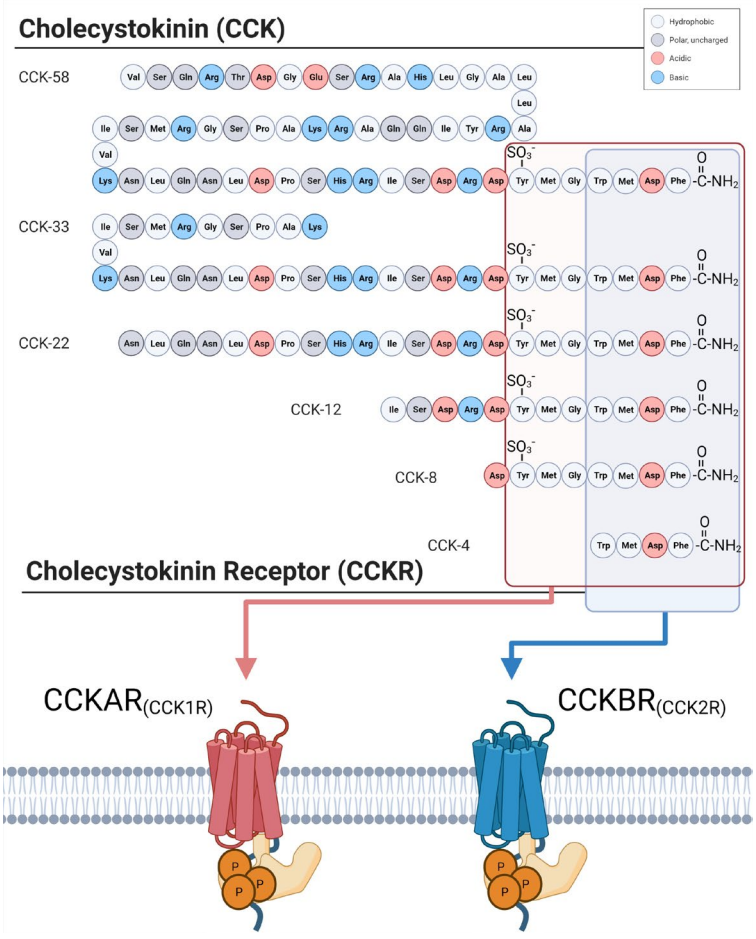

Figure 2. - Cholecystokinin and Cholecystokinin Receptors – Various lengths of CCKs are endogenously produced but the O-sulfation on tyrosine residue 7th position away from the common C-terminus determines the binding selectivity to CCKAR vs CCKBR.

gastrointestinal and central nervous systems functions. Thus far, CCK is known to regulate digestion (gut motility, hepatic bile production, bile secretion, relaxation of sphincter of Oddi, secretion of digestive enzymes from the exocrine pancreas), satiety (suppression of hunger and food intake), and various neurological functions (memory,

nociception control, anxiety, and mood regulation). More recent studies of CCK have provided new insight into additional roles for CCK within the pancreatic islets. I will expand on this matter later in this chapter after introducing the CCK ligand and its receptors. Translation of *Cck* mRNA yields a 115 amino acid pre-pro-CCK protein, and subsequent modifications occur through endoproteolytic activity by prohormone convertase 1/3 (PC1/3), resulting in CCK peptides of various lengths, including CCK-58, CCK-33, CCK-22, CCK-8, and CCK-4/5 (Fig.2). In humans, CCK-58 is the most predominant form found in the intestine and circulation (7). Varying lengths of post-translationally matured CCK peptide species are all amidated at the C-terminus. 62% to 90% of newly synthesized CCK peptides also have sulfate attached to the tyrosine residue located seven residues from the C-terminus. Sulfation is a critical step for receptor affinity, as discussed below.

Essentially all CCK peptides share the C-terminal sequence of Gly-His-Met-Arg-Phe-NH_2. CCK also shares this identical C-terminal sequence with gastrin and other naturally found hormones in mammals, amphibians, and insects (7). Gastrin is another peptide hormone produced by a completely separate gene with independent regulation of expression. This high level of homology to other natural peptides makes CCK a particularly poor target for performing specific antibody-based immunoassays.

Confirming the expression patterns and circulating levels of CCK peptides is challenging, given conflicting reports and the use of antibodies that may recognize both CCK and gastrin in humans and animals.

Once released into the blood, the bioavailability of CCK peptides in the systemic circulation of humans is estimated to range from femtomolar to picomolar, slightly less than other mammals (79–81). CCK starts to degrade in the human plasma in a matter of seconds. Interestingly, *in vitro* half-life of circulating CCK in humans are slightly longer ($t_{1/2}$= 2.5 mins) in comparison to pigs, dogs, and rodents ($t_{1/2}$ < 1 min) (82). The majority of circulating CCK is derived from diet-induced secretion of CCK by endocrine I-cells in the intestinal mucosa. I-cells are adjacent to intestinal capillaries where CCK is readily released into circulation through exocytosis of secretory granules upon stimulation by ingested protein and fat (80,81,83,84). The significance of low levels of circulating CCK and its ability to affect the pancreatic islet and β-cell have not been studied thus far. Furthermore, while CCKAR and CCKBR are expressed widely, their function has not been extensively studied β-cell (85).

Cholecystokinin Receptors (CCKRs) are part of a class A rhodopsin-like receptor family of G-protein coupled receptors (GPCRs). CCK is known to signal through two endogenous receptors: CCKA receptor (CCKAR or CCK1R) or CCKB Receptor (CCKBR or CCK2R). CCKAR and CCKBR are expressed widely. Traditionally, CCKAR was thought to be most abundantly found in the gut, whereas CCKBR is highly expressed in the brain and enterochromaffin-like (ECL) cells of the stomach (86). However, similar to detecting exact locations of CCK peptides using an immunoassay,

CCKAR and CCKBR share high homology in their amino acid sequence and 3D structure. Antibodies to GPCRs are classically challenging to develop with high specificity (87). Thus, previous studies of receptor expression patterns in different tissues are complicated by poor antibody specificity and overlap of downstream signaling pathways. More recent studies using RT-qPCR to detect the CCKAR and CCKBR

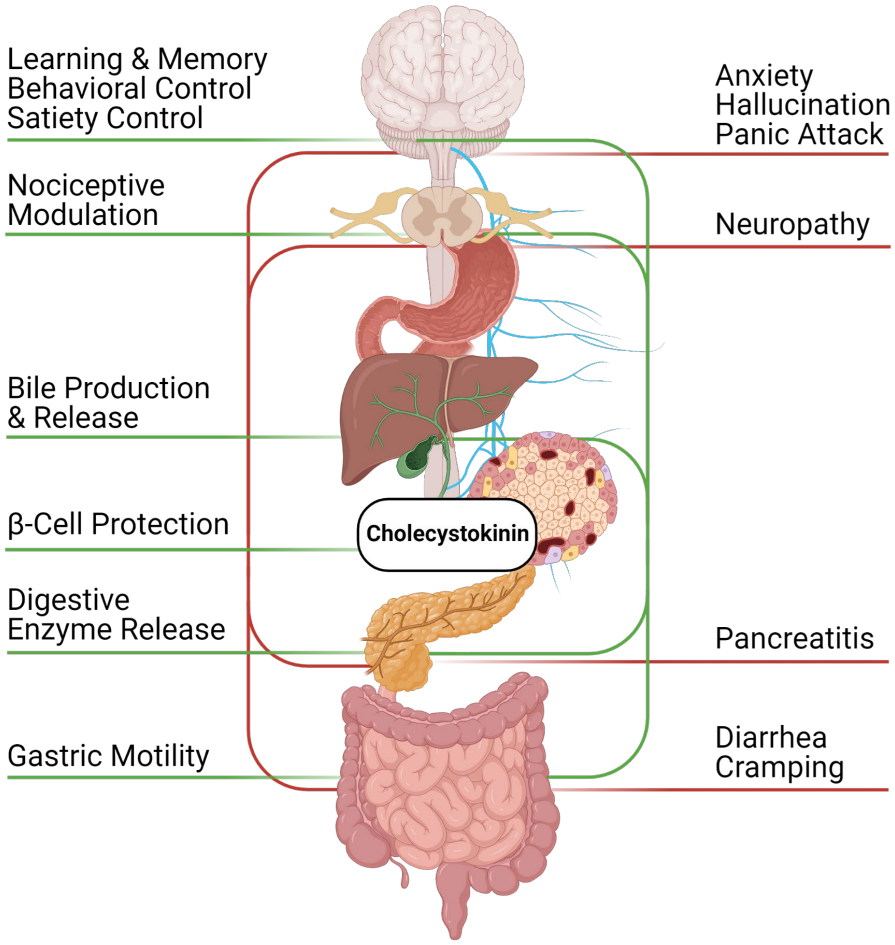

Figure 3. - Cholecystokinin can be both beneficial and detrimental – CCK modulates various normal physiological functions in our body as a neuroendocrine peptide hormone. However previous studies show supraphysiological doses of CCK given to humans and animals systemically have various detrimental effects and this makes using the CCK peptide as a useful drug difficult.

specific mRNA have better mapped where these receptors are expressed in animals, although mRNA expression is not always directly tied to receptor protein expression (88).

More recent profiling of CCKR mRNA expression patterns confirms that CCKARs are predominantly expressed in pancreatic acinar cells, gallbladder smooth muscles, gastric mucosal chief, and D cells, as well as cerebral and peripheral neurons. In contrast, CCKBR is expressed in the brain and selected gastrointestinal tract regions, including gastric epithelial parietal cells, ECL cells, D cells, pancreatic acinar cells, myenteric neurons, monocytes, and T- lymphocytes, and human blood mononuclear cells (89). In these various organs, CCK is known to have beneficial physiological functions and a pathogenic contribution when exogenic CCK is systemically given at supraphysiological concentrations in humans (Fig.3).

For this study, we focus on the pancreatic expression of CCK and CCKRs. We and others, either using RT-qPCR or single-cell RNA sequencing, have demonstrated that CCK expression occurs in obese rodent β-cells and at varying levels in human donor islets (35,90–92) while mature β-cells also express gastrin, a CCKBR ligand, in hyperglycemic mice and human islets from T2DM donors (93,94). Mice, rats, and humans express CCKAR in pancreatic β-cells (35,36,85,95,96). CCKBR is expressed in human pancreatic islets and at very low or undetectable levels in mouse islets (35,97,98). Human islet expression of both CCK receptors is variable. Pancreatic stellate cells (99,100) also express both CCKAR and CCKBR (101,102) but the implications of CCKRs in a stellate cell are still poorly understood.

Different forms of CCK ligand have various binding biases towards the two CCK receptors partially determined by their length and sulfation status. Only the CCK peptide species with O-sulfation on tyrosine positioned 7th amino acid away from the C-terminus can bind to the CCKAR. CCKBR does not discriminate the sulfation status for binding and binds unsulfated CCK peptides with relatively equal affinity to sulfated peptides (196, 210). The shortest form of CCK, CCK-4/5, does not have tyrosine that can be sulfated and therefore can only bind to CCKBR (209). For CCKAR, sulfated CCK-8 to CCK-58 have the highest CCKAR binding affinity (K_i = 0.6~1 nM). The unsulfated form of CCK-8 has a 1,000-to-10,000-fold reduction in its ability to bind to CCKAR. Gastrin-17 and sulfated and unsulfated CCK-8 and CCK-58 bind to the CCKBR with a similar affinity (K_i = 0.3~1 nM).

Accumulating evidence in cell culture and laboratory animals indicates that gastrin and CCK, and thus their physiological signaling partners CCKAR and CCKBR, integrate and coordinate a rich signaling network to modulate proliferation and apoptosis that can benefit normal physiological processes (89). In fact, an overwhelmingly large body of literature from different fields suggests that cholecystokinin has a protective effect on cells. These reports are found in a wide breadth of literature, including neuroscience (103–106), cardiology (107,108), immunology (109–111), oncology (112–115), and endocrinology (116) documenting that CCK has either anti-inflammatory (107,109,110,117–126), proliferative (127,128) or anti-apoptotic (129,130) effects against various insults in a broad range of different pathophysiological models. However, the molecular mechanism mediating the CCK response is still elusive.

CCKRs have also been implied in the pathogenesis and progression of cancer (239) as increased CCK and CCKRs have shown positive correlations to adenocarcinoma development in organs, including the brain and various gastrointestinal systems (131,132). Furthermore, exogenous CCK administration is previously shown to cause pancreatitis in experimental mouse models (133).

However, we and others have demonstrated that overexpression of β-cell-selective CCK (MIP-Cck, mouse insulin promoter driven-*Cck*) in mice alone does not result in evidence of increased pancreatic inflammation (Cd45+ leukocyte infiltration) or fibrosis (Sirius red collagen staining, presence of smooth muscle actin (SMA)-positive fibroblasts) compared to wild-type controls even up to one year of age (90,91).

In contrast, a recent study by Chung *et al*. concludes that β-cell overexpression of CCK can be an independent driver of pancreatic ductal tumorigenesis (90). However, this conclusion is confounded because both transgenic mice used in comparison already have *Cre*-activated Kras(G12D), a potent oncogenic mutation. Therefore, although there is an increase in tumors in K-ras mutant mice that also overexpress CCK in the β-cell, one cannot conclude that β-cell over expression of CCK is a primary driver for oncogenesis in the absence of predisposing mutations. Again, this argument is further supported by our data and their data that MIP-*Cck* mice do not develop pancreatitis or adenocarcinoma. However, the potential concern of tumorigenesis and/or promotion of cancer growth will need to be carefully investigated for any CCK-derived therapeutics. Anytime an agent promotes cell survival, this risk to aid the survival of cancerous cells remains a possibility.

Interestingly, Egozi et al. report that CCK originating from islets in db/db diabetic mice could drive pancreatic acinar cell remodeling. They conclude that CCK promotes trypsin secretion in the acinar cells immediately adjacent to the islet (peri-islet cells), which may facilitate the islet β-cell mass expansion in the setting of obesity(134). Nevertheless, the exact physiological role of β- cell-secreted CCK is still elusive.

Moving on to the signaling pathways of CCK, both CCK receptors couple with the Gq heterotrimeric G-protein. Gq coupling results in activation of phospholipase Cβ (PLCβ) and subsequent rapid hydrolysis of phosphatidyl inositol-bisphosphate (PIP2). The resulting release of inositol triphosphate (IP3) triggers intracellular ER calcium release (77,135). Additionally, and possibly more importantly in affecting the long-term nuclear transcriptional changes, diacylglycerol (DAG) release leads to phosphorylation of protein kinase C (PKC) followed by PKC-mediated mitogen-activated protein kinase (MAPK) pathway activation (a dominant regulator of proliferation, differentiation, apoptosis) (77). Gq coupled p38 MAPK activation degrades IκBβ by phosphorylating it and thus leads to subsequent activation and nuclear translocation of NFκB. NFκB is a significant regulator of STZ induced β-cell death (136) and inflammation and cytokine-mediated apoptosis (137).

CCKAR and CCKBR can also induce PKC-independent activation of MAPK and PI3K-AKT-mTOR signaling axis, resulting in NFκB and trypsinogen activation (77,138–140). Other signals that can be activated by both CCKAR and CCKBR include Src kinase (protooncogene), focal adhesion tyrosine kinase (FAK), Janus kinase (JAK) -

signal transducer, and activator of transcription proteins (STAT) (cell proliferation, differentiation, apoptosis) (77,89).

Additionally, and supported by older observations of viability studies in neurons (discussed later in this chapter), CCKBR activation can lead to trans-activation of the

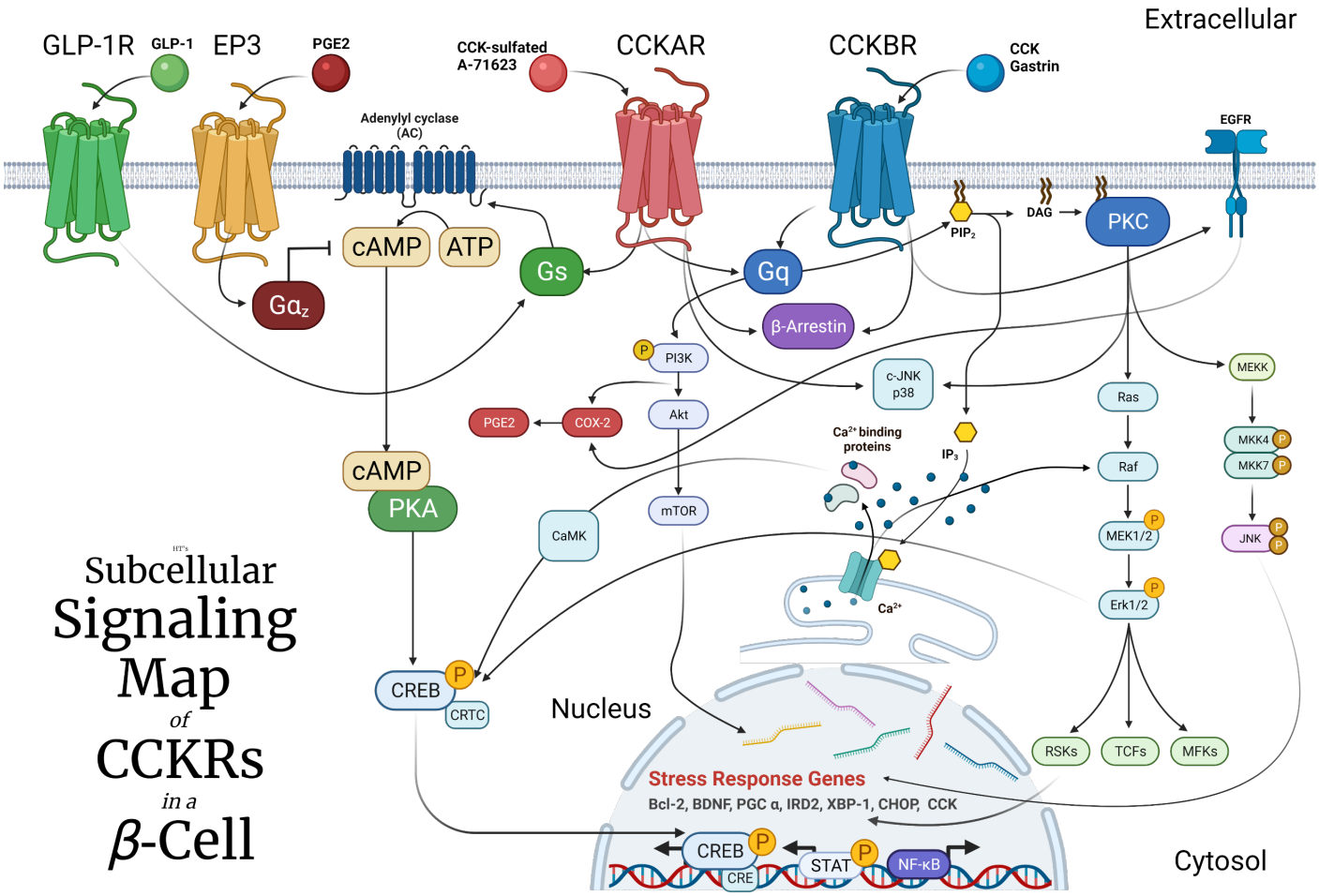

Figure 4. - Possible Subcellular CCKR signaling cascade in a β-cell - CCK receptor signaling pathways all converge and share multiple intracellular second messengers and kinases, signaling cascades that are also shared by various surface receptors and GPCRs to relay extracellular information to the cell

EGF receptor (EGFR), a transmembrane tyrosine kinase receptor that activates c-Fos, c-Jun, upregulation of cyclooxygenase-2 (COX-2) mRNA and protein and subsequent release of prostaglandin E_2 (PGE2) (141,142).

CCKAR alone can additionally couple with Gs protein to yield cAMP by promoting the conversion of ATP into cAMP. cAMP is a second messenger of PKA signaling, which promotes phosphorylation of cAMP response element-binding protein (CREB). CREB phosphorylated on Ser-133 interacts with its nuclear partner CREB-binding protein (CBP) and drives the transcription of a large number of genes. Interestingly, CREB and cAMP are among the primary targets responsible for the therapeutic benefit (anti-apoptotic, insulinotropic) of GLP-1 analogs and DPPIV inhibitors in the β-cell (143).

Additionally, it is worthwhile looking at the downstream consequences of CREB/CBP nuclear signaling to comprehend that CCKAR unique signaling Gs also converges with many of the transcriptional regulatory components signaled through CCKBR and Gq signaling. The classical sequences known to be recognized explicitly by CREB are the palindromic octa nucleotide sequences TGACGTCA, which are referred to as cAMP response elements (CRE). CREB is a prominent transcription factor family member that binds to the CRE element with a common structural motif, the basic region-leucine zipper (b-ZIP) domain. While various transcription factors that contain the b-ZIP domain interact with DNA to act as transcription factors, the most notable regulations that occur through these interactions include transcriptional upregulation of UPR target genes, including C/EBP homologous protein (CHOP) (144), ER chaperones, XBP-1 (145) and

genes that promote ER expansion (146). However, CREB signaling activation is a final consequence of many upstream signaling pathways. A signal that lands on CREB are like landing in Chicago Union Station or Seoul International Airport, a relay hub that virtually sends everyone and everything everywhere. In essence, both Gq and Gs-coupled signaling pathways can activate pro-survival, growth-promoting, apoptosis regulating transcriptional master regulators (77,89,147,148).

CCK receptor signaling pathways all converge and share multiple intracellular second messengers and kinases, signaling cascades that are also shared by various surface receptors and GPCRs to relay extracellular information to the cell (Fig.4). For example, the direct activation of PKA by cAMP can also occur via calmodulin (CaM)/CaM kinase IV (CaMKIV)-mediated pathway. At the same time, the Gq downstream PKC mediated MAPK branches such as Ras-Raf-MEK-ERK also have crosstalk that converges to CREB phosphorylation(149). Therefore, unless all other signalings can be temporarily suspended experimentally, a single cellular event cannot confidently trace back to confirm cause/effect relation. The efficiency of a cell in employing such a vast yet converging signaling cascade is admirable. Unfortunately, this complexity of GPCRs makes the study of distinguishing and dissecting out the resultant effect of CCKAR or CCKBR signaling profoundly more difficult.

Circling back, let us redirect our focus on pancreatic CCK. Similar to the CCK expressing neurons and I-cells, CCK is also locally produced and released in the pancreatic islets of humans and rodent β-cells. However, CCK is released independent of insulin secretion (36,37). Although the basal expression level of CCK in the β-cell in

lean animals and humans is low, the expression of CCK is increased up to 500-fold by obesity and hyperglycemia.

To elaborate in the context of diabetes, several reports have consistently demonstrated that metabolic stress such as obesity and insulin resistance increases pancreatic islet expression of CCK (35,92,150,151). CCK mRNA is upregulated in leptin-deficient *ob/ob* mouse islets (35,92). The regulation of CCK expression in the islet is not delineated *(89).* The Davis lab has recently demonstrated that incubation of C57BL/6J mouse islets in islet media containing 25mM (≈ 450 mg/dL) glucose for 24 hours induces a 7-fold increase in mouse islet *Cck* mRNA expression. One report states a positive correlation between β-cell CCK expression and BMI in humans (90). However, in our hands, no correlation between mRNA levels of CCK, CCKA receptor, or CCKB receptor to age, sex, and BMI were identified in human donor islets (35–37).

Mouse models have been developed to determine what role CCK has when expressed in the β-cell, mouse models have been created. Obese whole-body KO mice (*Cck*^{lacZ}-*ob*/*ob)* develop diabetes due to increased β-cell apoptosis and reduced β-cell mass (92), suggesting that CCK is necessary for islet mass expansion and survival in the setting of obesity. Furthermore, mice with mouse insulin promotor-driven β-cell CCK expression are protected against β-cell death after low dose streptozotocin injection (91) and retain more β-cell mass with aging. The Davis lab has shown that overexpression of CCK within INS-1 cells protects cells from cytokine-induced apoptosis (91). Therefore, there is evidence that endogenously produced CCK is important in β-cell mass regulation.

CCK also has the potential as a therapeutic agent for obesity and diabetes. Twice daily *i.p* injections of the stable CCK analog (pGlu-Gln)-CCK-8 can ameliorate weight gain and alleviate hyperglycemia in high-fat-fed or *ob/ob* mice models without significant side effects (152). Furthermore, multiple studies have shown synergistic properties between glucagon-like peptide-1 (GLP-1) and CCK in mitigating obesity and restoring euglycemia (153). Fusion peptides consisting of (pGlu-Gln)-CCK-8/exendin-4 (154) or (155) GLP-1R agonist/CCKA receptor-selective agonist A170222 demonstrated higher therapeutic efficacy attenuating obesity-related diabetogenic and STZ induced diabetes in mouse models (156). Rat BRIN BD11 cell lines had reduced TUNEL staining in response to cytokines when treated with CCK or CCK-exendin-4 fusion peptides (156) In summary, pharmacologic doses of CCK appear to promote weight loss, improve hyperglycemia, and reduce β-cell apoptosis and may synergize with GLP-1R signaling despite having similar downstream signaling pathways.

1.10 Outline of Dissertation: Revisiting Neurons & β-Cells

Before introducing diabetes and cholecystokinin, I began by drawing an analogy between neurons and β-cells and elaborated on how they share a functional similarity as signaling cells and thus share related cellular burdens originating from such homology. During the following sections, I first briefly touched on the slow but progressive nature of diabetes and neurodegenerative diseases and mentioned the heterogeneity of the β-cell susceptibility. I then elaborated on the pathophysiology of diabetes and covered the

current status of clinically used anti-diabetes drugs, emphasizing the lack of β-cell signaling targeted therapies, and later introduced that liraglutide, a GLP-1R agonist, is effective in pancreatic β-cell survival and neuron survival. I further elaborated on how diabetes has a wide therapeutic window and how employing a therapeutic approach to protect a weaker subpopulation of cells within a system could prevent or reverse the disease progression entirely. The last section of this introduction was a comprehensive introduction about cholecystokinin, a gut hormone and a neuropeptide known to be endogenous to both gut and brain. I have elaborated on how various fields ranging from neuroscience, immunology, and islet biology have independently reported cholecystokinin's potential to counter cytotoxicity and cellular apoptosis over the past three decades. I further highlight some potential cholecystokinin receptor downstream signaling pathways that could have resulted in these pro-survival and anti-apoptotic effects by administering exogenous cholecystokinin. I finally pointed out that the mechanism of how cholecystokinin protects pancreatic β-cells is unknown. It turns out that although still poorly understood in its mechanism, cholecystokinin protects neurons against N-methyl-D-aspartate-receptor (NMDAR) mediated glutamate toxicity (103,157,158) and methamphetamine-induced dopamine neurotoxicity (106,159,160). However, currently, the ability of CCK to protect human β-cells also remains unclear.

Studies on cholecystokinin being a key mediator of β-cell survival were limited to a handful of labs worldwide. Surprisingly despite being one of the most extensively studied proteins since the early 20th century, there is a significant knowledge gap about cholecystokinin in the scope of β-cell survival. It is necessary to identify the mechanism

of CCK's pro-survival impact to grasp its full potential to become an anti-diabetic pharmacotherapy target. To that end, in this work, I have aimed to tackle the following challenges.

I. Explore CCK's pharmacological efficacy and prerequisites for β-cell protection.
II. Elucidate the CCK receptor that is essential or dispensable for β-cell protection.
III. Examine the therapeutic potential of selective CCK receptor signaling to promote human pancreatic β-cell survival.

Chapter two provides evidence that CCK can protect rat β-cell lines, mouse pancreatic islets, and human β-cells from apoptosis, both in culture and transplanted *in vivo*.

Chapter three delineates findings from knockout mouse models that the CCKA receptor, but not the CCKB receptor, is essential for β-cell protection in mouse islets. In the remainder of chapter three, I present current evidence from an ongoing study where CCKR mediated protection in human donor islets has a variable protective result that may depend on the preexisting conditions or receptor expression levels. I also present compelling evidence that both CCKAR and CCKBR agonists can promote survival of human islet grafts to reduce hyperglycemia in diabetic mice, and even short-term pre-treatment of islets with CCK may have sustained benefits.

My work provides evidence that CCK protects both mouse, rat, and human islets through CCKA receptor signaling. It also hints that CCKA receptor activation before

introducing cytotoxic stressors may be sufficient to provide an extended cytoprotective effect in β-cells.

Chapter 2: Cholecystokinin Suppresses β-Cell Apoptosis, Including in Human Islets in a Transplant Model

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The work presented in this chapter is a product of a collaborative effort by the authors listed above. HungTae Kim's contribution to this work includes Experiments designed, conducted, data collection, and analysis to make Figure 1. A and B, Figure2. B and C, Figure 3. C, and Appendix Table 1.

The overall hypothesis aims to this project were developed by the research proposal written by the corresponding author Dawn B. Davis in her R01 and HungTae Kim's F31.

The manuscript was written by Hung Tae Kim, Arnaldo de Souza, and Carly Kibbe, with equal contributions from these authors. The contribution to the writing of abstract, introduction, results, and discussion was divided equally among the co-first authors.

2.0 Abstract

Loss of functional pancreatic β-cell mass and increased β-cell apoptosis are fundamental to the pathophysiology of type 1 and type 2 diabetes. Pancreatic islet transplantation has the potential to cure type 1 diabetes but is often ineffective due to the death of the islet graft within the first few years after transplant. Therapeutic strategies to directly target pancreatic β-cell survival are needed to prevent and treat diabetes and to improve islet transplant outcomes. Cholecystokinin (CCK) is a peptide hormone produced by enteroendocrine cells in the gut after food intake, with positive effects on weight regulation and glucose metabolism in mouse models and human subjects. We have previously shown that pancreatic islets also produce CCK. The production of CCK within the islet promotes β-cell survival in rodent models of obesity, diabetes, and aging. Now, we demonstrate a direct effect of CCK to reduce cytokine-mediated apoptosis in a β-cell line and in isolated mouse islets in a receptor-dependent manner. Whether CCK could protect human β-cells was previously unknown. Here, we report that CCK can reduce cytokine-mediated apoptosis in isolated human islets. CCK treatment *in vivo* decreases β-cell apoptosis in human islets transplanted into the kidney capsule of diabetic NOD/SCID mice. Collectively, these data identify CCK as a novel therapy that can directly promote β-cell survival in human islets and has therapeutic potential to preserve β-cell mass in diabetes and as an adjunct therapy after islet transplantation.

2.1 Introduction

Diabetes mellitus (DM) is a multifactorial disease that results from impairment of β-cell function and loss of β-cell mass. Type 1 DM (T1D) develops from autoimmune destruction of the pancreatic β-cells, resulting in insulin dependence (44). Type 2 DM (T2D) results from both increased insulin resistance and decreased insulin production (46). Failure of adaptive pancreatic β-cell proliferation and increased apoptosis under diabetic stress leads to β-cell mass reduction (161). Epidemiological studies show that there has been an alarming rate of growth in the prevalence of both types of diabetes during the past three decades, consequently driving diabetes-related complications and mortality (162). In 2020, the Centers for Disease Control reported that 34.2 million Americans are diagnosed with diabetes, while 88 million more Americans have prediabetes (163). Yet, no treatment strategies are specifically designed to protect against a decrease in pancreatic islet mass and prevent the loss of insulin-producing β-cells (164–166). Many patients with T2D eventually require insulin therapy due to a decline in functional β-cell mass, yet exogenous insulin delivery cannot replicate the dynamic insulin production of a functional islet, and dysglycemia often persists.

One possible curative therapy for diabetes is the transplantation of purified human pancreatic islets. The first human islet cell transplantation (ICT) took place in Minneapolis in 1974 (167). With the landmark clinical trial of the Edmonton protocol in 1999, allogenic-ICT became a potential solution to restoring glucose homeostasis for

type 1 diabetes (T1D), while auto-ICT became an option of preserving endocrine function for patients requiring total pancreatectomy for chronic pancreatitis (168).

Considerable improvements in transplant methods were made over the past three decades. However, the major problem with any ICT, particularly for a hepatic intraportal graft which is the primary site for transplantation in humans, is the significant loss of islet viability both early and late after transplant (169). Allogenic ICT only results in about 25-50% insulin independence after five years. New therapeutic approaches to protect and preserve β-cell mass in transplanted islets are necessary to improve outcomes.

Cholecystokinin (CCK) is a peptide hormone produced in the small intestine and brain, stimulating the release of digestive enzymes from the exocrine pancreas and inducing satiety (170). A large body of literature from different fields, including neuroscience, cardiology, immunology, and endocrinology, documents that CCK has anti-inflammatory (107,109,110), proliferative (127,128,171), and anti-apoptotic (36,85,91,130) effects against various insults in a wide range of pathophysiologic models. Combined, there is accumulating evidence that CCK receptor signaling can attenuate the onset and progression of several modes of cell death in various mammalian cell types and different organs. CCK receptor agonists were once extensively studied as a possible anti-obesity agent due to their potential modulation of leptin signaling (170–172) and their role in central nervous system-mediated appetite suppression (172,173). In the context of diabetes, systemic injection of CCK analogs in humans and rodents (84,152,154,174) improves glucose tolerance. While this has previously been thought to be due to an ability for CCK to stimulate insulin secretion, we have recently shown that CCK does not

directly enhance glucose-stimulated insulin secretion in mouse or human islets (35). In addition to its role as a circulating endocrine hormone from the enteroendocrine cells of the intestinal epithelium, CCK production is highly upregulated in mouse pancreatic β-cells under conditions of obesity and insulin resistance, and CCK is also produced and secreted from human islets (36,91,92,134). Mice with β-cell-specific CCK overexpression are protected against β-cell death after injection of the β-cell toxin streptozotocin and have preserved β-cell mass with aging (91). Conversely, CCK null obese mice have decreased islet mass and increased β-cell apoptosis (92). In mice and murine Min6 cells (85), as well as in 1.1B4 human β-cell lines (94), exogenous cholecystokinin treatment has been demonstrated to have anti-apoptotic effects.

Despite the accumulated observations of a pro-survival effect of CCK in various cell types and its overall potential benefits in weight regulation and glucose homeostasis, the ability of CCK to directly protect human pancreatic β-cells has not been previously tested. Here we show that CCK treatment results in a protection against β-cell apoptosis in various models ranging from *in vitro* insulinoma cell lines and *ex vivo* intact mouse and human islet. We demonstrate that this is a direct effect of CCK on its receptors in the islet. Additionally, we show that CCK-8 can improve human islet graft survival following transplant. The translational impact of this study lies in the identification of CCK-based therapeutics as a viable target for preventing β-cell apoptosis in humans, which also has relevance for T1D and T2D, including in the setting of transplant or β-cell replacement therapy.

2.2 Results

CCK Treatment Protects INS1E Cells Against Cytokine-Induced Apoptosis.

We have previously shown that the localized production of CCK in pancreatic β-cells leads to improved β-cell survival in mouse models of obesity, aging, and diabetes (36,91,92). However, in these experiments, locally produced intra-islet CCK could indirectly affect other cell types to mediate its pro-survival effects. To look more specifically at the direct pro-survival effects of CCK peptide treatment on the β-cell *in vitro*, we first turned to the rat insulinoma cell line, INS1E. In all experiments described, we used a stable analog of sulfated CCK-8, s-Glu-Gln-CCK-8, with high biologic activity *in vitro* and *in vivo* (152,174,175). We will refer to this peptide simply as CCK or CCK-8 throughout the manuscript. A time course of INS1E cell viability over 72 hours of exposure to mouse pro-inflammatory cytokine cocktail demonstrates that CCK-8 (100 nM) treatment mitigates cytokine-induced cell death, as measured by trypan blue exclusion, (n=6-19, p <0.05) up to 48 hours (Fig.1A). The viability of INS1E cells treated with CCK-8 was superior to those treated with GLP-1 (100nM), another peptide hormone with known pro-survival effects in β-cells *in vitro* and a widely used therapeutic strategy for type 2 diabetes (Fig.1A). We further demonstrate that CCK-8 also protected INS1E cells from cytokine-induced apoptosis in a concentration-dependent manner, using image flow cytometry with Annexin V and propidium iodide staining to identify cells in various stages apoptosis (Fig.1B, n=3-6, p <0.001).

CCK Treatment Protects Mouse Pancreatic β-Cells from Apoptosis.

To determine whether CCK also directly affects survival in primary mouse β-cells, we measured β-cell apoptosis of primary mouse islet cells by TUNEL assay. Intact mouse islets were pre-incubated with CCK-8 or vehicle for 24 hours. Mouse islets were then co-incubated with CCK-8 or vehicle and mouse cytokine cocktail for an additional 24 hours. Mouse islets treated with CCK-8 (100 nM) had a 27% reduction in β-cell apoptosis after cytokine treatment compared to vehicle control (3.18% ± 0.27 CCK vs. 4.38% ± 0.29 vehicle vs. 0.36% ± 0.15 non-treated) (Fig.2A). In addition to the ability to protect from cytokine-mediated apoptosis, which is due to a combination of cell stressors, we wanted to test whether CCK also reduced β-cell apoptosis specifically due to endoplasmic reticulum (ER) stress. ER stress is elevated in β-cells in the models of both type 1 and type 2 diabetes (176,177). Mouse islets that were treated with thapsigargin (10 uM) to induce ER stress and islets co-treated with CCK-8 (100 nM, including pre-treatment for 24 hours) had significantly fewer TUNEL positive cells than islets treated with vehicle alone (Fig.2B). To demonstrate that the reduction in apoptosis was a direct, CCK-receptor mediated effect; we tested whether CCK-8 could still protect from apoptosis in islets from mice with knockout of both the CCKA and CCKB receptors (ABKO). Using propidium iodide and annexin V staining via imaging flow cytometry, we demonstrate that the protective effect of CCK-8 (100 nM) seen in the WT islets disappears in islets from ABKOs, indicating that protection of mouse islets against cytokines by CCK is CCK receptor-dependent (Fig.2C). Taken together, these findings suggest that activation of CCK receptors protects rodent β-cells from apoptosis due to

multiple stressors directly through the CCKAR, CCKBR, or a combination of both receptors.

CCK Protects Human Pancreatic β-cells from Apoptosis *In Vitro*.

Human islets express and secrete CCK and express both CCK receptors, although at highly variable levels (Fig.3A) (35,36) suggesting that they may also be amenable to protection from CCK receptor-mediated treatments. Intact human islets from deceased organ donors were pretreated with 100 nM CCK-8 or vehicle for 24 hours and then treated with a pro-inflammatory human cytokine cocktail. Human islet donor information is provided in Appendix Table 1. β-cells from human islets treated with CCK-8 before cytokine cocktail exposure had significantly reduced TUNEL staining in comparison to those treated with vehicle (1.57-fold ± 0.3 vs. 2.36-fold ± 0.26, $p<0.05$) (Fig.3B). CCK-8 treatment also decreased apoptosis in dispersed human islet cells, as measured by cytokine-induced caspase 3/7 activity (1.7-fold ± 0.23 vs. 2.31-fold ± 0.36, $p<0.05$) (Fig. 3C). Together, these results demonstrate the efficacy of CCK treatment in promoting β-cell survival in human islets.

Systemic CCK-8 Does Not Alter Body Weight or Blood Glucose Levels in Mouse.

To provide evidence that CCK treatment could protect human islets *in vivo*, we transplanted a sub-therapeutic number of human islets (donor information in Appendix Table 1) under the kidney capsule of immunodeficient (NOD/SCID) mice with

streptozotocin (STZ)-induced diabetes and treated these mice with CCK-8 or saline via an osmotic pump for three weeks. Human islets were pre-treated with CCK-8 or vehicle control for 24 hours before transplant. In this xenograft model, we transplanted less than 1,000 islet equivalents (IEQs) of human islets per mouse, which we predicted would be insufficient to restore euglycemia. Our goal was to keep the islets in a metabolically unfavorable environment of persistent hyperglycemia, similar to that seen in human diabetes. The amount of CCK-8 delivered by an osmotic pump was calculated to approximate the daily dose previously administered to obese mice by twice-daily injection (174). We confirmed that the animals that received the CCK-8 treatment had increased levels of circulating serum CCK and achieved absolute serum concentrations of approximately 20 pM, which is only modestly higher than the physiologic postprandial levels of CCK in humans(178,179) (Fig.4A). However, blood glucose levels (Fig.4B) and body weight (Fig.4D) of the human islet transplant recipient animals receiving CCK-8 infusion were unaltered. Finally, circulating human insulin was measured and was not different in the two treatment groups (Fig.4C). Because the systemic circulation of CCK-8 did not alter the body weight or blood glucose levels in comparison to the control group, any changes in the viability or apoptosis of transplanted human islets is not due to differences in insulin demand or glucotoxicity between different treatment groups and should represent a direct effect of CCK-8 on the human islet cells.

CCK Protects Human Pancreatic β-Cells from Apoptosis Following Transplant.

We measured β-cell apoptosis in the islet grafts three weeks after transplantation. We found reduced TUNEL-positive β-cells in transplanted human islet grafts from mice with CCK treatment (0.4%) in comparison to islet grafts from saline-treated mice (1.97%). This result suggests that exogenous CCK-8 treatment protects human β-cells from apoptosis (Fig.5A&B). Taken together, our study indicates that *in vivo*, CCK protects human β-cells from apoptosis under hyperglycemic conditions and in the transplant setting.

2.3 Discussion

Pancreatic islet transplantation represents a potential cure for diabetes. The fulfillment of this goal is ultimately contingent on achieving the long-term survival of the islet graft. Allogenic-ICT can be a curative therapy for T1D, while auto-ICT can preserve the endocrine function of patients undergoing pancreatectomy. However, the major problem with any islet transplantation, particularly for T1D, is a significant loss of islet viability both early and late after the transplant (169,180,181). In T1D patients, only ~50% of successful islet transplant recipients maintain insulin independence after two years. The loss of islets in the early stages after a transplant requires many islets to restore glucose homeostasis in the patient, often requiring more than one donor per recipient. Inflammation and autoimmunity are one of the causes for the initiation of β-cell destruction during the development of T1D. Transplanted pancreatic islets themselves can also release pro-inflammatory cytokines, which play a significant role in cell failure and death during and post-islet transplantation (180,181). Thus, identifying factors that can help improve the preservation of pancreatic β-cell mass is necessary to improve islet transplant outcomes.

In rodents, administration of the stable CCK analog (pGlu-Gln-CCK-8) protects mice from obesity-induced diabetes in both high fat-fed and leptin-deficient models (152,174). Notably, a study by Irwin et al. (174) demonstrates that twice-daily injection of pGlu-Gln-CCK-8 (25 nmol/kg body weight) in high fat-fed mice and *ob*/*ob* mice reduced body weight, improved glucose tolerance, improved insulin sensitivity, and

lowered non-fasting glucose, demonstrating the potential of a CCK receptor agonist as an anti-obesity/anti-diabetic agent. However, this study has inherent limitations in isolating the direct beneficial effect of CCK receptor agonism on β-cell survival. As CCK-treated animals had improved glucose tolerance and insulin sensitivity in this study, these metabolic improvements can indirectly reduce the diabetogenic stress in the islets. Additionally, the high-fat diet feeding in their model did not induce the typical compensatory increase in β-cell mass. While these authors reported increased β-cell turnover with CCK treatment, a direct effect of CCK on β-cell survival could not be assessed. Our current study demonstrates direct *ex vivo* effects of CCK to reduce β-cell apoptosis and show reduced apoptosis of human (-cells *in vivo* despite persistent hyperglycemia comparable to controls.

In this study, we show that CCK-mediated protection is occurring in a pharmacologically validated mechanism via receptor-mediated effects. We achieve this by first demonstrating a dose-response relationship of the protective effects of CCK in INS1E cells across a pM to μM range (Fig.1B). The wide range of effective concentrations hints that CCK treatment to target β-cell survival has a large therapeutic index. Using CCK at lower doses will likely minimize the gastrointestinal side effects that impeded progress in prior clinical trials. Notably, the protection of human islets *in vivo* was achieved with circulating levels of CCK only in the 20 pM range. Additionally, we show that this protection disappears in islets from mice devoid of both types of CCK receptors (Fig.2C), confirming a direct, receptor-mediated effect.

This current study demonstrates the relevance of islet CCK signaling in humans by showing that human pancreatic islets express CCK, CCKAR, and CCKBR (Fig.3A), albeit at highly variable expression levels. This is distinct from mouse islets, which primarily express CCKAR with very low CCKBR expression (36,91). Therefore, this difference in CCK receptor expression is important in assessing species-specific effects of CCK-based therapies on the islet. CCK-8 peptide can activate both CCKAR and CCKBR with high affinity. We demonstrate that the CCK-8 analog directly protects isolated human islets from cytokine-induced apoptosis (Fig.3B&C). While these *in vitro* studies were encouraging, it was essential to study this effect under *in vivo* settings. We turned to the xenograft human islet model as it is extremely difficult to study β-cell mass regulation in living human subjects due to our inability to accurately measure β-cell mass *in vivo* and the morbidity of obtaining a biopsy. We find that continuous CCK-8 infusion for three weeks successfully reduced human β-cell apoptosis by over threefold compared to the vehicle-treated group (Fig.5). Not only does this support the promise of CCK-based therapy to promote β-cell survival in human islets, but it also demonstrates that CCK is effective in protecting from the pathophysiologic stressors relevant in diabetes and transplantation such as hyperglycemia, hypoxia, and ER and oxidative stress.

The results we present from the transplant study are encouraging, but there are some intrinsic limitations. We acknowledge that due to study design, including pre-treatment of islets with drug, having a single drug dose, and a single late endpoint for islet graft harvesting, it is impossible to determine whether the protection from apoptosis

we see in our transplant model is due to the CCK treatment of the islets before the transplant, the systemic CCK infusion, or a combination of the two. We also do not know if the protective effects of CCK treatment most are pronounced in the early post-transplant period or later in response to persistent hyperglycemic stress. To fully harness the protective effects of CCK and reduce unwanted side effects, it will be essential to determine the appropriate timing and dose of CCK treatment necessary to provide adequate β-cell protection.

All experiments in the current study used a non-biased CCK analog that presumably results in pharmacological activation of both CCK receptors. CCK signals through two G-protein coupled receptors, the CCKA receptor (or CCK1R) and the CCKB receptor (or CCK2R), to activate many different signal transduction pathways (77,85,89). Previously, others have demonstrated the direct effect of CCK on the survival of mouse insulinoma Min6 cells in response to hyperglycemia and suggested that this effect depended on β-arrestin-mediated signaling (85). However, the CCK receptor signaling pathways employed by β-cells are not fully understood. Since human islet cells express both the CCKA receptor and the CCKB receptor, future studies will be needed to address specific CCK signaling pathways in the human endocrine pancreas. Identifying the particular CCK receptor subtype activation that affords protection against β-cell apoptosis in human islets will further develop specific CCKR agonists to improve transplant outcomes with minimal adverse effects.

2.4 Conclusion

In conclusion, we demonstrate here, for the first time, that CCK protects human β-cells from apoptosis. We provide evidence through *in vitro*, *ex vivo*, and *in vivo* studies using isolated human islets to suggest that this protection occurs through direct effects on the β-cell and in response to several forms of apoptotic stress. CCK has been studied as a potential therapeutic against obesity due to its CNS-directed effects on satiety. Our work demonstrates that CCK has directly positive effects on protecting human β-cell mass, adding to its benefits for utilization in diabetes therapy. The endogenous function of CCK in human pancreatic islets remains an open question. A role for CCK in intra-islet autocrine and paracrine signaling is suggested (36,91) and other studies also show CCK's potential role in exocrine-endocrine crosstalk for islet mass expansion and regulation (134). Future studies will be essential to discover the CCK signaling pathways activated in β-cells and explore how these pathways can be targeted to develop an effective therapy with few adverse effects.

2.4 Materials and Methods

Viability Assay

INS1E rat insulinoma cells were cultured in RMPI 1640 (Thermo Fisher Scientific, #11875093) with 2.05 mM Glutamax (Cellgro, 35050079) supplemented with 5% (v/v) heat-inactivated FBS, 1% P/S, 10 mM HEPES, 1 mM sodium pyruvate, and 50 μM freshly added beta-mercaptoethanol (Sigma, M7522) at 37 °C and 5% CO2. INS1E

cells were seeded in 12 well plates at a density of 0.1 x 10^6 cells/well and were incubated for 24 hours before any treatment. Cells were then incubated in fresh media containing either 100 nM CCK (sulfated-(pGlu-Gln)-CCK-8, American Peptide Company) , 100 nM GLP-1 (1851, Tocris) or 100 nM saline vehicle control up to 72 hours with concurrent treatment with a mouse cytokine cocktail containing 50-ng/mL TNFα (Miltenyi Biotec, #139-101-687), 10-ng/mL IL-1β (Miltenyi Biotec, #130-101-680), and 50 ng/mL IFN-γ (Miltenyi Biotec, #130-105-785) as described (182). Cell media containing the treatment was refreshed at 36 hours of incubation for the time course study groups. At each time point, the growth media containing the suspended cells was collected and centrifuged and combined with the adherent cells that were released with 0.25% trypsin (Sigma, 59428C) and then resuspended in 100 ul of growth media containing FBS. 10 ul of suspended cells were then added to a 10ul solution of 0.4% trypan blue in a buffered isotonic salt solution (Bio-Rad, #1450021), pH 7.3, and measured for viability using TC10 automated cell counter (Bio-Rad, #145-0010) – Figure 1A.

Apoptosis Assay – Imaging Flow cytometry

Apoptosis was measured using image flow cytometry in INS1E cells and islets from wild-type (WT), and CCK receptor KO mice (ABKO) treated with mouse cytokine cocktail as above, GLP-1, and/or CCK-8 for 24 hours (Figure 1B and 2C). As above, all cells were pretreated with peptide hormone for 24 hours before the addition of cytokines. Cultured mouse islets or INS-1 cells were transferred to a 15 mL conical tube with the

incubation media. The culture plate was rinsed with 2 mL PBS and this was added to the tube to ensure complete transfer. Islets and cells were pelleted at *800xg* for 3 min at four °C followed by a 1-minute wash using cold PBS. Islets were resuspended in 1 ml Sigma Dissociation Solution (Sigma), then shaken horizontally in a 37°C water bath for 8 min at 100 RPM followed by placement of the tube on ice and addition of 2 mL of media to stop the dissociation process. Cells were then gently disrupted and incubated on ice for another 5 minutes before being pelleted again at low speed for 5 min at 4°C. The supernatant was removed, and the pellet was resuspended in 1x Annexin Binding Buffer containing 0.5% BSA. 5x Annexin binding buffer was made with 50 mM HEPES, 700 mM NaCl, 12.5 mM CaCl2 in water. The cells were then pipetted up and down 30 times, then 1 µl of this cell suspension was placed on a microscope slide to check for dissociation. Depending on the level of clumping, the cells were pipetted up and down for another 20-30 more times or until the majority of the cells were single cells. Cells were then counted on a hemocytometer and were resuspended in 1x Annexin Binding Buffer containing 0.5% BSA to a final concentration of $1x10^6$ cells/ml. An aliquot of 100 µl of cells ($1x10^5$ cells) was mixed with Propidium Iodide (50 µg/mL) and Annexin V-AF488 (Invitrogen #A13201). Unstained and individually stained control samples were prepared in separate tubes. All tubes were incubated at room temp in the dark for 15 min. Samples were diluted immediately before reading with the addition of 400 ul 1x Annexin Binding Buffer containing 0.5% BSA. Imaging Flow data was acquired by imaging flow cytometer (Amnis EMD Millipore, Image Stream Mark II). During the analysis, 10,000 live cell images were captured at 40x magnification. Data were

analyzed using ImageStream (Image Stream®X, Amaris) to quantify propidium iodide and annexin V stains that indicate the different stages of apoptosis.

Ex vivo Islet TUNEL and Caspase 3/7 Activity Assays

Using serum-free media supplemented with 5 g/L BSA fraction V (Roche, #107351080001), intact islets were pre-treated with 100 nM CCK or saline vehicle control for 24 hours before additional 24 hours of cytokine or 10 uM thapsigargin treatment to induce apoptosis (Figure 2A-B and 3B). Human islets were treated with a cytokine cocktail containing human 1,000 U/ml TNFα, 75 U/ml IL-1β, and 750 U/ml IFN-γ (PeproTech, #200-01B, #300-02, #300-01A) (Figure 3B-C). After treatments, islets were dispersed, plated on Poly-L-lysine pre-coated glass coverslips, and fixed. β-cell apoptosis was measured using TUNEL (Promega, #G3250) followed by insulin staining (Dako, A0564) as described in detail below. Imaging was performed using an EVOS FL Autofluorescence microscope (Life Technologies). ImageJ (NIH) or Adobe Photoshop (Adobe) was used to count DAPI (Vector Labs, H-1200-10) and quantify insulin-positive/TUNEL-positive cells in at least nine randomly chosen fields per treatment group for each replicate. For Caspase 3/7 activity (Figure 3C), dispersed human islets were plated in a 96-well plate at 20,000 cells per well and incubated for 24 hours before treatment. Dispersed islet cells were pre-treated with 100 nM CCK or vehicle control for 1-hour prior to treatment with human cytokines as above. Caspase 3/7

activity was measured 24 hours post-cytokine treatment using the Caspase-Glo® 3/7 Assay System (Promega, #G8090).

Animals, Islet Isolation and Culture

Animal care and experimental procedures were performed with approval from the University of Wisconsin and William S. Middleton Veterans Hospital Animal Care and Use Committees to meet acceptable standards of humane animal care. Male C57BL/6J (~10 to 15-week-old) (Stock Number #000664) and NOD/SCID (9-week-old) mice (NOD.Cg-Prkdc<scid>/J, Stock Number #001303) were purchased from The Jackson Laboratory for islet isolation and transplant studies, respectively. CCK receptor double knockout mice, 129-*Cckar*^{tm1Kpn} *Cckbr*$^{tm1Kpn/J}$ (Stock Number #006365) (183,184) age 14-20 weeks were used in the studies. These mice are in a mixed 129 background and were obtained from Jackson Laboratories after cryorecovery. WT controls used were littermates from 129-*Cckar*^{tm1Kpn} *Cckbr*$^{tm1Kpn/J}$ after breeding with 129S1/SvImJ (Stock Number #002448). Mice were housed in facilities with a standard light-dark cycle and fed ad libitum. Mouse pancreatic islets were isolated using collagenase digestion and hand-picked as previously described (185). Isolated mouse islets were cultured at 37°C and 5% CO_2 in RPMI 1640 media (Thermo Fisher Scientific, #11879020) containing 8 mM glucose, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin (1% P/S) (Thermo Fisher Scientific).

Human islet culture and mRNA Levels

Human islets were obtained through the Integrated Islet Distribution Program (IIDP). Upon arrival, islets were handpicked and then cultured in RPMI 1640 media as described above but without antibiotics and antimycotics. Islets were cultured overnight to confirm viability and sterility before treatments. Islets were cultured for up to 7 days, and the media was renewed every other day. After culture, RNA was isolated, and gene expression was quantified via quantitative PCR with SYBR and primers for human *CCK (hsCCK forward* TGA GGG TAT CGC AGA GAA CGG ATG*,* hsCCK *reverse* TGT AGT CCC GGT CAC TTA TCC TGT*)*, *CCKAR (hsCCKAR forward* TGG AAG CAA CAT CAC TCC TC, hsCCKAR *reverse* CAC GCT GAG CAG GAA TAT CA), and *CCKBR (hsCCKBR forward* GAT GTG GTT GAC AGC CTT CT, hsCCKBR *reverse* GGG CTG ATC CAA GCA GAA A) normalized to Beta-Actin (*forward* TCA AGA TCA TTG CTC CTG AGC, *reverse* TCA AGA TCA TTG CTC CTG AGC) – Figure 3A.

Human Islet Transplantation and Graft Harvest

A modified version of human islet transplantation described by Montanya *et al*. was used for the study (186). A single high dose (200 mg/Kg) of streptozotocin (Sigma, #S0130) was administered via intraperitoneal injection two days before transplant to induce hyperglycemia. Hyperglycemia (glucose >300 mg/dL) was confirmed with a tail vein blood sample using a glucometer before proceeding with the transplant. Human

islets were cultured in media supplemented with 100 nM pGlu-Gln-CCK-8 or saline vehicle control for 24 hours before transplant. Confirmed hyperglycemic mice were anesthetized using isoflurane, and approximately 1,000 islet equivalents were placed under the kidney capsule. While our initial study design was to have a paired sample of both CCK-treated and saline-treated from each human islet donor, in some cases, we had death of a mouse during or post-surgery or technical difficulties during the transplant that led to insufficient islet delivery in one animal. Therefore, the final data do not always represent paired treatment groups. An infusion pump (Azlet 1004) containing saline or CCK was placed subcutaneously in the back of the mice at the same time as the islet transplant to have continuous CCK infusion (52 pmol/hr) for three weeks. We calculated the concentration of infused CCK to recapitulate the amount of CCK delivered through twice-daily intraperitoneal injection dosing done in studies by Irwin et al. that led to beneficial effects on glucose and weight in obese mice (152,174). Mice were monitored during recovery and checked for surgical complications in the post-operative period. Bodyweight and randomly fed blood glucose were measured every 2-3 days following the transplant for a total of three weeks. Blood glucose was measured using a tail nick blood sample and glucometer (Bayer Contour Next EZ). Three weeks post-transplant, mice were anesthetized using Avertin (2,2,2-TRIBROMOETHANOL, 97% T48402, 500mg/kg), and terminal serum was collected by cardiac puncture and stored for human insulin and CCK assays. Kidneys containing islet grafts were harvested and fixed in 10% formalin (Fisher SF100) for 48 hours. 10% Formalin-fixed kidneys were paraffin-embedded and sectioned for immunofluorescence staining as described below.

CCK and Human Insulin Measurement

Serum CCK levels were measured three weeks post-transplant using a CCK radioimmunoassay (Alpco Diagnostics - now discontinued) as described by Rehfeld (80) (Figure 4A). Plasma was extracted by mixing with 96% ethanol and evaporating to dryness using a vacuum centrifuge. The dry extracts were dissolved in Alpco diluent (reagent D) and stored at -20 °C until assayed. The procedure provided by Alpco was followed for the radioimmunoassay. Briefly, samples were mixed with anti-CCK-8 (reagent A) and incubated for two days at 2-8 °C followed by the addition of I^{125}-CCK-8 (reagent B) and another 4-day incubation at 2-8 °C. Finally, the double antibody solid phase (reagent C) was added, incubated 60 minutes at 2-8 °C, centrifuged, and the supernatant discarded. A gamma counter was used to measure radioactivity with a counting time of 2-4 minutes. Insulin secreted from the islet grafts was measured in the mouse serum three weeks post-transplant using a specific human insulin ELISA (Millipore, EZHI-14K) – Figure 4C.

Immunofluorescence Staining

Paraffin-embedded islet grafts within the kidneys or intact mouse or human islets were stained for insulin using polyclonal guinea pig anti-insulin antibody (Dako, A0564), and apoptosis was measured using the DeadEnd Fluorometric terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) system

(Promega, #G3250). Images from each islet graft were obtained using an EVOS FL Autofluorescence microscope (Life Technologies). ImageJ software was used to quantify total insulin-positive cells and TUNEL-insulin co-staining cells. Image quantification was done blinded to the experimental group.

Statistics

Assessment of statistical significance between groups was determined using GraphPad Prism by 2-tailed Student's t or ANOVA tests as the non-parametric equivalent. Bonferroni posthoc test was performed to correct for multiple comparisons where appropriate. A paired t-test was used when samples were from the same islet prep to account for differences in baseline viability (Figures 2A-C, 3B-C). A probability of error less than 5% was considered significant (i.e., $P < 0.05$). Statistical information for experiments (data representation, P values, and n numbers) can be found in the figure legends. In all panels, data are represented as mean ± SEM.

2.5 Acknowledgements

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2.6 Figures

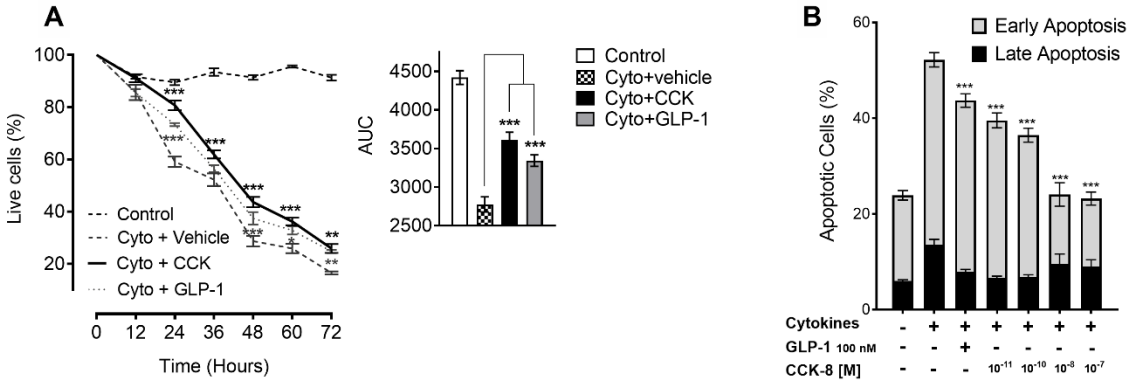

Fig 1. CCK protects INS1E cells from cytokine-induced apoptosis. Time-course (A) of INS1E cells treated with cytokine cocktail and co-treated with 100 nM CCK or 100 nM GLP-1 (n = 6-19). CCK dose-response effects (B) on the percentage of annexin V and propidium iodide (PI) double-positive cells (Late apoptosis – black bars) and Annexin V positive cells (Early Apoptosis - gray bars) treated with cytokines (n = 3-6). Data are means ± SEM; ***P < 0.001.

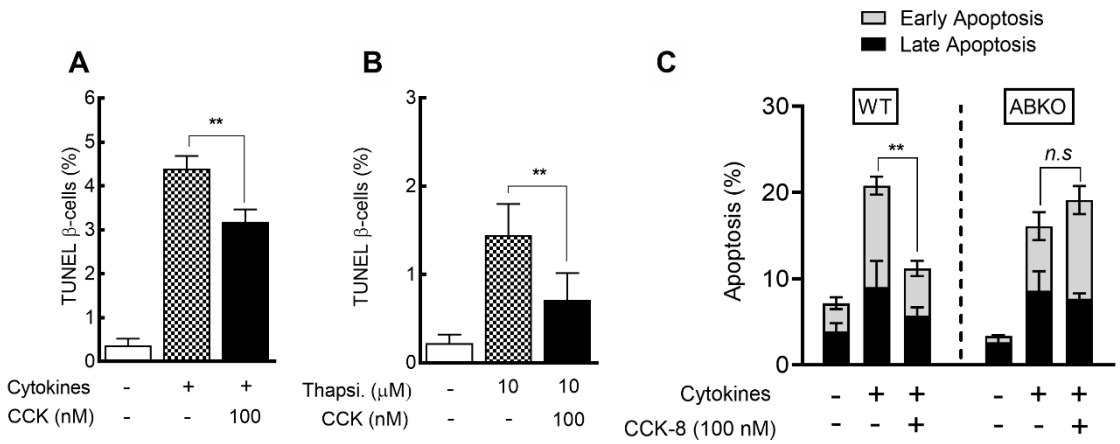

Fig 2. CCK protects mouse islets from apoptosis. Percentage of TUNEL-positive insulin-positive cells of mouse islets treated with mouse cytokine cocktail (A) or thapsigargin (B) is reduced when treated with 100 nM CCK (n = 3-4). (C) Percentage of Annexin V and propidium iodide (PI) double-positive cells (Late apoptosis - black bars) and Annexin V positive mouse islet cells (Early Apoptosis - gray bars) co-treated with cytokines and CCK is reduced in wild type controls but not in double CCK receptor KO islets (ABKO) (n = 6). Data are means ± SEM; $^{**}P < 0.01$; n.s, non-significant.

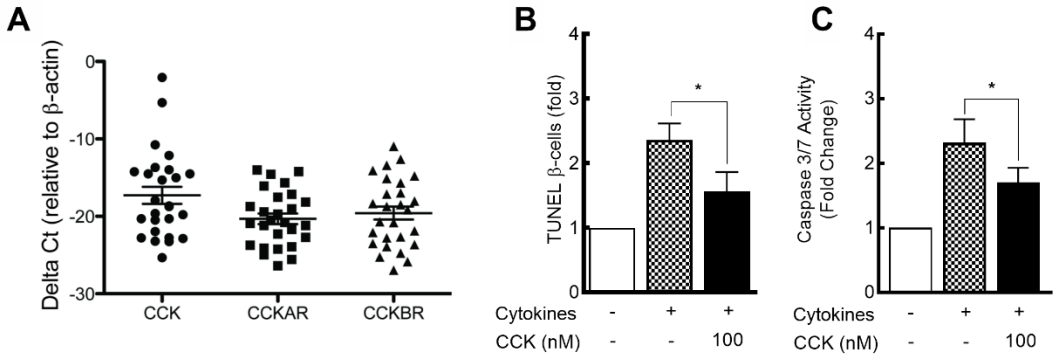

Fig 3. CCK protects human islets from cytokine-induced apoptosis. Human islets express CCK, CCKAR, and CCKBR mRNA at variable levels (n = 25) (A). Fold increase of TUNEL-positive/insulin-positive cells (B, n=5), and caspase 3/7 activity (C, n=6) of human islets treated with human cytokine cocktail is reduced when co-treated with CCK. Data are means ± SEM; *$P < 0.05$.

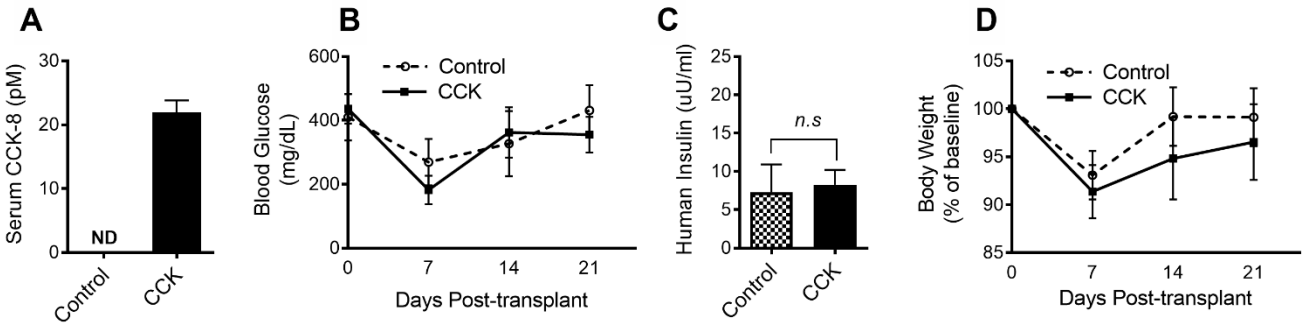

Fig 4. Treatment with CCK-8 does not alter the body weight or blood glucose of diabetic mice. Circulating levels of CCK (A) are increased in CCK-treated mice 21 days after transplant. Non-fasted blood glucose levels (B) measured weekly during 21 days post-transplant do not differ between treatment groups. Human insulin (C) is detected in the serum of mice 21 days after transplant and does not differ between control and CCK-treated mice. Body weight was measured weekly and was used to calculated percent change (D) during 21 days post-transplant; no significant differences were detected. Data are means ± SEM (n = 4-6). ND, not detectable; n.s, non-significant.

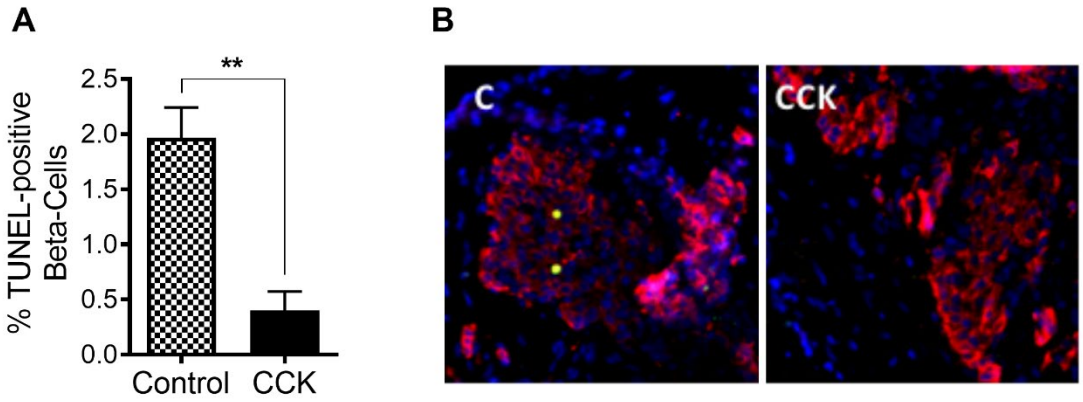

Fig 5. Human islet grafts in mice receiving CCK-8 treatment contain fewer apoptotic β-cells. Quantitative analysis (A) reveals a reduced percentage of TUNEL-positive insulin-positive cells in human islet grafts from CCK-treated mice 21 days after transplant. Representative images of islet grafts (B) from control (left) and CCK-treated (right) transplant mice. DAPI (blue), insulin (red), and TUNEL (green). Data are means ± SEM (n = 3-5); $^{**}P < 0.01$.

2.7 Appendix

Islet Preparation	Study	Unique identifier	Donor Age (years)	Donor Sex (M/F)	Donor BMI (kg/m²)	Donor HbA1c	Source	Islet isolation center	Donor history of diabetes? Yes/No	Cause of death	Cold ischemia time (h)	Estimated purity (%)	Estimated viability (%)	Glucose-stimulated insulin secretion or other functional measurement
1	Dispersed islet TUNEL	SAMN08744571	32	M	32.3	5.6	IIDP	University of Wisconsin	No	Anoxia	5	80	95	SI (G2.8mM-G28mM)= 5.2
2	Cytokine Study Caspase-Glo® 3/7 Assay	SAMN08769807	20	M	19.8	4.5	IIDP	University of Pennsylvania	No	Anoxia	7.3	80	92	SI (G2.8mM-G28mM)= 1.6
3	Cytokine Study Caspase-Glo® 3/7 Assay	SAMN08769808	54	F	30.1	5.8	IIDP	University of Wisconsin	No	Cerebrovascular/stroke	11.3	95	98	SI (G2.8mM-G28mM)= 1.0
4	Cytokine Study Caspase-Glo® 3/7 Assay	SAMN08769822	20	M	24.4	4.8	IIDP	University of Wisconsin	No	Anoxia	14.3	75	99	SI (G2.8mM-G28mM)= 8.8
5	Cytokine Study Caspase-Glo® 3/7 Assay	SAMN08769823	53	F	34	5.3	IIDP	University of Miami	No	Cerebrovascular/stroke	12.8	90	90	SI (G2.8mM-G28mM)= 3.6
6	NOD/SCID Kidney Capsule Transplant	SAMN08769826	30	M	56.8	5.6	IIDP	University of Wisconsin	No	Anoxia	8	95	98	SI (G2.8mM-G28mM)= 10.0
7	NOD/SCID Kidney Capsule Transplant	SAMN08773446	69	F	22.1	5.3	IIDP	The Scharp-Lacy Research Institute	No	Cerebrovascular/stroke	9.2	90	95	SI (G2.8mM-G28mM)= 2.3
8	NOD/SCID Kidney Capsule Transplant	SAMN08773838	45	F	22.6	5.5	IIDP	University of Miami	No	Cerebrovascular/stroke	12.4	85	92	SI (G2.8mM-G28mM)= 5.0
9	NOD/SCID Kidney Capsule Transplant	SAMN08774209	29	M	30.9	5.5	IIDP	University of Wisconsin	No	Head trauma	5.4	65	95	SI (G2.8mM-G28mM)= 2.4
10	NOD/SCID Kidney Capsule Transplant	SAMN08774210	38	M	25.3	Not Reported	IIDP	University of Pennsylvania	No	Cerebrovascular/stroke	4.6	93	90	SI (G2.8mM-G28mM)= 2.6
11	NOD/SCID Kidney Capsule Transplant	SAMN08774213	20	M	21.7	Not Reported	IIDP	University of Pennsylvania	No	Head trauma	11.3	90	90	SI (G2.8mM-G28mM)= 5.1
12	NOD/SCID Kidney Capsule Transplant	SAMN08774797	36	F	42.7	Not Reported	IIDP	University of Wisconsin	No	Anoxia	7.4	85	99	SI (G2.8mM-G28mM)= 2.8
13	Dispersed islet TUNEL	SAMN08832066	58	M	31.8	5.9	IIDP	The Scharp-Lacy Research Institute	No	Cerebrovascular/stroke	9.8	90	95	SI (G2.8mM-G28mM)= 3.3
14	Cytokine Study Caspase-Glo® 3/7 Assay	SAMN08930702	43	M	36.4	5.4	IIDP	University of Illinois	No	Cerebrovascular/stroke	5.3	95	95	SI (G2.8mM-G28mM)= 1.8
15	Dispersed islet TUNEL	SAMN08934018	43	F	30.9	5.5	IIDP	The Scharp-Lacy Research Institute	No	Head trauma	6.5	90	95	SI (G2.8mM-G28mM)= 1.4
16	Dispersed islet TUNEL	SAMN09091256	45	M	29.3	5	IIDP	The Scharp-Lacy Research Institute	No	Cerebrovascular/stroke	10.9	90	95	SI (G2.8mM-G28mM)= 1.8
17	Dispersed islet TUNEL	SAMN09370567	32	M	28.5	5.2	IIDP	University of Wisconsin	No	Head trauma	11.5	95	91	SI (G2.8mM-G28mM)= 1.8
18	NOD/SCID Kidney Capsule Transplant	SAMN13570019	37	F	24	5.2	IIDP	University of Pennsylvania	No	Anoxia	15.3	95	96	SI (G2.8mM-G28mM)= 1.2

Suppl. Table 1. Human islet donors
Adapted from Hart NJ, Powers AC (2018) Progress, challenges, and suggestions for using human islets to understand islet biology and human diabetes. Diabetologia.

Chapter 3: CCK Protects Human β-Cells Through Both CCKAR and CCKBR

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3.0 Abstract

Diabetes mellitus is an unsolved clinical problem with ever-increasing prevalence in the global population. The functional decline of β-cell and eventual reduction of islet mass by β-cell apoptosis is the hallmark of diabetes pathogenesis. However, only a few pharmacotherapeutic targets are known to protect β-cell mass and function, and there is no clinically available pharmacotherapy to reverse established diabetes. Thus, a mechanism-based therapeutic approach to diabetes prevention is necessary to address this epidemic. Identifying drug targets that can safely and effectively promote pancreatic β-cell survival will add a critical new approach to current diabetes therapies. We have previously identified a novel role for cholecystokinin in promoting human β-cell survival and demonstrated that both types of cholecystokinin receptors are expressed in human pancreatic islets. This study aimed to understand the islet endogenous cholecystokinin receptor responsible for the previously seen anti-apoptotic effect seen in the β-cells. This work seeks to identify the potential for receptor-specific agonists that may be safer, more effective therapeutics for diabetes.

3.1 Introduction

Despite the remarkable advancements in clinically available drugs for treating hyperglycemia in diabetes during the past 30 years, there are currently no cures for type 1 diabetes (T1D) or type 2 diabetes (T2D). Reflecting this reality, the global population living with diabetes has increased by over 301% from 108 million to 422 million during those three decades and is still growing. Current trends project 54.9 million cases of both types of diabetes in the United States alone by 2030 (187). Therapies that can promote β-cell survival and halt the progression of developing T2D are in dire need. Similarly, identification of better agents that can protect β-cells during the early stages of T1D and improve the graft efficiency and longevity of transplanted whole pancreas or islets for β-cell replacement is needed (188).

Cholecystokinin (CCK) is one of the most studied peptide hormones known for its physiological function in both the gut and brain for nearly a century (7). Despite the well-established understanding of its endogenous function as a critical modulator of digestion, food intake, and anxiety, CCK and its receptor-mediated signaling and specific downstream functions at the pancreatic β-cell level are still largely elusive. We and others have recently demonstrated that cholecystokinin is expressed endogenously in the human pancreatic β-cells (35,90). It is now well documented that CCK and gastrin transcripts are highly upregulated in mouse and human β-cells during diabetes and obesity (35,90–92). Data from the past decade, such as *in vivo* CCK β-cell-selective overexpression and knockout studies, indicate that the elevated levels of CCK are

probably a compensatory mechanism for metabolic stress and are thus beneficial for β-cell mass preservation (35–37,91,92).

Additionally, we have demonstrated that pretreatment of human islets and continuous treatment with CCK-8 protects transplanted human islets from apoptotic β-cell death in an immunodeficient diabetic mouse host. CCKA receptor (CCKAR) and CCKB receptor (CCKBR) are the only known GPCRs to which CCK naturally binds to signal at a cell surface (77,170). CCKAR and CCKBR are highly homologous and share many downstream signaling pathways upon stimulation. Yet, they are distinct GPCRs capable of different signal transduction in various mammalian tissues in healthy and diseased states (89,97).

Contrary to popular belief that CCKAR is the only dominantly expressed isoform outside of the brain, recent discoveries document that both forms of CCKRs are expressed in human islets and the pancreatic β-cells (93). Mature CCK peptides with O-sulfation on tyrosyl residue can bind to the CCKAR or CCKBR. During the pre-pro-CCK protein endoproteolytic maturation process, up to ~47% of CCK peptides remain non-sulfated, and these non-sulfated peptides can only bind to the CCKBR. Therefore, physiological CCK upregulation in the β-cells will likely result in increased local sulfated and non-sulfated CCK isoforms in the islet microenvironment. However, no prior study has focused on whether a CCK receptor isoform-selective role exists in β-cell survival. Whether there is a varied pro-survival response to selective CCKR receptor stimulation in the β-cell is still unanswered. Thus, we set out to explore the different functional outcomes of the CCKAR or CCKBR selective stimulation of β-cells under diabetogenic

stress. To answer this question, we utilized pancreatic islets from CCKAR or CCKBR whole-body knockout animals. We then repeated the experiment on human donor islet β-cells and tested if selective stimulation of one receptor using a selective agonist would change the previously observed pro-survival effect of CCK on β-cells.

3.2 Results

CCK concentration that protects INS-1E cells do not protect Min6 cells against cytokines

Ning *et al.* have reported that CCK can protect mouse (Min6) β-cell lines against hyperglycemic glucotoxicity (85) while we have demonstrated that CCK can protect rat (INS-1E) cells against pro-inflammatory cytokines (Chapter 2). Out lab has verified that loss of CCK via siRNA-mediated silencing of the *Cck* gene attenuated IL-1β and TNF-α mediated cell death in MIN6 cells *in vitro* (92). However, to our surprise, contradicting the report by Ning *et al.* (85) we could not detect significant improvement in Min6 cell survival during extended incubation with exogenous CCK-8 against pro-inflammatory cytokine cocktail-induced cell death (Fig.1A&B). In contrast, we confirmed that a substantial delay in cell death was observed in INS-1E cell lines under the same experimental conditions as previously reported (Fig.1C&D). We had previously demonstrated that 24-hour exposure of proinflammatory cytokines in INS-1E cells consistently induces around 55.02 ± 1.46 % Annexin V positive cells when individual cells were screened using imaging flow cytometry. The maximum drug effect is

achieved once all the receptors are occupied. The binding of a drug to a receptor is principally the same as a drug binding to an enzyme (189). To verify that we are using enough concentrations of CCK-8 to see the protective effect, we performed a measure of INS-1E viability changes as a function of CCK-8 concentration. Vehicle-treated control cells going through the flow cytometry had 23.86 ± 0.85% of baseline Annexin V positive population. Therefore, the difference in signal from the cytokine group and the vehicle indicates the maximum observable drug effect (Apoptotic signals from cytokine treated - apoptotic signals from vehicle = $\Delta E_{viability\ max} \approx \Delta 31\%$). In other words, biologically meaningful detectable changes in Annexin V positive cells in this experiment are limited to 31% ($E_{viability\ maximum}$ = 76.1%±0.85). Since we define the change in viability as a function of CCK-8 drug, pharmacodynamic (PD or dose-response) model of a receptor binding to ligand to elicit a biological effect (E) at the ligand concentration of (C) for an unknown dose-response (θ_{PD}CCK-8) can be estimated (189) by an equation:

$$E_{viability}(C) = f(C, \theta_{PD}) = \frac{E_{max}[C]}{EC_{50}+[C]} = \text{Biological response at drug concentration (C)}$$

where E_{max} is the maximal effect of a drug, C is the drug concentration, and EC_{50} is the drug concentration ([M]), resulting in half-maximal impact. In (Fig.1E), our result shows that a CCK concentration that attains half of the observed (½ $\Delta E_{viability\ max}$ *or* EC_{50}) against proinflammatory cytokines is around 10^{-11} M of CCK-8.

In INS-1E cells, we observe that 24-hour preincubation of cells with 100 nM of pGlu-Gln-(CCK-8) before introducing cytokines results in maximum observable

protection. However, in Min6 cells, the same concentration and length of incubation did not elicit a similar cytoprotecting effect in Min6 cells (Fig.1C&D).

Similarly, mitogen-activated protein kinases (MAPK) transduction response to CCK-8 is observed in INS-1E cells through robust phosphorylation of ERK1/2 at 100 nM of CCK-8 (Fig.1F). However, the same experimental conditions did not elicit a similar response in Min6 cells (data not shown). We also confirm that Min6 cells express and secrete higher levels of CCK (Fig.1G) (91) but also have significantly higher levels of CCKBR expression (Fig.1H). On the other hand, βLox5 cells do not consistently express CCKAR or CCKBR, and the detected levels are at the borderline of the detection limit (Fig.1H). INS-1E cells show preferential expression of CCKAR similar to the low levels expressed in lean mouse islets (35,91) (Fig.1H). Taken together, these data suggest that in rodent β-cell lines, CCK signaling through the CCKAR provides the predominant effect to promote cell survival.

CCKAR is the primary receptor in mouse pancreatic islets

Islets from mice with a germline KO of either one or both CCK receptors were isolated to test the hypothesis that CCKAR plays a predominant role in CCK ligand-mediated protective effect in β-cells. mRNA isolated from the islets of *Cckar*$^{-/-}$, *Cckbr*$^{-/-}$, *and Cckabr*$^{-/-}$ animals (hereon mentioned as AKO, BKO, and ABKO respectively) confirmed the absence of corresponding receptor transcript using RT-qPCR (AKO ΔC_T = -15.32 ± 0.67; BKO ΔC_T = -24.49 ± 3.62; n=3 each) (Fig.2A). The control mouse used

in the experiment was generated by breeding 129-*Cckar*^{tm1Kpn} *Cckbr*$^{tm1Kpn/J}$ with 129S1/SvImJ counterpart (from here on mentioned as 129Sv/WT).

We also observed that *Cckar* transcript level was dynamically regulated in WT mouse pancreatic islets. *Cckar* expression decreased after 24 hours of pro-inflammatory cytokine treatment compared to vehicle control-treated islets ($\Delta\Delta C_T$ 4.862 ± 1.157, ****p=0.0009*) (Fig.2B). However, when concurrently treated with CCK-8 (100 nM), this cytokine-induced reduction was eliminated ($\Delta\Delta C_T$ 0.6885 ± 1.481, *p=0.6560*) (Fig.2B). Conversely, *Cckbr* expression remained low and unchanged regardless of CCK-8 or cytokine cocktail treatment (Fig.2B). These expression patterns further supported the premise that CCKAR is the primary CCKR in mouse islets.

CCKAR protects mouse islet cells from the onset of early apoptosis and prevents the progression of late-stage apoptosis

Isolated islets from AKO, BKO, ABKO, and control littermates were cultured for 24 hours with or without 100 nM of CCK-8, followed by 24-hour proinflammatory cytokine exposure. Dissociated islets were stained with Annexin V (A5) and propidium iodide (PI) as apoptotic markers (Fig2.E&F). Islets from WT and BKO mice treated with 100 nM of CCK-8 for 24 hours before cytokine exposure had a cumulative reduction of cells expressing apoptotic markers by 11.6% and 10.6% (*$p<0.005$, n=3 and n=4 animals, respectively) in comparison to cytokine only treated control islets from the same animal (Fig 2C). In contrast, CCK failed to protect AKO and double ABKO mouse islets (Fig

2C, n=5, and n=4 animals, respectively) under the same conditions. Imaging flow cytometry showed that at 24-hour incubation period with murine proinflammatory cytokine cocktail, the protective effect of CCK-8 pre-incubation was primarily through reduction of the early apoptotic population (A5+ only cells) in the WT (6.45% reduction, $**p<0.01, n=9$) and in BKO islets (8.318% reduction, $*p<0.05, n=3$). In contrast, late-stage apoptotic populations (A5+ and PI+) or very late/necrotic populations did not show significant changes (Fig.2C).

Furthermore, when these dissociated islets were incubated further for another 12 hours in culture (totaling 36 hours of cytokine incubation), there was a marked increase in the number of cells in early apoptosis (A5+ only) as well as necrotic/very late-stage apoptosis population (PI+ only), while Annexin and PI co-stained population had decreased overall (Fig.2D). The CCK-8 islet cells from WT and BKO had fewer cells with early apoptotic signals (3.7% ($*p<0.05$) and 19.27% ($***p<0.001$) fewer, respectively. Additionally, at the end of extended 36-hour incubation, the number of cells with necrotic/very late apoptotic signals (PI+ only) was also significantly attenuated by CCK treatment for both WT and BKO mice (12.20% reduction, $***p<0.001$ and 14.50% reduction, $***p<0.001$). Again, no reduction of apoptotic marker signals was observed in islets from ABKO or AKO islet cells (Fig.2D).

Mouse islets pre-treated with CCK have "protective priming" that reduces β-cell apoptosis.

A 24 hour or overnight incubation of WT and BKO mouse islets with CCK-8 was determined to be a prerequisite for protective seen in reducing apoptotic cells. Although concurrent administration of CCK-8 and proinflammatory cytokines to these islets did show mild attenuation of Annexin V+, it did not show significant differences between cytokine alone treated islets from the same animal. Similarly, the differences in TUNEL staining were not found between CCK-8 treated and non-treated islets animals when 24-hour preincubation of the mouse islets was omitted, and the CCK-8 was given simultaneously with the cytokine cocktail.

CCKAR is required to protect mouse β-Cells against apoptosis

To further confirm the impact of CCK treatment on CCKRKO mouse islets and verify that the apoptosis we observed was in β-cells rather than another islet cell type, we measured apoptosis with a different methodology. TUNEL staining was quantified in mouse islets dissociated and plated on microscope slides and co-stained with insulin after receiving all treatments. Isolated islets were again treated with proinflammatory cytokines for 36 hours, either with or without the 24-hour pre-exposure with 100nM of CCK-8, followed by continued CCK-8 treatment during the cytokine exposure using the same CCK-8 concentration. We observed that CCK-8 treatment again reduced β-cell apoptosis in WT and BKO mouse islets by 3.7% (**$p<0.01$, n=9 animals) and 7.2% (*$p<0.05$, n=9) respectively (Fig.2G). Interestingly, CCK treated islets from ABKO and

AKO animals slightly increased net TUNEL positive β-cells relative to the cytokine only treated β-cells from the same animal islet prep (+4.22% and +2.68%, *$p<0.05$).

Human islets have variable responses to specific CCKR agonists that may depend on the donor's baseline receptor expression or metabolic disease.

Islets isolated from two obese non-diabetic female donors with a family history of diabetes (BMI 39.8, SAMN11483342 and BMI 39.5, SAMN11244711), one obese non-diabetic male donor (BMI 31.3, SAMN12598151), and a non-diabetic overweight male donor (BMI 28.5, SAMN09370567) were incubated with saline vehicle or 100 nM of CCK-8. They were later exposed to a human pro-inflammatory cytokine cocktail for 48 hours before dissociation for imaging flow cytometry analysis (Fig.3A-D).

Flow cytometry results of vehicle-treated islet cells from all subjects demonstrated low baseline levels of early apoptotic (Annexin V+ only) population (20.08%±2.34, n=3) and late apoptotic (A5+ and PI+) population (8.99%±7.6, n=3). They demonstrated an increase in apoptotic cells with cytokine exposure, although the islets from the overweight donor did not show significant cytokine-mediated apoptosis (Fig 3D).

All donor islets treated with CCK-8 before cytokine exposure had, although not statistically significant, a tendency towards reduction of early apoptotic cells (4.65%±2.21 reduction, $p=0.13$, n=4). At the same time, no differences were observed in late apoptosis makers (Fig.3E). Raw values of the differences are illustrated in Table.1.

We also show that human donor islets for the two obese non-diabetic female donors with a family history of diabetes (BMI 39.8, SAMN11483342 and BMI 39.5, SAMN11244711) and one obese non-diabetic male donor (BMI 31.3, SAMN12598151) all had varied mRNA levels of both CCKAR and CCKBR while the non-diabetic overweight male donor (BMI 28.5, SAMN09370567) did not express either CCKAR or CCKBR (Fig.3F).

Interestingly, treatment with CCKAR selective agonist A-71623 reduced total apoptosis in all islet preparations, although when combined, there is no significant difference (early apoptosis reduced by 6.07%±5.72, p=0.37, n=4). Finally, treatment with Gastrin-17 as an agonist for the CCKBR led to no improvements in β-cell survival, either in individual preparations or in aggregate. Taken together, we demonstrate that human islets have variable responses to CCK treatment, but the trend for improvement in β-cell survival is only seen with CCK-8 or CCKAR agonist treatment.

Both CCKAR and CCKBR selective agonists improve glycemic control following human islet transplant

To test whether selective activation of CCKAR or CCKBR could protect human islets *in vivo*, we transplanted 2,500 islet equivalents (IEQ was defined as 125 μm diameter islet = 1 IEQ) of human islets (donor information in Supplementary Table.1-3) under the kidney capsule of immunodeficient (NOD/SCID) mice with streptozotocin (STZ)-induced diabetes. We treated these mice with saline, A-71623, or Gastrin-17 via

an osmotic pump for three weeks. Human islets were pre-treated with A-71623, Gastrin-17, CCK-8, or vehicle control 24 hours before transplant. In contrast to our previous study, we transplanted 2,500 IEQs of human islets per mouse in this xenograft model, a sufficient but not excessive amount to reverse and restore euglycemia. We based this number on previous studies of human islet transplant into mouse kidney capsules that demonstrated that a graft consisting of 2,500~4,000 IEQ successfully restored euglycemia in mice with no reported difference in glycemic control (190–192). In prior studies, 2,000 IEQ of human islets transplanted into kidney capsules cured diabetes in 75–80% of recipient mice (193). Our goal was to mimic clinical transplantation and track hyperglycemia, similar to that seen in human diabetes. The amount of CCKAR or CCKBR selective agonist (A-71623 and Gastrin-17) delivered by an osmotic pump was calculated to approximate the daily dose previously administered to obese mice by twice-daily injection (174). The experimental is depicted in Supplemental Fig.1.

Five out of six animals that received the A-71623 and all of the Gastrin-17 treatment reversed hyperglycemia four days after the transplant. On day 4 after the transplant A-71623 treated group had mean random glucose of 118.62 mg/dL±109.6 (n=5) compared to 76.88 mg/dL±7.122 in the Gastrin-17 group (n=5) and 338.13 mg/dL±184.89 in the saline-treated control group (n=4) (Fig.4B). Surprisingly, animals that received human islets that were only pre-incubated with CCK-8 but did not receive continuous CCKR agonist therapy also had restored euglycemia by four days after the transplant with a mean random glucose measure of 130.5 mg/dL±123.0 (n=5) (Fig.4D). The body weight of the human islet transplant recipient animals receiving A-71623 or

Gastrin-17 infusion was unaltered compared to the control group or the CCK-8 pre-incubation group throughout the 5-week duration of the study (Fig.4G).

Furthermore, the average value of 5-hour fasting glucose levels in a sham animal ranged from 385~577 mg/dL, while the saline-treated control transplant group ranged between 134~218 mg/dL throughout the study (Fig.4C&E). However, all three CCKR drug-treated groups had fasting glucose revolving around ~60 mg/dL starting from 7 days after the transplant until the end of the study (Fig.4C&E).

The saline-treated control transplant group had mild attenuation of hyperglycemia in the early days (338.13 mg/dL±184.89, n=4). Yet, the saline-treated control group gradually rebounded to overtly diabetic level by week 3 (415.62 mg/dL±196.92, n=4), comparable to a sham-operated animal that was made diabetic by STZ injection but did not receive a human islet transplant (blood glucose > 500mg/dL) (Fig.4B-E). In contrast, both A-71623 or Gastrin-17 infusion and the CCK-8 pretreated group with saline pump did not show post-graft glucose rebound and maintained euglycemia throughout the study (Fig4.B-E) until a survival nephrectomy was performed to remove the left kidney containing the graft.

To further define the graft β-cell function, intraperitoneal glucose tolerance tests (IPGTTs) were performed on all experimental animals post-graft day 21. As shown in Fig. 4F, saline pump containing control animals without any pre-transplant drug treatment showed improved glucose tolerance than the STZ-induced diabetic NOD-SCID sham mice but performed much worse than all three of A-71623, Gastrin-17, or CCK-8 pretreatment only groups (Fig.4F).

Upon removing the graft on post-transplant day 22, all animals surviving the nephrectomy lost glycemic control (blood glucose > 550mg/dL). Animals were then sacrificed 7 to 10 days after the survival nephrectomy to collect the endogenous pancreas, later histologically stained for insulin and glucagon. The remaining mouse islets mainly consisted of a glucagon-positive cell population with less than ten insulin-positive cells remaining in the islets with the highest number of insulin-positive signals (Fig.5G), confirming that the euglycemia observed in experimental groups were driven by human insulin.

Transplanted human islets are protected by both CCKAR agonist and islets pre-treated with CCK alone preserves graft mass and reduces β-cell apoptosis.

We measured the total insulin-positive area in each animal graft as well as TUNEL-positive-β-cells to determine the level of apoptosis in the islet grafts three weeks after the transplantation. We report a higher amount of total insulin-positive β-cell mass in animals treated with A-71623 to have (33,823), Gastrin-17 (29,808), and observed the highest number of β-cells remaining for the CCK-8 pre-treated group (49,476) in comparison to a saline-treated control group (20,700) (Fig.5C). The graft β-cell mass was quantified from 30 images taken total from each transplant group. Five to six images were taken from each animal harvested per animal (n=4). Photographs were taken from every 7^{th} section at a 20X magnification. Total pixels in the insulin-positive area were first determined manually. Then, the entire pixel area of DAPI positive pixels within the

insulin-positive area, as well as the pixel count of the average nucleus, was determined to be 550 pixels.

Finally, to obtain the total number of β-cells in the insulin-positive region of interest (ROI), the total DAPI occupying pixels in the ROI was divided by the average DAPI area surrounded by insulin-positive pixels per cell (Fig.5B). To ensure that the total β-cell count is not biased by a set of images that contains more cells than others, the average number of β-cells per image quantified were determined by choosing 30 images from each cohort at random, and the nucleus was manually counted (Fig.5D). Finally, the total number of insulin-positive cells co-localizing with TUNEL was quantified (Fig5.E&F).

Out of 10,328 β-cells in human islet grafts from four saline control mice, 5.492 % ±1.120 β-cells (547 cells) were co-localizing with TUNEL (n=4). The total %TUNEL-positive β-cell numbers for A-171623 (0.704 % ±0.320, n=4, ***$p<0.001$), Gastrin-17 (2.548 % ± 2.095, n=4,**$p<0.01$), and CCK-8 pretreatment only group (1.397 % ±0.797, n=4, ***$p<0.001$) (Fig5.E). The net raw count of each treatment group for TUNEL-positive β-cells was 179 out of 26,749 (A-71623), 442 out of 23,586 (Gastrin-17), and 418 out of 30,444 β-cells (CCK-8), showing a reduction of 4.787% or 7.999 fold reduction, 2.943% reduction or 2.155 fold reduction, and 4.094% or 3.930 fold reduction relative to the saline control respectively (Fig5.E). These results show that both A-71623 and Gastrin-17 treatment protect human β-cells from apoptosis (Fig.5E&F) and preserve total β-cell mass (Fig.5C&D) as reflected in the improved glycemic control in these animals. Interestingly, the total TUNEL-positive β-cells in the graft transplanted with CCK-8 pretreatment but without continuous infusion showed the highest mean β-cell

mass. Similarly, A-71623 treatment and infusion demonstrated the highest reduction in TUNEL positive β-cells at three weeks post-transplant than the saline-treated control group (Fig.5E). Together, our study indicates that *in vivo*, both CCKAR and CCKBR selective agonists can protect human β-cells from post-transplant apoptosis, preserve total β-cell mass, and aid in maintaining improved glycemic control. We report an exciting discovery that 24-hour CCK-8 treatment of human islets before transplantation is sufficient to provide apoptotic suppression up to three weeks following the transplant and preserves insulin-positive graft mass.

3.3 Discussion

This study is the first to test the functional differences between CCKAR and CCKBR in the scope of pancreatic β-cell protection in mice and humans. Many previous studies had characterized the physiological and pathological consequences of CCKAR KO (183,194–196) or CCKBR KO (197–201) rodents in the scope of gut motility, smooth muscle contraction, and neurological phenotypes (202).

The most notable differences in CCKBR KO mice were related to neurological systems. CCKBR KO animals had reduced learning capacity, attenuation of pain perception, increased vascularization and hyperemia in the ganglionic nerve and spinal cord and reduced overall inflammation after surgically induced inflammatory nerve damage(199).

The metabolic effects of CCKR double KO, despite the known regulatory role of CCK in the control of satiety, were not dramatic. CCKAR KO animals do not reduce food intake in response to exogenous CCK, but the absence of CCKR in animals did not lead to obesity (183). Collectively the reports employing CCKR KOs in mice reported that neither of the receptor animals had pathological changes in pancreatic islet morphology in acinar cells, β-cells, α-cells, and duct cells nor developed any signs of glucose regulatory malfunction (183,194,195,202).

It was not until recently that it was observed that CCK expression could increase up to 500-fold during obesity or hyperglycemic stress and that CCKRs, CCK, and gastrin are locally expressed within the mature pancreatic islet of humans and animals. The increase in islet CCK expression in response to obesity or other stressors was interpreted as an adaptive response (90,92,93). The characterization of CCK KO in obesity and overexpression in β-cells revealed CCK might have a direct beneficial effect on β-cells while treatment of β-cells with CCKR dual agonist pGlu-Gln-CCK-8 protected β-cells from apoptosis.

Discussion On CCKR Selective KO Mouse Experiments

Using CCKR knockout mice, two further intriguing observations were made. First, we report that CCKAR is the primary driver of β-cell protection in mice by demonstrating that islets lacking the CCKAR are not protected against proinflammatory cytokines by CCK treatment (Fig.2C, 2D, &2G). Reflecting this result, Min6 cells with relatively low CCKAR expression (Fig.1H) did not show increased viability with CCK

treatment. In contrast, INS-1E cells with 10-fold higher levels of CCKAR than CCKBR ($\Delta\Delta$Ct >3.33) had significant protection from CCK against pro-inflammatory cytokines.

Secondly, in imaging flowcytometry, we see that the significant difference between total apoptotic signaling shown in figure 2A is the sum of all apoptotic signaling. However, when this stack is deconstructed to subpopulations separately representing the *early apoptotic*, *late apoptotic*, and *very late apoptotic/necrotic* cells, we see that the primary driver of the total viability difference is seen in islets expressing CCKAR is a reduction in early apoptotic cells (Appendix Fig.3.2). Thus, CCK is either suppressing the onset of *early apoptosis* or preventing a subpopulation of cells from entering *late-stage apoptosis* (Fig.6). When the same islet cells that are now dissociated were collected back and incubated for another 12 hours in culture in cytokine cocktail containing media, the CCK-8s protective differences in CCKAR intact islets were significant in both *early apoptotic* and very *late apoptotic/necrotic* cells but not in *late apoptotic* cells (Appendix Fig.3.3). One possible interpretation of this change in *late apoptotic* to *very late/ necrotic* difference at 36 hours of cytokine incubation is that a population of cells that had already progressed into *late apoptosis* at 24 hours of cytokine incubation had further moved into complete cell death. This shift is then represented by necrosis-like morphology, where remnants of nuclear DNA are still detectable by PI+ signaling. Still, the cell membrane integrity is mainly lost, and the Annexin V+ signals seen on phosphatidylserine (PS) are partially stripped away by the microcapillary fluidic shear force exerted on the dead cells by the flow cytometer pressure (203). Therefore, the majority of *late apoptotic* cells shifting to *very late apoptotic/necrotic* might be an

artifact of the experiment. However, regardless of the presence of experimental artifact between these two designations, they can be collectively seen as dying or already dead cells, and different interpretations of our data between these two populations results do not disqualify the validity of our result since the combined values of both *late apoptotic* (A5+ & PI+) cells, and *very late apoptotic/necrotic* (PI+ only) cells are still significantly lesser at 36 hours.

Discussion On Human Islet Imaging Cytometry Results

We have previously shown that treating human islets with CCK-8 prevents cytokine-induced β-cell apoptosis (Chapter 2). Caspase 3/7 activity and TUNEL staining were reduced for CCK-8 treated human islets when the cells were exposed to cytokine for 24 hours (Chapter 2, Fig.2B&C). Here we report that when the experiment was repeated using imaging flow cytometry with a longer duration of cytokine exposure for 48 hours following the 24 hours of CCK-8 pre-incubation, we observed varying magnitude of suppression of apoptotic progression at the end of 48 hours by CCK-8, A-71623, and Gastrin-17 (CCKAR, and CCKBR selective agonists) (Fig.3A-E).

Notably, the untreated islet cells from the only overweight non-diabetic male donor (SAMN09370567) had similar levels of early apoptotic cells (18.03%±2.41 vs. 20.22%±2.29) but much higher levels of baseline late apoptotic cells (24.27%±2.21 vs. 2.62%±1.30) and necrotic cells (8.54%±1.32 vs. 2.82%±1.09) in comparison to the three other obese (BMI >30) donor islet cells (SAMN11483342, SAMN11244711, and SAMN12598151) (n=3). Inclusion or removal of the donor islets from the non-diabetic

overweight male (SAMN09370567) who had higher baseline apoptotic cells did not alter the overall result significantly (data not shown).

Interestingly, islets from the donors who had the highest BMI (>39) and higher average blood glucose at the time of inpatient admission (>140mg/dL) had the most reduction in overall apoptosis and early apoptotic signals when treated with CCK-8, A-71233, or Gastrin-17 (Table.1). Another observation is that Gastrin-17, a CCKBR selective agonist, had the highest variance in increasing or reducing apoptotic signals. In some cases, Gastrin-17 has been shown to increase apoptotic signals instead of lowering them. In all cases where donor islets dramatically reduced early apoptosis by CCK-8, it has also demonstrated a significant decrease in either early or late apoptosis by A-71623 and showed a unidirectional trend in protection by both CCK-8 and A-71623. No directional correlation was observed in changes of apoptotic signals between CCK-8 and Gastrin-17 within the same donor islets treated with different drugs. Furthermore, islet CCKAR, CCKBR, and CCK mRNA levels from the human donor islets did not significantly correlate with the donor BMI, Weight, or Age (Supplemental Fig.2).

Additionally, our previous TUNEL staining in dissociated mouse islets show that CCK only reduced the apoptotic β-cell but showed no difference in non-β cells. Therefore, since the flowcytometry reading of dissociated human islets did not distinguish between β-cell and other islet cells in counting for the dying population, the difference of β-cell death in the current experiment may have been deflated.

Although we have previously demonstrated that CCK protects β-cells against apoptosis (Chapter 2, Fig.3), the imaging flow result of human islets shows that human islets have variable responses to specific CCKR agonists that may depend on baseline receptor expression or metabolic disease in the donor.

Discussion On Human Islet Transplant Study

In contrast, to lean mouse islets containing little or no CCKBR, both CCKAR and CCKBR are present in the human islets. Our previous study reported that CCK protected human β-cells *in vivo* and provided valuable information that even under sustained hyperglycemic stress and the stress of transplantation, exogenous infusion CCK can protect human β-cells.

However, our previous study in chapter 2 was limited by its sub-therapeutic transplantation design of human islets, preventing us from examining the CCKs' effect on post-transplant glycemic control. Furthermore, our previous study could not ask if either selective activation of human CCKAR or CCKBR can provide similar protective benefits in transplanted humans β-cells.

Our current study addresses these limitations and uses a therapeutic amount of human islet IEQs transplanted into STZ indued diabetic NOD/SCID mice to examine the effect of CCK receptor-selective agonists. Furthermore, in designing the current study, we have ensured that the human donor islets used for the xenograft expressed both CCKAR and CCKBR through validation using an RTq-PCR (Fig.5A).

We report that for non-diabetic human islets that express both CCKAR and CCKBR, a 24-hour preincubation of the islets and co-transplantation of pumps that release either CCKAR selective agonist A-71623 or CCKBR selective agonist Gastrin-17 both significantly improve the post-transplant glycemic control in the diabetic NOD/SCID xenograft model. We report that human islet graft mass is considerably better preserved, and β-cell apoptosis is reduced when the islets were preincubated and transplanted with either of CCKAR or CCKBR agonist releasing pump. We also report a novel discovery that conditioning human islets with CCK-8 alone is sufficient to provide similar anti-apoptotic and improved glycemic control of the transplanted human islets *in vivo* (Fig.5). The translational implications of this result in the scope of allogenic islet transplant in the clinic will be discussed in conclusion.

The subcellular signaling mechanism of how CCK protects β-cells is still unsolved. Although our intention in using a CCKR subtype-selective agonist was to identify the CCKR responsible for the protective effect on human islets and thereby narrow down the candidate signaling pathways. However, our findings suggest that multiple paths may lead to a pro-survival effect, including CCKAR and CCKBR agonists and pre-treatment alone with CCK-8.

CCKR activation is sufficient to protect β-cells even if it is given before introducing the diabetogenic stress. We have preliminary evidence that pre-exposure of mouse and human islet to CCK before pro-inflammatory cytokines or transplant into a diabetic mouse is sufficient for β-cell protection. However, we have not performed a converse

transplant experiment to see if an omission of CCK-8 pre-incubation in human donor islets results in reduced or loss of protective effect demonstrated here. Thus, further investigations to determine if the CCK preincubation step is essential, and not just sufficient, for β-cell protection is needed.

3.4 Conclusion

CCKR downstream signaling converges on numerous nuclear transcription factors that regulate cellular stress response genes, apoptotic signaling genes, and cell proliferation regulators. Accordingly, cells respond to various types of stress through shared signaling pathways and adoptive transcriptional regulation, which almost always makes cellular responses to multiple stresses are synergistic.

However, so far, various literature on CCK on multiple tissues has been equivocal. CCK is beneficial to cell survival in some cases but can also promote maladaptive cellular responses such as induction of pancreatitis and adenocarcinoma. Pre-exposure to one form of stress can induce a stress-hardening in most cells. Could an overused phrase "*What doesn't kill you makes you stronger"* be partially true for CCK? Can exogenous CCK be providing an otherwise vulnerable β-cell to deploy a protective signaling mechanism?

If we can, for a moment, accept that CCK is a signaling molecule with a dual role. The idea fits well with the conflicting reports that systemic CCKR activation can be detrimental in some tissues. Many cells either do not express or express trace amounts of

CCK mRNA. Still, in other studies using when exogenous CCK stimulates them, they are protected from subsequent insults. This brings up an interesting question to obesity-driven upregulation of CCK seen in animal models. Could the obesity-driven upregulation of islet CCK in obese animals provide a *"protective priming"* of their β-cells to be more responsive to the exogenous CCK?

As seen in our current transplant study, even transient pretreatment of human islets, even just for 24 hours, is sufficient to elicit long-term protection of the graft function and mass. Therefore, islets that are already expressing larger quantities of endogenous CCK might perform better than the islets from a lean mouse when challenged to the same levels of diabetogenic stress. Few interesting experiments could be performed in the future to test whether if high CCK expressing islets from non-diabetic obese mice are more resistant to stress-induced death than the islets from their lean littermate counterparts. An additional experiment comparing the transplant outcomes using identical IEQ of isolated islets from high-fat diet (HFD) induced, non-diabetic obese animals and the islets isolated from lean littermates into NOD/SCID mice could further test if exogenous CCK treatment is needed. Islets from obese animals may not even need to be pre-incubated in CCK if the donor islets are already "primed" by their CCK production.

Interestingly, in sourcing the human islets for allogenic islet transplant, an Italian study in 2005 reported that analysis of 437 human pancreases processed for islet transplantation between 1989 to 2003 in Milan showed that donors' BMI and weight are positively correlated to islet yield (204). At the same time, a more recent histological

study shows that a higher insulin-positive area in the donor pancreas is a sufficient index to predict the likely success of islet isolation. They also report that BMI of less than 25 kg/m^2 is unfavorable to islet isolation outcomes, while islet isolations from donors with a BMI less than 20 kg/m^2 were entirely unsuccessful (205). Could there be involvement of CCK here?

To our knowledge, there has not been a study to see if the human islets from obese donors result in better glycemic control and improved graft longevity in comparison to the islets from lean donors. A post-hoc analysis of human islet transplant might be warranted to answer this question.

The requirement of pancreatic islets and β-cells to be pre-conditioned with CCK exposure may also explain the β-cell protective effect of MIP-CCK mice under multiple low-dose STZ. In contrast, overexpression of CCK in β-cell in a transgenic animal that already has an oncogenic mutation on *Kras* aggravates the pancreatic carcinoma if CCK is further augmenting the proliferative and pro-survival signaling in cells that are genetically modified to be hyperproliferative.

A β-cell could be deploying a counter-stress response that protects the β-cell through activation of UPR, ER Expansion, ROS protective paths, thus better preparing a cell for a possible subsequent insult. Interestingly, a similar observation of "*protective priming*" by CCK has been previously observed in neurons.

In fact, in studies during the early 90s, CCK was shown to have a similar "*protective priming*" effect in brain cells. Cultured rodent cortical neurons pretreated with CCK

protect them against NMDAR mediated glutamate neurotoxicity. Notably, a study reports that pre-exposure to CCK-8 suppresses superoxide generation malondialdehyde and prevents methamphetamine/dopamine-induced cell death in PC12 cells by suppressing nitric oxide (106). Furthermore, a pre-treatment of E19 Wistar female rat neuron cultures with CCK-8 inhibited cell death by subsequent exposure to high doses of glutamate (103,158). PC12 cells and cortical neurons are known to express high levels of CCK receptors. Finally, implantation of CCK secreting osmotic pump *in vivo* prevented the degeneration of cholinergic neurons in rats with basal forebrain lesions mimicking Alzheimer's disease (104).

If CCK is also providing protective priming in β-cells, current results have an exciting translational application. Allo-islet transplantation can be minimally invasive therapy for T1D, while auto-transplantation following a total pancreatectomy is a promising treatment for chronic pancreatitis (CP) to preserve endocrine function. However, the major problem of any islet transplantation for diabetic patients is the massive cell death occurring weeks following the transplantation, requiring multiple sources of islets donor to restore glucose homeostasis in one patient. Thus far, in chapter 2, we have shown that CCK can protect human β-cells after transplant, while in chapter 3, we report that short pretreatment of humans islets with CCK alone is likely protective for up to 3 weeks after the transplant. Suppose β-cell-protective priming by CCK pre-exposure before transplant has long-term improvement in post-transplant outcomes. In that case, CCKR agonists can be clinically used as a pre-transplant method to improve the longevity of islet graft, which has been one of the complex challenges that prevented

the islet transplantation from becoming an FDA-approved cure for diabetes in the United States.

In conclusion, our current study has focused on identifying which type of CCKR is driving the protection of β-cells. We report that while CCKAR is the primary driver for β-cell protection in mice, we conclude through *in vivo* study that in humans, either CCKAR or CCKBR activation alone is sufficient to protect β-cells. Our current result in CCK's ability to prevent β-cells death and its implications in improving the longevity of human islet transplantation is exciting and deserves further study.

3.5 Materials and Methods

CCK and CCKR Selective Agonists

Following CCKR targeting peptides or drugs were used throughout the study: pGlu-Gln-CCK-8 (denoted as CCK-8) was purchased from (American Peptide Company, #338663, Lot# 1504085X) as described previously (152) and was also later custom synthesized through GeneScript (GeneScript, custom order #U608DFH060-1). A-71623 (R&D Systems, #2411/1) and Gastrin-17-II (Millipore Sigma, #G1260) were purchased from the vendors.

Cell Culture

Min6 mouse insulinoma cells were cultured in DMEM (Gibco, 12800-017) supplemented 10% (v/v) heat-inactivated FBS (Hyclone, Fisher Scientific), 1% P/S, 70

uM freshly added beta-mercaptoethanol (Sigma, M7522) at 37 °C and 5% CO2. INS1E rat insulinoma cells were cultured in RMPI 1640 (Thermo Fisher Scientific, #11875093) with 2.05 mM Glutamax (Cellgro, 35050079) supplemented with 5% (v/v) heat-inactivated FBS (Hyclone, Fisher Scientific), 1% P/S, 10 mM HEPES, 1 mM sodium pyruvate, and 50 μM freshly added β-mercaptoethanol (Sigma, M7522) at 37 °C and 5% CO2. βLox5 cells ere cultured in Low glucose (1mg/mL) DMEM (Cellgro) supplemented with 10% FBS (HyClone, Fisher Scientific, Pittsburgh, PA), 1% MEM non-essential amino acids (Cellgro), 1% P/S, 0.02% BSA (Sigma), and 15mM HEPES (Cellgro) (VC-DMEM) [1M HEPES Solution] at 37 °C and 5% CO2. Min6 or INS1E cells were seeded in 12 well plates at a density of 0.1 x 10^6 cells/well or in 6 well plates at a density of 0.5 x 10^6 cells/well and were incubated for 24 hours before any treatment.

Viability Assay

Cells cultured as described above were incubated in fresh media containing either 100 nM CCK-8 or 100 nM saline vehicle control up to 72 hours with concurrent treatment with a mouse cytokine cocktail containing 50-ng/mL TNFα (Miltenyi Biotec, #139-101-687), 10-ng/mL IL-1β (Miltenyi Biotec, #130-101-680), and 50 ng/mL IFN-γ (Miltenyi Biotec, #130-105-785) as described (182). Cell media containing the treatment was refreshed at 36 hours of incubation for the time course study groups. At each time point, the growth media containing the suspended cells was collected and centrifuged and combined with the adherent cells that were released with 0.25% trypsin (Sigma, 59428C)

and then resuspended in 100 ul of growth media containing FBS. 10 ul of suspended cells were then added to a 10ul solution of 0.4% trypan blue in a buffered isotonic salt solution (Bio-Rad, #1450021), pH 7.3, and measured for viability using TC10 automated cell counter (Bio-Rad, #145-0010).

Apoptosis Assay – Imaging Flow cytometry

Apoptosis was measured using image flow cytometry in INS-1E cells (Fig.1E), and islets from wild-type (WT), CCK A receptor KO mice (AKO), CCK B receptor KO mice (BKO), CCK receptor double KO mice (ABKO) treated with mouse cytokine cocktail as above, CCK-8 for 24 to 48 hours (Fig.2C-F). Similarly for human donor islets, islets were treated with cytokine cocktail containing human 1,000 U/ml TNFα, 75 U/ml IL-1β, and 750 U/ml IFN-γ (PeproTech, #200-01B, #300-02, #300-01A) (Fig.4A-E). As above, all cells were pretreated with peptide CCK-8 for 24 hours before the addition of cytokines. Cultured mouse islets, human islets, or INS-1 cells were transferred to a 15 mL conical tube with the incubation media. The culture plate was rinsed with 2 mL PBS and this was added to the tube to ensure complete transfer. Islets and cells were pelleted at *800xg* for 3 min at 4°C followed by a 1-minute wash using cold PBS. Islets were resuspended in 1 ml Sigma Dissociation Solution (Sigma), then shaken horizontally in a 37°C water bath for 8 min at 100 RPM followed by placement of the tube on ice and addition of 2 mL of media to stop the dissociation process. Cells were then gently disrupted and incubated on ice for another 5 minutes before being pelleted

again at low speed for 5 min at 4°C. The supernatant was removed, and the pellet was resuspended in 1x Annexin Binding Buffer containing 0.5% BSA. 5x Annexin binding buffer was made with 50 mM HEPES, 700 mM NaCl, 12.5 mM CaCl2 in water. The cells were then pipetted up and down 30 times, then 1 µl of this cell suspension was placed on a microscope slide to check for dissociation. Depending on the level of clumping, the cells were pipetted up and down for another 20-30 more times or until the majority of the cells were single cells. Cells were then counted on a hemocytometer and were resuspended in 1x Annexin Binding Buffer containing 0.5% BSA to a final concentration of $1x10^6$ cells/ml. An aliquot of 100 µl of cells ($1x10^5$ cells) was mixed with Propidium Iodide (50 µg/mL) and Annexin V-AF488 (Invitrogen #A13201). Unstained and individually stained control samples were prepared in separate tubes. All tubes were incubated at room temp in the dark for 15 min. Samples were diluted immediately before reading with the addition of 400 ul 1x Annexin Binding Buffer containing 0.5% BSA. Imaging Flow data was acquired by imaging flow cytometer (Amnis EMD Millipore, Image Stream Mark II). During the analysis, 2,000 to 10,000 live cell images were captured at 40x magnification. For an extended incubation experiment performed for Fig.2D, cells from the flow cytometry were collected, centrifuged, washed, as described above. They were resuspended in growth media containing CCK-8 and cytokine cocktail for additional 12 hours in 37 °C and 5% CO2 before the flowcytometry was repeated on them. Data were analyzed using ImageStream (Image Stream®X, Amaris) for the quantification of propidium iodide and annexin V stains that indicate the different stages of apoptosis

Western blot

Cells cultured as described above were incubated in fresh media containing 100 nM CCK (sulfated-(pGlu-Gln)-CCK-8, American Peptide Company) for 5, 15,30, 45, and 60 minutes. Protein from the cells was harvested by asperating out the media followed by washing the cells with ice-cold 1xPBS followed by lysing cells in cold RIPA buffer (ThermoFisher, 89900) containing 1% Nonidet P-40 (Sigma, 21-3277) as well as protease and phosphatase inhibitors (Roche, 04693116001). The lysates were centrifuged in QIAshredder columns, and total protein was quantified via BCA assay (Sigma, BCA1). 20 ug of total protein homogenate were loaded per lane onto a 10% polyacrylamide gel (Biorad, 4561036) under constant voltage of 140v for 1 hour using a vertical electrophoresis cell (Biorad, 1658025FC) and were dry transferred onto a 0.2 µm PVDF membrane (Biorad, 1704156) using a Trans-Blot Turbo dry transfer system (Biorad, 1704150). Membranes were then blocked with 5% milk/TBST for 1hr , and incubated overnight with primary antibodies 1:2,000 Rabbit Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody (CST, 9101), 1:2,000 Rabbit p44/42 MAPK (Erk1/2) (137F5), 1: 5,000 mouse β-Actin (8H10D10) (CST, 3700), 1:10,000 mouse GAPDH (CST, 97166) in 1% BSA/TBST solution. Membranes were then washed and incubated with 1:10,000 of Goat Anti-Rabbit IgG H&L (HRP) (Abcam, ab205718), or Goat Anti-Mouse IgG H&L (HRP) (Abcam, ab205719) for 1 hour at room temperature before imaging using chemiluminescent substrate (Thermo, 34579) in an image analyzer (GE HealthCare, Imagequant LAS 4000).

CCK-8 Detection in Min6 and INS-1E Cells

CCK peptide levels were measured using a radioimmunoassay (Alpco Diagnostics, Salem, NH) as previously described (91) in growth media from Min6 cells and INS-1E cells cultured for 24 h as described above (Fig1.G).

mRNA level detection using RTq-PCR

RNA was isolated from Min6, INS-1E, βLox5 cells lines, and isolated mouse islets or human donor islets from cultures previously described. Total RNA was extracted using Rneasy Plus Kit (Qiagen, 74034), the first-strand cDNA was synthesized using iScript cDNA synthesis kit (Biorad, 1708891) from the total RNA extraction. Gene expression was quantified via quantitative PCR (Life Technologies, StepOnePlus Real-Time PCR System) with SYBR (Applied Biosystems, A25741) and the primers listed below:

Human

hsCCK forward TGA GGG TAT CGC AGA GAA CGG ATG
hsCCK reverse TGT AGT CCC GGT CAC TTA TCC TGT

hsCCKAR forward TGG AAG CAA CAT CAC TCC TC
hsCCKAR reverse CAC GCT GAG CAG GAA TAT CA

hsCCKBR forward GAT GTG GTT GAC AGC CTT CT
hsCCKBR reverse GGG CTG ATC CAA GCA GAA A

β-Actin forward TCA AGA TCA TTG CTC CTG AGC
β-Actin reverse TCA AGA TCA TTG CTC CTG AGC

Mouse

msCCKAR forward GCAGTCCTGGCAAACATTCC
msCCKAR reverse ATGAGTCCGTAAGCCACCAC

msCCKBR forward CCAACAAATGTGGTCCGTGC
msCCKBR reverse GGTAGAGTTCGCGGGAGATG

β-Actin forward TCAAGATCATTGCTCCTGAGC
β-Actin reverse TCAAGATCATTGCTCCTGAGC

Rat

ratCCKAR set 1 forward CCCAACCTGCTCAAGGATTT
ratCCKAR set 1 reverse CTACCAGGTTGAAGGTGGAAAC

ratCCKAR set 2 forward ATGCAGCAGTCCTGGCAAACATTC
ratCCKAR set 2 reverse TTTGGCAGATTTCTTCTGGCTGGC

ratCCKBR forward TAGTGCCGGGAACCTCA
ratCCKBR reverse TCACTGCATAAAGGGTGATTCT

β-Actin forward TCAAGATCATTGCTCCTGAGC
β-Actin reverse TCAAGATCATTGCTCCTGAGC

Islet TUNEL Assays

Using serum-free media supplemented with 5 g/L BSA fraction V (Roche, #107351080001), intact mouse islets were pre-treated with 100 nM CCK or saline vehicle control for 24 hours before additional 24 to 36 hours of mouse cytokine cocktail containing 50-ng/mL TNFα (Miltenyi Biotec, #139-101-687), 10-ng/mL IL-1β (Miltenyi Biotec, #130-101-680), and 50 ng/mL IFN-γ (Miltenyi Biotec, #130-105-785). After treatments, islets were dispersed, plated on Poly-L-lysine pre-coated glass coverslips, and fixed. β-cell apoptosis was measured using TUNEL (Promega, #G3250) followed by insulin staining (Dako, A0564) as described in detail below. Imaging was performed using an EVOS FL Autofluorescence microscope (Life Technologies) or DMi8 Inverted fluorescence microscope (Leica Microsystems). ImageJ (NIH) or Adobe Photoshop (Adobe) was used to count DAPI (Vector Labs, H-1200-10) and quantify insulin-

positive/TUNEL-positive cells in at least nine randomly chosen fields per treatment group for each replicate (Fig.2G)

Animals, Islet Isolation and Culture

Animal care and experimental procedures were performed with approval from the University of Wisconsin and William S. Middleton Veterans Hospital Animal Care and Use Committees to meet acceptable standards of humane animal care. NOD/SCID (8 to 12 week-old) mice (NOD.Cg-Prkdc<scid>/J, Stock Number #001303) were purchased from The Jackson Laboratory for transplant studies. CCK receptor double knockout mice, 129-*Cckar*^{tm1Kpn} *Cckbr*$^{tm1Kpn/J}$ (Stock Number #006365) (183,184) age 12-24 weeks were used in the studies. These mice are in a mixed 129 background and were obtained from Jackson Laboratories after cryo-recovery. WT controls used were littermates from 129-*Cckar*^{tm1Kpn} *Cckbr*$^{tm1Kpn/J}$ after breeding with 129S1/SvImJ (Stock Number #002448). Mice were housed in facilities with a standard light-dark cycle and fed ad libitum. Mouse pancreatic islets were isolated using collagenase digestion and hand-picked as previously described (185). Isolated mouse islets were cultured at 37°C and 5% CO_2 in RPMI 1640 media (Thermo Fisher Scientific, #11879020) containing 8 mM glucose, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin (1% P/S) (Thermo Fisher Scientific).

Human islet culture

Human islets were obtained through the Integrated Islet Distribution Program (IIDP). Upon arrival, islets were handpicked and then cultured in RPMI 1640 media as described above but without antibiotics and antimycotics. Islets were cultured overnight to confirm viability and sterility before treatments. Islets were cultured for up to 7 days, and the media was renewed every other day.

Human Islet Transplantation, survival unilateral nephrectomy, and Graft Harvest

A modified version of human islet transplantation described by Montanya *et al*. was used for the study (186). A single high dose (200 mg/Kg) of streptozotocin (Sigma, #S0130) was administered via intraperitoneal injection two days before transplant to induce hyperglycemia. Hyperglycemia (glucose >300 mg/dL) was confirmed with a tail vein blood sample using a glucometer 36 hours prior and immediately before proceeding with the transplant. Human islets were cultured in media supplemented with 100 nM pGlu-Gln-CCK-8 (American Peptide Company or GeneScript), A-71623 (R&D Systems, 2411), Gastrin-17 (G1260), or saline vehicle control for 24 hours before transplant.
Confirmed hyperglycemic mice were anesthetized by delivery of 3-4% isoflurane in oxygen at 3 L/m followed by subcutaneous injection of 0.25% (2 mg/mL) bupivacaine as an incisional line block. A small incision in the skin above the left kidney was made, exposing the peritoneum, followed by an additional incision in the peritoneum, exposing the kidney. Approximately 2,500 islet equivalents human islets were placed under the

kidney capsule on a ventral plain of the left kidney. An infusion pump Alzet 1004 (Alzet, 0009922) containing saline, A-71623, or Gastrin-17 was placed subcutaneously posterior to the scapulae of the mice at the same time as the islet transplant to have continuous drug infusion (52 pmol/hr) for three weeks at a nominal pump rare of 0.10 to 00.11 uL/hr. We calculated the concentration of infused drugs to recapitulate the amount of CCK delivered through twice-daily intraperitoneal injection dosing done in studies by Irwin et al. that led to beneficial effects on glucose and weight in obese mice (152,174). Mice were monitored during recovery and checked for surgical complications in the post-operative period. Bodyweight randomly fed blood glucose and 4-hour fasting blood glucose was measured on post-transplant days 4, 7, 14, 21, for a total of three weeks. Blood glucose was measured using a tail nick blood sample and glucometer (Bayer Contour Next EZ). Three weeks post-transplant, mice underwent a survival nephrectomy. General and local anesthesia was induced in mice as described above, following a 1 cm lumbar incision blunt dissection to separate the muscle and fascia to expose the left kidney using forceps. Left renal artery and vein were ligated using 5-0 gut suture, and the kidney containing the human islet graft was removed. Animals were then closed back using a 4-0 silk suture and were monitored for three days with post-operative treatment of 5 mg/kg twice daily dose of subcutaneous delivery of meloxicam. Once development of hyperglycemia was confirmed 5 to 8 days after post-nephrectomy, animals were anesthetized using Avertin (2,2,2-TRIBROMOETHANOL, 97% T48402, 500mg/kg), and terminal serum was collected by cardiac puncture and stored for human insulin and CCK assays. Kidneys containing islet grafts were harvested and fixed in 10% formalin (Fisher SF100) for 48

hours. 10% Formalin-fixed kidneys were paraffin-embedded and sectioned for immunofluorescence staining as described below.

Immunofluorescence Staining

OCT-embedded islet grafts within the kidneys or mouse pancreas containing the endogenous islets were stained for insulin using polyclonal Guinea Pig anti-insulin antibody (Dako, A0564), Rabbi anti-glucagon antibody (CST, #2760), Mouse monoclonal [P2B1] to anti-CD31 (Abcam, ab24590) and apoptosis was measured using the DeadEnd Fluorometric terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) system (Promega, #G3250). At least ten random images from each islet graft were obtained using a DMi8 inverted fluorescence microscope (Leica Microsystems). Adobe Photoshop (Adobe) software was used to quantify total insulin-positive cell area, total-glucagon positive cells area, and TUNEL-insulin co-staining cells. Total β-cells were counted in 30 images from each transplant group to determine the β-cell mass 3-weeks post-transplant. Five to six images were taken from each animal harvested kidney containing the human islet graft. The mean number of β-cells found per imaging field was determined and total β-cells and TUNEL positive β-cells were counted in five non-consecutive sections from each tissue. Image quantification was performed semi-automatically within the region of interest selected to isolate the grafted human cells from the kidney tissue was performed blinded to an experimental group.

Statistics

Assessment of statistical significance between groups was determined using GraphPad Prism by 2-tailed Student's t or ANOVA tests as the non-parametric equivalent. Bonferroni posthoc test was performed to correct for multiple comparisons where appropriate. A paired t-test was used when samples were from the same islet prep to account for differences in baseline viability (Figures 2A-C, 3B-C). A probability of error less than 5% was considered significant (i.e., $P < 0.05$). Statistical information for experiments (data representation, P values, and n numbers) can be found in the figure legends. In all panels, data are represented as mean ± SEM.

3.6 Acknowledgement

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3.7 Figures

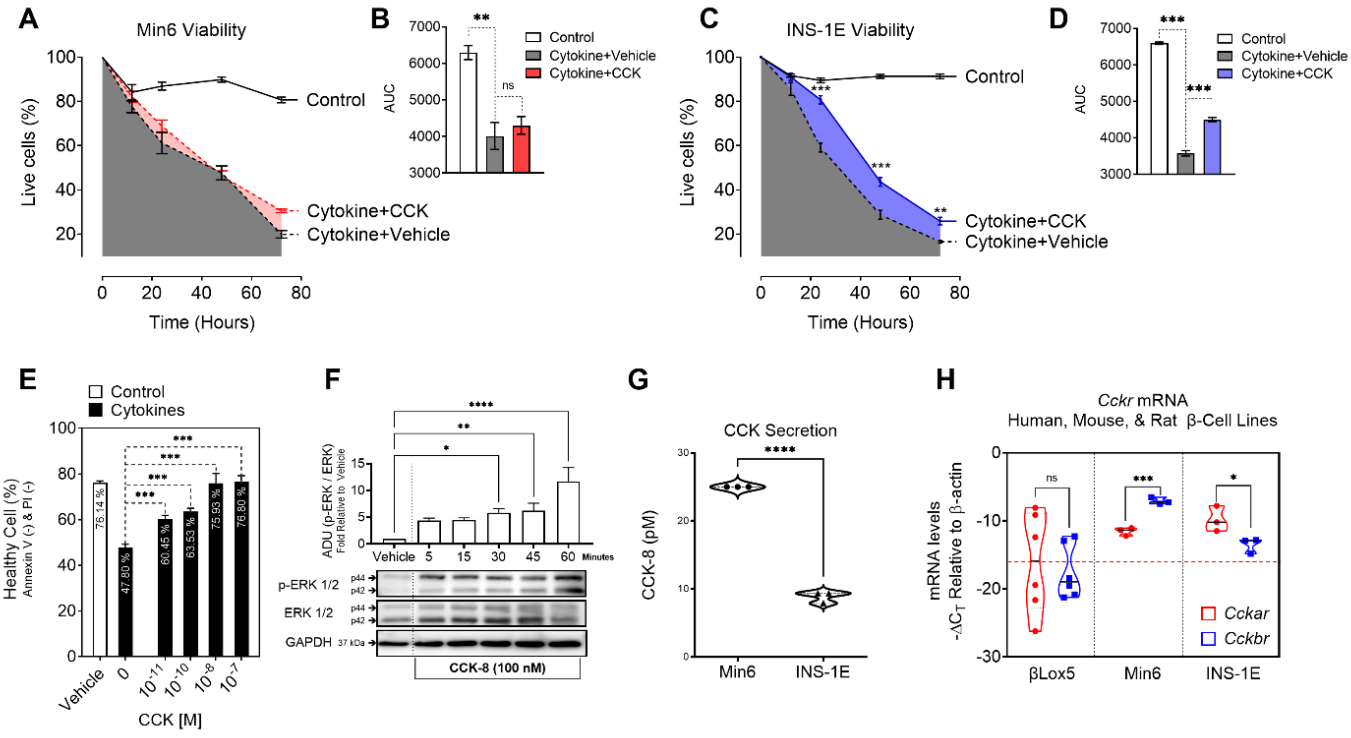
A
Min6 Viability
Live cells (%)
Time (Hours)
Control
Cytokine+CCK
Cytokine+Vehicle
B
AUC
ns
Control
Cytokine+Vehicle
Cytokine+CCK
C
INS-1E Viability
Live cells (%)
Time (Hours)
Control
Cytokine+CCK
Cytokine+Vehicle
D
AUC
Control
Cytokine+Vehicle
Cytokine+CCK
E
Control
Cytokines
Healthy Cell (%)
Annexin V (-) & PI (-)
76.14 %
47.90 %
60.45 %
63.53 %
75.93 %
76.80 %
Vehicle
0
10-11
10-10
10-8
10-7
CCK [M]
F
ADU (p-ERK / ERK)
Fold Relative to Vehicle
Vehicle
5
15
30
45
60
Minutes
p-ERK 1/2
ERK 1/2
GAPDH
37 kDa
CCK-8 (100 nM)
G
CCK Secretion
CCK-8 (pM)
Min6
INS-1E
H
Cckr mRNA
Human, Mouse, & Rat β-Cell Lines
mRNA levels
-ΔCT Relative to β-actin
ns
Cckar
Cckbr
βLox5
Min6
INS-1E

Fig 1. CCK protects cells CCKAR expressing INS-1E cells but not Min6 Cells - Time-course of Min6 cells (A&B) and INS-1E cells (C&D) treated with cytokine cocktail and co-treated with 100 nM CCK (n = 5 to 15). CCK dose-response (E) effects on the percentage of Annexin V (A5) and propidium iodide (PI) double-positive cells (Late apoptosis – black bars) and Annexin V positive cells (Early Apoptosis - gray bars) treated with cytokines (n = 3-6). MAPK activation by 100 nM of CCK-8 in INS-1E cells (F) as a function of time. (G) Min6 and INS-1E cells endogenously secrete CCK-8. (H) INS-1E cells express the highest amounts of CCKAR mRNA, whereas CCKBR mRNA is dominant in Min6 cells. Expression of either CCKAR or CCKBR is not consistently observed in βLox5 cells. less Data are means ± SEM; ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05

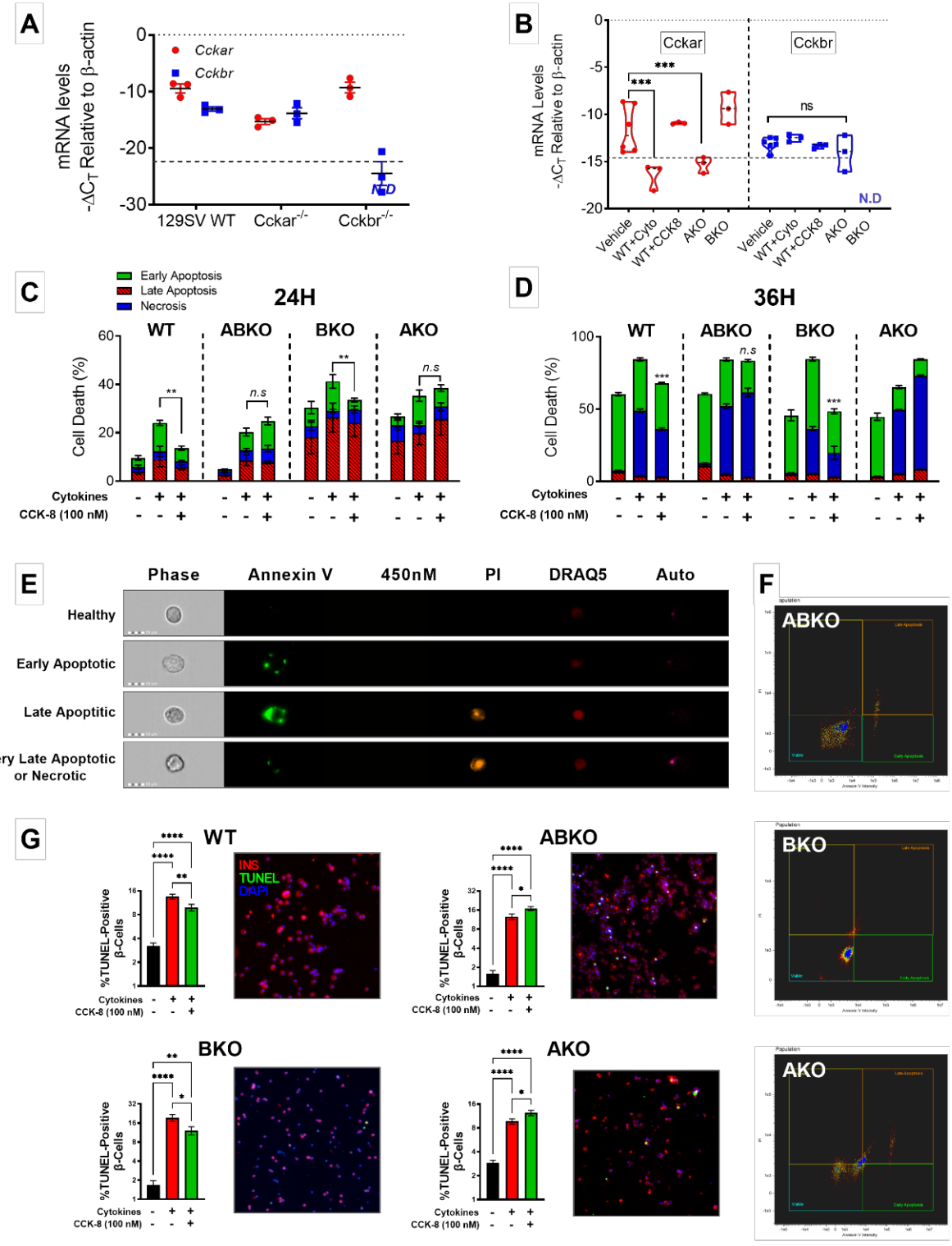
A
mRNA levels
-ΔCT Relative to β-actin
Cckar
Cckbr
N.D
129SV WT
Cckar-/-
Cckbr-/-
B
mRNA Levels
-ΔCT Relative to β-actin
Cckar
Cckbr
***
ns
N.D
Vehicle
WT+Cyto
WT+CCK8
AKO
BKO
C
Early Apoptosis
Late Apoptosis
Necrosis
24H
WT
ABKO
BKO
AKO
Cell Death (%)
n.s
Cytokines
CCK-8 (100 nM)
D
36H
E
Phase
Annexin V
450nM
PI
DRAQ5
Auto
Healthy
Early Apoptotic
Late Apoptitic
Very Late Apoptotic
or Necrotic
F
ABKO
BKO
AKO
G
WT
ABKO
BKO
AKO
%TUNEL-Positive
β-Cells
INS
TUNEL
DAPI

Fig 2. CCKAR, but not CCKBR, protects mouse islets from apoptosis - (A) mRNA levels of CCKAR and CKBR in selective receptor KO mouse islets (n=3). (B) mRNA levels of mouse islets from WT, CCKAR KO, and CCKBR KO animals showing that in WT animal's islets, CCKAR transcription is dynamically regulated. Cytokine treatment of the WT islet reduces the CCKAR transcript comparable to CCKAR KO (n=3). (C) CCK selective receptor KO effects on the percentage of Annexin V (A5) and propidium iodide (PI) double-positive cells when treated with cytokines at 24 hours (n=5 to 8) and 36 hours (D) (n=3). A representation of imaging flow cytometry image of each cell captured for *early apoptosis, late apoptosis,* and *very late apoptosis/necrosis* (E). In imaging flow cytometry, a representative image of apoptotic staining patterns shifts towards positive apoptotic markers for different CCKR KO animal islets. (F) Percentage of TUNEL-positive insulin-positive cells of mouse islets treated with mouse cytokine cocktail (n=8). Data are means ± SEM; ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05, *n.s*, non-significant.

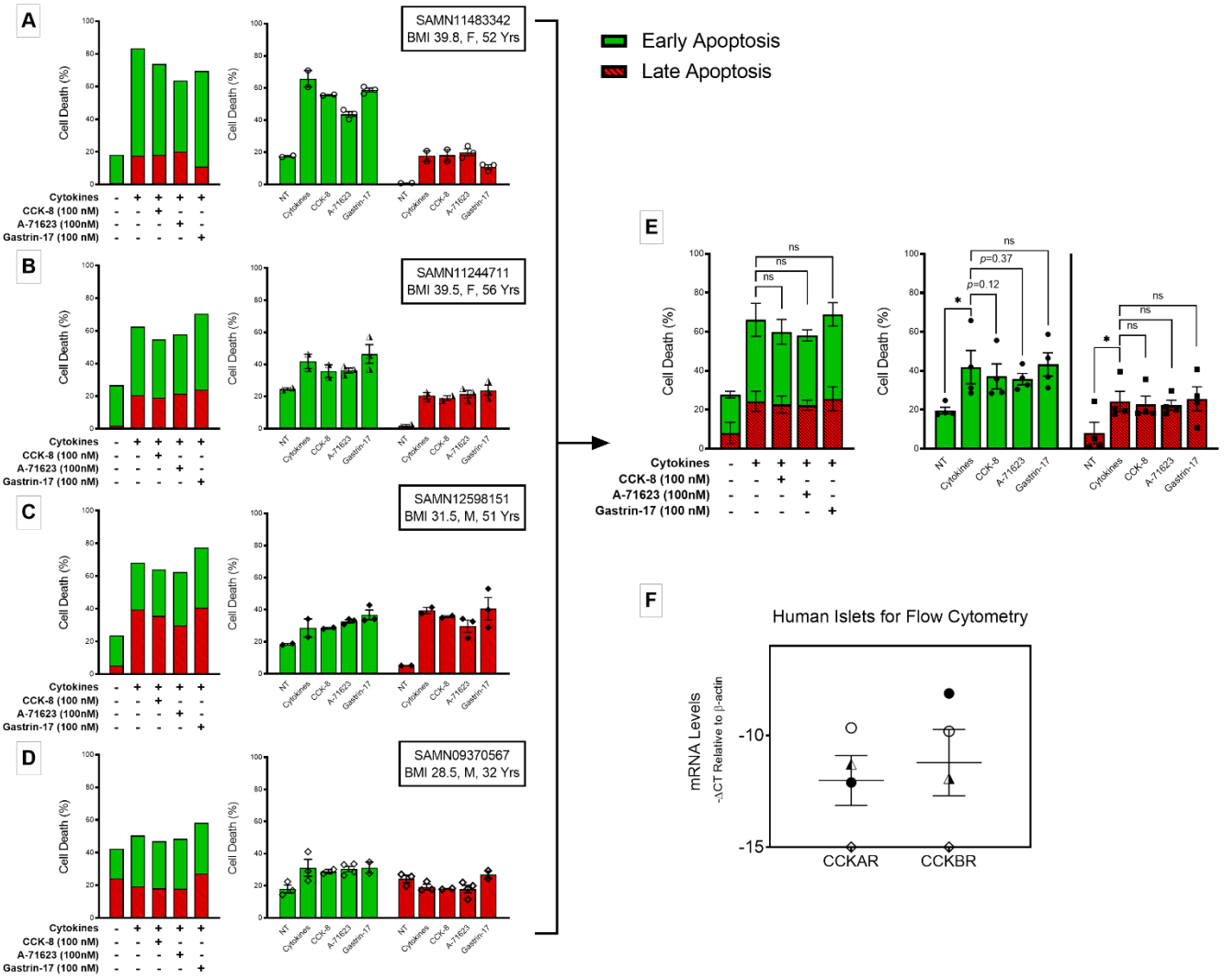
A
B
C
D
SAMN11483342
BMI 39.8, F, 52 Yrs
SAMN11244711
BMI 39.5, F, 56 Yrs
SAMN12598151
BMI 31.5, M, 51 Yrs
SAMN09370567
BMI 28.5, M, 32 Yrs
Cell Death (%)
Cytokines
CCK-8 (100 nM)
A-71623 (100nM)
Gastrin-17 (100 nM)
NT
Cytokines
CCK-8
A-71623
Gastrin-17
Early Apoptosis
Late Apoptosis
E
ns
p=0.37
p=0.12
F
Human Islets for Flow Cytometry
mRNA Levels
-ΔCT Relative to β-actin
-10
-15
CCKAR
CCKBR

Fig 3. Human islets have variable responses to specific CCK receptor agonists that may depend on baseline receptor expression or metabolic disease in donor - Isolated human islets treated with vehicle, cytokines, CCK-8, CCKAR agonist (A-71623), and CCKBR agonist (Gastrin-17). (A) Obese female with hyperglycemia and insulin treatment protection through CCKAR compared to cytokine. (B) Obese females with hyperglycemia and no insulin treatment also showed a trend towards protections through CCKAR. (C) Obese male Overweight male with no history of diabetes and (D) Overweight male exhibited minimal to no protection against cytokine-induced apoptosis from CCKAR agonist. (E) Combined graph representing the mean value of figures A-D (n=4). (F) mRNA levels of CCAR and CCKBR in human islets used for the flow cytometry. The blank circle denotes (F, BMI 39.8), Triangle denotes (F. BMI 39.5), Solid black circle represents (M, BMI 31.5), and Blank square indicates (M, BMI 28.5). Data are means ± SEM; *n.s*, non-significant.

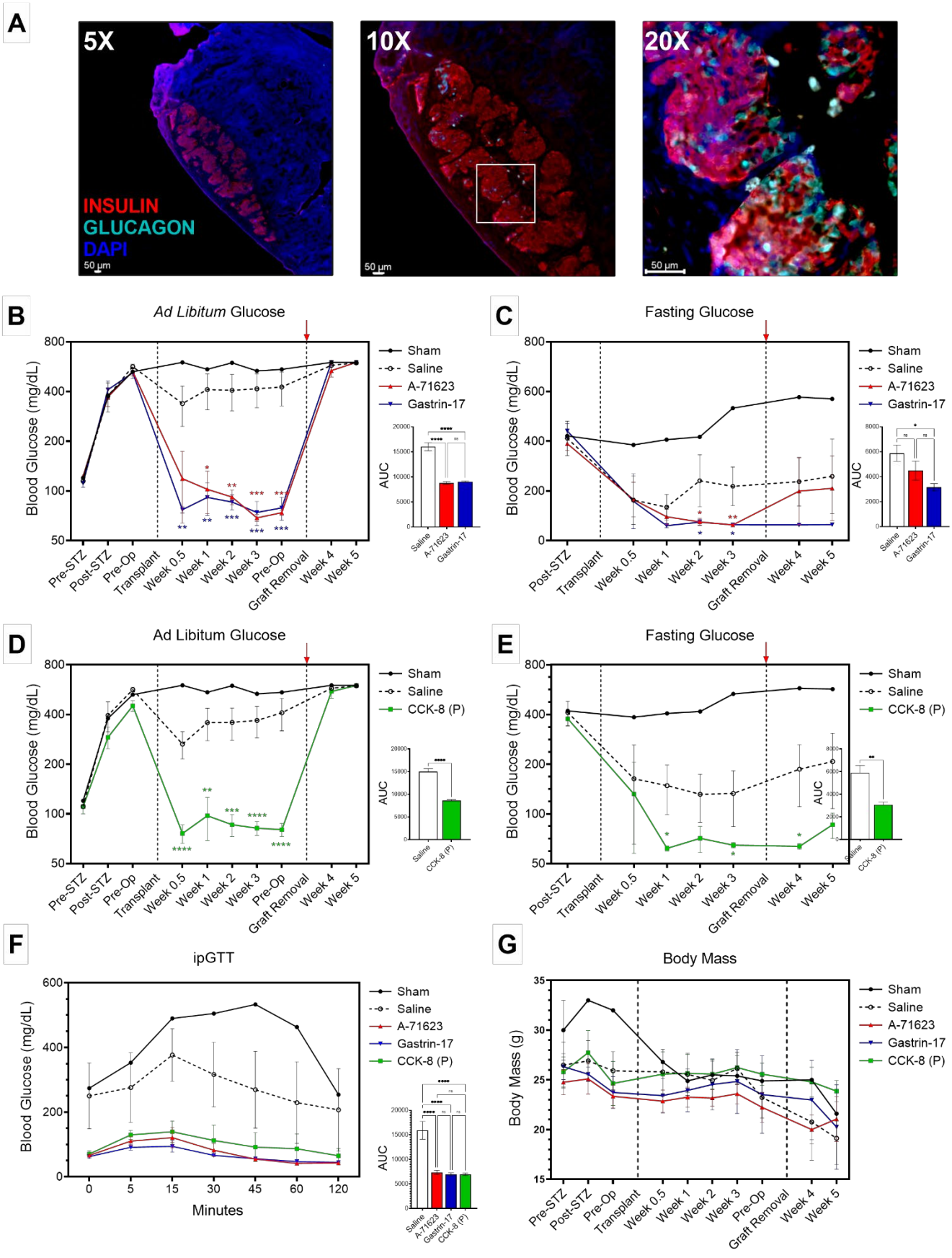
A
5X
10X
20X
INSULIN
GLUCAGON
DAPI
50 µm
B
Ad Libitum Glucose
Blood Glucose (mg/dL)
Sham
Saline
A-71623
Gastrin-17
AUC
Pre-STZ
Post-STZ
Pre-Op
Transplant
Week 0.5
Week 1
Week 2
Week 3
Pre-Op
Graft Removal
Week 4
Week 5
C
Fasting Glucose
D
Ad Libitum Glucose
CCK-8 (P)
E
Fasting Glucose
F
ipGTT
Minutes
G
Body Mass
Body Mass (g)

Fig 4. Both CCKAR and CCKBR selective agonists improve glycemic control following human islet transplant – (A) A representative immunofluorescence histology image of human islet graft in diabetic NOD/SCID mouse kidney capsule. Insulin (Red), Glucagon (Cyan), DAPI (Blue). (B) Random blood glucose remained elevated in the STZ induced sham animal (solid black line) (> 550 mg/dL). At the same time, A-71623 treated group (Red solid line, n=5) and Gastrin-17 group (Blue solid line, n=5) had restoration of glycemic control in comparison to mild attenuation of hyperglycemia in the saline-treated control group (Black dotted line, n=4). Improvement glycemic control in A-71623 and Gastrin-17 treated islet grafts continued over three weeks after transplant, while saline control did not attain euglycemia after a mild recovery. (C) The average value of 5-hour fasting glucose levels in a sham animal ranged from 385 to 577 mg/dL (Black solid line), while the saline-treated control transplant group ranged between 134 to 218 mg/dL throughout the study (Black dotted line, n=4). All three CCKR drug-treated groups had fasting glucose revolving around ~60 mg/dL starting from 7 days after the transplant until the end of the study (C&E). (D) Animals that received pre-incubated islets with CCK-8 but did not receive continuous CCKR agonist therapy (Green solid line) also had restored euglycemia by four days after the transplant and throughout the remainder of the study (n=5). (E) Fasting glucose for CCK-8 pretreatment group only (n=4). (F) ipGTT result on post-graft day 21 show saline pump containing control animals without any pre-transplant drug treatment showed improved glucose tolerance than the STZ-

induced diabetic NOD-SCID sham mice but performed much worse than all three of A-71623, Gastrin-17, or CCK-8 pretreatment only groups no significant differences were found between the drug treatments (n=4 to 5). (G) The body weight of the human islet transplant recipient animals receiving A-71623 or Gastrin-17 infusion was unaltered compared to the control group or the CCK-8 pre-incubation group throughout the 5-week duration study (n=5-6). Upon removing the graft on post-transplant day 22 (Red arrow above), all animals surviving the nephrectomy lost glycemic control (blood glucose > 550mg/dL) Data are means ± SEM; ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05, *n.s*, non-significant.

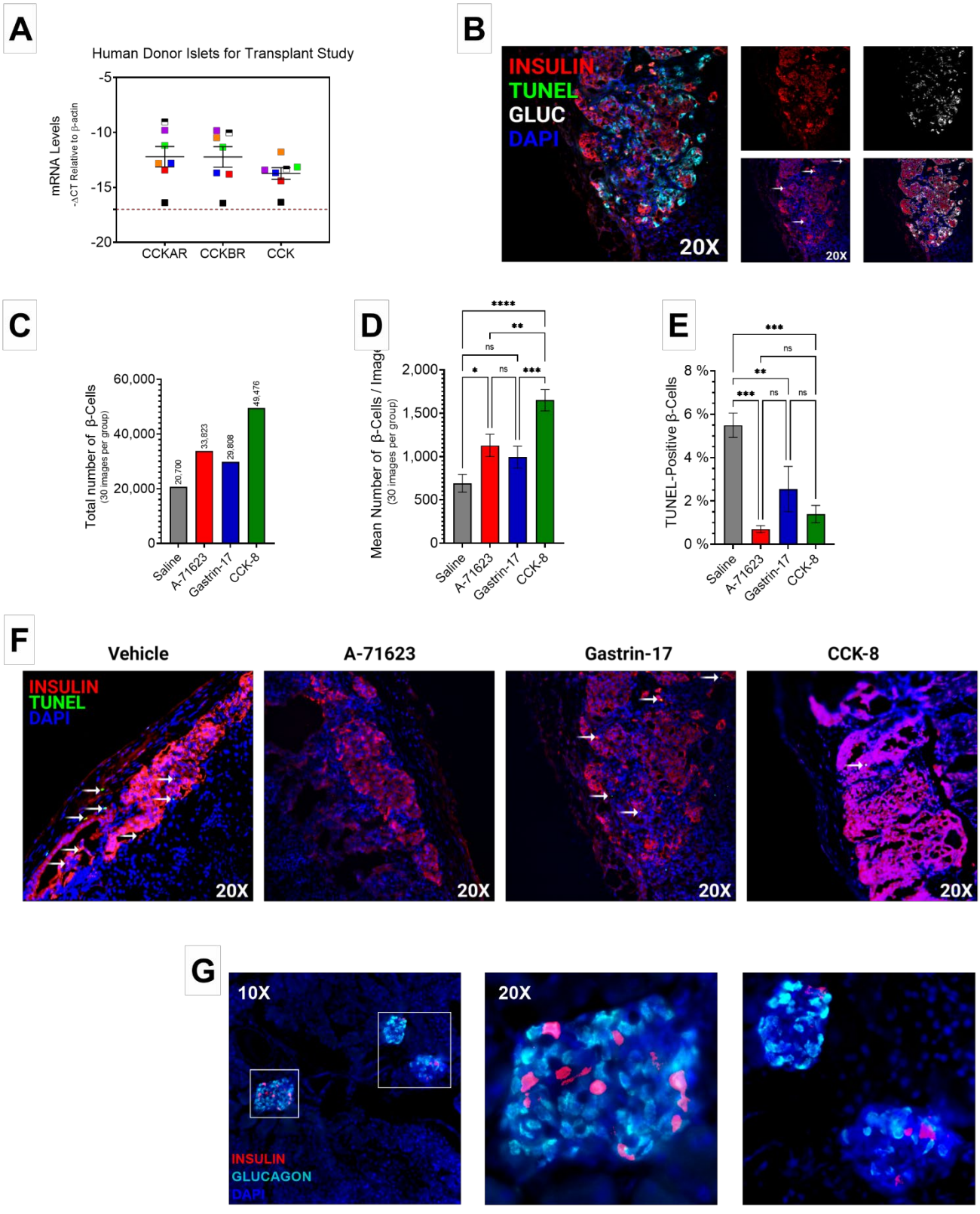
A
Human Donor Islets for Transplant Study
mRNA Levels
-ΔCT Relative to β-actin
-5
-10
-15
-20
CCKAR
CCKBR
CCK
B
INSULIN
TUNEL
GLUC
DAPI
20X
20X
C
Total number of β-Cells
(30 images per group)
60,000
40,000
20,000
0
20,700
33,823
29,608
49,476
Saline
A-71623
Gastrin-17
CCK-8
D
Mean Number of β-Cells / Imag
(30 images per group)
2,000
1,500
1,000
500
0
****
**
ns
*
ns
***
Saline
A-71623
Gastrin-17
CCK-8
E
TUNEL-Positive β-Cells
8 %
6 %
4 %
2 %
0 %
***
ns
**
***
ns
ns
Saline
A-71623
Gastrin-17
CCK-8
F
Vehicle
A-71623
Gastrin-17
CCK-8
INSULIN
TUNEL
DAPI
20X
20X
20X
20X
G
10X
INSULIN
GLUCAGON
DAPI
20X

Fig 5. Human islet grafts are protected by both CCKAR and CCKBR, while preincubation of islets in CCK is sufficient to induce the same level of protection as seen by CCKAR selective agonists. – (A) CCKAR and CCKBR mRNA level prescreening human donor islets upon shipment arrival shows varied endogenous CCKR and CCK expression levels in humans. All islets used for the transplant were only taken from the donor islets that showed CCKAR and CCKBR. Each color represents samples measured from the same donor. The red dotted line marks the threshold for biologically meaningful mRNA detection cutoff (-ΔCt =17 relative to β-actin). (B)Representative image of total β-cell number and TUNEL-positive cell counting. (C) The total number of β-cells found in 30 images from each transplant group. Five to six images were taken from each animal harvested graft (n=4). (D) Average number of β-cells found per imaging field. (E) % TUNEL positive β-cells in each group. A total of five non-consecutive sections was used to quantify the total β-cell and TUNEL+ β-cells (n=4 per group). Total β-cell counted for each group: Saline group (10,238), A-71623(26,749), Gastrin-17 (23,586), and CCK-8 (30,444). Data are means ± SEM; ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05, *n.s*, non-significant.

3.8 Appendix

Chapter 3 - Table 1 - Human Islet Donor Data for Imaging Flow										
					A Early Apoptosis (%)			A Late Apoptosis (%)		
Donor Islet ID	Family History of Diabetes	Average Blood Glucose	BMI	Insulin Therapy	Cytokines vs. CCK-8	Cytokines vs. A-71623	Cytokines vs. Gastrin-17	Cytokines vs. CCK-8	Cytokines vs. A-71623	Cytokines vs. Gastrin-17
SAMN11244711	Paternal T2D	172.80 mg/dL	39.5	Yes, 8.0 U	-10.1±5.16	-22.15±4.43	-7.885±4.16	-0.45±4.85	+2.35±5.66	-6.82±2.96
SAMN11483542	Paternal T2D	144.40 mg/dL	39.8	No	-6.18±6.2	-5.68±5.93	+4.60±5.27	-1.45±2.54	+0.95±5.28	+3.32±4.26
SAMN12598151	No	N/A	31.5	No	+0.1±5.668	+4.18±4.40	+8.22±5.74	+3.90±1.99	**-9.88±5.0884**	-1.33±9.102
SAMN09577067	No	N/A	28.5	No	+2.2±6.91	-0.72±4.88	+0.15±5.72	+1.20±2.21	-1.5±5.07	+7.70±2.84

Table 1. - Donor Islet Data for Figure 3

IIDP Human Islet Data for Agonist Transplant Study - Chapter 3

Adapted from Hart NJ, Powers AC (2018) Progress, challenges, and suggestions for using human islets to understand islet biology and human diabetes. Diabetologia https://doi.org/10.1007/s00125-018-4772-2.

Selected Islet Preparations for Alvin C. Powers
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Data provided by IIDP
Labels in blue text are additional fields or clarification provided by IIDP

Islet Preparation	1	2	3
Unique identifier	SAMN13570019	SAMN13972304	SAMN14255441
Donor Age (years)	37	49	48
Donor Sex (M/F)	F	M	M
Donor BMI (kg/m^2)	24.0	34.8	25.6
Donor HbA1c	5.2	5.5	5.9
Origin/source of islets	IIDP	IIDP	IIDP
Islet isolation center[a]	University of Pennsylvania	Southern California Islet Cell Resc	The Scharp-Lacy Research Institut
Donor history of diabetes? Yes/No	No	No	No
If Yes, complete the next two lines if this information is available			
Diabetes duration (years)	Not Applicable	Not Applicable	Not Applicable
Glucose-lowering therapy at time	Not Applicable	Not Applicable	Not Applicable
Cause of death	Anoxia	Cerebrovascular/stroke	Anoxia
Warm ischemia time (h)	Not Reported	Not Reported	Not Reported
Cold ischemia time (h)	15.3	6.3	11.1
Estimated purity (%) By islet isolation center at time of shipment with Dithizone (DTZ) staining per IIDP SOP	95	90	95
Estimated viability (%) By islet isolation center at time of broadcast with Fluorescence Diacetate/Propidium Iodide (FDA/PI) staining per IIDP SOP	96	96	95
Date/Time Islet Culture Began at islet isolation center (Pacific Standard Time, 24-hour format)	2019-12-13 13:00	2020-01-30 09:00	2020-02-29 20:00
Glucose-stimulated insulin secretion or other functional measurement[d] Stimulation Index (SI) by static incubation at islet isolation center pre-shipment per IIDP SOP	SI (G2.8mM-G28mM)= 1.2	SI (G2.8mM-G28mM)= 14.0	SI (G2.8mM-G28mM)= 4.7
Glucose-stimulated insulin secretion or other functional measurement Area Under the Curve (AUC) by perifusion at HIPP post shipment sample per HIPP SOP	AUC (G5.6mM-G16.7mM)= 22.3 (ng/100 IEQs)	AUC (G5.6mM-G16.7mM)= 45.2 (ng/100 IEQs)	AUC (G5.6mM-G16.7mM)= 15.8 (ng/100 IEQs)

Supplemental Figure 1-1. - Donor Islet Data for Figure 4 & 5, Part 1

IIDP Human Islet Data for Agonist Transplant Study - Chapter 3

Adapted from Hart NJ, Powers AC (2018) Progress, challenges, and suggestions for using human islets to understand islet biology and human diabetes. Diabetologia https://doi.org/10.1007/s00125-018-4772-2.

Selected Islet Preparations for Alvin C. Powers

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Data provided by IIDP

Labels in blue text are additional fields or clarification provided by IIDP

Islet Preparation	4	5	6
Unique identifier	SAMN15877725	SAMN17367763	SAMN18875704
Donor Age (years)	31	56	37
Donor Sex (M/F)	M	M	M
Donor BMI (kg/m^2)	27.4	33.0	28.4
Donor HbA1c	5.6	5.3	5.6
Origin/source of islets	IIDP	IIDP	IIDP
Islet isolation center[a]	University of Wisconsin	The Scharp-Lacy Research Institut	The Scharp-Lacy Research Institut
Donor history of diabetes? Yes/No	No	No	No
If Yes, complete the next two lines if this information is available			
Diabetes duration (years)	Not Applicable	Not Applicable	Not Applicable
Glucose-lowering therapy at time	Not Applicable	Not Applicable	Not Applicable
Cause of death	Head trauma	Head trauma	Head trauma
Warm ischemia time (h)	Not Reported	0.7	Not Reported
Cold ischemia time (h)	7.8	10.9	11.6
Estimated purity (%) By islet isolation center at time of shipment with Dithizone (DTZ) staining per IIDP SOP	95	90	90
Estimated viability (%) By islet isolation center at time of broadcast with Fluorescence Diacetate/Propidium Iodide (FDA/PI) staining per IIDP SOP	98	95	95
Date/Time Islet Culture Began at islet isolation center (Pacific Standard Time, 24-hour format)	2020-08-21 17:00	2021-01-15 14:30	2021-04-25 17:30
Glucose-stimulated insulin secretion or other functional measurement[d] Stimulation Index (SI) by static incubation at islet isolation center pre-shipment per IIDP SOP	SI (G2.8mM-G28mM)= 2.2	SI (G2.8mM-G28mM)= 1.5	SI (G2.8mM-G28mM)= 3.1
Glucose-stimulated insulin secretion or other functional measurement Area Under the Curve (AUC) by perifusion at HIPP post shipment sample per HIPP SOP	AUC (G5.6mM-G16.7mM)= Not Available	AUC (G5.6mM-G16.7mM)= 51.9 (ng/100 IEQs)	AUC (G5.6mM-G16.7mM)= Not Available

Supplemental Figure 1-2. - Donor Islet Data for Figure 4 & 5, Part 2

IIDP Human Islet Data for Agonist Transplant Study - Chapter 3

Adapted from Hart NJ, Powers AC (2018) Progress, challenges, and suggestions for using human islets to understand islet biology and human diabetes. Diabetologia https://doi.org/10.1007/s00125-018-4772-2.

Selected Islet Preparations for Alvin C. Powers

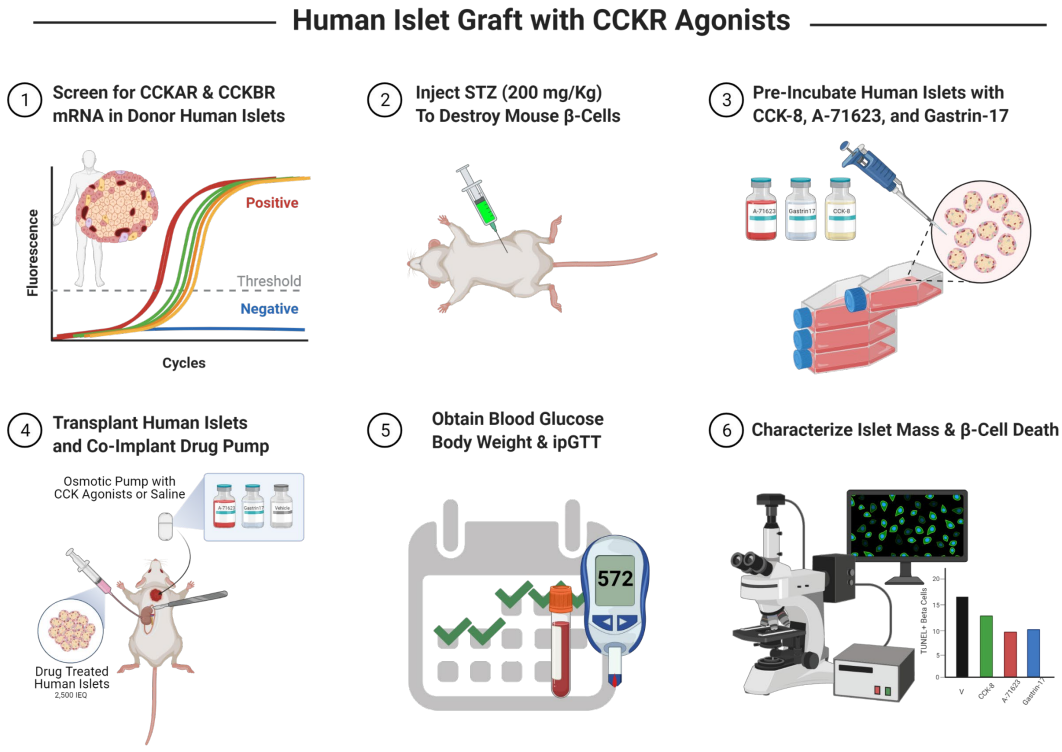
Report Downloaded on 7/31/2021 9:09:46 AM

Data provided by IIDP

Labels in blue text are additional fields or clarification provided by IIDP

Islet Preparation	7		
Unique identifier	SAMN19920583		
Donor Age (years)	54		
Donor Sex (M/F)	M		
Donor BMI (kg/m^2)	24.9		
Donor HbA1c	5.4		
Origin/source of islets	IIDP		
Islet isolation center[a]	The Scharp-Lacy Research Institute		
Donor history of diabetes? Yes/No	No		
If Yes, complete the next two lines if this information is available			
Diabetes duration (years)	Not Applicable		
Glucose-lowering therapy at time	Not Applicable		
Cause of death	Head trauma		
Warm ischemia time (h)	0.3		
Cold ischemia time (h)	9.0		
Estimated purity (%) By islet isolation center at time of shipment with Dithizone (DTZ) staining per IIDP SOP	90		
Estimated viability (%) By islet isolation center at time of broadcast with Fluorescence Diacetate/Propidium Iodide (FDA/PI) staining per IIDP SOP	95		
Date/Time Islet Culture Began at islet isolation center (Pacific Standard Time, 24-hour format)	2021-06-26 18:00		
Glucose-stimulated insulin secretion or other functional measurement[d] Stimulation Index (SI) by static incubation at islet isolation center pre-shipment per IIDP SOP	SI (G2.8mM-G28mM)= 2.7		
Glucose-stimulated insulin secretion or other functional measurement Area Under the Curve (AUC) by perifusion at HIPP post shipment sample per HIPP SOP	AUC (G5.6mM-G16.7mM)= 36.4 (ng/100 IEQs)		

Supplemental Figure 1-3. - Donor Islet Data for Figure 4 & 5, Part 3



Supplemental Figure 1. - Human Islet Graft Experimental Procedure

Chapter 4: Concluding Remarks

Cholecystokinin and gastrin are the two of the longest studied hormones since their discovery in 1928 and 1905. Many investigators have attempted to pharmacologically exploit these peptide receptors' physiological actions to counter obesity and gastric secretory disorders (206). However, CCKR- targeted therapies have been primarily thwarted as a meaningful therapeutic target due to concerns of these drugs' adverse neurological effects, GI side effects, and carcinogenic potential. Both CCKAR and CCKBR downstream signaling pathways converge on key nuclear transcription regulators that control cell proliferation, differentiation, stress management, and apoptosis (89).

Pharmacological manipulation of cell death is indeed almost always two-sided. In oncology, a vast majority of chemotherapy is based on the inhibition of cell proliferation (cytostatic drugs) and promoting targeted and controlled death (oncolysis) of aberrant cells that make up the tumor mass (207).

On the contrary, islet biologists aim to develop new ways to inhibit β-cell apoptosis and induce proliferation (181). Therefore, any therapeutic strategies directly targeting cell survival will always face a dilemma and need to be carefully examined for the risk of promoting tumor development. The development of drug delivery strategies to target β-cells more specifically has been a challenge in the field, but this is one possible way to improve the safety of such therapeutics. Alternatively,

identifying specific receptor agonists and signaling pathways that promote β-cell survival without having an oncogenic impact on other cell types would be an ideal goal.

An example of this dilemma can be found not very far. Since 1899, acetylsalicylic acid, an irreversible inhibitor of COX-2, has probably been one of the most used drugs. While the drug's famed sales slogan "*Take an aspirin a day and keep the doctor away"* is shown to have beneficial long-term health effects, a randomized clinical study of aspirin (ASPREE) recently reported a surprising result during early 2021. ASPREE study consisting of more than 19,000 healthy elderly population reports that daily aspirin intake is reported to be associated with a greater likelihood of death caused by advanced cancer (208). Aspirin works via a mechanism of action that inhibits major inflammatory pathways, uncoupling of mitochondrial OXPHOS, and NF-κB inhibition. Therefore, aspirin could also impede desirable cellular apoptosis for aging cells, manifesting as oncogenesis in the long term.

In other fields of medicine, while it may seem less apparent, an increase in undesirable cell death or mass apoptosis caused by an acute insult such as various forms of ischemia, stroke, heart attack, or liver failure leads to a cascade of sudden uncontrollable death of organs (209). Additionally, gradual and progressive loss of cells in the brain or central and peripheral nerves result in neurological diseases. Research efforts in these fields have resulted in many successful but clinically less viable approaches using gene delivery therapy, recombinant protein, and peptide delivery strategies. Together those studies have proved that targeting cell death

signaling pathways can be a tricky strategy against post-ischemic cell death and neurodegenerative diseases. Thus, the concept of developing an anti-diabetes drug that prevents or reverses diabetes through controlling β-cell survival and proliferation should also first come from attempts to carefully identify an intrinsic signaling receptor that plays a dynamic role in cell death or survival.

My dissertation has focused on identifying which type of CCKR is driving the protection of β-cells.

In chapter one, I have introduced diabetes and CCK and suggested the possibility of CCK as a β-cell protective agent by drawing a parallel between neuroscience and islet biology, the similar signaling responsibilities, and cellular stress that likely arise from those similarities between neurons and β-cells.

In chapter two, through careful observations of insulinoma cell lines, mouse islets, and a xenograft study, I showed that exogenous CCK protects mouse and human β-cells.

In chapter three, I report that in mouse CCKAR is the primary driver for β-cell protection. I also conclude through *in vivo* study that in humans, either CCKAR or CCKBR activation can protect β-cells. Furthermore, I provide a piece of preliminary evidence that the CCKR mediated protective effect seen in human islets may be inducible by pre-incubation of islets in CCK.

Finally, from the observations from these chapters, I suggest an exciting possibility that CCK-induced protective priming of the human β-cells might be

clinically translatable. A simple step during the pre-transplant processing of donor islets where the transient addition of CCK agonist before the transplant could be sufficient to provide improved graft longevity and function in clinical β-cell replacement therapy. However, additional work on carefully delineating the exact downstream effector of CCKR needs to be completed before utilizing a CCK as an anti-diabetic agent. When CCKR is activated without discretion, the CCK signaling can promote cell survival, even when the added resilience to apoptotic cell death is undesirable.

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