PANCREATIC SECRETIONS AND THEIR EFFECT ON CIRCULATORY AND IMMUNE CELLS

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ABSTRACT

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Diabetes mellitus is a metabolic disease characterized by chronic hyperglycemia and insulin dysfunction, which often results in secondary complications associated with blood flow. Four major types of diabetes are defined by the American Diabetes Association: type 1 diabetes (T1D, insulin insufficiency), type 2 diabetes (T2D, insulin resistance), gestational diabetes (occurring during pregnancy), and other types, which includes cystic fibrosis related diabetes (CFRD). Although each of these types of diabetes is associated with insulin dysfunction, it is important to note that the treatment of oral medications or exogenous insulin, although sufficient to effectively manage blood glucose levels, is not sufficient to completely cure diabetes. Secondary complications resulting from diabetes include retinopathy, nephropathy and neuropathy, along with dysfunctions in immune response. In recent years, a surge in the research of C-peptide, the 31 amino acid peptide co-secreted with insulin, has revealed that C-peptide may actually help to ameliorate some of these secondary complications, including neuropathy and immune response. Although initial studies revealed promising results, a phase 2b clinical trial was shut down in 2014 due to indistinguishable results in placebo and treatment groups in T1D patients.

To enhance our knowledge of these downstream problems, we here report the effect of C-peptide/zinc/albumin on RBCs and peripheral neutrophils (an immune cell) that mimic

those from CF/CFRD patients using a chemical inhibitor (CFTRinh-172). C-peptide and zinc binding to RBCs confirms previously published data, and binding to CFTR-inhibited RBCs is increased from the control, however, the molar binding ratio of C-peptide to zinc remains 1:1. ATP release is increased in control and CFTR-inhibited cells only in the presence of the ensemble of C-peptide/zinc/albumin, but is abolished when any one of these components is missing. Similarly, in neutrophils, we observe a 1:1 molar binding ratio of C-peptide to zinc when albumin is present in both control and CFTR-inhibited cells. We also observed a significant increase in intracellular calcium, intracellular NO, extracellular NO, glucose uptake, and rate of chemotaxis in neutrophils that had been treated with the ensemble, but not when any one component was missing. In both RBCs and neutrophils, we observed an increase in membrane expression of glucose transporter 1 (GLUT1) only when C-peptide, zinc and albumin were present. Insulin did not have this effect.

The results reported here are the first to indicate that C-peptide may be acting on cells that contain primarily GLUT1, and that the mechanism of action may be similar to the way that insulin acts in muscle and fat cells, by translocating GLUT4 to the plasma membrane. These results are also the first to suggest that C-peptide may directly be affecting peripheral neutrophils, which could have significant implications in the treatment of immune complications in all forms of diabetes. Additionally, these results are the first to examine the effect this peptide and its metal may have on patients who have CF, and may point to its use a potential therapy in both T2D and CFRD.

I dedicate this work to all of the doctors, nurses, researchers, and families who fight cystic fibrosis. As we keep fighting every day, for every breath, we are coming one step closer to making CF stand for Cure Found.

ACKNOWLEDGMENTS

Not to us, Lord, not to us but to your name be the glory,
because of your love and faithfulness.
Psalm 115:1

All glory and honor to God.

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KEY TO ABBREVIATIONS

ABC	ATP-Binding Cassette
AC	Adenylyl Cyclase
ADP	Adenosine Diphosphate
ANO1	Anoctamin 1
APS	Ammonium Persulfate
AS	Additive Solution
ATP	Adenosine Triphosphate
BBB	Blood Brain Barrier
BCIP	5-bromo-4-chloro-3'-indolyphosphate
cAMP	cyclic Adenosine Monophosphate
CAD	Computer Aided Design
CAP	Catabolite Activator Protein
CIS	Clinically Isolated Syndrome
CF	Cystic Fibrosis
CFRD	Cystic Fibrosis Related Diabetes
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CPD	Citrate Phosphate Dextrose
DAF-FM	4-Amino-5-Methylamino-2',7' Difluorofluorescein
DAF-FM DA	4-Amino-5-Methylamino-2',7' Difluorofluorescein Diacetate
DDW	Purified Water (18 MΩ)
DHAP	Dihydroxyacetone Phosphate
DPBS	Dulbecco's Phosphate Buffered Saline
E27A	Single Amino Acid Mutant of C-peptide
eNOS	endothelial Nitric Oxide Synthase
ELISA	Enzyme Linked ImmunoSorbent Assay

ER	Endoplasmic Reticulum
FITC	Fluorescein
fMLP	*n*-formyl-Methionyl-Leucyl-Phenylalanine
G3P	Glyceraldehyde-3-phosphate
GD	Gestational Diabetes
GLUT	Glucose Transporter
Gly3P	Glycerol-3-phosphate
GPCR	G-protein Coupled Receptor
GWAS	Genome Wide Association Studies
HbA_{1c}	Hemoglobin A_{1c}
HPLC	High Performance Liquid Chromatography
HVA	High Voltage Associated
IL	Interleukin
K_{ATP}	ATP Sensitive Potassium Channel
KRB	Krebs-Ringers Buffer
LVA	Low Voltage Associated
MAPK	Mitogen-Activated Protein Kinase
MHC	Major Histocompatibility Complex
MRI	Magnetic Resonance Imaging
MS	Multiple Sclerosis
MPO	Myeloperoxidase
NAD^+	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NBD	Nucleotide Binding Domain
NBT	Nitro-blue tetrazolium
NCV	Nerve Conduction Velocity
NF-κB	Nuclear Factor Kappa Beta

NO	Nitric Oxide
OGTT	Oral Glucose Tolerance Test
PAI-1	Plasminogen Activator Inhibitor-1
PBS	Phosphate Buffered Saline
PC	Prohormone Convertase
PEP	Phosphoenolpyruvate
PI3K	Phosphoinositide 3-Kinase
PKA	Protein Kinase A
PKB	Protein Kinase B
PKC	Protein Kinase C
PLC	Phospholipase C
PP cells	Pancreatic Polypeptide cells
PSS	Physiological Salt Solution
PVDF	Polyvinylidene difluoride
RBC	Red Blood Cell
RER	Rough Endoplasmic Reticulum
ROS	Reactive Oxygen Species
SDS-PAGE	Sodium Dodecyl Sulfide Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
shRNA	small hairpin RNA
SKRB	Stimulation Solution
SNARE	Soluble NSF Attachment Protein Receptor
SNP	Single Nucleotide Polymorphism
SOD	Superoxide Dismutase
SUR1	Sulfonylurea Receptor
STZ	Streptozotocin
T1D	Type 1 Diabetes

T2D	Type 2 Diabetes
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline with Tween-20
TEMED	Tetramethylethylenediamine
TGFβ	Transforming Growth Factor Beta
TMD	Transmembrane Domain
TSQ	6-methoxy-8-p-toluenesulfonamido-quinoline
VEGF	Vascular Endothelial Growth Factor
WBC	White Blood Cell

CHAPTER 1

INTRODUCTION

1.1 The Human Pancreatic System

The human pancreas is situated near the bottom of the stomach, surrounded by the spleen and small intestine. The pancreas has two major functions: first, it serves as an exocrine gland, secreting a variety of digestive enzymes; second, the pancreas has an endocrine function through clusters of cells known as pancreatic islets, or the islets of Langerhans.[1,2] The human pancreas is made up of about a million islets of Langerhans,[3] which consist of a number of cell types, including alpha cells, delta cells, pancreatic polypeptide (PP) cells, and beta cells.[1, 4] Dysfunction of the pancreatic beta cells causes a metabolic disease known as diabetes.

1.1.1 Alpha Cells

Alpha cells make up about 35-40% of the islets, and primarily secrete the hormone glucagon.[4] Glucagon secretion occurs in response to a decline in blood glucose levels, such as during exercise or fasting. Glucagon stimulates glycogenolysis in the liver, the conversion of glycogen to glucose; gluconeogenesis, the uptake and conversion of amino acids to glucose; and lipolysis, the breakdown of triglycerides to free fatty acids and glycerol.[1] Glucagon levels do not differ between healthy patients and patients with diabetes after fasting, however, the response to an arginine load showed that glucagon concentrations were significantly higher in diabetic than control patients.[5]

1.1.2 Delta Cells

Delta cells make up 10-15% of the islet, and primarily secrete the peptide hormone somatostatin.[4] Pancreatic somatostatin inhibits the release of other beta cell hormones, including glucagon.[1] In healthy patients, somatostatin release is stimulated by glucose, but this effect is abolished in patients with diabetes.[6] The lack of somatostatin in diabetic patients may contribute to the impaired suppression of glucagon secretion from alpha cells.[7, 8] Suppression of glucagon secretion from alpha cells in response to a glucose load is necessary to reduce blood glucose levels, and, when impaired, leads to hyperglycemic conditions.

1.1.3 Pancreatic Polypeptide (PP) Cells

PP cells are the least well-studied of the islet cell types, making up about 1% of the islet. These cells primarily secrete pancreatic polypeptide hormone, which is thought to play a role in regulation of both the exocrine and endocrine responses of the pancreas, and in regulation of appetite.[9]

1.1.4 Beta Cells

The remainder of the islet cell mass, about 50%, contains the pancreatic beta cells, which secrete a number of small molecules, including amylin, γ amino butyric acid (GABA), zinc, C-peptide, and insulin. Amylin, also known as islet amyloid polypeptide (IAPP), is thought to act as a synergistic partner to insulin, decreasing food intake. GABA functions in an autocrine manner, acting on a receptor in alpha cells to suppress glucagon secretion.[10-12] The following sections will deal with the current knowledge of the remaining secretions from beta cells: insulin, C-peptide, and zinc.

1.2 Insulin

Insulin is well known as the hormone that regulates glucose levels in the body, and is closely associated with the development and pathophysiology of diabetes. Insulin is a 5,800 Da hormone that acts to increase glucose uptake in cells that contain glucose transporter 4 (GLUT4), found in muscle and adipose tissues. Briefly, in these cell types, insulin binds to its specific receptor, causing an intracellular signaling cascade that translocates GLUT4 from its intracellular stores to the membrane, where it increases glucose influx. Insulin does not increase glucose uptake in cells that do not contain GLUT4.

1.2.1 Discovery of Insulin

Prior to the discovery of insulin, diabetes was an unmanageable disease that often ended in the death of patients due to starvation within a year of diagnosis. As early as the 1860s, Paul Langerhans discovered the existence of groups of cells within the pancreas whose function was unknown. These clusters of cells eventually became known as the islets of Langerhans. In 1889, the link between the pancreas and development of diabetes was discovered by a German physiologist, Oscar Minkowski, and a physician, Joseph von Menring, when they removed the pancreas from a dog and it developed diabetes. However, until the 1920s, no specific molecule could be linked to the development of diabetes. While working in laboratory space provided by Professor John Macleod at the University of Toronto, Dr. Frederick Banting and his medical student, Charles Best, isolated a substance from the pancreas that kept a diabetic dog healthy. They decided to call this extract insulin, and brought in Bertram Collip, a biochemist, in order to purify insulin for testing in humans.

Banting and Best first tested the insulin extract on themselves, and in January 1922, a 14 year old boy with diabetes (type 1) was the first to receive insulin. Insulin rapidly improved the boy's condition, and more testing on diabetic subjects continued. The pharmaceutical company Eli Lilly began producing insulin in large quantities to supply all those in North America by 1923. That same year, Banting and Macleod were awarded the Nobel Prize in Physiology or Medicine. Both men shared their prize money with their colleagues, Best and Collip.[13]

Since the discovery of insulin, much research has been done to understand the molecular mechanism of insulin secretion. Fred Sanger's sequencing of insulin in the 1950s[14] won him the Nobel Prize in Chemistry in 1958 and consequently inspired the study of the mechanism of the assembly of insulin. Steiner discovered the structure of the single-chain precursor, proinsulin in 1967.[15]

1.2.2 Processing of Insulin

Preproinsulin is the initial translation product of insulin mRNA and contains a 24 residue signal peptide, which interacts with the signal recognition particle in the cytosol (Figure 1.1).[16-18] The preproinsulin peptide is translocated across the membrane of the rough endoplasmic reticulum (RER) into the lumen, where the signal sequence is cleaved and rapidly degraded.[19] In the RER, proinsulin is folded by the formation of three disulfide bonds, then, within 10-20 minutes of this occurrence, proinsulin is transported to the Golgi apparatus for further processing.[20] Once in the Golgi apparatus, proinsulin is transported into immature secretory vesicles, where the pH drops to about 5.5,[21] and proinsulin is cleaved to produce insulin and the 31 amino acid connecting peptide, C-peptide.[22] Within the secretory vesicles, conversion of

proinsulin to insulin begins about 20 minutes after initial synthesis of preproinsulin and continues for 1-2 hours.[23-25] Intermediate cleavage products are generated within the vesicle, and only small amounts of proinsulin (1-2% of total beta cell contents) are contained in mature secretory granules, along with other beta cell products.[25-27] Proinsulin that exists in these granules is not prevented from forming dimers and zinc-stabilized hexamers, which means that C-peptide, when connected in the proinsulin molecule, does not inhibit hexamer formation.[28, 29] Furthermore, studies have shown that proinsulin and insulin are similar in their solubility, isoelectric point,[22] reactivity with antisera,[15, 30] and structural conformation.[22, 31]

Two Ca^{2+}-dependent, acidic prohormone convertases (PCs) are expressed in pancreatic beta cell granules, PC2 (Type II secretory granule protease) and PC1/3 (Type I secretory granule protease).[32, 33] PC2 preferentially cleaves proinsulin at the A-chain junction, while PC1/3 cleaves it at the B-chain junction. PC2 is thought to cleave the intact prohormone first, while PC1/3 cleaves the product much more rapidly than the intact prohormone.[34] Removal of residues exposed by endoproteolytic cleavage by PC2 and PC1/3 are removed by carboxypeptidase E or H.[35, 36] These enzymes are active at pH 5.5, and show little activity at pH 7.4. At a pH of 8.0 or above, these enzymes are irreversibly inactivated.[37]

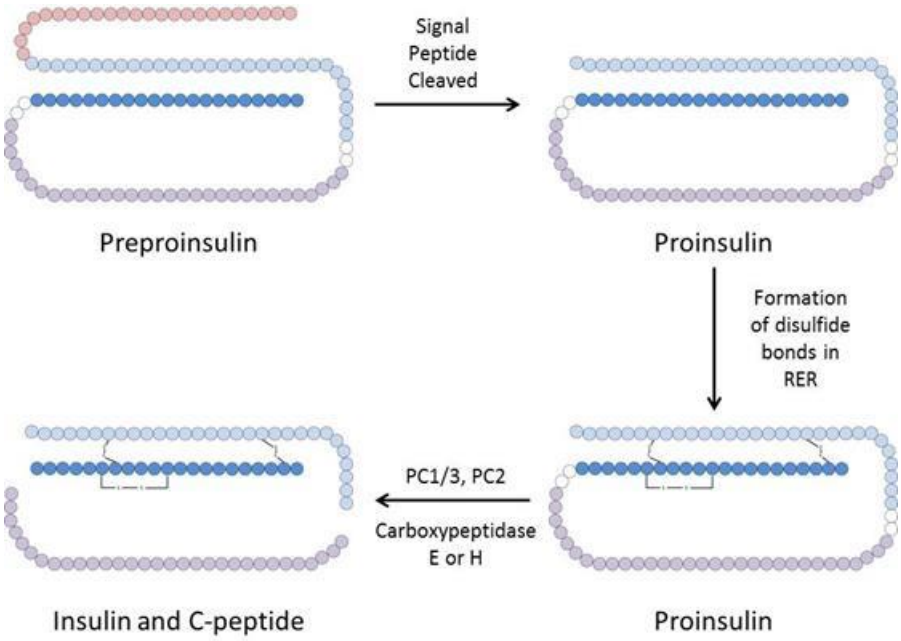

Figure 1.1: The processing of insulin, from preproinsulin to insulin. Preproinsulin contains a signal peptide (pink) which is cleaved in the lumen. Proinsulin is then transported to the rough endoplasmic reticulum, where three disulfide bonds form between the A and B chains of insulin (dark blue and light blue, respectively). Proinsulin is transported into immature secretory vesicles, where two prohormone convertases cleave the residues between insulin and C-peptide (purple). Carboxypeptidase E or H then removes the exposed residues, resulting in insulin and C-peptide.

This processing of preproinsulin (Figure 1.2) results in free insulin and C-peptide within the mature secretory vesicle. Other small molecules, proteins, and ions also exist in the secretory vesicle, including zinc.

1.2.3 Insulin

Insulin is highly conserved between species[2] and promotes glucose uptake from adipose cells and muscle cells while inhibiting hepatic glucose production.[38] In healthy humans, peripheral blood insulin levels rise and fall with the intake of glucose, from a fasting level of about 50 pM to a postprandial level of 200-500 pM.[39] Release

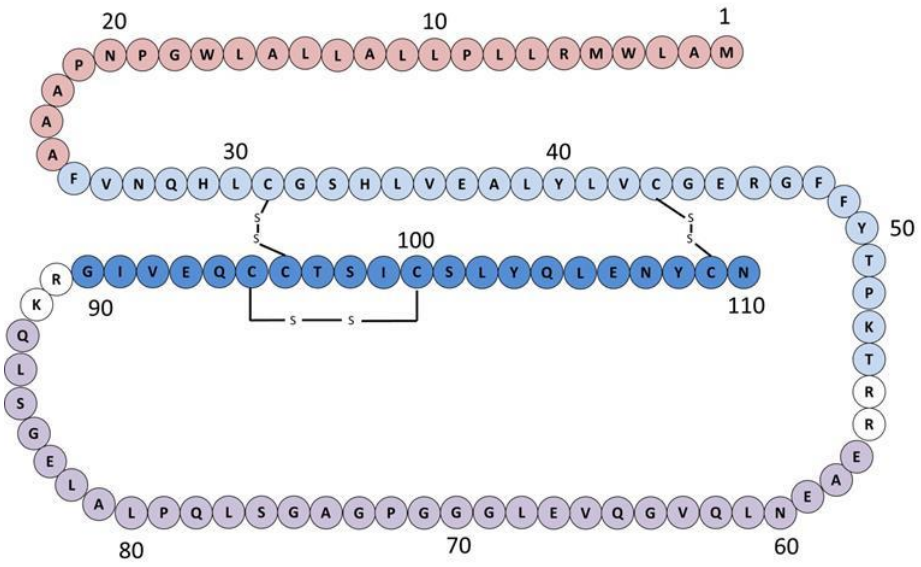

Figure 1.2: The amino acid sequence of preproinsulin. The signal sequence (1-24) is shown in red, the B chain of insulin (25-54, light blue) and the A chain of insulin (90-110) are held together by three disulfide bonds. The 31 amino acid connecting peptide, C-peptide (57-87, purple) is cleaved from insulin and the connecting residues (88-89, 55-56) are removed to yield insulin and C-peptide.

of insulin from pancreatic beta cells is biphasic, where the first phase insulin release occurs 5-6 min after stimulation, and the second phase insulin release is a gradual increase in insulin release over 60 minutes.[40]

There are an estimated 13,000 secretory granules per beta cell, which contain a sufficient amount of insulin to regulate glucose levels for several days.[41] In the secretory granules of pancreatic beta cells, insulin forms dimers, which eventually form stable crystalline hexamers [42-44] and are zinc complexed[45, 46] by Histidine 10 on the B chains of insulin.[47, 48]

Interestingly, the monomers in the insulin hexamer can adopt one of two conformations: T, where the first nine residues of the B chain are elongated, or R, where the first nine

residues of the B chain are in an alpha helix. Different forms of the hexamer are stabilized by water molecules, and other coordinating cations and anions, including Zn^{2+}, Ca^{2+}, Na^{+}, and Cl^{-}.[48]

The mature secretory granules contain a central dense core of insulin molecules in crystalline-like arrays suspended in an aqueous halo of ions, small molecules, and C-peptide.[41] The crystalline insulin hexamer is stable at pH 5.5, however, upon exocytosis, the granules are exposed to blood pH, which is about 7.4, destabilizing the crystalline form.[49] The release of the secretory granule contents includes insulin and C-peptide in a 1:1 molar ratio.

1.3 C-peptide

C-peptide is a negatively charged 31-amino-acid peptide with no ordered structure that begins as the connecting peptide in proinsulin, connecting the A and B chains of insulin.[2] In humans, C-peptide is primarily metabolized and excreted by the kidneys.[50-52] Plasma concentrations of C-peptide after an overnight fast range from 0.3 to 0.6 nM in healthy subjects; postprandial levels of C-peptide rise to 1-3 nM.[53]

For many years after its discovery in 1967, C-peptide was thought to be a biologically inactive molecule that assisted only in the folding of insulin in the secretory vesicles.[54] However, its half-life (30 minutes) is significantly higher than that of insulin (5 minutes), making it an ideal marker for the function of the pancreas.[55, 56]

It was initially hypothesized that C-peptide had the same biological function as insulin,[57, 58] such as increasing glucose uptake in muscle and adipose tissues. Although no effect on glucose uptake was discovered, skeletal muscle exhibited a

significant net uptake of C-peptide.[59] Reports in recent years have shown that C-peptide is a bioactive peptide,[60] with functions in blood flow, kidney function, and utilization of glucose in skeletal muscle,[61-64] though its mechanism of action is poorly understood.

Only 25% of the amino acid sequence of C-peptide is conserved between species (Figure 1.3); the most well conserved include the glutamic acids at positions 3 and 27 (E3 and E27).[2] The C-terminal pentapeptide, EGSLQ, has been shown to be able to elicit biological effects independent of the rest of the peptide, and is thought to be important for binding to the cell surface or to a receptor.[65, 66]

C-peptide's mechanism of action in cells is unclear, however, much research has been done to discover a potential mechanism. Initial studies determined that C-peptide remains disordered in aqueous solvent in the presence of lipid vesicle, which therefore makes it highly unlikely that C-peptide would be able to insert into lipid bilayers and form ion channels.[67] C-peptide has also been shown to be internalized to the cytosol and exhibit some transportation into the nuclei in Swiss 3T3 (fibroblast) and HEK-293 (embryonic kidney) cells.[68] The most likely mechanism of action involves C-peptide binding to the cell membrane to an unknown receptor.

C-peptide has been shown to specifically bind to renal tubular cells (k_{ass}=3.3x10^{9} M^{-1}), fibroblasts (k_{ass}=2.5x10^{9} M^{-1}), endothelial cells (k_{ass}=2.0x10^{9} M^{-1}),[65] and red blood cells (approximately 1,800 molecules/cell).[69] Difficulty in explaining the binding of C-peptide to cell membranes has been compounded by the lack of an identified receptor for C-peptide. Binding of C-peptide and its downstream effects are abolished by

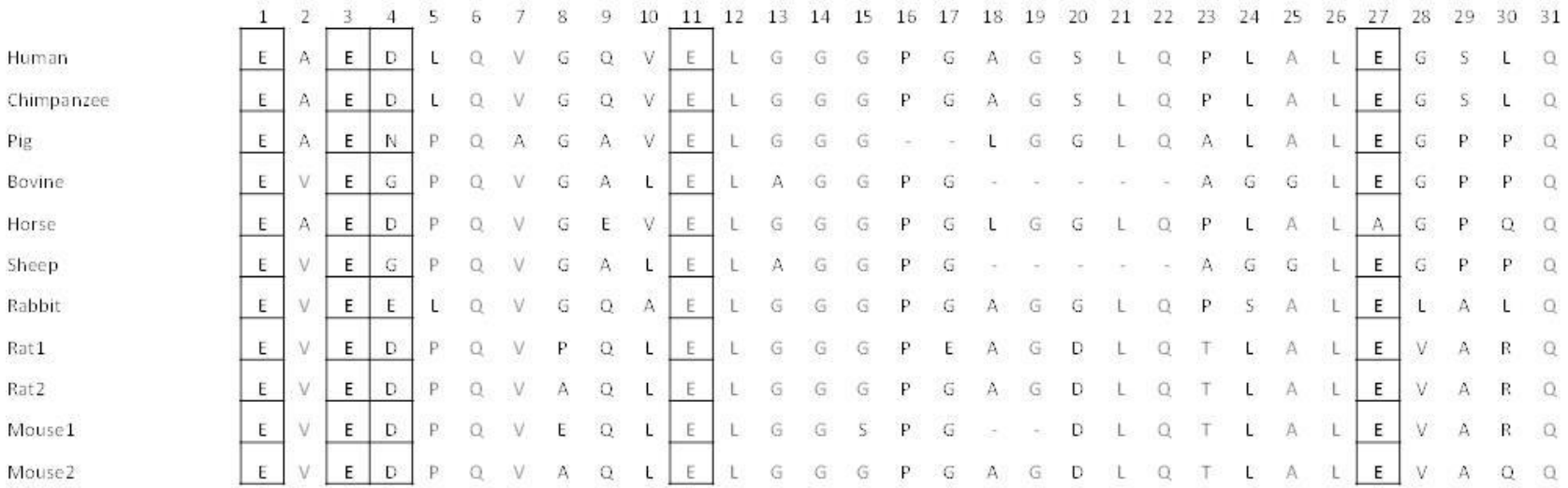

Figure 1.3: Amino acid sequence conservation of C-peptide. Conservation of the amino acid sequence of C-peptide across some species, including human, chimpanzee, pig, bovine, horse, sheep, rabbit, rat, and mouse. Residues that are 50% conserved are shown in gray and residues that are 70% conserved are in bold. Acidic residues are shown in boxes. Adapted from [2]

pertussis toxin, which suggests that C-peptide signaling is through a G protein-coupled receptor (GPCR).[70-72]

A likely candidate is an orphan GPCR, GPR146.[73] Initial studies by Gina Yosten's group, using a Deductive Ligand-Receptor Matching Strategy, determined a short list of orphan GPCRs that could be possible receptor candidates. Selective knockdown of the expression of these orphan GPCRs in three cell lines revealed that C-peptide induced cFos expression was abolished only with the selective knockdown of GPR146.[73] In 2016, Gina Yosten filed a patent identifying GPR146 as the C-peptide receptor.[74] GPR146 has been found to be expressed in the kidney,[75] human retinal pigment epithelial cells,[76] human KATOIII cells (stomach), HEK293 cells (embryonic kidney), and TF-1 cells (erythroblast).[73]

Intracellular C-peptide signaling leads to an elevation of intracellular calcium concentrations,[71, 77] phosphorylation of phospholipase C (PLC) and protein kinase C (PKC) isoforms,[78-80] phosphoinositide 3-kinase (PI3K) activation,[80, 81] and mitogen activated protein kinase (MAPK) signaling.[78, 79] *In vitro* studies have shown that C-peptide stimulates the release of nitric oxide (NO) in endothelial cells,[77] and has a direct effect on Na^+, K^+-ATPase activity in renal tubular cells.[71]

C-peptide's anti-oxidant, anti-inflammatory, and anti-apoptotic effects have also been described: the reduction of reactive oxygen species (ROS) through inhibition of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, inhibition of caspase-3, and slowing of apoptosis,[82-84] downregulation of nuclear factor nuclear factor κB (NF-κB) activity, decreased secretion of pro-inflammatory cytokines and chemokines,[85]

and reduction of cellular adhesion molecules such as vascular endothelial growth factor (VEGF),[86] transforming growth factor β (TGF-β)[87, 88] and plasminogen activator inhibitor 1 (PAI-1).[89]

1.1 Zinc

Zinc is required for the function of nearly 300 proteins in the body, as well as for insulin synthesis and storage.[90] Additionally, zinc content in the pancreatic beta cells is among the highest in the body, reported to be about 30 mM;[10, 91] with about one third of that zinc present in the secretory vesicles in beta cells,[92] and large amounts being secreted from the beta cells.[93]

Zinc homeostasis in the pancreatic beta cell is critical and is modulated by a complicated network of transporters on the cell membrane, endoplasmic reticulum, Golgi, mitochondria, and secretory granules (Figure 1.4).[94] The ZnT family of transporters is responsible for transporting zinc from the cytosol into the extracellular space or into intracellular compartments, such as the secretory vesicles. The ZIP family of transporters is responsible for zinc entering into the cytoplasm from outside the cell or from intracellular compartments into the cytosol.

Zinc is required for insulin synthesis and storage in the beta cell granule,[90] with two zinc ions complexed to the insulin hexamer. Additionally, zinc regulates the secretion pathway of beta cells at many levels: through ATP-sensitive K^+ channel (K_{ATP}) channels,[95, 96] synthesis and storage,[42] and at the alpha cell level.[97] At rest, beta cell cytosolic free zinc is 400-450 pM.[98]

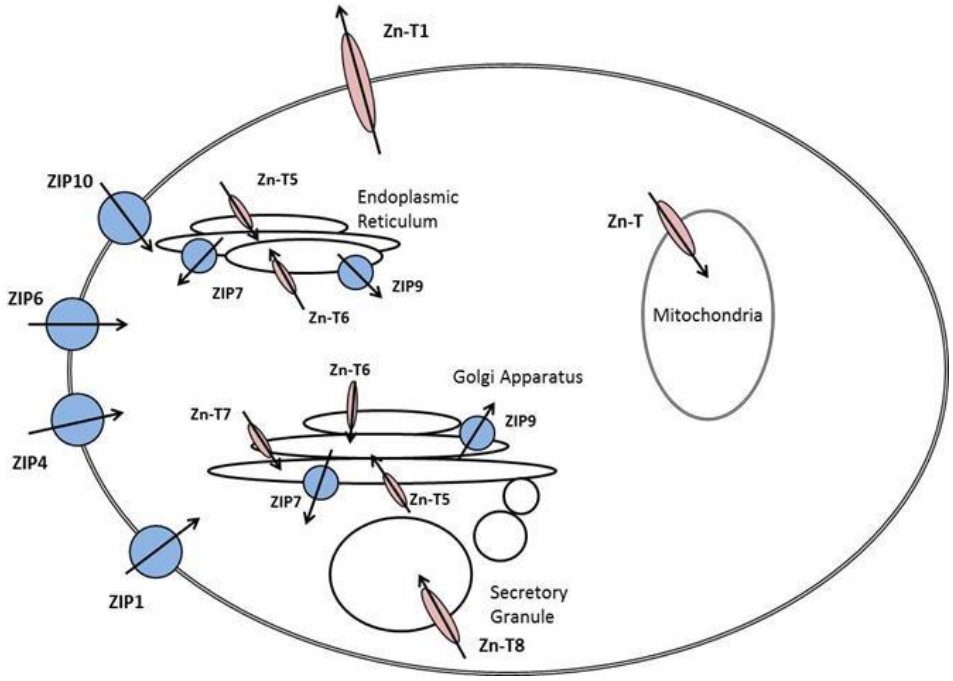

Figure 1.4: Zinc homeostasis in the pancreatic beta cell. Zinc homeostasis in the pancreatic beta cell is mediated by a network of ZIP and ZnT family proteins present on the plasma membrane, secretory granule, endoplasmic reticulum, golgi apparatus, and mitochondria. Adapted from [94].

1.5 Glucose Stimulated Hormone Secretion from Pancreatic Beta Cells

Insulin, C-peptide and zinc are secreted from pancreatic beta cells following the intake of food. The breakdown of food increases the glucose levels in the bloodstream, while these secretions from pancreatic beta cells decrease blood glucose levels through various mechanisms. As shown in Figure 1.5, glucose transporters on the surface of beta cells are activated at high levels of glucose, stimulating glucose transport into the cell. Three glucose transporters have been reported to be present on human beta cells: GLUT1 (K_m=6 mM), GLUT2 (K_m=11 mM), and GLUT3 (K_m=1 mM).[99] Upon glucose entering the beta cell, glycolysis is initiated by glucokinase (a hexokinase). Studies of

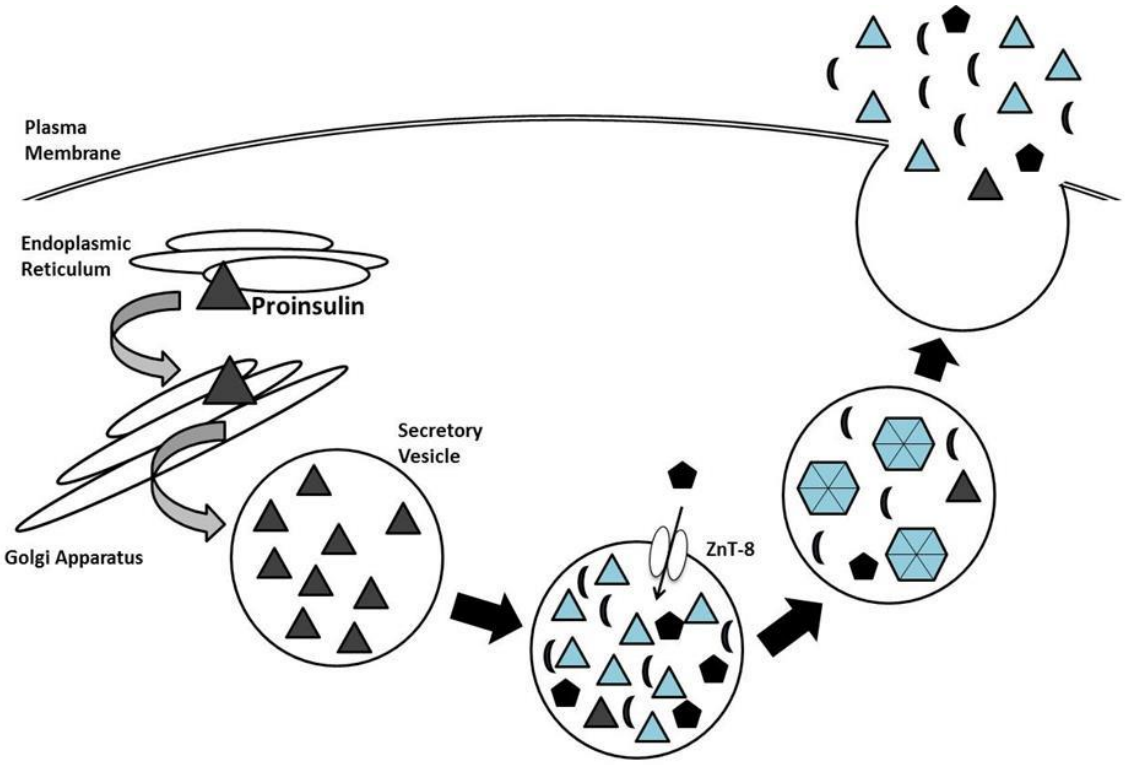

Figure 1.5: The mechanism of hormone secretion from pancreatic beta cells. Proinsulin is packaged into immature secretory vesicles, where it is cleaved into insulin and C-peptide. The ZnT-8 transporter brings zinc into the secretory vesicle and allows the crystalline insulin hexamer to form. Upon intracellular calcium increase, the secretory vesicle is translocated to the plasma membrane, where its contents is exocytosed.

islets from healthy human subjects revealed that glucose oxidation increases threefold when glucose concentrations increased from 1 to 6 mM, but only increase another 25% when the glucose concentration increases to 12 mM.[100]

Upon glucose entering the beta cell and the increase in glycolysis, the [ATP]/[ADP] ratio increases,[101, 102] which closes the K_{ATP} channel. The K_{ATP} channel is responsible

for controlling electrical activity in the beta cell.[103, 104] In the absence of glucose, the membrane potential of the beta cell is about -70 mV. At 6 mM glucose, the resting conductance is reduced by 70%, triggering membrane depolarization.[105] This membrane depolarization triggers the opening of voltage dependent calcium channels, which leads to exocytosis of the granule contents.

Human pancreatic beta cells contain two types of calcium channels: low-voltage-activated (LVA) and high-voltage-activated (HVA). LVA Ca^{2+} channels likely are involved in regulation and initiation of regenerative electrical activity, while HVA Ca^{2+} channels are activated during depolarizations.[106] The resultant increase in intracellular calcium ($[Ca^{2+}]_i$) triggers the cell to exocytose its secretory vesicles,[107] which ultimately releases insulin, C-peptide, zinc and other contents of the beta cell granules.[108-112] Insulin secreted from the islets of Langerhans flows directly into the portal vein. About half of the insulin is cleared, while the rest flows into systemic circulation.[113]

Dysfunction of pancreatic beta cells affects not only insulin secretion, but also C-peptide and zinc secretions, which leads to chronic hyperglycemic conditions. Patients who exhibit this type of pancreatic dysfunction have the disease known as *diabetes mellitus.*

1.6 Diabetes mellitus

Diabetes mellitus is a metabolic disease characterized by chronic hyperglycemia, and affects 9.3% of the American population. Chronic hyperglycemia leading to diabetes results from the body's inability to produce or utilize insulin. A patient is considered diabetic if their fasting blood glucose level is above 126 mg/dL (5.9 mM), or if after an

oral glucose tolerance test, their blood glucose levels remain above 200 mg/dL (10 mM). This hyperglycemia can be treated in different ways depending on the patient's type of diabetes. Four types of diabetes are recognized by the American Diabetes Association: type 1 diabetes (T1D), type 2 diabetes (T2D), gestational diabetes (GD), and other types of diabetes.[114] A long term indicator of glycemic levels, hemoglobin A1c (HbA_{1c}), measures the amount of glycated hemoglobin in a blood sample, giving an average blood glucose level over three months. HbA_{1c} is the most common test for how well a diabetic patient is controlling blood glucose levels with their current therapy.

1.6.1 Type 1 diabetes (T1D)

T1D, traditionally known as juvenile diabetes or insulin-dependent diabetes, accounts for only 5% of the total number of diabetes diagnoses.[114, 115] These patients produce no insulin or C-peptide from their pancreas and must administer exogenous insulin in order to control blood glucose levels. This lack of insulin production is thought to be caused by the autoimmune destruction of the pancreatic beta cells.[116, 117] Evidence for the autoimmune basis of T1D includes lymphocyte infiltration into and around the islets, presence of autoantibodies to some islet autoantigens, and the presence of major histocompatibility complex (MHC)-linked and non-MHC-linked disease susceptibility genes.[47, 114] T1D has multiple associated genetic and environmental factors and patients are prone to developing other autoimmune disorders.[114]

Patients with T1D must administer exogenous insulin in order to survive and manually check their blood glucose levels with a blood glucose monitor. The first analogues of insulin that were given to patients were made without zinc. However, *in vitro* studies

suggest that insulin alone binds to liver membranes, leading to substantial degradation and very little efficacy, and that there is less degradation when zinc is co-administered with insulin.[118] An analogue of insulin, known as protamine zinc insulin (PZI), has zinc and lente crystalline insulin (an intermediate acting insulin), which prolongs the duration of action by delaying absorption.[119] Currently, there are analogues of insulin which don't form hexamers with zinc and insulin, resulting in a more rapid absorption from the injection site.[120]

Insulin and C-peptide levels in T1D patients are virtually nonexistent, as the majority of beta cells have been destroyed. However, the predominant effect on zinc homeostasis in diabetes is hypozincemia, resulting from hyperzincuria or decreased gastrointestinal absorption of zinc.[119] Hyperglycemic conditions have been shown to decrease the islet cell labile zinc,[91] and higher levels of urinary zinc excretion have been correlated to HbA_{1c} levels.[121]

In T1D patients, there is a 20% decrease in serum zinc levels.[122] Studies in streptozotocin (STZ)-induced rats (T1D model) showed that administration of insulin reduced hyperglycemia but does not ameliorate hyperzincuria.[123, 124] STZ-induced rats showed increased OH radical production,[125] lipid peroxidation and decreased superoxide dismutase.[126] A decrease in pancreatic superoxide dismutase (SOD) and catalase antioxidant activity precede loss of beta cell function.[127]

Zinc levels in T1D patients may be related to increased metallothionein production,[128] or decreased zinc absorption[129] A zinc supplementation study in T1D patients found that patients exhibited hyperzincuria, but there was no difference in zinc excretion and

mononuclear zinc concentrations after zinc supplementation. However, an increase in HbA_{1c} was observed in all individuals, both control and T1D patients, indicating a possible mechanism of zinc toxicity.[130]

Secondary complications of diabetes include retinopathy, nephropathy, and neuropathy, which are responsible for blindness, non-traumatic limb amputations, and kidney failure, respectively. Blood glucose control and insulin injections, do not preclude patients from developing these complications. The Diabetes Control and Complications Trial found that, with an intensive glucose control therapy, secondary complications were reduced by 47% (retinopathy), 54% (nephropathy), and 60% (neuropathy) but were not completely abolished. Intensive control therapy was defined as achieving blood glucose levels as close to nondiabetic levels as possible, 3 or more daily injections of insulin, self-blood glucose monitoring four times daily, and monthly outpatient visits.[131] Much of this intensive control therapy has been adopted today as the conventional method for treatment, however, although exogenous insulin is effective to lower hyperglycemic blood glucose levels, it does not preclude T1D patients from developing secondary complications.

Inadequate zinc may contribute to tissue damage in diabetes, owing to the fact that zinc is a necessary factor in antioxidant enzymes. This has a contribution to secondary complications, specifically, retinopathy.[117] Additionally, zinc deficient STZ-induced rats demonstrate an increase in progression of nephropathy, which indicates that a lack of zinc may indeed contribute to the progression of secondary complications.[132]

1.6.2 Type 2 diabetes (T2D)

T2D accounts for 90-95% of all cases of diabetes, and is traditionally referred to as non-insulin dependent diabetes. T2D patients exhibit insulin resistance, meaning that their bodies cannot adequately use the insulin secreted from their pancreas.[114] T2D is traditionally associated with increased obesity and reduced physical activity.[133] Pancreatic beta cells (at least initially) secrete normal and higher amounts of insulin (hyperinsulinemia) and C-peptide, which may contribute to insulin resistance in the tissues.[134]

Treatment for T2D patients often begins with diet and lifestyle changes, however, many patients will also be prescribed oral medications that aim to increase the amount of insulin secreted from the pancreas (sulfonylureas, meglitinides), decrease the amount of glucose produced from the liver (biguanides, thiazolidinediones), or inhibit specific processes involved in glucose breakdown (DpP-4 inhibitors, SGLT2 inhibitors, alpha-glucosidase inhibitors).[135] For many T2D patients, after prolonged hyperglycemia and the inability of the beta cell to make enough insulin, there is a loss of islet cells completely, leading to the necessity of exogenous insulin.[136]

T2D patients are also prone to developing secondary complications of diabetes. As of 2015, 28.5% of diabetic patients (T1D and T2D) had developed retinopathy, 44% had developed nephropathy, and 60-70% had mild to severe forms of neuropathy.[115] Clearly, insulin and oral medications alone are not sufficient to control blood glucose levels and protect patients from developing secondary complications in both T1D and T2D. Many researchers believe that other molecules secreted from pancreatic beta cells, such as C-peptide and zinc, may be the answer to this problem.

As in T1D, zinc levels in T2D patients are also dysfunctional. One study showed a 40% decrease in serum zinc in diabetic patients, with no difference in treatment type.[137] However, another study found that insulin treatment reduces hyperzincuria while oral medications had no effect in T2D patients.[138] These zinc dysfunctions in both T1D and T2D have downstream effects due to the fact that zinc affects neighboring endocrine cells in both autocrine and paracrine fashions.[95, 97, 139-141]

In recent years, evidence has surfaced of genetic abnormalities in T2D. In 2007, a genome wide association study (GWAS) of type 2 diabetic patients found a synonymous single nucleotide polymorphism (SNP) in SLC30A8,[142] the gene that encodes for ZnT-8, which is responsible for zinc uptake into the secretory granules of pancreatic beta cells. Other GWAS experiments have confirmed this discovery.[143-147] This sparked much interest in ZnT-8, but the literature since then has been filled with contradictory results from mouse knockout and cell knockdown studies. These studies will be explored in later chapters.

1.6.3 Gestational diabetes (GD)

Glucose intolerance during pregnancy has been known for many years, and has led to the classification of GD.[148] The American Diabetes Association recommends that all pregnant women take a 75 g OGTT at 24-28 weeks of gestation,[149] which results in the diagnosis of diabetes in 15-20% of all pregnancies.[114]

1.6.4 Other types of diabetes

Other specific types of diabetes (~1% of all cases) recognized by the American Diabetes Association include monogenic diabetes syndrome and cystic fibrosis related

diabetes (CFRD). Monogenic defects cause beta cell dysfunction, resulting in neonatal diabetes and maturity-onset diabetes of the young.[114] CFRD is diagnosed in about 50% of adult patients with an autosomal recessive genetic disorder known as cystic fibrosis (CF).[114]

1.7 Cystic Fibrosis (CF)

Cystic fibrosis (CF) is a life-shortening autosomal recessive genetic disorder that affects 1 in 3,500 live births, which amounts to approximately 30,000 people in the United States.[150] The defective gene in CF, discovered in 1989, is located on the long arm of the seventh chromosome and encodes for the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR).[151, 152]

CFTR is a 1,480 amino acid protein classified as a member of the family of ATP-binding cassette (ABC) transporter ATPases. It is a cyclic adenosine monophosphate (cAMP) regulated chloride channel found in the apical membrane of epithelial cells lining organs such as the airways, intestinal tract, pancreatic ducts and sweat glands.[153] CFTR (Figure 1.6) contains two transmembrane domains (TMD1 and TMD2), each with six transmembrane spanning α-helices, two nucleotide binding domains (NBD1 and NBD2) and one regulatory domain (R domain). CFTR remains the only ABC transporter ATPase to be identified as an ion channel, as well as the only one to contain a R domain.[154] The chloride pore is formed by the two TMDs, which are opened when one ATP binds to each NBD, causing a conformational change that brings the two NBDs into close proximity to each other. In addition to this conformational change, cAMP-dependent protein kinase A (PKA) mediated phosphorylation of the R domain is

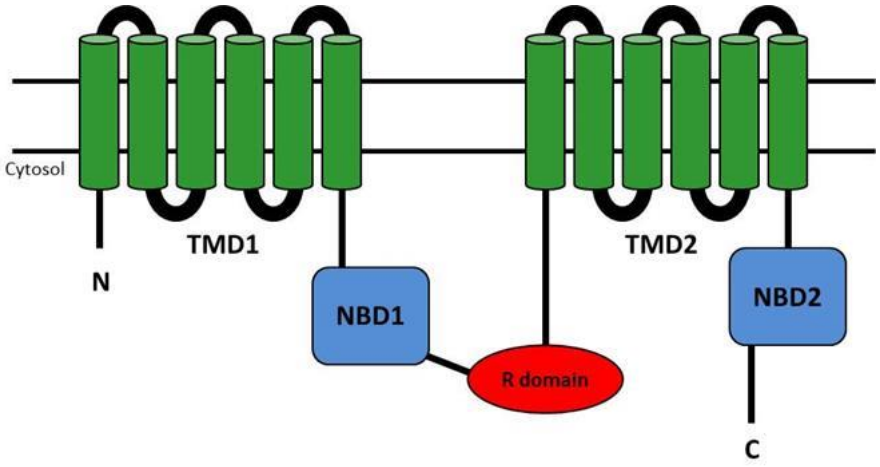

Figure 1.6: Cartoon structure of the CFTR protein. The secondary structure of CFTR contains two transmembrane domains (TMD1 and TMD2), each with six transmembrane spanning alpha helices. Two nucleotide binding domains (NBD1 and NBD2) and an unstructured R domain lie on the cytosolic side of the membrane. The most common mutation in CFTR, delF508, lies in NBD1.

necessary for the channel to function properly.[155] Hydrolysis of one ATP molecule closes the channel.

Under normal conditions, the immature CFTR polypeptide is then incorporated into the endoplasmic reticulum (ER) membrane, and is *N*-glycosylated during co-translational transport, requiring chaperone molecules to ensure that the polypeptide is folded properly.[153] The protein is resistant to proteases and is then transported to the Golgi stacks, and is further modified by glycosylation. This mature protein is transported to the cell membrane of epithelial cells to function as a chloride ion channel. The C-terminal tail of CFTR contains a tyrosine internalization signal and a PDZ binding

motif.[156] The mature, full-length CFTR protein has a half-life of 16 hours, and is eventually targeted to the lysosomes for degradation.

The misfolding and increased targeting for degradation of CFTR occurs in CF as a result of mutations in the gene, causing abnormally thick, viscous secretions in the airways, intestinal tract, pancreatic ducts, and sweat glands. These secretions can cause blockages in these organs, and forms a prime breeding ground for bacterial infections in the lungs, leading to pulmonary complications, which is the leading cause of death in CF patients.

Over 1,000 mutations of the CFTR gene have been identified and categorized into six classes.[157] Class I mutations include nonsense and frameshift mutations that result in no production of CFTR. The most common mutation, ΔF508 (a deletion of phenylalanine at position 508, residing in NBD1), is a class II mutation that results in abnormal processing of CFTR such that less than normal amounts accumulate at the apical plasma membrane. Class III mutations result in altered regulation of the chloride ion transport and gating properties of the channel, although the whole protein accumulates at the apical plasma membrane. Mutations that produce full-length CFTR, but have defective ion permeation through the channel are class IV mutations. Class V mutations cause reduced synthesis of full-length CFTR, and Class VI mutations produce CFTR that have truncated C-termini and shortened residence times at the apical plasma membrane.

As medical technologies and drug therapies have advanced, the life expectancy for CF patients has risen to nearly 38 years.[150] With this increase, more complications of CF

have become important to diagnose and study, including CFRD, which is diagnosed in more than 30% of CF patients over the age of 18, though some report as high as 50% incidence.[150, 158, 159] CFRD is currently the most common co-morbidity with CF and is associated with a nearly six-fold greater mortality rate.[160]

CFRD was originally thought to be a consequence of pancreatic insufficiency, duct obstruction and fibrosis, but not all patients with pancreatic insufficiency develop CFRD.[161, 162] However, abnormalities in islets and beta cell loss have been observed. Additionally, CFRD has been shown to correlate with first-phase insulin and overall insulin secretion deficiencies,[161-164] indicating that some beta cell intrinsic factors may also play a role. Nearly two-thirds of all CF patients have some sort of impaired glucose tolerance.[165] These patients have normal fasting glucose levels, but suffer from post-prandial hyperglycemia, indicating that the insulin response from the beta cells is insufficient.[155] Additionally, a common treatment in CF is high dose, long regimen oral steroids, which are known to increase blood glucose levels.[166]

CFRD is unique in that it has characteristics of both T1D and T2D, including low insulin production[167] and insulin resistance.[168, 169] CFRD patients also have random bouts of hypoglycemia,[170] which makes glycemic levels difficult to control. To date, the major accepted therapy used for treatment of CFRD is recombinant insulin injections.[171] Due to its unique characteristics, the glycemic control of CFRD patients is difficult and not entirely controlled by insulin in many cases. Additionally, microvascular complications are still common among CFRD patients.[172]

CFRD has nutritional consequences of concern: protein catabolism and weight loss;[173] as well as pulmonary consequences including lung function decline and increased mortality.[165, 174, 175] Using isotope enrichment studies following an intracellular leucine metabolite, it was found that control and CF patients did not have any difference in the fasting state.[176] However, after insulin treatment, control patients showed an expected suppression of amino acid appearance rates. CF patients did not have suppression of amino acid appearance, indicating that inhibition of protein breakdown in these patients remains, despite bloodstream glycemic control. A study of free fatty acid flux in CF patients revealed that lipolysis is impaired compared to control subjects even after insulin treatment and bloodstream glycemic control.[177]

Only recently has a link between CFTR and the pancreatic beta cells been studied. When CFTR was chemically inhibited, beta cells showed reduced cAMP dependent exocytosis and a decreased number of docked insulin granules.[155] Other studies with CFTR mutant and wild-type mice have shown reduction or abolition of insulin and calcium secretions in CFTR mutant mice.[178]

C-peptide secretion in CF patients is decreased and delayed, concurrent with increased blood glucose levels after an oral glucose tolerance test.[161] Holl *et al.* found that first-phase insulin secretion is also decreased and delayed in CF patients with impaired glucose tolerance as well as those with CFRD.[179] Control patients had a peak insulin concentration at 30 minutes after an oral glucose tolerance test, while CF patients with normal glucose tolerance had a delayed peak insulin concentration at 60 minutes. Peak insulin concentration in CF patients with impaired glucose tolerance or diagnosed CFRD

did not obtain peak insulin concentration until 90 minutes after an oral glucose tolerance test.

Zinc levels in CF patients are also known to be low.[180, 181] Studies have shown that absorption of zinc seems to be increased with pancreatic enzyme treatment.[181] However, plasma zinc levels are an underestimation of the amount of zinc in the body, as 99% of zinc is intracellular. A study of the amount of zinc within the red blood cell (accounting for 80-90% of total zinc) showed that more than one-third of the CF population studied had low zinc content.[180] To date, most studies concerning zinc levels in CF patients, whether in serum or intracellular, have been correlated to nutritional status only. Recent work has also indicated that some secondary damage to the pancreatic beta cells in CF patients is possible, based on the presence of ZnT-8.[182]

1.8 Microvascular Complications of Diabetes

CFRD, T1D, and T2D patients are all prone to developing secondary microvascular complications, including nephropathy, neuropathy, and retinopathy. Diabetic nephropathy is the leading cause of kidney failure, with nearly 50,000 Americans beginning treatment for kidney failure every year.[115] Nephropathy begins with the development of microalbuminuria due to glomerular hyperfiltration.[183, 184] There is also evidence of an increase in glomerular basement membrane thickness, microaneurysm formation, and an increase in VEGF.[185, 186]

Diabetic neuropathy is the leading cause of non-traumatic lower-limb amputations in the United States, with about 73,000 amputations performed annually.[115] The most

common form of diabetic neuropathy is chronic sensorimotor distal symmetric polyneuropathy, which results in a burning or tingling feeling or numbness in the limbs.[187] A second type of diabetic neuropathy is known as diabetic autonomic neuropathy, which causes neurological dysfunction in most organ systems.[188]

Diabetic retinopathy leads to blindness, and 4.2 million Americans over the age of 40 with diabetes have symptoms of diabetic retinopathy.[115] Diabetic retinopathy can be classified as background or proliferative. Patients with background retinopathy exhibit small hemorrhages in the retina, while those with proliferative retinopathy exhibit the formation of new blood vessels on the surface of the retina.[189]

All of these secondary microvascular complications have their roots in blood flow, which involves the human cardiovascular system, primarily red blood cells and endothelial cells.

1.9 The Human Cardiovascular System

One major element of the cardiovascular system is the blood, which is made up of red blood cells (RBCs), white blood cells (WBCs), platelets and plasma. The functions of blood include transportation of molecules and drugs, defense against pathogens, and maintenance of homeostasis. Centrifugation of a blood sample will separate the components into RBCs, plasma, and the buffy coat. The major component of blood is RBCs, which make up about 45% of whole blood, while about 55% is referred to as the plasma, which contains water, proteins, nutrients, and other small molecules. The buffy coat contains the remaining WBCs and platelets.[1]

1.9.1 Red Blood Cells (RBCs)

RBCs are the major component of the bloodstream, with about 2.6 x 10^{13} cells *in vivo*.[190] RBCs are anucleated and lack all formed organelles, including mitochondria. They are approximately 6-8 μm in diameter with a volume of about 87 fL, lasting about 120 days in circulation.[191] RBCs primarily metabolize glucose via glycolysis. *In vivo*, the main function of RBCs is to deliver oxygen to the organs and tissues, however, it also plays a role in regulating vascular tone through the release of ATP.[190] RBCs metabolize glucose via glycolysis and fermentation, and contain primarily the glucose transporter 1 (GLUT1).

The mechanism of ATP release from RBCs is a well-defined mechanism (Figure 1.7), with high nM to μM levels released during hypoxia, deformation, decrease in pH, and molecular stimuli.[192] Briefly, a heterotrimeric G-protein is activated and the $G_{\alpha i}$ subunit inhibits activation of adenylyl cyclase (AC), while the $G_{\beta\gamma}$ subunit activates other forms of AC. AC activation converts ATP into cyclic AMP (cAMP), activating protein kinase A (PKA). PKA phosphorylates CFTR, causing an influx of chloride ions. Downstream from CFTR lies the ATP release channel, Pannexin 1.[193]

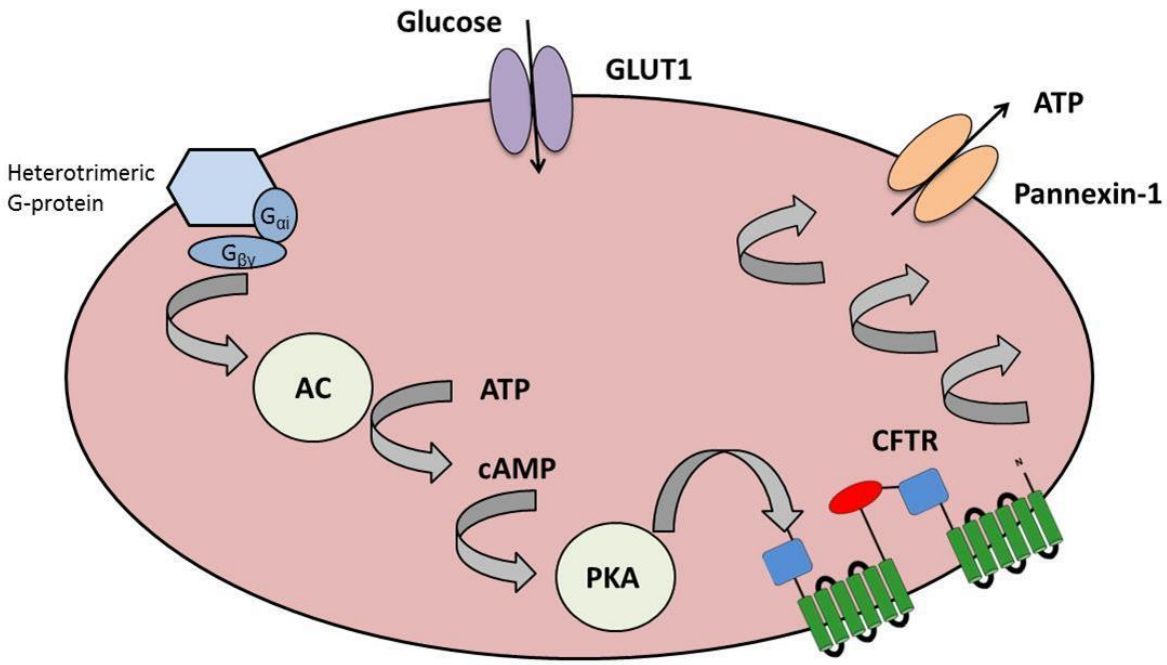

Figure 1.7: The mechanism of ATP release from RBCs. A heterotrimeric G-protein becomes activated, releasing the βγ subunit to activate adenylyl cyclase (AC). AC converts ATP to cyclic adenosine monophosphate (cAMP), which activates protein kinase A (PKA). PKA phosphorylates CFTR. Somewhere downstream of CFTR lies the ATP release channel, Pannexin-1.

In patients with *diabetes mellitus*, RBCs show decreased deformability, effects in ageing, and decreased Na^+, K^+-ATPase and Mg^{2+}-ATPase activities, leading to hypertension.[194-197] Patients with CF also have RBCs with decreased deformability.[192]

1.10 The Human Immune System

The human immune system is responsible for protecting the body from foreign pathogens, such as viruses and bacteria. Once a pathogen has penetrated the initial barriers, such as the skin, mucous membranes, oral cavity, and stomach, there are two

responses of the immune system to capture and destroy pathogens: the innate immune response, and the adaptive immune response.[1]

1.10.1 Innate and Adaptive Immunity

The innate immune response is the first phase of defense against pathogens, occurring between 0 and 12 hours after infection. Innate immunity consists of cellular responses from phagocytes (macrophages and neutrophils) and natural killer cells. The adaptive immune response, on the other hand, occurs between 12 hours and days after the infection occurs, and contains the element of memory, allowing the body to fight off the same infection again if it returns. The cells involved in the adaptive immune response are B cells and T cells.[198] Here, we are concerned with the adaptive immune response, primarily neutrophils, which can be isolated from the bloodstream.

1.10.2 Neutrophils

Neutrophils are the first line of defense in the immune system. They are the most abundant white blood cell, have a diameter of 8-9 μm and contain intracellular machinery that human RBCs do not. They survive only 5 days inactivated in the bloodstream,[199] and a healthy human has between 2 and 7 million neutrophils per mL of whole blood.[200] Neutrophils primarily metabolize glucose via glycolysis and create glycogen stores for later use.[201] The primary function of neutrophils is to travel to the site of infection (chemotaxis), phagocytose bacterial pathogens, and kill pathogens through cytotoxic mechanisms.[199]

Activation of neutrophils is a multistep process that begins with the translocation of neutrophils from the bone marrow in response to a pro-inflammatory signal. After

release from the bone marrow, neutrophils attach to the endothelium and travel along it until translocation into the tissue, a process known as neutrophil priming.[199, 202] Neutrophils respond to pro-inflammatory stimuli (chemoattractants) in the tissue, such as lipopolysaccharides, which causes the release of chemokines from neutrophils, the most common of which is interleukin 8 (IL-8).[202]

Neutrophils can kill pathogens by three different mechanisms: phagocytosis, production of reactive oxygen species (ROS), and degranulation. Neutrophil phagocytosis is rapid, with internalization of IgG-coated targets occurring within 20 seconds, followed by fusion of the phagocytic vacuole with granules that contain hydrolytic enzymes.[199] Production of ROS is generated by the activation of NADPH oxidase, and nitric oxide (NO) production by inducible nitric oxide synthase (iNOS), which is induced upon neutrophil priming, which complements ROS production.[199, 202] Zinc is a known activator of NADPH oxidase in neutrophils at low concentrations, while higher concentrations of zinc result in generation of hydrogen peroxide, which is then used by myeloperoxidase to generate another ROS, hypochlorous acid.[203] Degranulation occurs during neutrophil activation, releasing the antimicrobial peptides contained in the granules.[199]

It has long been established that patients with *diabetes mellitus* have changes in blood neutrophils, including chemotaxis,[204] phagocytosis,[205] and production of ROS.[206] Some studies have suggested that insulin eliminates these changes and increases the amount of neutrophils in the bloodstream, though the mechanism is unknown and is not linked to glycemic control.[207, 208] Neutrophil function in CF is also markedly changed. The lack of CFTR causes an increase in production of pro-inflammatory

cytokines in submucosal gland cells, causing an increase in the production of IL-8.[209] Additionally, the deficient expression of CFTR in neutrophils causes alterations in cytokine production, chemotaxis, ROS production, phagocytosis, and apoptosis.[209, 210]

1.11 Insulin is Insufficient to Control Secondary Complications in Diabetes

When insulin was discovered, it was thought to be the cure to a disease that claimed the lives of many children. However, over time it has become clear that insulin is not solely responsible for glucose homeostasis in the human body. Although insulin is sufficient to lower blood glucose levels and increase glucose utilization by skeletal muscle and adipose cells, it is not sufficient to alleviate the secondary complications of diabetes, many of which are related to blood flow. The bulk of the current research to ameliorate these secondary complications lies with C-peptide. Experiments in animal models of T1D and human clinical trials have shown promising results.

Significantly reduced blood flow in the nerves of diabetic rats has been reported, mimicking neuropathy in human diabetic subjects.[211, 212] In BB/Wor rats (T1D mimic), C-peptide administration for 2 months resulted in the prevention of acute nerve conduction velocity (NCV) defects and a 55% increase in neural Na^+,K^+-ATPase activity.[213] C-peptide has also been reported to improve nerve blood flow, probably through a stimulation of endothelial nitric oxide synthase (eNOS), increasing NO bioavailability.[77, 214] STZ-induced diabetic rats with 2 weeks of C-peptide replacement showed 62% increase in sciatic motor NCV and 78% increase in saphenous sensory NCV.[211] Clinical studies in humans have also shown beneficial

effects of C-peptide.[215] In both 3-month and 6-month C-peptide replacement therapy trials, patients with T1D showed significant improvements in sensory NCV.[215, 216]

C-peptide replacement has also been shown to decrease or prevent nephropathy symptoms in STZ-induced diabetic rats. After 2 weeks of C-peptide replacement therapy, glomerular filtration rate, glomerular hyperfiltration and albuminuria were all significantly reduced.[217, 218] Human clinical trials have shown no effect in albuminuria or glomerular filtration rate,[219] but have shown increased renal Na^+,K^+-ATPase activity, eNOS expression and NO production.[77, 220]

As mentioned previously, C-peptide's anti-oxidant, anti-inflammatory, and anti-apoptotic effects have also been described.[82-89] Taken together, these studies indicate that C-peptide may indeed be a necessary secretion from pancreatic beta cells that is missing in current diabetes therapy.

A pharmaceutical company in California, Cebix, raised almost $50 million dollars in order to conduct a phase 2b trial on the effects of a PEGylated C-peptide replacement therapy (Ersatta) for treatment of diabetes-related microvascular problems. However, although small scale human clinical trials and animal studies showed promise, the trial was shut down in phase 2b trials when results from patients taking Ersatta and those taking the placebo were indistinguishable. The company has now folded, and no ongoing clinical trials are in progress for Ersatta.[221, 222]

Two developments in *in vitro* studies with C-peptide may give insight into why this trial failed. Firstly, Meyer *et al.* was the first to show that C-peptide was only able to induce ATP release from RBCs when complexed to a metal (Cr^{3+} or Fe^{2+}). ATP release when

RBCs were incubated with 10 nM C-peptide and equimolar Cr^{3+} showed about a 74% increase in ATP release from control RBCs, while RBCs incubated with 10 nM C-peptide and Fe^{2+} showed a 95% increase in ATP release after a 6 hour incubation.[223] After further biological investigation, Meyer *et al*. realized that the most likely metal that C-peptide would bind to is zinc due to its high concentrations in the pancreatic beta cell.[43] Subsequent studies indicated that zinc was able to bind to HPLC-purified C-peptide and induce RBC ATP release and consequently NO release from endothelial cells.[224, 225] The second major development in recent years was the discovery of the necessity of albumin for C-peptide efficacy. Liu *et al*. showed that C-peptide was able to bind to RBCs in the presence or absence of zinc, but that zinc was only able to bind to RBCs in the presence of C-peptide. Furthermore, binding of both molecules was abolished when albumin was not present in the buffer. Isothermal titration calorimetry (ITC) analysis showed no binding of C-peptide alone to zinc, but showed specific binding of C-peptide to albumin ($N=0.53 \pm 0.03$, $K_a=1.75 \pm 0.64 \times 10^5\ M^{-1}$). Additionally, specific binding of both C-peptide and zinc to albumin was shown (Zinc: $N_1=0.33 \pm 0.01$, $K_1=5.08 \pm 0.98 \times 10^7\ M^{-1}$; C-peptide: $N_2=1.15 \pm 0.01$, $K_2=2.66 \pm 0.25 \times 10^5\ M^{-1}$). These results indicate a two-phase binding event, with zinc binding first and then two molecules of C-peptide binding.[69] Interestingly, diabetic patients (T1D or T2D) have albumin in their bloodstream that is extensively glycated due to the excess glucose in the bloodstream.[226, 227] This glycated albumin causes altered binding to transition metals[228] and drugs,[229] and could therefore alter the binding of C-peptide as well. Taken together, these results indicate that in order for C-peptide to have a

biological effect, not only must healthy albumin be present, but zinc must also be present.

1.12 Glucose Transporters

Glucose transporters (GLUTs) belong to the major facilitator superfamily of membrane transporters. To date, 14 isoforms of GLUTs have been identified, and most catalyze the transmembrane facilitative bidirectional transfer of substrates with either symmetric or asymmetric transport kinetics. GLUT proteins are made up of about 500 amino acids and are all structurally similar, with 12 transmembrane spanning alpha helices and one N-linked oligosaccharide.[230] The two most extensively studied transporters are GLUT1 and GLUT4.

GLUT1 was the first glucose transporter isoform identified, purified and cloned.[231-233] It is expressed at high levels in RBCs, cells forming the blood-tissue barriers, astrocytes, and cardiac muscle, though it is ubiquitously expressed in almost all cell types.[234, 235] Like other glucose transporters, GLUT1 is an asymmetric[236] bidirectional transporter with a catalytic turnover of over 1,200/second,[237] and transports glucose, galactose, and ascorbic acid.[238, 239] GLUT1 protein expression is regulated at a developmental level,[235] due to perturbations in circulating glucose concentrations,[240] and in hypoxic conditions.[241] On SDS-PAGE, GLUT1 runs as a broad band due to heterogeneous N-linked glycosylation at Asn-45.[242] There is evidence to suggest that cytoplasmic ATP inhibits RBC GLUT1-mediated glucose transport and is mediated by conformational changes in the protein itself.[243, 244]

Studies of GLUT1 translocation have shown that IL-3 and activation of a tyrosine kinase promote GLUT1 translocation in a haemopoietic cell line.[245] More recent evidence suggests that GLUT1 translocation is regulated by IL-3, PI3K activity and activation of Akt.[246, 247]

GLUT4 is found in muscle and adipose cells, and was discovered in the 1980s. It has been studied extensively in regard to insulin signaling and membrane translocation.[248] The insulin signaling pathway is a well characterized mechanism. Insulin binds to the tyrosine kinase insulin receptor (IR), triggering a complex downstream cascade of signals. Phosphorylation and recruitment of adaptors initiates the activity of PI3K, which activates Akt/PKB and PKC cascades. The PI3K/Akt pathway, phosphorylation of CAP, and formation of a CAP complex lead to the translocation of GLUT4 vesicles to the plasma membrane, which allows glucose to enter the cell. This translocation of GLUT4 in response to insulin signaling is crucial to allow the cell to remove excess glucose from the bloodstream and metabolize it.

Cells that do not contain GLUT4, but are primarily GLUT1-containing, including RBCs, neutrophils, and endothelial cells, none of which are known to contain an insulin receptor. Consequently, these cell types do not metabolize glucose from the bloodstream in response to insulin. Some groups have assumed that there is an insulin receptor present on RBC membranes, however, no biochemical evidence of this has been presented.[249]

1.13 Goals of This Research

Taking all of the present research into account, it is necessary to determine the effects of C-peptide and zinc on cell types that do not respond to insulin, namely the GLUT1-containing cell types, RBCs and neutrophils, which will have implications in vascular and immune health.

First, we chose to study the effect of two proteins on pancreatic secretogogues that are known to be compromised in diseases: ZnT-8 in T2D patients and CFTR in CF and CFRD patients. Next, we looked at the effect of pancreatic secretogogues on RBCs and neutrophils in healthy and disease-mimic states. Ultimately, we will describe a molecular mechanism of C-peptide and zinc action on these cell types independent of insulin, and provide insights into the molecular mechanism of disease in T1D, T2D, and CF/CFRD.

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CHAPTER 2

PANCREATIC SECRETIONS

2.1 Normal Pancreatic Secretions

Hormone and small molecule secretion from pancreatic beta cells is a fairly well understood mechanism, as described in the previous chapter. Briefly, when the glucose level outside the beta cell rises, a glucose transporter (GLUT) shuttles glucose into the cell, increasing the [ATP]/[ADP] ratio. This increase closes the ATP-sensitive K^+ channel (K_{ATP} channel), causing a membrane depolarization, which opens voltage dependent calcium channels, promoting exocytosis of the secretory granule contents.

In rodent beta cells, GLUT2 is the principal transporter for glucose,[1] and was therefore for many years the only known GLUT in human pancreatic beta cells.[2] However, recent studies have shown that three GLUTs are present on human pancreatic beta cells: GLUT1, GLUT2, and GLUT3.[3] Expression levels of GLUT2 are reported to be modest in human islets,[4] and studies of GLUT2 variants did not show any effect on beta cell function, which indicates that it may not be the primary transporter for glucose uptake in human beta cells.[5] GLUT1 and GLUT3 are expressed at 2.8 and 2.7 fold higher levels than GLUT2 in human islet cells respectively, and expression levels did not change after one hour of stimulation with 20 mM glucose. In mouse pancreatic islets, expression of GLUT2 was 10 fold higher than GLUT1. Results from this same study confirmed the expression of GLUT4 on skeletal muscle and adipose tissue.[3] These results by McCulloch *et al.* are the first to demonstrate that GLUT1 and GLUT3

are present at similar levels in human pancreatic beta cells, suggesting important roles for both transporters. The kinetic properties of GLUT1 (K_m=6.9 mM) are much more indicative of the dose-response curve for glucose stimulated insulin secretion in humans (K_m=6.5 mM), while GLUT2 (K_m=11.2 mM) is not. GLUT3, which is typically limited to neuronal glucose transport, appears to have a role in the pancreatic beta cell in humans, though perhaps not in rodents.

Recently, genome wide association studies (GWAS) have revealed many genes that may be linked to diabetes, including the gene for GLUT2.[6] However, a single nucleotide polymorphism (SNP) in this gene did not correlate to any effects on insulin processing, secretion, or sensitivity.[5] This result may be explained by the observation that the pancreatic beta cell may not contain significant amounts of GLUT2 as compared to GLUT1 and GLUT3, and that GLUT2 may be more involved in hepatic and renal glucose metabolism, rather than in insulin secretion.[3, 7]

2.1.1 Fates of Glucose in the Pancreatic Beta Cell

Three possible fates of glucose exist in pancreatic beta cells that are linked to insulin secretion: glucose oxidation, anaplerosis, and the provision of glycerol-3-phosphate (Gly3P).[2] Glucose that is metabolized via glucose oxidation enters glycolysis (Figure 2.1) to make pyruvate, which is metabolized in the mitochondria.[8, 9] Pyruvate is metabolized to acetyl-CoA, which enters the citric acid cycle and then oxidative phosphorylation to increase the [ATP]/[ADP] ratio in the beta cell.[10] As previously mentioned, this increase closes the K_{ATP} channel and triggers the opening of a voltage dependent calcium channel, allowing exocytosis of the granules that contain insulin.

Glucose that is metabolized via anaplerosis also enters glycolysis to create pyruvate. Pyruvate is then metabolized in the mitochondria to oxaloacetate, replenishing the carbon pool in the citric acid cycle, and exits via cataplerosis.[8, 9, 11] The major accepted cataplerotic signals include NADPH, malonyl-CoA and glutamate. An increase in NADPH corresponds with an increase in extracellular glucose, and NADPH can promote exocytosis in permeabilized cells, which may act through the thioredoxin system.[12] These cataplerotic signals amplify the effects of the K_{ATP} channel, including calcium influx and membrane depolarization, which directly affect insulin secretion.[2]

The third mechanism of glucose metabolism is the provision of Gly3P for glycerol/fatty acid cycling. Glucose enters glycolysis, where the intermediate dihydroxyacetone phosphate is metabolized to Gly3P by cytosolic Gly3P dehydrogenase, which is thought to replenish the NAD^+ required for glycolysis in the beta cell.[13, 14] This mechanism of glucose metabolism, however, is somewhat controversial due to a study showing that mice that lack mitochondrial or cytosolic Gly3P dehydrogenase have normal insulin secretion; however, an increase in the aspartate/malate shuttle may explain this result.[13, 15]

Each fate of glucose in the pancreatic beta cell contributes to insulin secretion: through increasing the [ATP]/[ADP] ratio, which closes the K_{ATP} channel; through increasing the concentration of NADPH, which amplifies the calcium influx into the cell and membrane depolarization; or through the replenishing of NAD^+ used for glycolysis. The three fates of glucose in the beta cell are outlined in Figure 2.2.

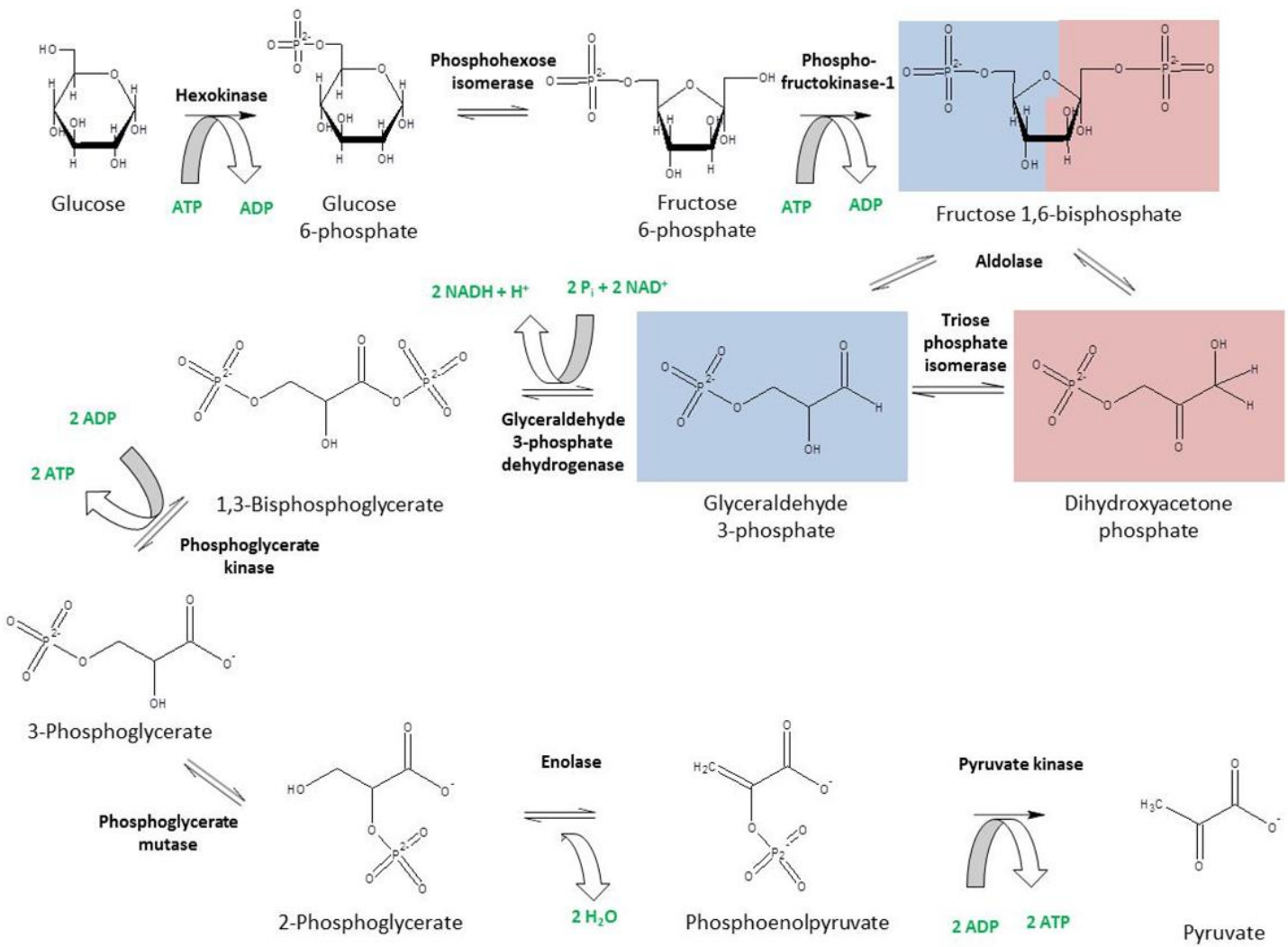

Figure 2.1: The process of glycolysis. Glucose is initially converted to glucose 6-phosphate (G6P) by hexokinase, using one ATP molecule. Phosphohexose isomerase converts G6P to fructose 6-phosphate (F6P).

Figure 2.1 (cont'd): Phosphofructokinase-1 uses one ATP molecule to convert F6P to fructose 1,6-bisphosphate, where an aldolase converts it to dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (G3P). DHAP is converted to G3P by triose phosphate isomerase. Two molecules of G3P are converted to 1,3-bisphosphoglycerate by G3P dehydrogenase, creating 2 molecules of NADH. Phosphoglycerate kinase converts two molecules of 1,3-bisphosphoglycerate to 3-phosphoglycerate, creating two molecules of ATP. Phosphoglycerate mutase converts 3-phosphoglycerate to 2-phosphoglycerate, where enolase converts it to phosphoenolpyruvate (PEP). PEP is converted to pyruvate by pyruvate kinase, producing 2 molecules of ATP. Pyruvate can then be metabolized through different pathways for use by the cell.

2.1.2 The K_{ATP} Channel

Once glucose is metabolized and the [ATP]/[ADP] ratio inside the cell increases, the K_{ATP} channel is closed. The K_{ATP} channel is an octamer made up of 4 pore-forming Kir6.2 subunits and 4 regulatory SUR1 (sulfonylurea receptor) subunits.[16] Binding of ATP to the Kir6.2 subunit closes the channel, while Mg-ADP binds to SUR1, stimulating channel activity. Below stimulatory concentrations of glucose, K_{ATP} is open, and K^+ efflux maintains the cell membrane's resting potential at -70 mV, which is a hyperpolarized state. Upon glucose stimulation, K^+ efflux through K_{ATP} is reduced, while an inward current increases, which causes the membrane to depolarize.[17] Mutations in the K_{ATP} channel have been linked to different disease states, including diabetes. Gain of function mutations in K_{ATP} cause neonatal diabetes, while loss of function mutations cause congenital hyperinsulinism.[18-20] Sulfonylureas, a class of drugs commonly used to treat type 2 diabetes (T2D), are used to increase insulin secretion even in the absence of glucose by binding to a sulfonylurea receptor (SUR1) on the cell. Binding to this receptor directly leads to closing of the K_{ATP} channel.[17] Conversely, diazoxides are drugs that open the K_{ATP} channel.[21] Along with ATP and ADP levels,

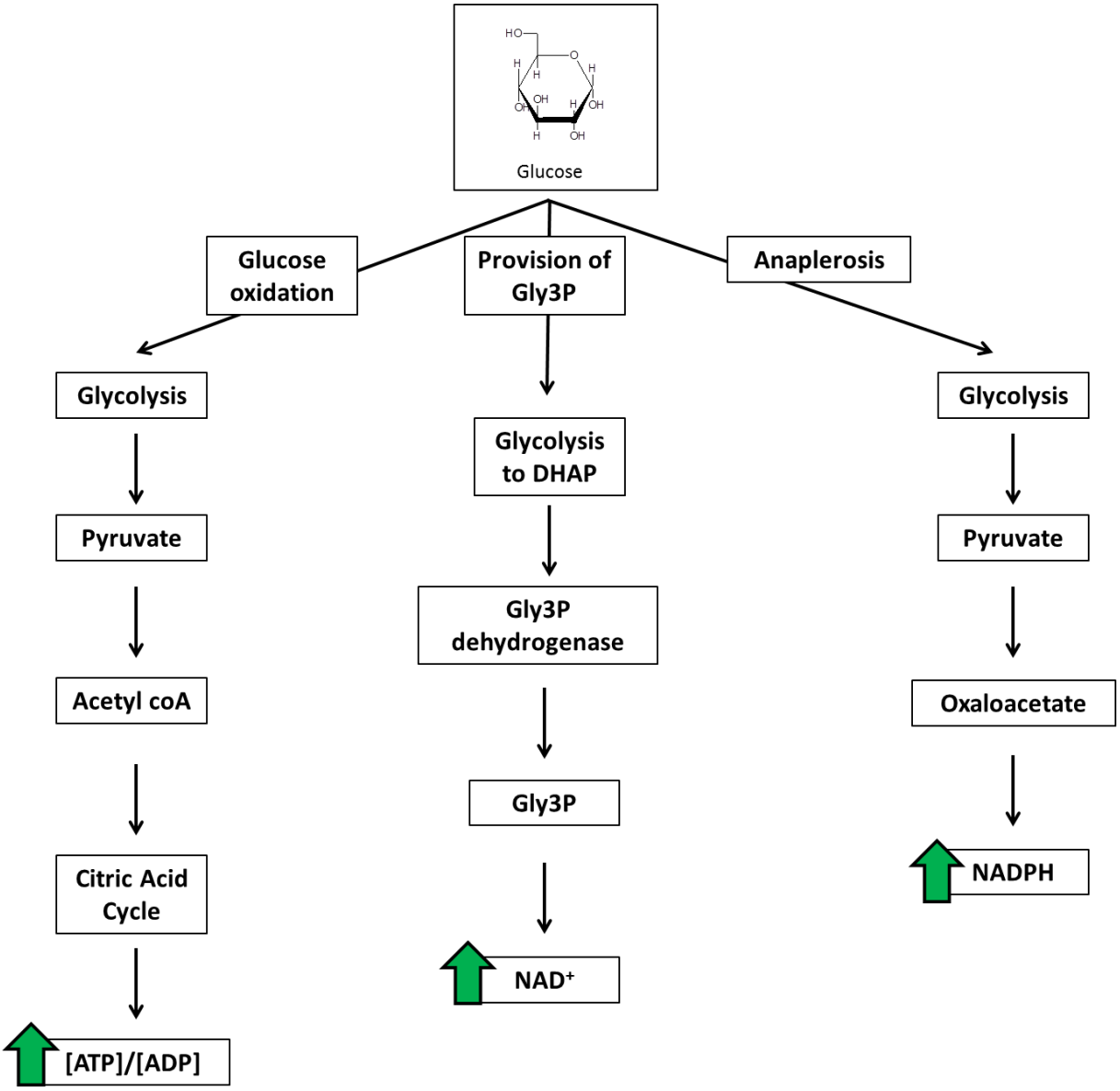

Figure 2.2: The three fates of glucose in the pancreatic beta cell. Glucose can be metabolized via glucose oxidation, which increases the [ATP]/[ADP] ratio. Glucose metabolized via anaplerosis increases the concentration of NADPH in the cell. Glucose that is metabolized for glycerol/fatty acid cycling increases the concentration of [NAD^+].

the K_{ATP} channel is also influenced by lipids[22, 23] and long chain acyl coA esters.[24-26]

Closure of the K_{ATP} channel causes a membrane depolarization, which opens a voltage gated calcium channel, inducing exocytosis of the beta cell secretory granules. The secretory granules, as outlined in chapter 1, are the site of proinsulin conversion. Proinsulin conversion to insulin (Figure 2.3) is dependent on two calcium and pH-dependent endopeptidases.[27] PC2, a type II endopeptidase, cleaves proinsulin preferentially at the A chain junction and requires μM levels of calcium. PC2 functions over a broad pH range, and cleaves proinsulin first. PC1/3 is a type I endopeptidase, and requires mM levels of calcium. It functions optimally at pH 5.5 and cleaves des-64,65-proinsulin (the PC2-cleaved product of proinsuliln) at the B chain junction.[27] Based on the knowledge of the calcium requirements for each endopeptidase, the intragranular calcium is estimated to be between 1 and 10 mM.[28] Quantitative intracellular calcium levels have not been reported, however, based on the intragranular calcium requirements, intracellular levels should be in the mM range. Immature secretory granules are less acidic than the mature granules, which is due in part to the clathrin-coated nature of the immature granule.[29, 30] Intragranular acidification is necessary for the activity of endopeptidases and therefore the complete conversion of proinsulin to insulin. This acidification is achieved by a secretory granule Mg^{2+}-dependent ATP-dependent proton pump.[31] Proinsulin conversion to insulin is abolished in the presence of beta cell granule proton pump inhibitors, but not in the presence of mitochondrial protein pump ATPase inhibitors, indicating that it is indeed the secretory granule proton pump that is responsible for this acidification.[32]

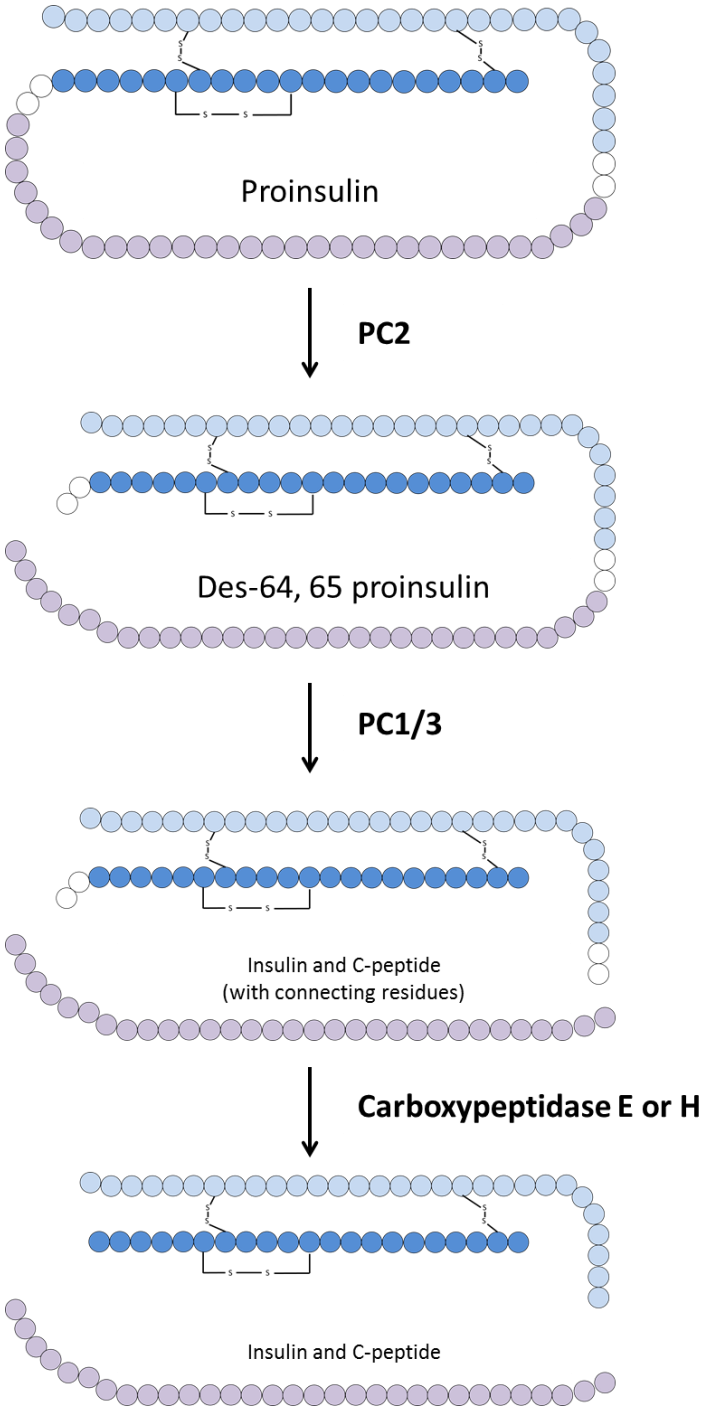

Figure 2.3: The conversion of proinsulin to insulin by PC2 and PC1/3. PC2 cleaves preferentially at the A chain junction, while PC1/3 cleaves preferentially at the B chain junction.

An important protein on the plasma membrane of the secretory granule is ZnT-8, which is responsible for the influx of zinc into the granule for insulin hexamer formation. Zinc content in the beta cell is among the highest in the body (10-20 mM),[33, 34] and about 70% is found in the secretory granule.[35] In addition to cationic channels on the beta cell and the secretory vesicle, anionic channels are also present. One of the major anionic channels that has been linked to insulin secretion is the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride ion channel. Upon 16.7 mM glucose stimulation and activation of CFTR by cAMP, insulin is released. When CFTR is inhibited, there is a decrease in the amount of insulin secreted from the beta cells, a decrease in cAMP-dependent exocytosis of granules, and a decrease in the number of docked insulin granules.[36] In order to study the direct effect of proteins on insulin, C-peptide and zinc secretion, researchers must turn to the utilization of beta cells.

2.2 Pancreatic Beta Cell Lines

The utilization of human and rodent primary beta cells in research is limited by the availability of pancreatic endocrine tissue. This tissue can only be obtained from deceased donors, however, even when these tissues can be obtained, cellular and hormonal heterogeneity between individual donors makes reproducible research difficult. For these reasons, much research involving pancreatic beta cells has been performed using cultured cells rather than using procured primary cells.

Cell cultures can be derived from primary tissues and often contain tumor cell factors that allow for prolonged cell proliferation, making them immortal. These immortal cell lines enable the researcher to obtain highly uniform cell lines, give them the ability to

test drugs or toxins under a variety of conditions, and the ability to manipulate various genes for specific study; however, there are some disadvantages of using cell lines. First, the cell characteristics may change over time. Second, manipulation of the cells to make them immortal may result in responses to stimuli and activities that differ from those measured *in vivo*. Many beta cell lines exist from different species, however, some have defective secondary characteristics, making them unable to respond to physiological levels of glucose.

Mouse and rat pancreas secretions differ in one significant way from human pancreas secretions: instead of producing one proinsulin, which, upon cleavage, results in one C-peptide and one insulin molecule, mice and rats produce two different proinsulins, which result in two C-peptides and two insulins, which differ from each other by only one amino acid. They have no difference in biological activity; for all intents and purposes, they are equal. Hamster, mouse, rat and human cell lines are the most commonly used in beta cell research.[2] The βTC1 cell line is a mouse insulinoma cell line isolated from a transgenic mouse. These cells produce both types of proinsulin and insulin and have hexokinase activity, but are not responsive to glucose stimulation.[37] IgSV195 is another mouse insulinoma cell line isolated from transgenic mice that retains morphological and physiological characteristics of beta cells, but needs to be exposed to fetal bovine serum, glutamine, and 3-isobutyl-1-methylxanthine in order to proliferate. These cells also do not respond to glucose in the physiological range.[38] The CM cell line is a human insulinoma cell line derived from a patient with liver metastasis of a malignant insulinoma. These cells express GLUT2 and glucokinase, but do not secrete insulin in response to glucose stimulation.[39] TRM-1 is a human foetal pancreas cell

line that contains an oncogenic vector for meiotic maturation. These cells express GLUT2 and release small amounts of insulin and glucagon, but do not respond to glucose stimulation.[40] The RINm cell line is a rat insulinoma cell line that is radiation induced. The insulin level in these cells decreases with passage number and they do not respond to glucose stimulation.[41] The INS-1 cell line is a radiation induced rat insulinoma cell line. These cells respond to glucose in the physiological range and have relatively high insulin content, but require mercaptoethanol in the culture medium for cell proliferation.[42] Mercaptoethanol is toxic at high concentrations and irreversibly denatures proteins in culture media; however, INS-1 cells are unable to propagate and lose essential characteristics when mercaptoethanol is not present in the culture media. Merglen *et al*. have created an INS-1E cell line, which is a clonal isolate from the parent cells. INS-1E cells stably differentiate over a high number of passages, but they require supraphysiological concentrations of glucose in order to trigger insulin secretion.[43]

For the present research, we chose to use the INS-1 cell line due to its relatively high insulin content – and therefore C-peptide content – and its response to physiological glucose concentrations. Additionally, utilization of a cell line allows us to manipulate proteins of interest in two different diseases: the ZnT-8 protein in T2D, and the CFTR (cystic fibrosis transmembrane conductance regulator) protein in cystic fibrosis/cystic fibrosis related diabetes (CF/CFRD).

2.3 GWAS in Diabetes

Multiple GWAS of T2D patients and matched controls have discovered genes that may be linked to the development of T2D in many different ethnicities of patients, including

Icelandic, Japanese, British, Finnish, Swedish, and French.[44-49] GWAS were made possible by the completion of the Human Genome Project,[50, 51] progress in high-throughput genotyping technology, and analytical tools to assist in data mining. GWAS aim to determine SNPs (single nucleotide polymorphisms) that may be associated with different diseased states. SNPs occur approximately every 300 base pairs, rarely recur as a mutation, and are often binary.[52] SNPs that can be associated with a particular disease must be associated with the disease beyond reasonable statistical doubt. The search for SNPs that are associated with T2D has led to almost 100 identified SNPs from about 30 different studies (reviewed in [53]). In 2007, five separate GWAS discovered a missense mutation in *SLC30A8*, which encodes for the ZnT-8 transporter present on the secretory vesicles of pancreatic beta cells.[45-48, 54] The polymorphism identified by these studies (rs13266634) results in a non-conservative substitution of arginine to tryptophan (R325W). R325W is associated with altered glucose homeostasis due to beta cell dysfunction or overt T2D in many[55-60] but not all[61, 62] study populations. Additionally, ZnT-8 may also be an autoantigen in T1D.[63] These results have sparked much interest in ZnT-8, but the literature since then has been filled with some contradictory results from mouse knockout and cell knockdown studies.

2.4 ZnT-8

ZnT-8 is a cation diffusion facilitator protein that is encoded by the *SLC30A8* gene and was discovered in 2004.[64] It has the same topology as other known ZnT proteins, with six-transmembrane spanning domains and a histidine-rich intracellular loop between the fourth and fifth helices. Human ZnT-8 expression is the highest in the pancreatic beta cells,[65] but has also been found in the retina,[66, 67] adipocytes[68]

and lymphocytes[69] (only mRNA, not protein expression), and in pancreatic alpha cells.[65, 70, 71] In the pancreatic beta cell, ZnT-8 has been shown to co-localize with insulin in the beta cell granules,[64, 72] and its expression is downregulated in INS-1 cells upon cytokine exposure.[73] The sequence of human ZnT-8 is relatively well conserved with rodent species: mice share 80% identity with humans, while rats share 76% identity with humans.[72]

Selective deletion of ZnT-8 in the beta cells or global ZnT-8 deletion in mice have resulted in some consistent results, namely that glucose tolerance is impaired by ZnT-8 elimination.[74-80] However, the effects on glucose stimulated insulin secretion from isolated islets remain unclear, as some have reported an increase,[78-80] while others report a decrease,[74, 75] and still others report no change.[76] Interestingly, in one study, ZnT-8 knockout mice exhibited higher levels of insulin secretion from beta cells, but low peripheral blood insulin levels.[80] Overexpression of ZnT-8 in INS-1E cells increases glucose stimulated insulin secretion and zinc accumulation.[72]

Fu *et al.* was the first to report the shRNA-mediated downregulation of ZnT-8 in a cell line (INS-1).[81] They found that downregulation of ZnT-8 mRNA reduced uptake of exogenous zinc, insulin content, and insulin secretion in response to glucose, as well as that there were fewer dense core vesicles present in the beta cell. They did confirm that ZnT-4 and ZnT-5 were not downregulated by ZnT-8 shRNA, however, they did not report the downregulation of protein expression of ZnT-8, only the mRNA decrease. A decrease in mRNA does not necessarily correspond to a decrease in protein expression. Additionally, they assumed protein expression had been downregulated

based on the amount of intracellular zinc present in the cells. Another study in INS-1E cells showed that zinc depletion decreased ZnT-8 gene expression.[82]

2.5 Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Protein

CFTR expression was first reported in pancreatic beta cells by Boom *et al.* in 2007.[83] Prior to this discovery, evidence existed for the dysfunction of pancreatic beta cells in CF. Autopsy studies of 23 CF patients showed a significantly decreased insulin to beta cell ratio as compared to controls, and a rise in other pancreatic cell types (alpha, delta and PP).[84] The prevailing theory of the development of CFRD is a combination of chronic pancreatitis and loss of the islet cells, which results in low insulin production and some insulin resistance.[85-87]

Since the discovery of CFTR presence in the beta cells, studies have focused primarily on the mechanism by which CFTR may be participating in insulin exocytosis and glucose stimulated insulin secretion. One study of note that has not focused on this mechanism is one that has linked CFTR mutations in the beta cells and immune reactivity in a murine model of CF, which found that hyperglycemia may adversely affect CF by impacting immune reactivity.[88] Two major studies on CFTR in the beta cells have linked CFTR activity to exocytosis of secretory granules and glucose-induced electrical activities.[36, 89]

Guo *et al.* hypothesized that since CFTR is gated by intracellular ATP, which is metabolized from glucose, CFTR might be sensitive to changing glucose concentrations. They used RINm5F cells, which are a radiation induced rat insulinoma cell line.[90] This cell line has abnormal properties of glucose transport and

phosphorylation,[41, 91] which makes it a less than ideal cell line in which to study the effect of CFTR on glucose induced insulin secretion. Guo *et al.* report that chemical inhibition of CFTR attenuates the intracellular calcium increase induced by glucose and reduces insulin secretion cells. They also speculate that CFTR could hyperpolarize the membrane, counteracting the closure of the K_{ATP} channel.[89]

In contrast to these results in cell culture, islets from human donors have revealed only some similar results. Chemical inhibition of CFTR in human islets resulted in a significant decrease in insulin secretion, a reduction in cAMP-activated current, and a decrease in exocytosis.[36] However, in contrast to the results from Guo *et al*., they found that the calcium current in beta cells was unaffected by CFTR inhibition, which suggests that CFTR may directly affect the exocytotic machinery, possibly interacting with the SNARE-protein syntaxin 1A, which is crucial for granule docking and exocytosis in beta cells.[36, 92, 93] These studies have also revealed that CFTR likely acts as a regulator of other chloride channels in beta cells, specifically Anoctamin 1 (ANO1), in insulin secretion.[36, 94-96] The effect of CFTR on glucose stimulated hormone secretion from pancreatic beta cells will also affect the intragranular chloride channel, ClC-3.[97, 98]

2.6 Present Study

In an effort to expound upon the existing literature evidence in the effect of dysfunction ZnT-8 and CFTR in pancreatic beta cells, we here present evidence of the successful, stable downregulation of ZnT-8 and CFTR (separately) in INS-1 cells, and their effect on secretions from these cells, including C-peptide 2, zinc, and insulin. In addition, we

show the effect of the downregulation of these proteins on intracellular functions, including intracellular pH and Ca^{2+} levels, which provide evidence for their involvement in granule exocytosis and proper cleavage of granule products. Fully elucidating the function of both of these proteins in the pancreatic beta cell is crucial to the understanding and treatment of T2D and CFRD.

2.7 Materials and Methods

2.7.1 Buffer Preparation

All buffers were prepared in purified water (DDW, 18.2 MΩ). All reagents were purchased from Sigma Aldrich (St Louis, MO, USA) unless otherwise noted.

2.7.1.1 Phosphate Buffered Saline (PBS)

Phosphate Buffered Saline (PBS) contained 137 mM NaCl, 2.7 mM KCl (Fisher Scientific, Waltham, MA, USA), 10.1 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 (Spectrum Chemical, New Brunswick, NJ, USA).

2.7.1.2 Krebs-Ringers Buffer (KRB)

Krebs-Ringers Buffer (KRB) contained 135 mM NaCl, 5 mM KCl (Fisher Scientific), 1 mM $MgSO_4$ (Fisher Scientific), 0.4 mM K_2HPO_4 (Spectrum Chemical), 5.5 mM glucose, 20 mM HEPES (Tocris Bioscience, Bristol, UK), and 1 mM $CaCl_2$ (Fisher Scientific). The pH was adjusted to 7.4.

2.7.1.3 Stimulation Solution (SKRB)

Stimulation Solution (SKRB) contained KRB supplemented with glucose and bovine serum albumin (BSA) to final concentrations of 12 mM glucose and 1% BSA.

2.7.1.4 INS-1 Cell Lysis Buffer

INS-1 Cell Lysis Buffer contained 1% Triton X-100, 20 mM HEPES (Tocris Bioscience), 100 mM KCl (Fisher Scientific), 2 mM EDTA (Fisher Scientific), 1 mM phenylmethane sulfonyl fluoride (PMSF), 10 μg/mL leupeptin, 10 μg/mL aprotinin, 10 mM NaF, 2 mM Na_3VO_4, and 10 nM okadaic acid in PBS. PMSF and okadaic acid were diluted in ethanol. Preparation of Na_3VO_4 was performed in a fume hood by boiling a 100 mM solution at pH 9.0 until colorless, cooling to room temperature and readjusting the pH, and then boiling again until the pH remained stable at 9.0 after cooling.

2.7.2 INS-1 Cells

2.7.2.1 INS-1 Cell Maintenance Media

Parental INS-1 cells were a gift from Dr. L. Karl Olson in the Department of Physiology at Michigan State University. INS-1 cells were maintained in maintenance media containing 1 mM sodium pyruvate, 100 U/mL penicillin (Life Technologies, Carlsbad, CA, USA), 100 μg/mL streptomycin (Life Technologies), 55 μM β-mercaptoethanol, 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA, USA), 2 mM L-glutamine, and 10 mM HEPES in RPMI 1640 (Lonza, Allendale, NJ, USA).

2.7.2.2 Growth and Maintenance of INS-1 Cells

INS-1 cells were grown to confluence in T-75 flasks (MIDSCI, Valley Park, MO, USA). Media was removed and cells were washed with Dulbecco's Phosphate Buffered Saline

Solution (DPBS, Gibco, Waltham, MA, USA). Trypsin-EDTA (0.25%, Life Technologies) was added and allowed to rest for 3 minutes at 37 °C to remove the cells from the bottom of the flask. Maintenance media was added to stop trypsinization and the cells were pelleted by centrifugation. Media and trypsin were removed and cells were resuspended in fresh maintenance media and seeded on T-75 flasks for further growth, 6-well plates for shRNA mediated downregulation, or 96-well plates for analysis.

2.7.2.3 shRNA-mediated downregulation of INS-1 cells

Cells were stably transfected with pRS vectors containing specific ZnT-8-directed 29-mer oligos (Table 2.1) or pRS vectors containing specific CFTR-directed 29-mer oligos (Table 2.2) (Origene, Rockville, MD, USA) using Lipofectamine-3000 (ThermoFisher Scientific) according to the manufacturer's specifications.

Table 2.1: 29-mer target sequences of the four vectors obtained from Origene for shRNA mediated downregulation of ZnT-8. Vectors were stably transfected into INS-1 cells via Lipofectamine 3000.

Name	29-mer target sequence
Empty vector	none
A	ATGAGTCCAAGTGATCATCCAAGAAGACC
B	CTGCTACCATGGAGTTTCTTGAGAGGACT
C	TGTGAGCGCCTCTTGTATCCTGATTACCA
D	AGTGAACCAAGTGATTCTCTCTGTTCATG

For each gene, one pRS vector contained a 29-mer sequence of nonsense nucleotides that served as a control (empty vector), while four pRS vectors contained specific 29-mer target sequences (A through D). Each targeted vector was used alone and

together with one other vector, with the goal of achieving the most downregulation possible (A, B, C, D, E: A and B, F: A and C, G: A and D, H: B and C, I: B and D, and J: C and D). After transfection, cells were maintained in maintenance media containing 0.2 μg/mL puromycin.

Table 2.2: 29-mer target sequences of the four vectors obtained from Origene for shRNA mediated downregulation of CFTR. Vectors were stably transfected into INS-1 cells via Lipofectamine 3000.

Name	29-mer target sequence
Empty vector	none
A	GGCGATTATCGCAAGATAGCACACTGAAC
B	TTGCCAATACCTCCTATGTTGTGGTCATC
C	TCCTCTGTGAACACAGGATAGAAGCAATG
D	TGGTGTCTCCTGGAACTCAATGACCTTAC

2.7.2.4 Freezing of shRNA downregulated cells

After cells were grown to confluence for several passages, they were trypsinized as previously described. After centrifugation, media and trypsin were removed and the cells resuspended in fetal bovine serum containing 0.05% DMSO. Aliquots of 1 mL cell suspension were placed in cryovials and stored at -80 °C until use.

2.7.3 SDS-PAGE and Western blot analysis of downregulation

INS-1 cells and transfected INS-1 cells were lysed in INS-1 Cell Lysis Buffer on ice for 30 minutes. After pelleting the cell lysate by centrifugation (15,000 *g* for 10 minutes), the supernatant was collected and analyzed for protein content using a standard BCA assay (ThermoFisher Scientific, Waltham, MA, USA).

2.7.3.1 BCA Assay

Standards were made in a working range (0-1 μg/μL) from a BSA stock of 2 mg/mL. The working reagent was prepared as specified by the company (ThermoFisher Scientific) in a 50:1 ratio (Reagent A:Reagent B). Samples and working reagent were incubated together at 37 °C for 30 minutes and then absorbance was recorded at 562 nm on a UV-Vis spectrophotometer. Samples were compared to the standard curve to determine the total protein content.

2.7.3.2 Buffers for SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) running buffer contained 192 mM glycine (Invitrogen), 25 mM Tris (Invitrogen), and 3.5 mM SDS. Transfer buffer contained 25 mM Tris (Invitrogen), 192 mM glycine (Invitrogen), and 20% methanol (Macron Fine Chemicals, Center Valley, PA, USA) at pH 8.3. TBS contained 20 mM Tris-HCl (Invitrogen) and 500 mM NaCl. TBST contained TBS with 0.05% Tween-20. Bicarbonate buffer contained 0.1 M $NaHCO_3$ (Jade Scientific, Westland, MI, USA) and 5 mM $MgCl_2$. Blocking solution contained TBST with 5% dry milk.

2.7.3.3 Casting of SDS-PAGE

The 10% resolving gel was made by mixing DDW with 1.5 M Tris (pH 8.8, Invitrogen), 30% bis-acrylamide (BioRad), 20% SDS, 10% APS (Ammonium Persulfate), and TEMED (tetramethylethylenediamine). The stacking gel was fixed at 5% acrylamide and was made by mixing DDW with 0.5 M Tris (pH 6.8, Invitrogen), 30% bis-acrylamide (BioRad), 20% SDS, 10% APS, and TEMED.

2.7.3.4 SDS-PAGE

A 10% SDS gel was cast and 10 μg (total protein) samples loaded with loading dye (1:1 ratio, BioRad) onto the gel. A protein ladder was also loaded onto the gel (Precision Plus Protein All Blue Prestained Protein Standards, BioRad). After successful separation of the proteins via electrophoresis (SDS-PAGE Running buffer, 150 V, 1 hour), one gel was transferred to a PVDF membrane (Thermo Fisher Scientific) on ice overnight at 30 V in transfer buffer. If necessary, a second gel was stained with Coomassie Brilliant Blue R-250 (BioRad, Hercules, CA, USA) and destained with Coomassie Brilliant Blue R-250 Destain Solution (BioRad). Gels were scanned at 600 dpi using a desktop scanner for further analysis.

2.7.3.5 Western Blot

The PVDF membrane was blocked with blocking solution and then washed before incubation with primary (β-actin: mouse monoclonal anti-beta actin antibody; CFTR: rabbit polyclonal anti-CFTR antibody; ZnT-8: rabbit polyclonal anti-ZnT-8 antibody; Abcam, Cambridge, UK) and secondary (Anti-Mouse IgG (whole molecule)−Alkaline Phosphatase antibody produced in goat; Anti-Rabbit IgG (whole molecule)–Alkaline Phosphatase antibody produced in goat; ThermoFisher Scientific) antibodies. Membranes were washed (with TBS or TBST) prior to the addition of each antibody and then again prior to addition of 5-Bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma Aldrich) and Nitro blue tetrazolium chloride (NBT, Sigma Aldrich) in bicarbonate buffer. After color development, the blots were washed with water, dried and then were scanned at 600 dpi and analyzed using Image J (NIH).

2.7.4 Stimulation of secretions from INS-1 cells

All cell lines were cultured in 96-well plates and grown to confluency in maintenance media with or without puromycin. Prior to experimentation, cells were incubated with low glucose (5.5 mM) maintenance media overnight and then washed in KRB twice for 30 minutes each. Cells were then washed three additional times for two minutes each with KRB and then incubated with SKRB at 37 °C. Aliquots of the SKRB were collected at different time points from 0 minutes to 3 hours.

2.7.5 C-peptide ELISA

Samples were diluted to a concentration within the standard curve and were analyzed for the amount of C-peptide released from the INS-1 cells via ELISA (Enzyme Linked ImmunoSorbent Assay, EMD Millipore, Billerica, MA, USA) for C-peptide 2.

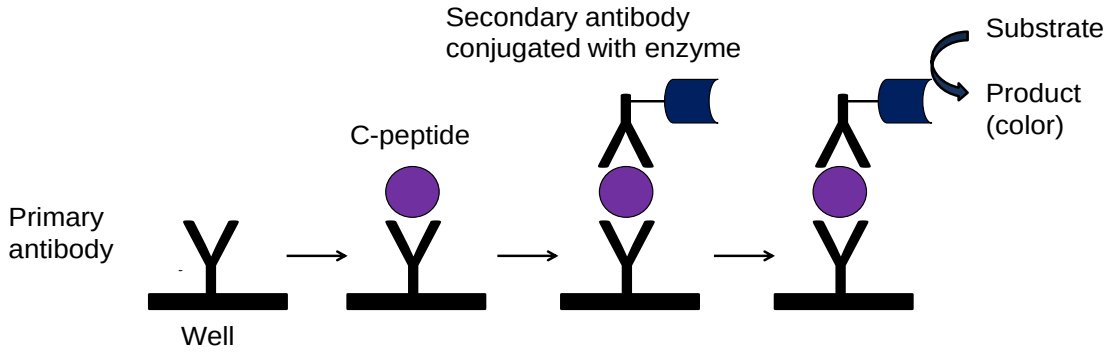

Figure 2.4: Sandwich ELISA scheme. A primary antibody against C-peptide is incubated with the sample, and the excess antibody is washed off. A secondary antibody conjugated to an enzyme is incubated with the bound primary antibody, which then converts a substrate into the colored product, with an absorbance at 450 nm. The absorbance directly correlates to the amount of C-peptide in the sample.

Absorbance values were recorded on a SpectraMax M4 Spectrophotometer (Molecular Devices LLC, Sunnyvale, CA, USA). Briefly, a primary antibody against C-peptide is incubated with the sample. Residual antibody is washed off and then an enzymatically-tagged secondary antibody catalyzes a colorimetric reaction that is measured using a standard plate reader at 450 nm. This reaction is shown in Figure 2.4.

2.7.6 Insulin ELISA

Samples were diluted to a concentration within the standard curve and were analyzed for the amount of insulin released from the INS-1 cells via ELISA (EMD Millipore, Billerica, MA, USA) for insulin. Absorbance values were recorded on a SpectraMax M4 Spectrophotometer.

2.7.7 Free Zinc Measurements

Analysis for zinc secretion from INS-1 cells was performed using 50 μM 6-methoxy-8-p-toluenesulfonamido-quinoline (TSQ, AnaSpec, Fremont, CA, USA) in methanol. Samples were mixed for 45 seconds with TSQ before the fluorescent signal (excitation 365 nm) was measured from 430 to 520 nm.

2.7.8 Intracellular calcium measurements

Cells were grown to confluence in 96-well plates and incubated with low glucose maintenance media overnight. After washing twice (30 minutes each wash) with KRB, cells were incubated with Fura-2 (5 μM, Life Technologies, Carlsbad, CA, USA) for 30 minutes. Cells were then quick washed (one minute each wash) three times with KRB

before stimulation with SKRB. Plates were placed in a SpectraMax M4 at 37°C for three hours and measured at excitation wavelengths of 340 nm and 380 nm and an emission wavelength of 510 nm. The following equation allows for the quantification of intracellular calcium, where K_d is the calcium dissociation constant of Fura-2, which is determined by intracellular calibration, Q is the ratio of the minimum fluorescence to the maximum fluorescence at 380 nm, R is the ratio of bound calcium to free calcium ($\lambda_{340}/\lambda_{380}$), R_{min} is the ratio of ion-free value of Fura-2, and R_{max} is the ratio of ion-saturated value of Fura-2.

$$[Ca^{2+}] = K_d Q \frac{(R - R_{min})}{(R_{max} - R)}$$

2.7.9 Intracellular pH measurements

Cells were grown to confluence in 96 well plates and incubated with low glucose maintenance media overnight. After washing twice with KRB, cells were incubated with 5-(and-6)-Carboxy SNARF-1 AM (8.8 μM, Life Technologies) for 30 minutes. Cells were then quick washed three times with KRB before stimulation with SKRB. Plates were placed in a SpectraMax M4 at 37°C for three hours and read at an excitation of 488 nm and emission at 580 and 640 nm. *In situ* calibration was performed by using the ionophore nigericin (50 μM) in the presence of 150 mM K^+. Quantification can be achieved using the following equation, where R is the ratio of the fluorescence at both wavelengths ($F_{\lambda 1}/F_{\lambda 2}$) and the subscripts A and B represent the limiting values at the acidic and basic ends of the titration.

$$pH = pK_a - log\left[\frac{R - R_B}{R_A - R} \times \frac{F_{B(\lambda 2)}}{F_{A(\lambda 2)}}\right]$$

2.7.10 Statistical Analysis

Data is presented as average ± SEM, and all statistical analyses were performed using Student's t-test , where p values less than 0.05 were considered significant.

2.8 Results

2.8.1 ZnT-8

Successful downregulation of ZnT-8 in INS-1 cells was achieved using the four pRS vectors (Figure 2.5) obtained from Origene (refer to Table 1 for 29-mer sequences of each vector). The empty vector contains a set of 29 scrambled bases that serve as a control. Table 2.3 shows the Western blot results of each of the eleven downregulated cell lines. Cell lines A through D were created using the vectors obtained from Origene alone, while cell lines E through J were created using two of those vectors to achieve higher downregulation of the gene (E: A and B; F: A and C; G: A and D; H: B and C; I: B and D; J: C and D). Three cell lines are approximately 40% downregulated (A, B, and E), while four are approximately 50% downregulated (C, D, F, and G), and three are greater than 60% downregulated (H, I, and J) (Table 2.3). This library of cell line variants allows for experimentation of cells with varying levels of ZnT-8.

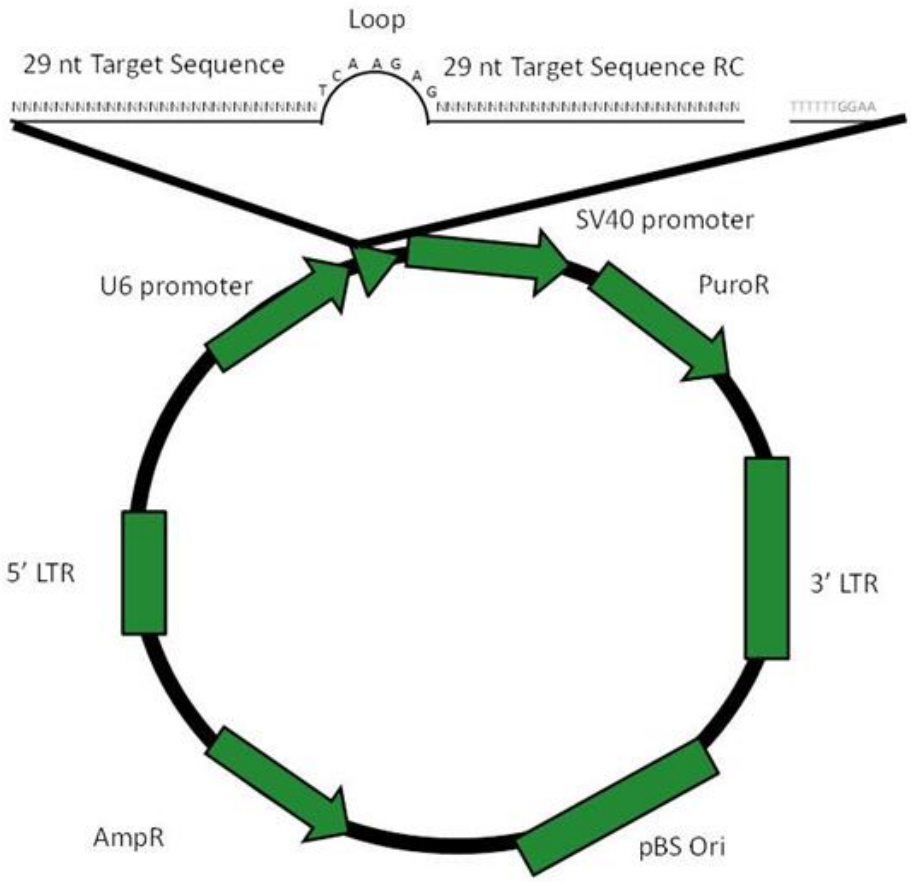

Figure 2.5: The pRS vector. The puromycin resistance gene can be used to selectively grow only cells that have taken up the vector. The 29 nt (nucleotide) target sequences in forward and reverse (RC) order can be found in Table 2.1 for ZnT-8 and Table 2.2 for CFTR.

Table 2.3: Results of shRNA mediated downregulation of ZnT-8 in INS-1 cells. Cell lines A, B, C, and D were created using the original vectors obtained from Origene. Cell lines E through J were obtained using a combination of two these vectors (E: A and B; F: A and C; G: A and D; H: B and C; I: B and D; J: C and D). n=3, error=SEM.

Cell Line	% ZnT-8 Downregulated
Empty vector	0 ± 1
A	43 ± 6
B	46 ± 5
C	59 ± 7
D	59 ± 6
E	39 ± 3
F	53 ± 2
G	51 ± 3
H	74 ± 6
I	66 ± 4
J	76 ± 6

Zinc secretions from INS-1 cells and those transfected with the empty vector show no significant difference from each other (Figure 2.6A). At 30 minutes, some of the cell lines show a significant decrease in the amount of zinc released compared to the controls (Figure 2.6B), while at 90 minutes all cell lines above 60% downregulation release significantly less zinc than controls (Figure 2.6C). At 180 minutes, all cell lines that are at least 50% downregulated release significantly less zinc than controls (Figure 2.6D). The three cell line variants that are approximately 40% downregulated show

some difference from the control, but only after 90 minutes. Variant A is significantly different from the control at 90 minutes only, while B is significantly lower than the control at 90 and 180 minutes, and E is not statistically different than the control. Three of the four cell lines that are approximately 50% downregulated (C, F, and G) have significantly lower zinc secretions at all time points, while D simply does not increase over time. Cell lines that are greater than 60% downregulated are significantly different from the control at all time points.

Insulin secretions are not significantly different in INS-1 cells and those transfected with the empty vector (Figure 2.7A). Cell line variants B and E, which are approximately 40% downregulated in ZnT-8, are have significantly lower insulin secretion than the control at all time points, while A, which is also approximately 40% downregulated, is not significantly different from the control (Figure 2.7B, 2.7C, 2.7D). Cell line variants that are greater than 60% downregulated in ZnT-8 are significantly different than the control at and after 90 minutes. Cell lines C and D show significantly lower insulin secretion than the control cells at all time points, while cell lines F and G mimic those cell lines that are greater than 60% downregulated.

C-peptide 2 secretions from INS-1 cells and cells transfected with the empty vector show no significant difference from each other (Figure 2.8A). Cell lines B and E mimic insulin secretion patterns with significantly lower secretions at all time points (Figure 2.8B, 2.8 C, 2.8D). Cell line A is only significantly lower than the control at 30 and 90 minutes. Cell line variants that are greater than 60% downregulated also mimic insulin secretions, showing significantly lower C-peptide 2 secretions at and after 90 minutes.

Cell lines C, F and G show significantly lower C-peptide 2 secretions at all time points, while cell line D mimics those cell lines that are greater than 60% downregulated.

Intracellular calcium levels are not significantly different between INS-1 cells and those with the empty vector over the course of three hours (Figure 2.9A). At all time points, none of the cell lines with downregulated ZnT-8 show a significant difference from the controls (180 minutes, Figure 2.9B). Intracellular pH measurements shown here agree with literature values[99] of about 7.2 over the course of three hours (Figure 2.10A), but when ZnT-8 is at all downregulated, intracellular pH increases significantly at all time points (180 minutes, Figure 2.10B).

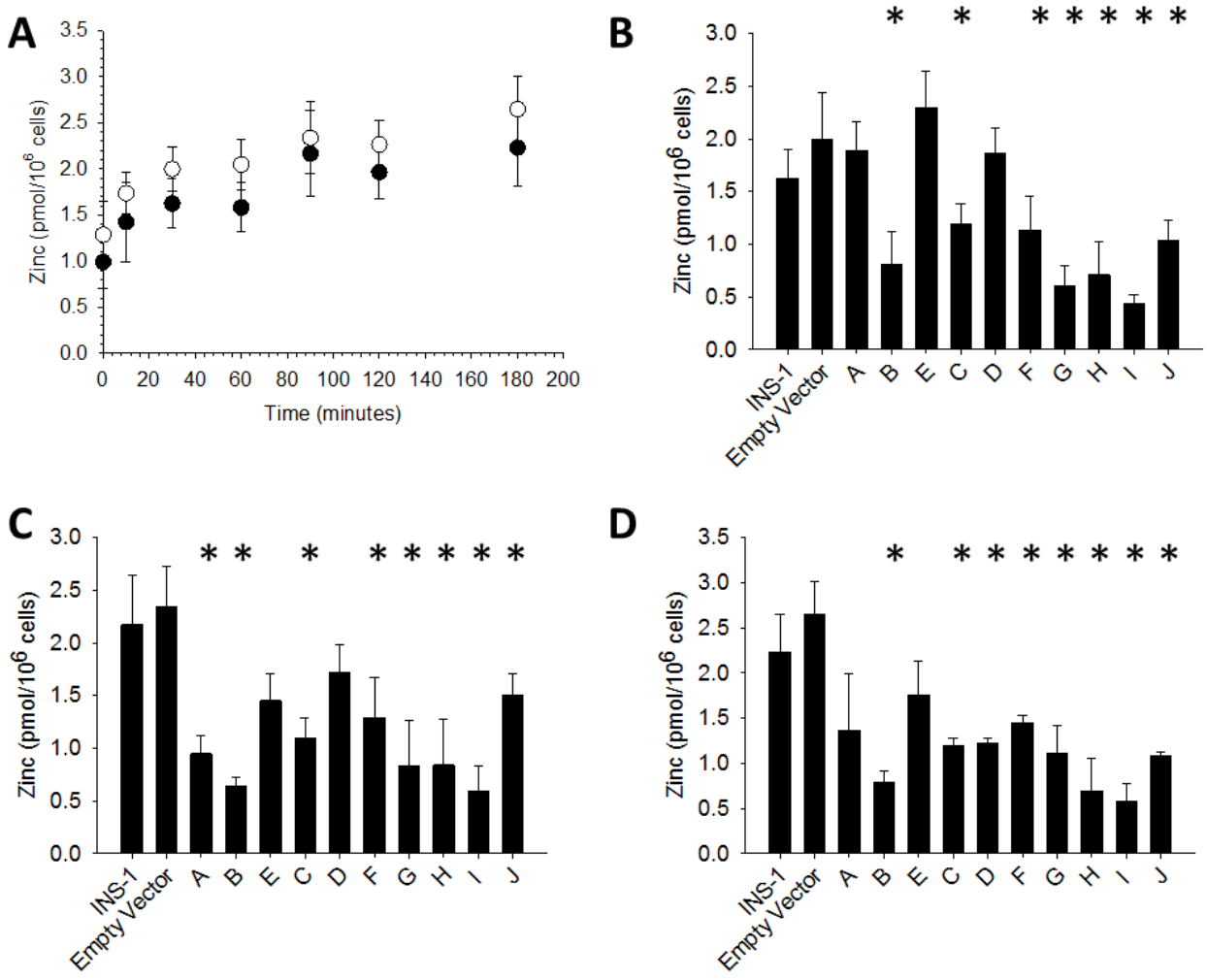

Figure 2.6: Zinc secretions from INS-1 cells and their ZnT-8 downregulation variants. (A) INS-1 cells transfected with the empty vector release zinc over the course of three hours after stimulation (Black circles, INS-1; white circles, empty vector). (B) At 30 minutes, ZnT-8 downregulation variants show some significant decrease in zinc secretions. (C) At 90 minutes, ZnT-8 downregulation variants still show some significant decrease in zinc secretions. (D) At 180 minutes, all cell lines that are greater than 50% downregulated show significant decreases in the amount of zinc released. n=4, error=SEM, *p<0.05 from the empty vector

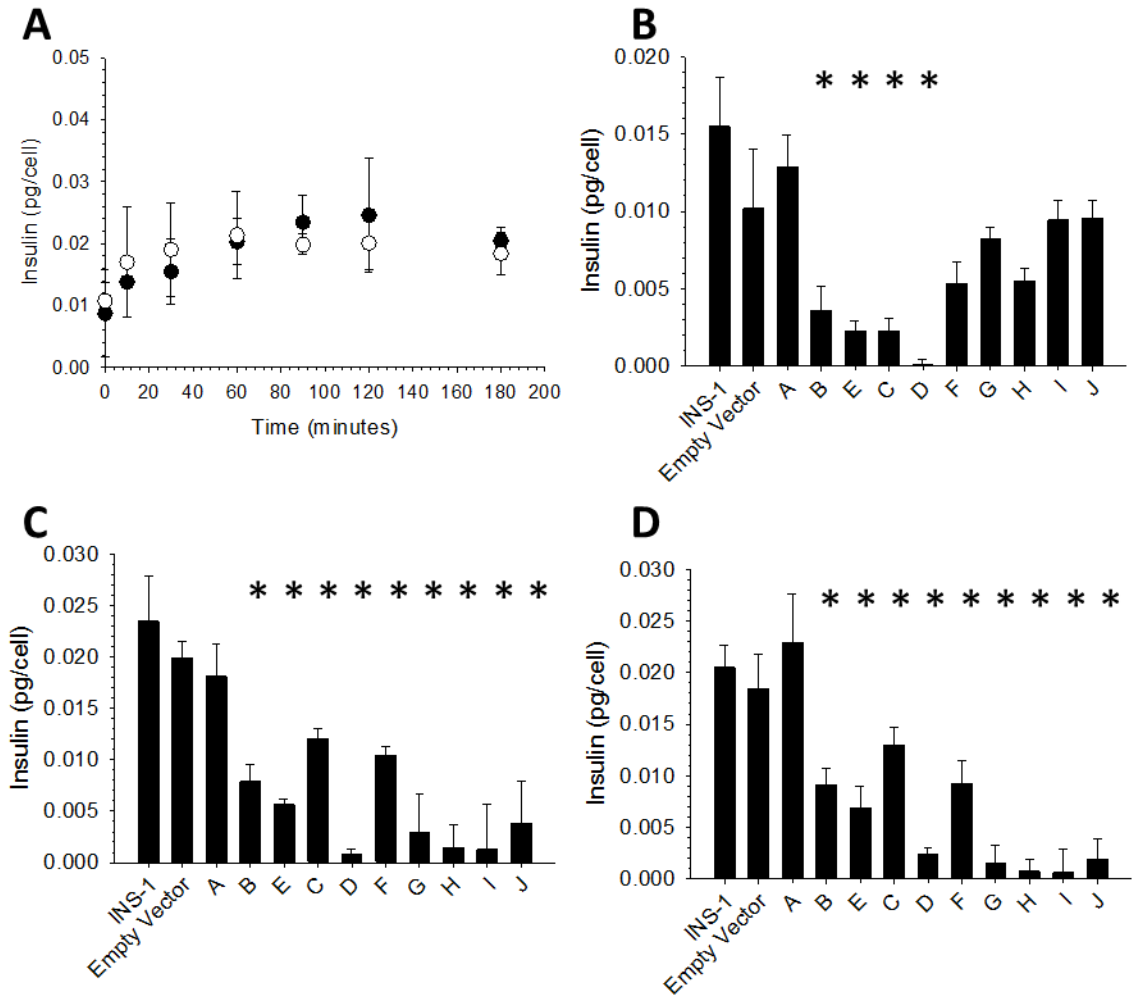

Figure 2.7: Insulin secretions from INS-1 cells and their ZnT-8 downregulation variants. (A) INS-1 cells transfected with the empty vector release insulin over the course of three hours after stimulation (Black circles, INS-1; white circles, empty vector). (B) At 30 minutes, ZnT-8 downregulation variants show some significant decrease in insulin secretions. (C) At 90 minutes, ZnT-8 downregulation variants show a significant decrease in insulin secretions for those cell lines with greater than 50% downregulation. (D) At 180 minutes, all cell lines that are greater than 50% downregulated show significant decreases in the amount of insulin released. n=4, error=SEM, *p<0.05 from the empty vector

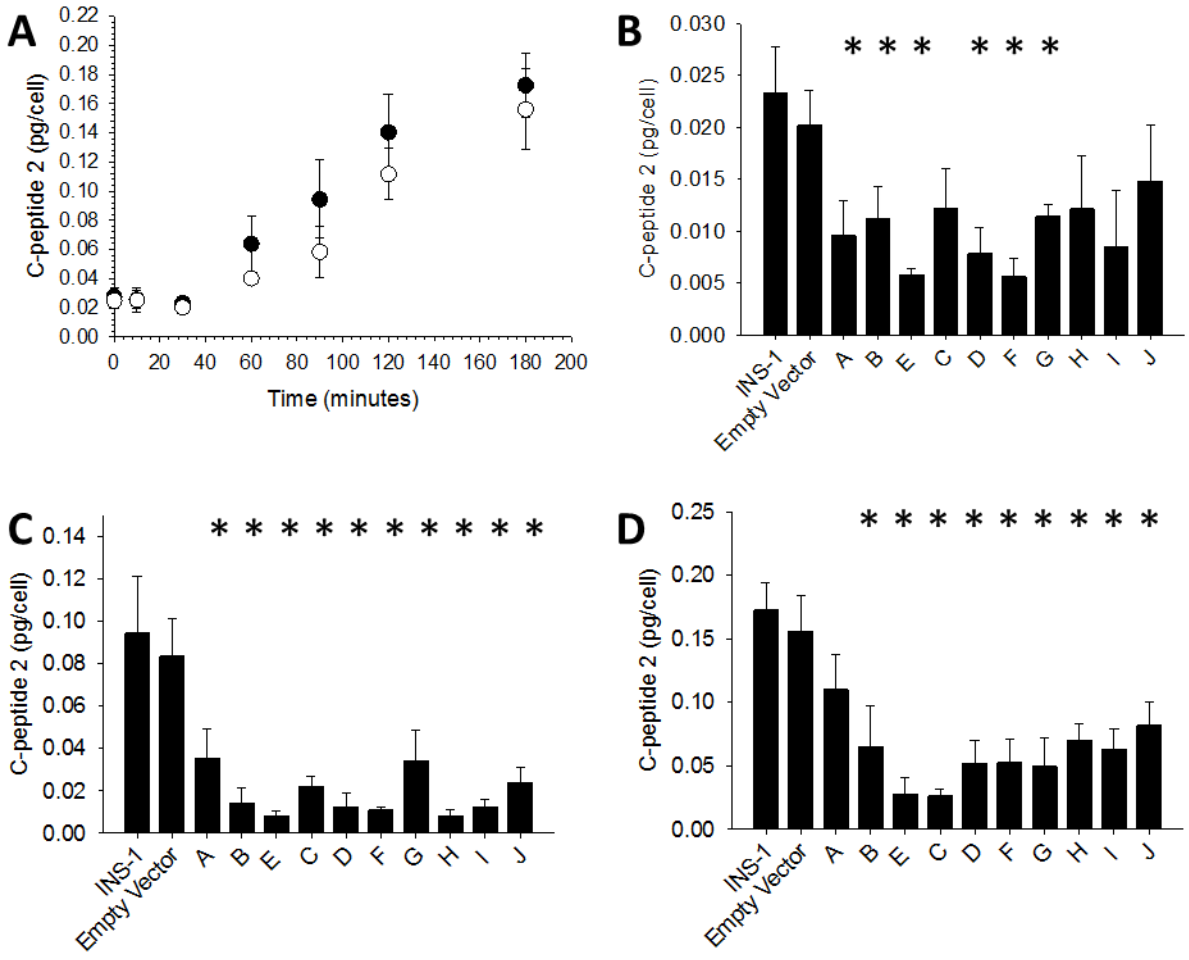

Figure 2.8: C-peptide 2 secretions from INS-1 cells and their ZnT-8 downregulation variants. (A) INS-1 cells transfected with the empty vector release C-peptide 2 over the course of three hours after stimulation (Black circles, INS-1; white circles, empty vector). (B) At 30 minutes, ZnT-8 downregulation variants show some significant decrease in C-peptide 2 secretions. (C) At 90 minutes, ZnT-8 downregulation variants show a significant decrease in C-peptide 2 secretions at greater than 50% downregulation. (D) At 180 minutes, all cell lines that are greater than 50% downregulated show significant decreases in the amount of C-peptide 2 released. n=4, error=SEM, *p<0.05 from the empty vector

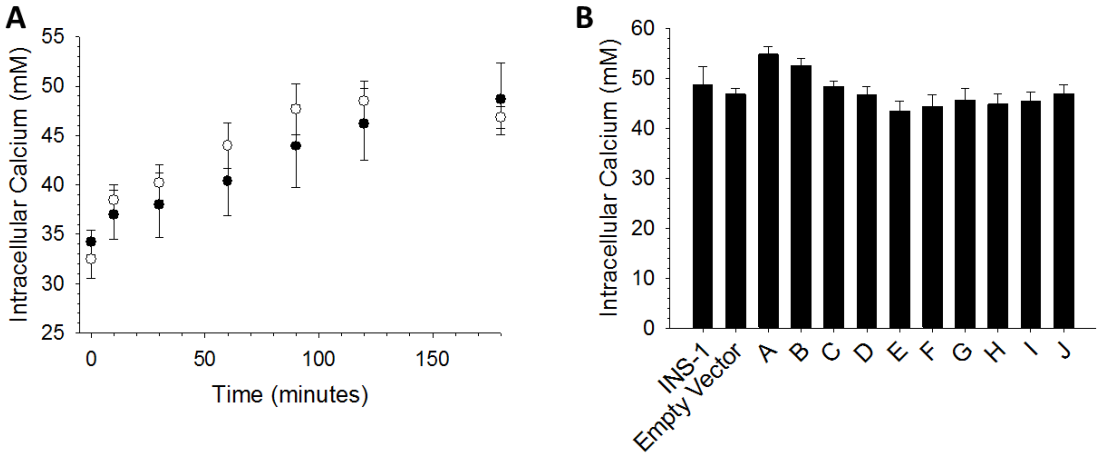

Figure 2.9: Intracellular calcium levels of INS-1 cells and their ZnT-8 downregulation variants. (A) Intracellular calcium release over time from INS-1 cells (black circles) and INS-1 cells with the empty vector (white circles). There is no statistically significant difference between the two. (B) Intracellular calcium levels of all ZnT-8 downregulation variants at 180 minutes. There is no significant difference between any of the cell lines. n=4, error=SEM

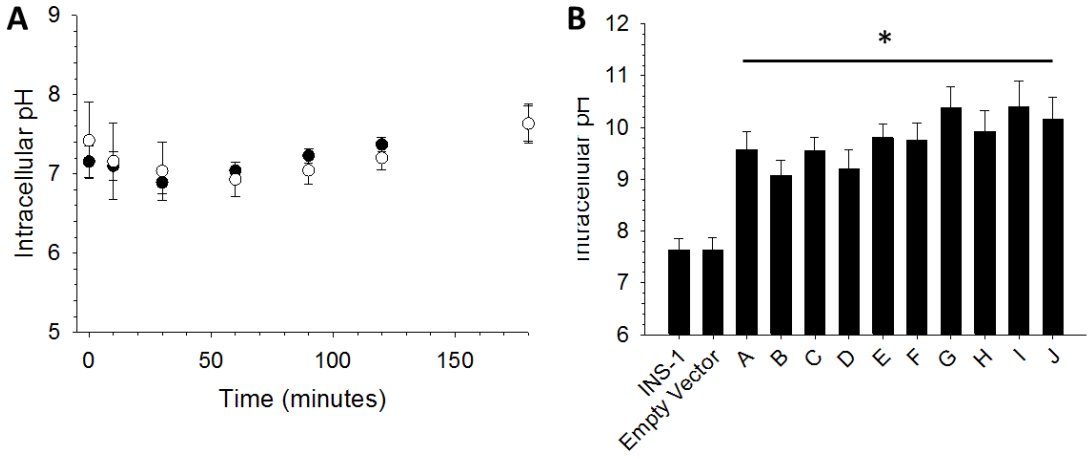

Figure 2.10: Intracellular pH of INS-1 cells and their ZnT-8 downregulation variants. (A) Intracellular pH over time from INS-1 cells (black circles) and INS-1 cells with the empty vector (white circles). There is no statistically significant difference between the two. (B) Intracellular pH of all ZnT-8 downregulation variants at 180 minutes. There is a statistically significant difference between all ZnT-8 downregulation variants and the controls. n=4, error=SEM, *p<0.05 from the empty vector

2.8.2 CFTR

Successful downregulation of INS-1 cells using the vectors obtained from Origene for CFTR are shown in Table 2.4. Rather than studying the secretion patterns from all cell line variants, three representative variants were chosen: J, which has the lowest downregulation (about 40%); C, which is about 50% downregulated; and F, which has the highest degree of downregulation with the lowest error, at about 70%.

Table 2.4: CFTR downregulation results as determined by SDS-PAGE and Western blot in INS-1 cells. Cell lines A, B, C, and D were created using the original vectors obtained from Origene. Cell lines E through J were obtained using a combination of two these vectors (E: A and B; F: A and C; G: A and D; H: B and C; I: B and D; J: C and D). n=3, error=SEM

Cell Line	% CFTR Downregulated
Empty vector	0 ± 2
A	59 ± 2
B	59 ± 2
C	52 ± 6
D	44 ± 5
E	68 ± 15
F	69 ± 5
G	51 ± 20
H	40 ± 3
I	53 ± 1
J	37 ± 6

Zinc secretions from INS-1 cells and cells transfected with the empty vector show no significant difference from each other (Figure 2.12A). Secretions from downregulated cells at 30 minutes are all statistically different from the control (Figure 2.12B), but at 90 minutes show no significant difference from the control (Figure 2.12C). At 180 minutes, only cell line variants that are greater than 50% downregulated are significantly different from the control.

C-peptide 2 secretions from INS-1 cells and those transfected with the empty vector show no significant difference from each other over three hours (Figure 2.13A). All CFTR downregulated cell lines exhibit significantly less C-peptide 2 secretions at all time points compared to the control (Figure 2.13B, 2.13C, 2.13D). Insulin secretions similarly mimic the C-peptide 2 results, as expected (Figure 2.14).

Intracellular pH levels in INS-1 cells and those transfected with the empty vector show no statistically significant difference from each other (Figure 2.15A). However, CFTR downregulated cells show significantly lower intracellular pH levels than the control (Figure 2.15B), and these levels appear to decrease over time.

Intracellular calcium levels in CFTR downregulated cells are not statistically different from the control (INS-1 cells) or INS-1 cells transfected with the empty vector over the course of time (Figure 2.16).

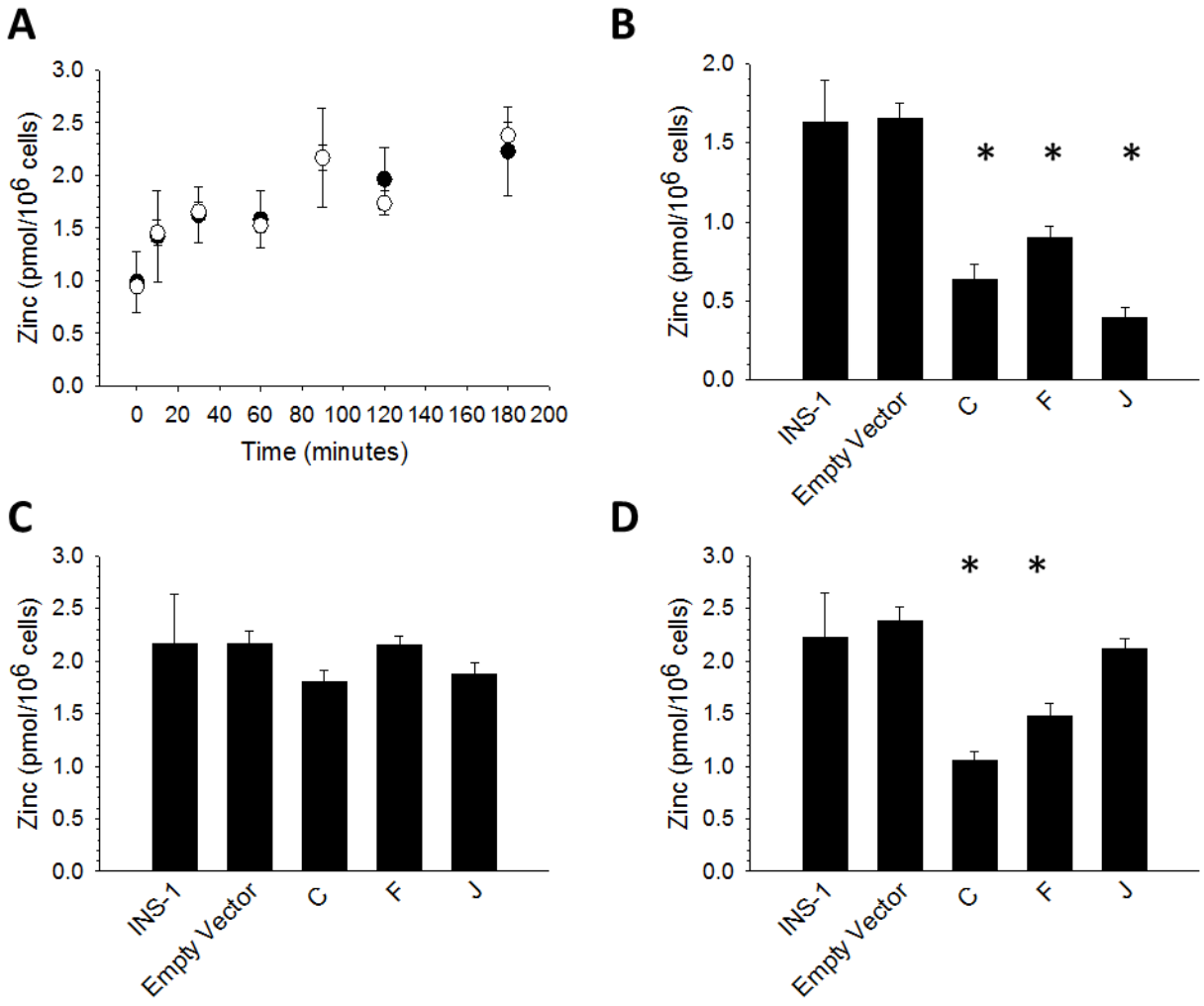

Figure 2.11: Zinc secretions from INS-1 cells and their CFTR downregulation variants. (A) INS-1 cells transfected with the empty vector release zinc over the course of three hours after stimulation (Black circles, INS-1; white circles, empty vector). (B) At 30 minutes, CFTR downregulation variants show significant decreases in zinc secretions. (C) At 90 minutes, CFTR downregulation variants show no significant decrease in zinc secretions. (D) At 180 minutes, cell lines C and J show significant decreases in the amount of zinc released. n=4, error=SEM, *p<0.05 from the empty vector

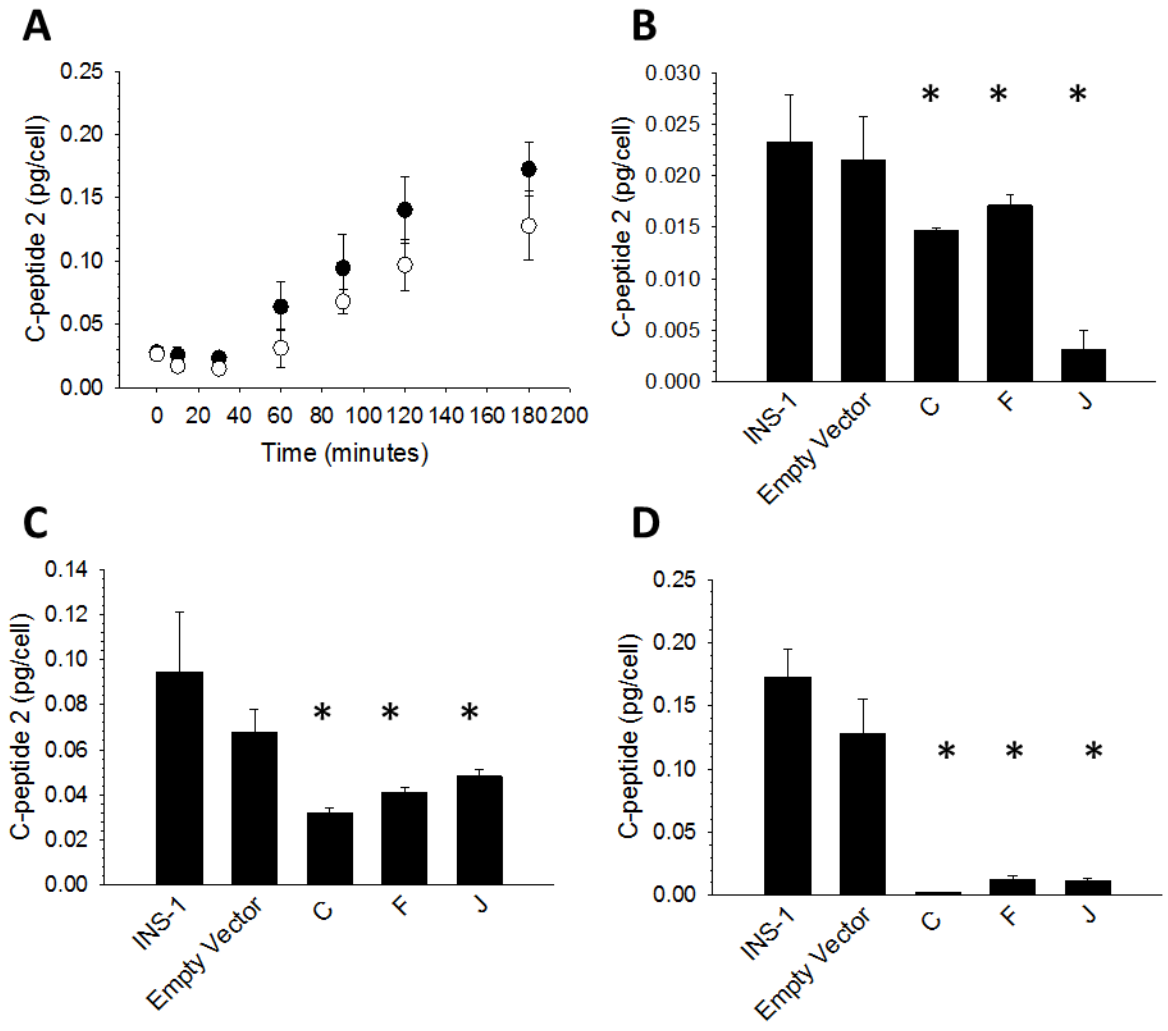

Figure 2.12: C-peptide 2 secretions from INS-1 cells and their CFTR downregulation variants. (A) INS-1 cells transfected with the empty vector release C-peptide 2 over the course of three hours after stimulation (Black circles, INS-1; white circles, empty vector). (B) At 30 minutes, all cell lines show significant decreases in C-peptide 2 secretions. (C) At 90 minutes, all cell line variants show significant decrease in C-peptide 2 secretions. (D) At 180 minutes, all cell lines show significant decreases in the amount of C-peptide 2 released. n=4, error=SEM, *p<0.05 from the empty vector

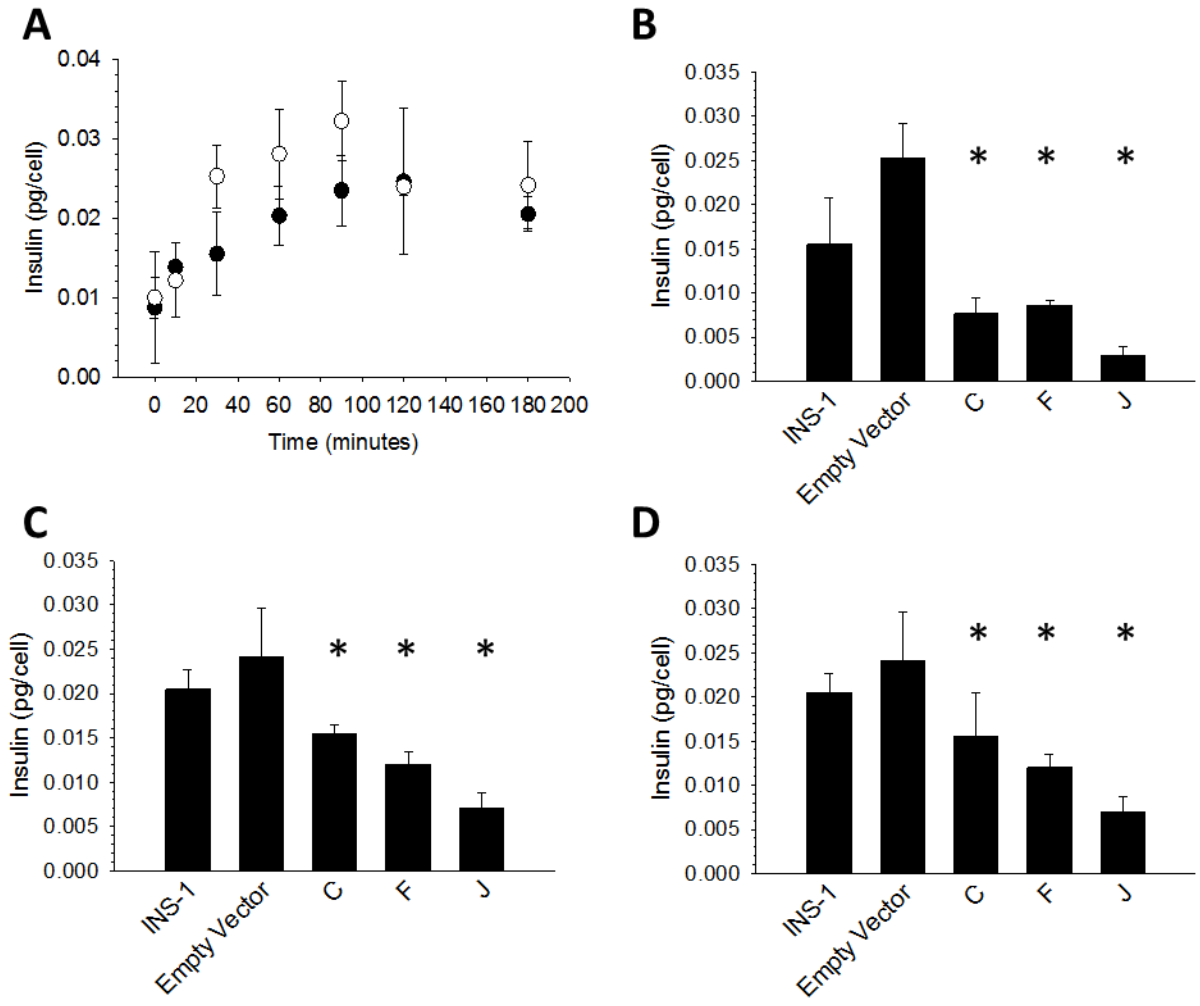

Figure 2.13: Insulin secretions from INS-1 cells and their CFTR downregulation variants. (A) INS-1 cells transfected with the empty vector release insulin over the course of three hours after stimulation (Black circles, INS-1; white circles, empty vector). (B) At 30 minutes, all cell lines show significant decreases in insulin secretions. (C) At 90 minutes, all cell line variants show significant decrease in insulin secretions. (D) At 180 minutes, all cell lines show significant decreases in the amount of insulin released. n=4, error=SEM, *p<0.05 from the empty vector

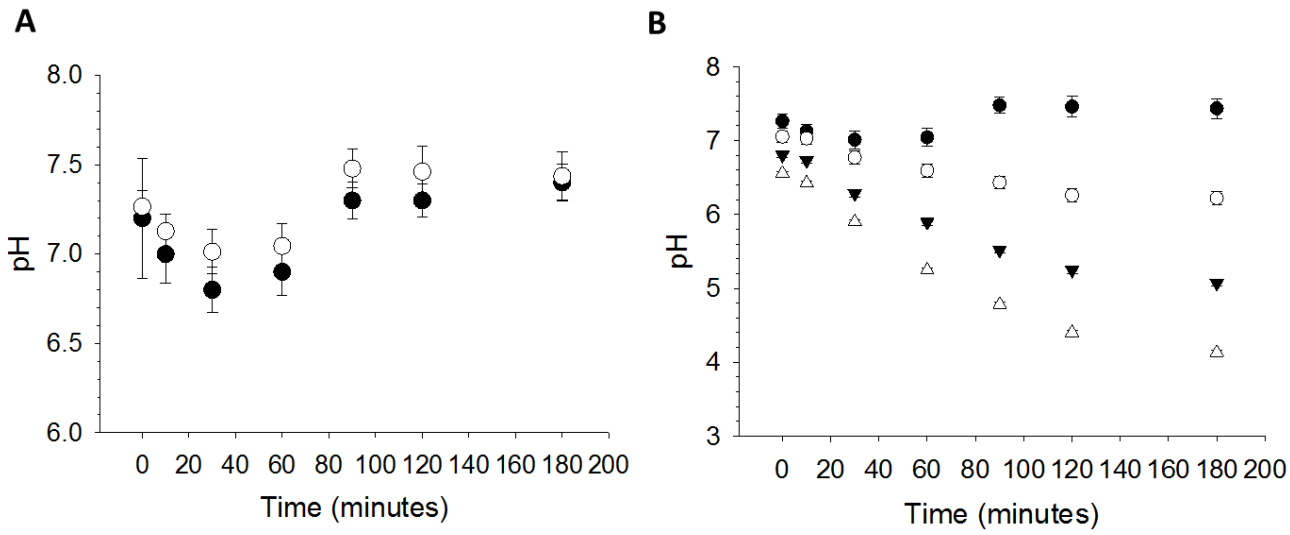

Figure 2.14: Intracellular pH of INS-1 cells and their CFTR downregulation variants. (A) Intracellular pH over time from INS-1 cells (black circles) and INS-1 cells with the empty vector (white circles). There is no statistically significant difference between the two. (B) Intracellular pH of all CFTR downregulation variants over time (Black circles, empty vector; White circles, C; Black triangles, F; White triangles, J). There is a statistically significant difference between all CFTR downregulation variants and the controls at all time points. n=4, error=SEM (error bars too small to be seen)

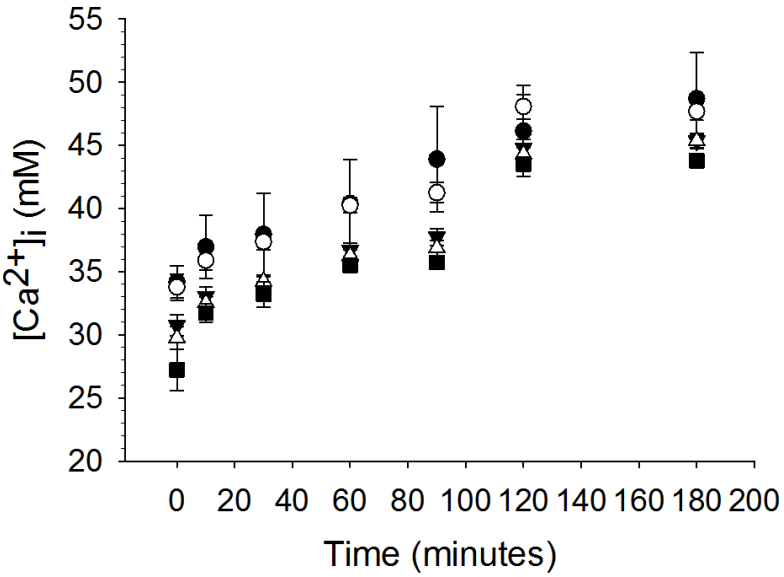

Figure 2.15: Intracellular calcium levels of INS-1 cells and their CFTR downregulation variants. Intracellular calcium increases over time in both the INS-1 cells (black circles) and cells transfected with the empty vector (white circles). Cell line variants C (black triangles), F (white triangles) and J (black squares) show no significant difference from the control. n=4, error=SEM

2.9 Discussion

2.9.1 ZnT-8

Successful downregulation of ZnT-8 in INS-1 cells was achieved using shRNA constructs, with downregulation ranging from about 40 to 75% (Table 2.3). For the purpose of better understanding the large amount of data collected in these experiments, ZnT-8 downregulated cell line variants were grouped into three categories: about 40% downregulation, about 50% downregulation, and greater than 60% downregulation. Cell line variants A, B, and E are approximately 40% downregulated; cell line variants C, D, F, and G are approximately 50% downregulated; and cell line

variants H, I and J are greater than 60% downregulated. Downregulation of ZnT-8 resulted in changes in the secretion patterns of all cell line variants for all three analytes measured: C-peptide 2, insulin, and zinc.

All measured secretions from INS-1 cells were affected by this downregulation (Figures 2.6, 2.7, 2.8), with all cell lines greater than 50% downregulated expression releasing significantly less C-peptide 2, zinc, and insulin at 180 minutes. At 90 minutes, cell lines with greater than 50% downregulation also released significantly less C-peptide 2 and insulin, while those cell lines with greater than 70% downregulation released significantly less zinc. ZnT-8 performs an essential function to the secretory vesicles of pancreatic beta cells, namely, to bring in zinc to facilitate formation of the insulin hexamer. Therefore, as this data shows, the downregulation of that function causes deficiencies in all measured secretions from these cells.

For cell line variants that are approximately 40% downregulated, zinc, insulin, and C-peptide 2 secretions are irregular, with some being lower than the control, and others being unchanged from the control. Total insulin and C-peptide (twice the amount of C-peptide 2) secretions in INS-1 cells and those transfected with the empty vector give the expected and approximate 1:1 molar ratio (insulin: C-peptide) secretions (Table 2.5). This ratio is unchanged in some cell lines that have been downregulated in ZnT-8 (A and F), and lower in others (B, C, D, E, G, H, I and J). Higher secretions of insulin in these cell lines may indicate that exocytosis of the secretory vesicles is altered or that C-peptide is not undergoing proper cleavage from insulin in the granule. Exocytosis of the granules is partially controlled by the rise in intracellular calcium, while the

conversion of proinsulin to insulin requires a drop in pH in the granule to allow endopeptidases to properly cleave and free insulin and C-peptide.

Table 2.5: Molar ratios of Insulin to C-peptide secreted from ZnT-8 downregulated cells. Ratios are presented as insulin to C-peptide, i.e. in the control, 1 insulin to 0.9 C-peptide. In all but two cell lines (A and F) the ratios are significantly changed.

Cell Line	Ratio Insulin:C-peptide
Control	0.9 ± 0.2
Empty Vector	0.9 ± 0.2
A	$1._{16} \pm 0.04$
B	$0.4_3 \pm 0.07$
C	$0.1_4 \pm 0.01$
D	$0.1_4 \pm 0.02$
E	0.5 ± 0.1
F	$1._1 \pm 0.1$
G	0.6 ± 0.1
H	0.6 ± 0.2
I	0.5 ± 0.2
J	0.6 ± 0.3

The rise in intracellular calcium that triggers exocytosis of the granules from the pancreatic beta cell is unchanged with the downregulation of ZnT-8 (Figure 2.9), indicating that exocytosis of the granules is unaffected. The decrease in insulin, zinc,

and C-peptide secretions and altered molar ratios is therefore likely due not to the lack of exocytosis, but to another mechanism entirely.

The intracellular pH of mammalian pancreatic beta cells is reported to be around 7.2, [99, 100] while the pH of the granules is 5.5.[101] Here we report the intracellular pH of INS-1 cells and those transfected with the empty vector to agree with that value (Figure 10). However, when ZnT-8 expression is downregulated by any amount, the intracellular pH significantly increases. Since intracellular calcium levels remain unchanged in the downregulated cells, a mechanism involving H^+ ion transport into and out of the secretory vesicle is probable. As previously mentioned, Hutton and Peshavaria reported the evidence of a membrane-associated proton-translocating Mg^{2+}-dependent ATPase in beta cell secretory granules.[31]

Other groups have reported that downregulation of ZnT-8 expression in INS-1 cells by shRNA did indeed reduce insulin secretion from the cells as well as decreased intracellular zinc content and fewer vesicles were present in the cells, indicating that downregulation of ZnT-8 affects glucose stimulated insulin secretion from these cells.[81] A decrease in intracellular zinc as a result of ZnT-8 downregulation involves a complex network of regulation through ZnTs and ZIPs on the beta cell plasma membrane.[102] Both intracellular and extracellular zinc concentrations affect insulin secretion from beta cells. Intracellular micromolar zinc levels have been shown to hyperpolarize the plasma membrane via activation of K_{ATP}, which results in decreased insulin secretion from beta cells,[103] while extracellular micromolar zinc activates K_{ATP} and is pH dependent.[104] Binding of zinc to amino acids is affected by changes in H^+ concentration, which will affect the activation/inhibition of K_{ATP}.[105] According to

Bancila *et al.*, at an extracellular pH below 6.4, zinc inhibited K_{ATP}, while at higher pH, zinc activates K_{ATP}.[104]

In normal beta cells, intracellular pH should remain stable at about 7.2, while the intragranular pH changes due to the pH activity requirements of endopeptidases PC2 and PC1/3. The presence of multiple ion channels and proteins at the plasma membrane of the cell and on the membrane of the secretory vesicle are sufficient to retain this pH. Here we report an increase in intracellular pH when ZnT-8 is downregulated, and a decrease in secretions from these cells, but also a change in the ratio of products produced. Although overall secretions decrease, in many cell lines, the molar ratio of insulin: C-peptide decreases, which indicates that there is more insulin released from these cells than C-peptide. If the pH in the granules does not decrease properly, endopeptidases will not function, keeping C-peptide attached to insulin. This points to a mechanism involving inhibition of the proton pump present on secretory granules, which causes an increase in intracellular pH. One potential reason for this increase in pH is the cell trying to balance out its ion concentrations. Less intracellular free zinc would necessitate an increase in other positively charged ions, such as H^+.

Taken together with the data presented here, a mechanism is proposed by which downregulation of ZnT-8 decreases the secretions from INS-1 cells, not by reduced exocytosis, but by altering the intracellular pH, and therefore the intragranular pH. This change in intracellular pH has the potential to cause downstream problems, including changes in osmotic pressure and salt concentration balance, which could lead to the destruction of the beta cells.

The data presented here indicates that ZnT-8 plays an important role in regulating the homeostasis of the pancreatic beta cell as well as in the regulation of molecules present in the secretory granules. This data helps to shed some light on how this transporter may be affecting molecular mechanisms crucial to glucose stimulated insulin secretion and the development and progression of T2D.

2.9.2 CFTR

Successful downregulation of CFTR by shRNA in INS-1 cells is shown in Table 2.4 from Western blot analysis. Three cell lines of various downregulations were selected for further analysis: J, which is 37 ± 6% downregulated; C, which is 52 ± 6% downregulated; and F, which is 69 ± 5% downregulated. These three cell line variants were chosen for their varying downregulations and their low SEM between three different passages.

In all secretions that were measured, there is no statistically significant difference between INS-1 cells and those transfected with the empty vector (Figure 2.12A, 2.13A, 2.14A). Cell line variant J has the lowest downregulation of the three variants studied here. Zinc secretion from this cell line is only significantly different from the control at 30 minutes (Figure 2.12B), which indicates that this cell line is deficient in initial or first-phase hormone release. C-peptide 2 and insulin secretions from J are significantly lower than the control at and after 90 minutes (Figure 2.13 and 2.14).

Zinc secretions from cell line variant C are significantly different from the control at 30 and 180 minutes, which indicates that there is a deficiency in first-phase and prolonged hormone release. C-peptide 2 secretions are significantly lower than the control at and

after 90 minutes and insulin secretions are significantly lower than the control at all time points (Figure 2.13 and 2.14).

Zinc secretions from cell line variant F are significantly different from the control at 30 and 180 minutes (Figure 2.12). C-peptide 2 secretions are significantly different from the control at and after 90 minutes, while insulin secretions are significantly different than the control at all time points (Figure 2.13 and 2.14).

Table 2.6 Insulin to C-peptide molar ratios in CFTR-downregulated cells. Ratios are presented as insulin to C-peptide, i.e., in the control, 1 insulin to 0.8 C-peptide. Ratios are unchanged from the control in cell lines C and F, while ratios are significantly lower in cell line J.

Cell Line	Ratio Insulin:C-peptide
Control	0.8 ± 0.2
Empty Vector	0.9 ± 0.1
C	$1._1 \pm 0.1$
F	$1._1 \pm 0.2$
J	0.6 ± 0.1

Studies of C-peptide and insulin levels after a glucose tolerance test in patients with CF have shown that CF patients with both impaired and normal glucose tolerance exhibit lower C-peptide and insulin levels than healthy, age-matched controls.[106] Interestingly, this study also showed significantly increased levels of proinsulin in patients with impaired glucose tolerance from controls, which could indicate that the

endopeptidases responsible for cleavage are not correctly functioning. One would expect that this data would be reflected in total insulin to C-peptide molar ratios.

Total insulin to C-peptide molar ratios (Table 2.6) are unchanged in cell lines C and F from the control, indicating that the intragranular pH is stable, and the activity of endopeptidases is unchanged. However, cell line J, which is the least downregulated, does appear to show lower amounts of C-peptide as compared to insulin, which may indicate higher levels of proinsulin than in other cell lines. Therefore, we investigated the effect of downregulating CFTR on intracellular calcium levels and intracellular pH.

Intracellular calcium levels do not differ from the control over time (Figure 2.16), however, intracellular pH levels do show a significant decrease from the control, decreasing over time (Figure 2.15). Taken together these data indicate that though the intracellular calcium levels are not decreasing, which would be indicative of decreased exocytosis, the change in intracellular pH may be contributing to decreased exocytosis.

Although rising intracellular calcium levels are necessary for exocytosis of the granules, there is mounting evidence in the literature that CFTR is coupled to the exocytotic machinery in the beta cell. In human islets, chemical inhibition of CFTR in human islets resulted in a significant decrease in insulin secretion and a decrease in exocytosis.[36] The same study also found that the calcium current in beta cells was unaffected by CFTR inhibition, as we confirm here, suggesting that CFTR may directly affect the exocytotic machinery. This action could be through interaction with the SNARE-protein syntaxin 1A, which is crucial for granule docking and exocytosis in beta cells.[36, 92, 93]

These studies have also revealed that CFTR likely acts as a regulator of other chloride channels in beta cells, specifically Anoctamin 1 (ANO1), in insulin secretion.[36, 94-96]

2.10 Conclusions

Separate downregulation of ZnT-8 and CFTR in INS-1 cells has been presented here, as well as their effects on C-peptide, zinc, and insulin secretions. Both genes have negative effects on the secretion patterns of these important molecules, though through apparently different mechanisms.

INS-1 cells downregulated in ZnT-8 appear to have no difference in the ability to exocytose granules, as evidenced by the unchanged intracellular calcium levels as compared to controls. However, the intracellular pH increases significantly from the control at any amount of ZnT-8 regulation, and the typical 1:1 molar ratio of insulin to C-peptide is perturbed, indicating that the endopeptidases contained within the secretory vesicle are unable to correctly function. This dysfunction could lead to lower amounts of C-peptide circulating in the bloodstream, thereby contributing to blood flow and immune complications in T2D patients.[107]

INS-1 cells downregulated in CFTR also appear to have no difference in the ability to exocytose granules, however, they may have dysfunction in the docking and fusing of granules to the plasma membrane. Lower amounts of insulin, C-peptide, and zinc are released from these cells, which could contribute to the development of CFRD in these patients. The intracellular pH in these cells decreases as compared to the control, which indicates that the docking and fusing of granules may indeed be affected by CFTR dysfunction, agreeing with other literature reports.[36, 92, 93]

The successful downregulation of INS-1 cells for both ZnT-8 and CFTR opens up a number of possibilities for further studies, including using these cells to study the docking of granules to the plasma membrane and their direct effect on other cell types in the bloodstream that C-peptide is known to affect. Prior to performing these experiments, however, it is necessary to determine the effect that pancreatic secretions have on these cell types important in secondary complications of diabetes, namely, blood flow and immune complications.

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CHAPTER 3

THE EFFECT OF PANCREATIC SECRETIONS ON RED BLOOD CELLS

3.1 Red Blood Cells (RBCs)

Red blood cells (RBCs) are anucleated cells lacking all formed organelles. They are the major component of the bloodstream, with about 2.6 x 10^{13} cells *in vivo*.[1] They are approximately 6-8 μm in diameter with a volume of about 87 fL, lasting about 120 days in circulation.[2] *In vivo*, RBCs deliver oxygen to the organs and tissues, however, they also plays a role in regulating vascular tone through the release of ATP.[1]

ATP is released from RBCs in response to a number of stimuli, including hypoxia[3] and shear-induced mechanical deformation,[4, 5] as experienced by RBCs as they travel through the vascular system. ATP is also released from RBCs in response to lower pH[6] and changes in osmotic pressure.[7] Abnormal ATP release from RBCs has been identified in diseases such as cystic fibrosis (CF),[5] pulmonary hypertension,[8] diabetes,[9, 10] and multiple sclerosis (MS).[11]

ATP released from RBCs has the ability to stimulate nitric oxide (NO) production in the endothelial cells that line blood vessels. ATP binds to the purinergic receptor, P2Y, to stimulate endothelial nitric oxide synthase (eNOS), which converts L-arginine to L-citrulline, releasing NO as a byproduct (Figure 3.1). NO is a well-established vessel relaxing factor, and is a determinant in the control of vasodilation.[12, 13]

The mechanism of ATP release from RBCs has been previously described in chapter 1. Briefly, a heterotrimeric G-protein is activated, activating adenylyl cyclase (AC). AC converts ATP into cyclic AMP (cAMP), which activates phosphokinase A (PKA). PKA phosphorylates the cystic fibrosis transmembrane conductance regulator (CFTR), which promotes the release of ATP through Pannexin-1.

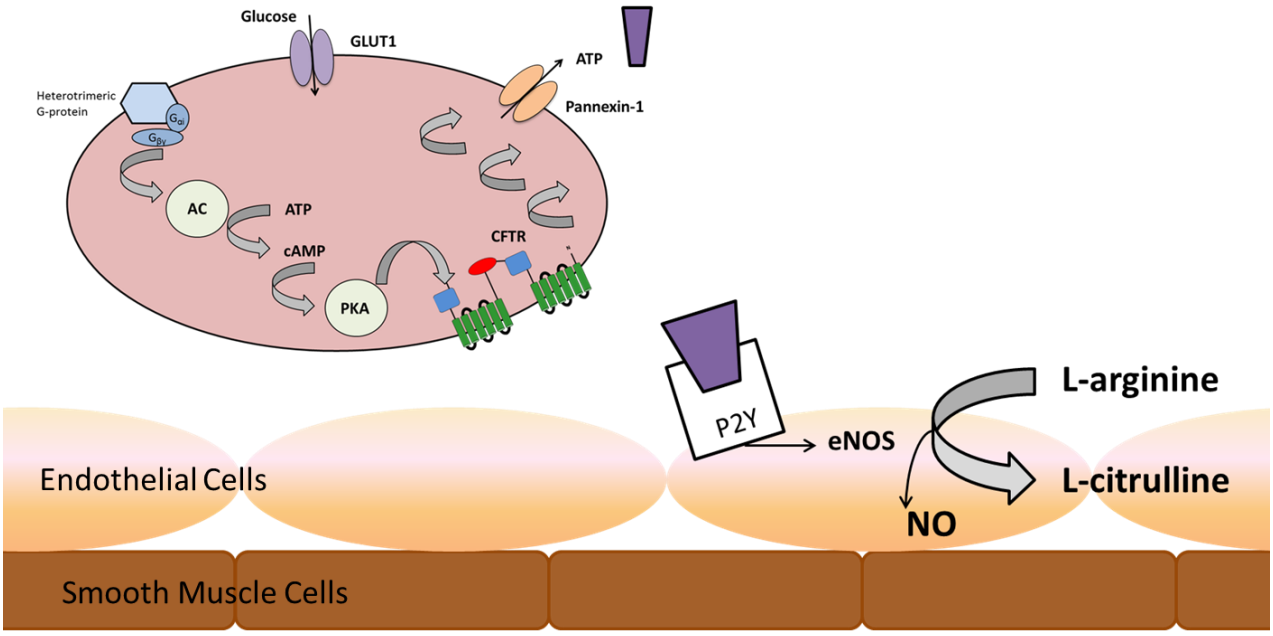

Figure 3.1: The mechanism of RBC derived ATP stimulating NO release from endothelial cells. RBC derived ATP binds to the P2Y receptor on endothelial cells, which stimulates eNOS to convert L-arginine to L-citrulline, which gives NO as a byproduct.

RBCs that are exposed to increasing concentrations of glucose, as in *diabetes mellitus*, show altered conductivity of the cell membrane.[14] RBCs of patients with diabetes also exhibit less deformability than those of healthy controls,[15] which may lead to a decreased amount of ATP release. Intracellularly, RBCs from patients with diabetes

contain increased amounts of ATP as compared to control patients.[16] These results indicate that there is likely a defect in the mechanism of ATP release from RBCs in these patients. Sprague *et al*. were the first to show that ATP release is modulated by CFTR in the RBC, but that it was not due to decreased deformability. [5]

3.1.1 GLUT1

GLUT1 (Figure 3.2) was the first glucose transporter isoform identified, purified and cloned.[17-19] It is expressed at high levels in RBCs, making up 10-20% of all membrane proteins in RBCs[20] and is the most abundant glucose transporter present on the RBC. As previously mentioned, GLUT1 is an asymmetric[21] bidirectional transporter with the capability of transporting glucose, galactose, and ascorbic acid.[22, 23] On SDS-PAGE, GLUT1 runs as a broad band due to N-linked glycosylation at Asn-45.[24] There is evidence to suggest that cytoplasmic ATP inhibits RBC GLUT1-mediated glucose transport and is mediated by conformational changes in the protein itself.[25, 26]

GLUT1 has been shown to translocate from the intracellular vesicles to the plasma membrane in response to IL-3, and activation of a tyrosine kinase.[27] More recent evidence suggests that GLUT1 translocation is regulated by IL-3, PI3K activity and activation of Akt.[28, 29] Since GLUT1 is the primary glucose transporter in RBCs, it is important to elucidate its role in the present studies, namely in the effect that C-peptide and zinc have on RBCs. As previously mentioned, insulin affects adipose and muscle cells, which contain GLUT4, but not on cells that contain GLUT1, such as RBCs. Previous research in the Spence group has provided evidence that C-peptide and zinc's

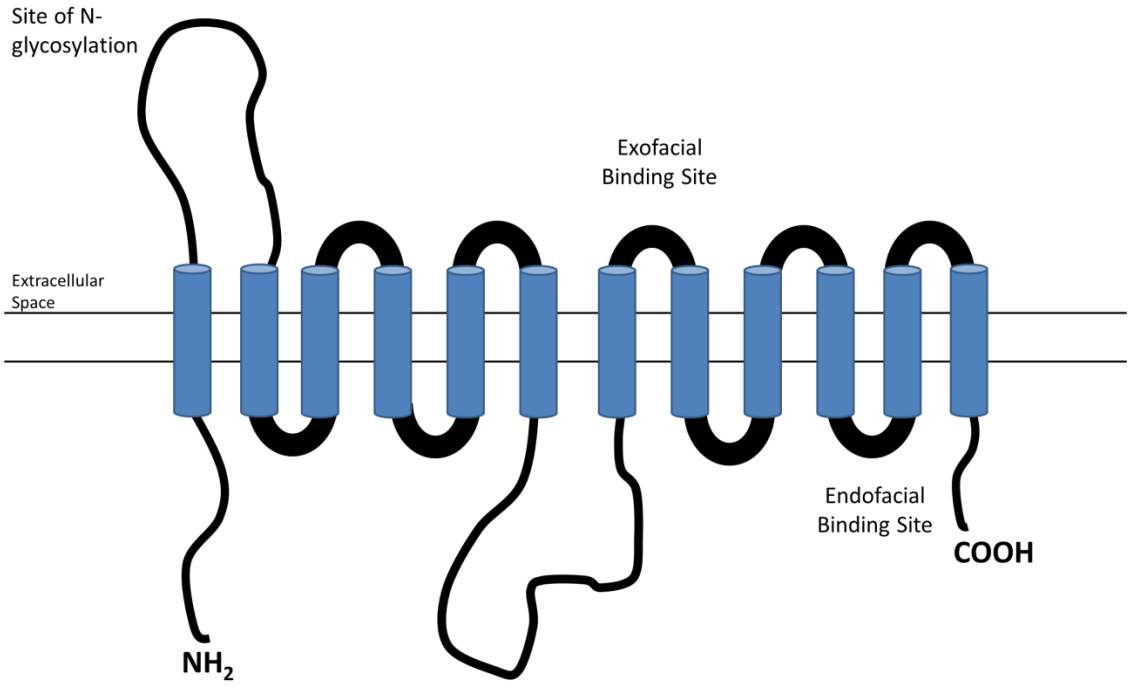

Figure 3.2: Cartoon structure of GLUT1. There are 12 transmembrane spanning domains, with both the amino and carboxy termini in the intracellular space. The site of N-glycosylation is in the extracellular loop between helices 1 and 2. The endofacial and exofacial binding sites are also shown in this diagram.

action is linked to GLUT1, and research presented here will further elucidate the insulin-like effect that C-peptide and zinc have on GLUT1-containing cells.

3.1.5 GLUT1 Inhibitors

There are a number of commonly used chemical inhibitors for GLUT1, including forskolin, cytocholasin B,[30] and phloretin.[31] The exofacial and endofacial binding sites are the docking sites for these inhibitors, blocking either one or both directions of glucose transport. Phloretin has been shown to bind to both the endofacial and exofacial sites, therefore inhibiting both import and export of glucose. Forskolin also

binds to both the endofacial and exofacial sites, while cytocholasin B binds to a second intracellular site near the transport channel.

Although both forskolin and phloretin block both the import and export of glucose from GLUT1, phloretin binds more tightly to GLUT1, making it a more useful chemical inhibitor in these studies.[32]

3.2 Previous Work in the Spence Group

Previous work in the Spence group has focused on RBCs and their response to pancreatic secretions, namely C-peptide and zinc. Initial experiments with crude C-peptide were successful in increasing ATP release from RBCs, however, after approximately 24 hours in an aqueous solvent, C-peptide no longer increased ATP release. Mass spectrometric analysis of the commercially available crude peptide revealed that the crude C-peptide was contaminated (~2% by weight) with Fe^{2+}.[33] After purification of the peptide and subsequent addition of a metal (Fe^{2+} or Cr^{3+}), ATP release was again increased from control RBCs.[34] Further examination of the metal that might be necessary in C-peptide's action revealed that, although chromium might be biologically relevant in the treatment of diabetes, the much more likely choice was zinc, due to its high abundance in the pancreatic beta cell.

Further studies explored the ability of the RBC to uptake or bind C-peptide and zinc. This terminology (binding or uptake) has been disputed, however, there is evidence of C-peptide binding specifically to renal tubular cells, fibroblasts, endothelial cells.[35] However, binding of C-peptide is abolished by pertussis toxin, which suggests that C-peptide signaling is through a G protein-coupled receptor (GPCR).[36-38] As explained

in chapter 1, the advent of research surrounding a probable receptor (GPR146)[39, 40] makes it much more likely that what we are actually seeing in this research and previous research in the Spence group is binding of C-peptide, not uptake. Zinc is likely binding to the receptor with C-peptide as well. Uptake of these molecules may occur, but it is likely after a binding event occurs.

C-peptide binding to RBCs is specific, and saturates at approximately 2 picomoles in a 7% RBC sample, and does not change in the presence or absence of zinc. Zinc binding to RBCs is also specific and saturates at about 2.5 picomoles, but only in the presence of C-peptide. In the absence of C-peptide, no zinc binding to RBCs is observed. In the absence of albumin, no binding occurs.[41]

In 2015, Liu *et al*. showed that C-peptide alone was unable to bind to zinc, but that C-peptide was able to bind to albumin ($N=0.53 \pm 0.03$, $K_a=1.75 \pm 0.64 \times 10^5\ M^{-1}$). Additionally, specific binding of both C-peptide and zinc to albumin was shown (Zinc: $N_1=0.33 \pm 0.01$, $K_1=5.08 \pm 0.98 \times 10^7\ M^{-1}$; C-peptide: $N_2=1.15 \pm 0.01$, $K_2=2.66 \pm 0.25 \times 10^5\ M^{-1}$). These results indicate a two-phase binding event, with zinc binding occurring first.[41] Taken together, these results indicate that in order for the binding C-peptide to have a biological effect, not only must healthy albumin be present, but zinc must also be present.

ATP release from RBCs under shear stress that had been treated with C-peptide alone or zinc alone is not significantly different from the control; however, in the presence of C-peptide and zinc together with albumin, ATP release increases by about 65%. In a

3D-printed microfluidic device, this increase in RBC derived ATP increased NO release from downstream endothelial cells by approximately 75%.[41]

These results in healthy control RBCs set the stage for analysis of RBCs in diseased states, including in diabetes, CF, MS, and stored RBCs. Here we present the results of RBCs inhibited with CFTRinh-172 as a model of CF, and these results' connection to two other projects in the lab involving RBCs from MS patients and stored RBCs.

3.2.1 Multiple Sclerosis (MS)

MS is a neurodegenerative disease that affects 2.1 million people worldwide.[42] MS is not a genetic disease, though much work has been done to determine if there is a genetic predisposition.[43] It is most often referred to as an autoimmune disease in which the body's immune system attacks the central nervous system, but no specific antigen has yet been identified for MS. The hallmark feature of MS is the breakdown of the myelin sheath, which covers the axon of nerve cells. Demyelination causes lesions to form in the central nervous system, and slows down nerve signals. Two important features of MS are the breakdown of the blood brain barrier (BBB) and the appearance of lesions in the brain (diagnosed by magnetic resonance imaging (MRI)).[44] Destruction of the BBB occurs prior to the appearance of lesions, which have been shown to contain nitric oxide (NO), which has been shown to be disruptive to cerebral capillaries.[45]

Patients with MS typically experience a variety of neurological symptoms including fatigue, difficulty walking, numbness or tingling of the face, body, arms or legs, muscle spasms, vision problems, dizziness, and vertigo.[42] These symptoms alone are not

enough to diagnose MS. Diagnosis of MS requires one year of disease progression, and two of the following: the appearance of at least on lesion in the brain, at least two lesions in the spinal cord, or cerebral spinal fluid that tests positive for IgG.[46]

According to the National Multiple Sclerosis Society, MS is categorized into four disease courses: relapse-remitting MS (RRMS), primary progressive MS (PPMS), secondary progressive MS (SPMS), and clinically isolated syndrome (CIS). The most common form of MS is RRMS, which is characterized by alternating periods of health (remission) and exacerbated symptoms (relapse). RRMS commonly progresses into SPMS, which still has alternating periods of health and exacerbated symptoms, but there is a worsening of neurological function between attacks. PPMS is characterized by a worsening of neurological function over time without remissions.[42]

CIS is not criteria for the diagnosis of MS due to the fact that those who have CIS may or may not develop MS. CIS is defined as an episode of neurologic symptoms that must last for at least 24 hours. When CIS is accompanied by lesions on the brain (diagnosed via MRI), development of MS is likely.[42]

There are only a handful of drugs available to slow the rate of disability of MS, and some that help with symptom management, but there is no cure for MS. When disease-modifying therapies are unsuccessful, patients are often treated with steroids. Steroids are thought to impact the immune system, reducing lymphocyte and pro-inflammatory cytokines.[47]

Despite a century of searching,[48] a biomarker for MS has not been clinically utilized. A biomarker would be useful in MS to allow for earlier treatment and simpler diagnosis.

Previous work in the Spence lab has identified a potential biomarker in RBCs. This work began with Letourneau *et al.*, who published that RBCs under shear stress from MS patients release significantly more ATP than control patients.[11]

This increase in ATP release RBCs of MS patients, coupled with reports from Dore-Duffy in the 1980s that zinc is more prevalent in the RBC membranes of patients with MS in comparison to those RBCs obtained from controls, led the Spence group to begin investigating the effect that C-peptide and zinc may have on MS RBCs. A start-up company from the Spence lab has recently put forth a potential biomarker for MS: the binding of C-peptide.

C-peptide binding in control patients ($n > 40$) in a pilot study by this start-up company were consistent with previous results at approximately 2 picomoles in a 7% RBC solution. RBCs from MS patients in varying stages of the disease ($n > 50$) is approximately 3.5 picomoles, which is significantly higher than control patients (approximately a 75% increase).[49] Two major hypotheses exist for this increase in C-peptide binding to the MS RBC: first, that MS RBCs contain a more significant amount of receptors for C-peptide; second, that these receptors have higher turnover in MS RBCs than in healthy controls.

3.2.2 Blood Storage

According to the World Health Organization (WHO), over 100 million units of blood are collected and utilized in hospitals for surgery, trauma-related injuries, and severe anemia.[50] When a healthy and consenting donor donates blood for storage, approximately 450 mL (~1 pint) of whole blood is collected into about 70 mL of an

anticoagulant solution (citrate phosphate dextrose, CPD). RBCs and plasma are separated, and the RBCs are stored in an additive solution (AS) for up to 40 days. The components of FDA-approved AS and CPD solutions can be found in Table 3.1. The high levels of glucose in each solution are necessary to allow the cells to live undisturbed for up to 40 days, however, stored RBCs experience an extremely hyperglycemic environment during storage (approximately 40 mM in CPD/AS-1) which is significantly higher than that of a diabetic patient's bloodstream. This extreme hyperglycemic environment may cause permanent changes to the RBCs, resulting in transfusion related complications.

Transfusion related complications are a result of the red cell storage lesion, including oxidative stress[51] and advanced glycation endproducts.[52] One study showed that patients who received RBCs stored longer than 14 days exhibited increased morbidity and mortality.[9, 53] One potential mechanism for this increase in morbidity and mortality is insufficient NO bioavailability in patients who have received transfusions.[54] Lower bioavailability of NO, which could be due to decreased ATP release from RBCs, has an effect on blood flow.[55] Previous research in the Spence group has linked this decrease in ATP release from stored RBCs to the hyperglycemic environment in which they are stored.[50]

The Spence group has developed normoglycemic storage solutions in which the final glucose level of stored RBCs is in the normal range (~5.5 mM), and requires feeding of the RBCs every 3-5 days.[50] All other components in the storage solutions remained the same (CPD-N, CP2D-N, AS-1N, AS-3N, AS-5N).

Wang *et al.* reported that RBCs stored in hyperglycemic, FDA-approved solutions released significantly less ATP (approximately 150 nM) than RBCs stored in normoglycemic solutions (approximately 400 nM), and that the ATP level remained steady over 29 days of storage.[50]

Table 3.1: Components of FDA-approved AS and CPD solutions. A fourth AS has been recently approved (AS-7), which will not be discussed here. Note the high concentrations of glucose in each solution, higher than even those levels seen in the bloodstream of diabetic patients.

Component	CPD	CP2D	AS-1	AS-3	AS-5
Sodium citrate	89.4	89.4	-	20	-
Citric acid	15.6	15.6	-	2.0	-
NaH_2PO_4	16.1	16.1	-	20	-
Glucose	**129**	**258**	**111**	**55**	**45**
NaCl	-	-	154	70	150
Adenine	-	-	2.0	2.2	2.2
Mannitol	-	-	41	-	29
pH	5.6	5.6	5.8	5.8	5.8

Liu went on to show that decreased ATP release from RBCs stored in hyperglycemic conditions could be reversed when placed in normoglycemic conditions (mimic of

transfusion) for up to 12 days of storage.[56] Additionally, C-peptide could bind to RBCs stored in normoglycemic conditions over the whole storage period (36 days) at amounts that agreed with previous data (about 2 picomoles per 7% solution). C-peptide binding to RBCs stored in hyperglycemic conditions were unable to statistically bind the same amount of C-peptide as those in normoglycemic solutions, and this binding was reversible into normoglycemic solutions for up to 12 days of storage.[56]

There has been some research on the effect of hyperglycemia on GLUT1 expression in varying cell types. In placental tissue from patients with gestational diabetes, basal membrane GLUT1 density was approximately 2-fold higher than in control patients, and this increase in GLUT1 expression persisted postpartum.[57] *In vitro* studies of trophoblast cells have found that GLUT1 expression is reduced by 16% when they are cultured in hyperglycemic conditions.[58] Studies of the effect of hyperglycemia on GLUT1 expression in the cerebral cortex has been controversial, with no change[59, 60], increased GLUT1,[61] and decreased GLUT1[62] expression being reported. Other studies have focused solely on glucose transport.[63, 64] These studies have likely been controversial due to the fact that many of them have been performed *in vitro* and in cell culture, not using cells from human patients. However, there has yet been no study on how hyperglycemic conditions, whether *in vivo* or in storage conditions, affect GLUT1 expression on RBCs.

3.3 CFTR inhibitors

Glibenclamide is one of the most well-known inhibitors of CFTR, and has been shown to be a chloride channel inhibitor, but is not specific for CFTR.[65-68] Another common inhibitor is niflumic acid, which is an open channel blocker of CFTR.[69] Two more

recent inhibitors, CFTRinh-172 and GlyH-101, have been extensively studied to determine the mechanism by which they inhibit CFTR. GlyH-101 and CFTRinh-172 are both potent inhibitors of CFTR, however, GlyH-101 also inhibits the outwardly rectifying chloride conductance channel and calcium dependent chloride conductance, while CFTRinh-172 does not.[70] For these reasons, we chose to use CFTRinh-172 for the present studies.

3.4 Materials and Methods

3.4.1 Buffer Preparation

All buffers were prepared in purified water (DDW, 18.2 MΩ). All reagents were purchased from Sigma Aldrich (St Louis, MO, USA) unless otherwise noted.

3.4.1.1 Physiological Salt Solution (PSS)

Physiological Salt Solution (PSS) contained 4.7 mM KCl (Fisher Scientific), 2.0 mM $CaCl_2$ (Fisher Scientific), 140.5 mM NaCl, 12 mM $MgSO_4$ (Fisher Scientific), 21.0 mM tris(hydroxymethyl) aminomethane (Invitrogen), 5.5 glucose, and 0.5% bovine serum albumin adjusted to a final pH of 7.4.

3.4.1.2 PBS

Phosphate Buffered Saline (PBS) contained 137 mM NaCl, 2.7 mM KCl (Fisher Scientific, Waltham, MA, USA), 10.1 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 (Spectrum Chemical, New Brunswick, NJ, USA).

3.4.2 Purification of C-peptide

Synthesized human C-peptide (~80 % pure) was purchased from Peptide 2.0 (Chantilly, VA, USA), and purified using reverse phase high performance liquid chromatography (RP-HPLC) with a Shimadzu (Columbia, MD, USA) LC-20AB solvent system and a SPD-20AV UV-Vis absorbance detection system with a C18 preparative HPLC column (Grace, Deerfield, IL, USA) (25 cm column length, 10 µm particle size and 10 mm i.d.). C-peptide was purified by gradient elution (Table 3.2). Collected C-peptide peaks were lyophilized overnight and then stored at -20 ºC. Purity of C-peptide was verified by mass spectral analysis (Thermo Scientific LTQ Orbitrap (Thermo, SanJose, CA, USA) equipped with an NanoMate electrospray source (Advion, Ithaca, NY, USA)). C-peptide was purified (Mass Spectra in Figure 3.3) by the author and frozen at -20 °C.

Table 3.2: Gradients and conditions for HPLC purification of C-peptide and its single amino acid mutant E27A. Solvent A contained 0.1% trifluoroacetic acid (TFA) in HPLC-grade water, Solvent B contained 0.089% TFA in 60% acetonitrile in HPLC-grade water. The flow rate was set at 5 mL/min.

C-peptide	
Time (min)	**%B**
0	0
5	40
30	50
40	100
45	100
50	0
55	stop

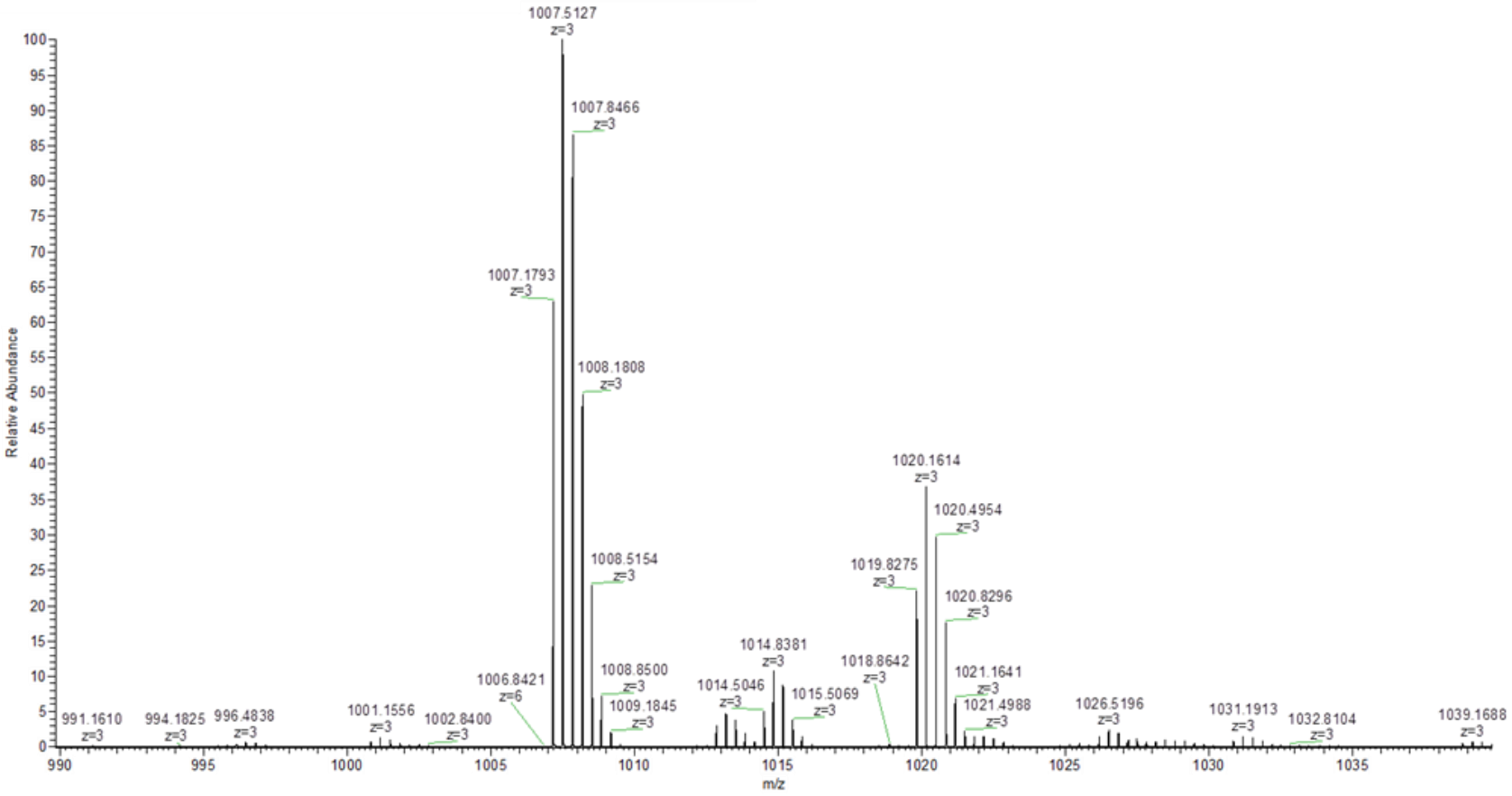

Figure 3.3: Mass Spectrum of purified C-peptide (January 2015). The fragmented peak at 1007.5127 is C-peptide (triply charged) and agrees with previously published data in the Spence group. The peak at 1014.7381 is likely a sodium adduct, and the peak at 1020.1614 is likely a potassium adduct, neither of which will affect C-peptide's activity in *in vitro* experiments.

3.4.3 Isolation of RBCs

Whole blood was collected from healthy, consenting donors into heparinized tubes. Whole blood was centrifuged at 500 *g* for 10 minutes and the buffy coat and plasma were removed by aspiration. The resulting packed RBCs were washed three times in PSS prior to determining the hematocrit of the sample (StatSpin CritSpin, Beckman Coulter Inc., Brea, CA, USA).

3.4.4 Experiment Setup

C-peptide, zinc, and insulin were diluted to working concentrations in DDW and allowed to incubate for 3 minutes at room temperature. PSS was added to the sample followed by purified RBCs of a known hematocrit to create a 7% RBC sample in a total volume of 1 mL.

3.4.5 C-peptide binding studies

Determination of C-peptide binding to RBCs was performed using a commercially available enzyme linked immunosorbent assay (ELISA) kit (Millipore, Billerica, MA, USA). All samples were prepared in PSS. For studies of C-peptide binding in the presence of zinc (Zn^{2+}), zinc was added to the C-peptide in a 1:1 molar ratio 3 minutes prior to the addition of buffer and RBCs. All samples were incubated at 37 °C for one hour. Samples were centrifuged to pellet the cells and the supernatant collected and used in the ELISA-based determination of C-peptide concentrations. An enzymatically-tagged secondary antibody catalyzes a colorimetric reaction that is measured using a standard plate reader at 450 nm (Molecular Devices LLC, Sunnyvale, CA, USA), which is directly proportional to the amount of C-peptide present in the sample. The number

of picomoles of C-peptide taken up by each sample was determined by subtracting the number of moles remaining in the supernatant from the original number of moles added to the 7% RBC sample.

3.4.6 Zinc binding studies

Zinc binding to RBCs was performed using radioisotopic zinc ($^{65}Zn^{2+}$; 5.7 Bq/molar weight, PerkinElmer Inc., Waltham, MA, USA). All samples were prepared in either PSS or albumin-free PSS. For studies of zinc binding to RBCs in the presence of C-peptide, C-peptide was added to the zinc in a 1:1 molar ratio 3 minutes prior to the addition of buffer and RBCs. All samples were incubated at 37 °C for one hour, centrifuged to pellet the cells and the supernatant collected. After addition of 100 µL of scintillation cocktail (PerkinElmer Inc., Waltham, MA, USA), the amount of $^{65}Zn^{2+}$ remaining in the supernatant was measured using a scintillation counter (1450 MicroBeta PLUS, Wallac Inc., Finland). The amount of $^{65}Zn^{2+}$ uptake by the RBCs was determined by subtracting the amount of $^{65}Zn^{2+}$ left in the supernatant from the amount originally added.

3.4.7 Luciferin/Luciferase Assay for ATP

ATP release from RBCs was quantitatively measured using a Luciferin/Luciferase chemiluminesence assay (Figure 3.4). Firefly luciferase (100 mg) is added to 20 mg of D-luciferin (Gold Biotechnology, St. Louis, MO, USA) in 10 mL DDW. Aliquots of this Luciferin/Luciferase mixture are frozen at -20 °C for further use. At the time of experimentation, 10 µL of the Luciferin/Luciferase mixture was added to 100 µL of the

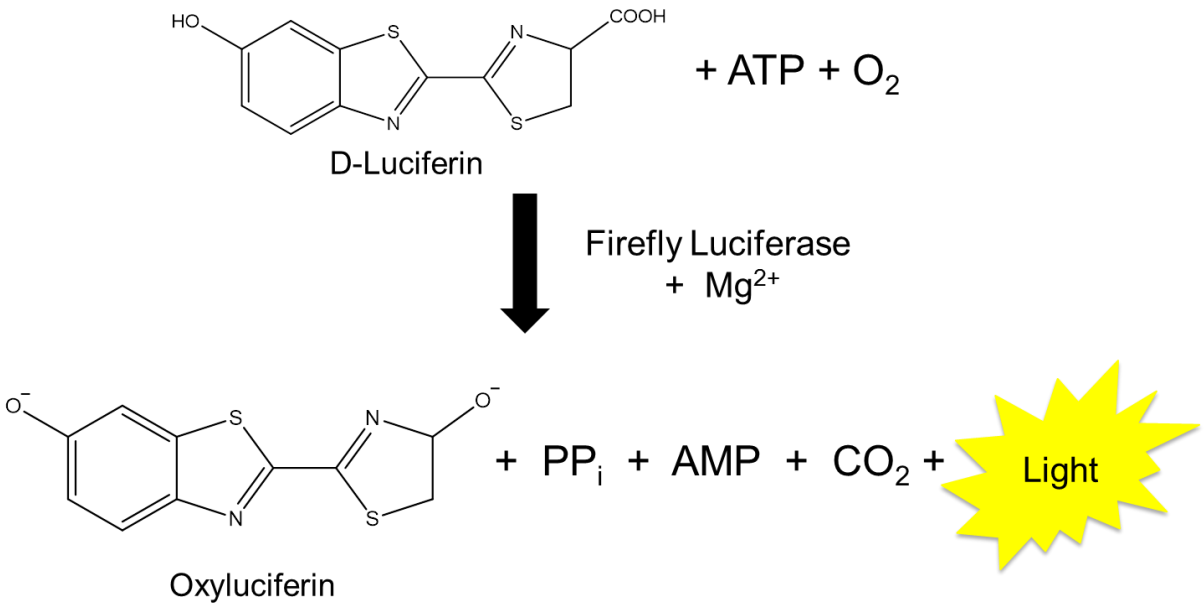

Figure 3.4: Overview of the Luciferin/Luciferase reaction for measuring ATP. D-Luciferin reacts with ATP, oxygen firefly luciferase and magnesium to form oxyluciferin, inorganic phosphate, adenosine monophosphate, carbon dioxide, and light. This resulting light is read at all wavelengths and corresponded to the ATP concentration in the sample based on a standard curve.

sample in a black 96-well plate and placed in the plate reader to be read at all wavelengths.

3.4.8 Determination of Glucose Uptake

RBCs were incubated with PSS containing 11 mM glucose. After pelleting by centrifugation, the supernatant was collected and analyzed for the amount of glucose present using an indirect fluorescent glucose assay. The working reagent contains glucose 6-phosphate dehydrogenase (0.9 U/mL), hexokinase (1.7 U/mL), $NADP^+$ (1.25 mM), $MgCl_2$ (2 mM), and ATP (2 mM) in TEA (triethanolamine, 0.3M) buffer. An equal volume of the working reagent and the sample are incubated in a black 96-well plate for

15 minutes before reading the fluorescence at an excitation of 340 nm and emission of 460 nm and interpreting with a standard curve.

3.4.9 Chemical inhibition of GLUT1

Phloretin (25 mM) was diluted in DDW and added to RBCs at a final concentration of 0.5 mM for 30 minutes prior to incubation with the appropriate reagents for analysis.

3.4.10 Chemical inhibition of CFTR

CFTRinh-172 was purchased from Tocris and diluted in DMSO and then buffer to a working concentration. RBCs were incubated in 1 µM CFTRinh-172 for 30 minutes and then washed to remove excess inhibitor. Cells were then incubated with the appropriate reagents for analysis as previously described.

3.4.11 Stored blood

Whole blood (approximately 7 mL per tube) was collected from consenting donors into non-heparanized tubes containing 1 mL of anticoagulant solution (CPD, CP2D, CPD-N or CP2D-N). After 30 minutes, tubes were centrifuged at 2,000 *g* for 10 minutes. The buffy coat and plasma were removed and packed RBCs were stored in PVC bags in a 2:1 ratio with the appropriate storage solutions (AS-1, AS-3, AS-5, AS-1N, AS-3N, AS-5N). RBCs stored in normoglycemic solutions were fed with 200 mM glucose in 0.9% NaCl every 5 days to maintain extracellular glucose levels at 5.5 mM (~30-50 µL). Cells were removed from the bags on day 0, day 1, week 1, week 2, week 3, week 4, and week 5 for analysis.

3.4.12 RBC Ghosts

RBCs were lysed in lysis buffer (10 mM TrisHCl, 0.2 mM EDTA, pH 7.2) on ice for 30 minutes (50 µL cells in 1.5 mL lysis buffer). Lysates were then centrifuged at 22000 *g*

for 15 minutes at 4 °C and the supernatant was removed. Cells were washed three more times in lysis buffer (22000 *g* for 5 minutes) until all hemoglobin is removed.

3.4.13 SDS-PAGE and Western Blot for GLUT1

3.4.12.1 BCA Assay

Standards were made in a working range (0-1 μg/mL) from a BSA stock of 2 mg/mL. The working reagent was prepared as specified by the company (ThermoFisher Scientific) in a 50:1 ratio (Reagent A:Reagent B). Samples and working reagent were incubated together at 37 °C for 30 minutes and then absorbance was read at 562 nm on a spectrophotometer.

3.4.12.2 Buffers for SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) running buffer contains 192 mM glycine (Invitrogen), 25 mM Tris (Invitrogen), and 3.5 mM SDS. Transfer buffer contains 25 mM Tris (Invitrogen), 192 mM glycine (Invitrogen), and 20% methanol (Macron Fine Chemicals, Center Valley, PA, USA) at pH 8.3. TBS contains 20 mM Tris-HCl (Invitrogen) and 500 mM NaCl. TBST is TBS with 0.05% Tween-20. Bicarbonate buffer contains 0.1 M $NaHCO_3$ (Jade Scientific, Westland, MI, USA) and 5 mM $MgCl_2$. Blocking solution contains TBST with 5% dry milk.

3.4.12.3 Casting of SDS-PAGE

The resolving gel was made by mixing DDW with 1.5 M Tris (pH 8.8, Invitrogen), 30% bis-acrylamide (BioRad), 20% SDS, 10% APS (Ammonium Persulfate), and TEMED (tetramethylethylenediamine). The stacking gel was fixed at 5% acrylamide and was

made by mixing DDW with 0.5 M Tris (pH 6.8, Invitrogen), 30% bis-acrylamide (BioRad), 20% SDS, 10% APS, and TEMED.

3.4.12.4 SDS-PAGE

A 10% SDS gel was cast and 10 μg (total protein) samples loaded with loading dye (1:1 ratio, BioRad) onto the gel. A protein ladder was also loaded onto the gel (Precision Plus Protein All Blue Prestained Protein Standards, BioRad). After successful separation of the proteins via electrophoresis (SDS-PAGE Running buffer, 150 V, 1 hour), one gel was transferred to a PVDF membrane (Thermo Fisher Scientific) on ice overnight at 30 V in transfer buffer. If necessary, a second gel was stained with Coomassie Brilliant Blue R-250 (BioRad, Hercules, CA, USA) and destained with Coomassie Brilliant Blue R-250 Destain Solution (BioRad). Gels were scanned at 600 dpi using a desktop scanner for further analysis.

3.4.12.5 Western Blot

The PVDF membrane was blocked with blocking solution and then washed before incubation with primary (β-actin: mouse monoclonal anti-beta actin antibody; GLUT1: rabbit polyclonal anti-GLUT1 antibody; Abcam, Cambridge, UK) and secondary (Anti-Mouse IgG (whole molecule)−Alkaline Phosphatase antibody produced in goat; Anti-Rabbit IgG (whole molecule)–Alkaline Phosphatase antibody produced in goat; ThermoFisher Scientific) antibodies. Membranes were washed (with TBS or TBST) prior to the addition of each antibody and then again prior to addition of 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) and Nitro blue tetrazolium chloride (NBT) in

bicarbonate buffer. After color development, the blots were washed with water and dried and then were scanned at 600 dpi and analyzed using Image J.

3.4.14 GLUT1 FITC antibody

A FITC-labeled antibody against human GLUT1 was purchased from R&D Systems (Minneapolis, MN, USA). RBCs were isolated as previously described and treated with appropriate combinations of C-peptide, zinc, and insulin. RBC ghosts were made as previously described. Cells were pelleted and then incubated with 5 μL of the antibody (stock 0.5 g/mL in PBS) for 30 minutes on ice. Cells were washed three times in ice cold PBS prior to reading the fluorescence of the FITC-labeled antibody (excitation: 485, emission: 515). All samples were compared to the control for a relative amount of GLUT1 on the plasma membrane of the RBCs.

3.4.15 Statistical Analysis

All data was compared by Student's t-test and expressed as mean ± standard error of the mean (SEM) and $p < 0.05$ was considered statistically significant.

3.3 Results

Here, we report 2.14 ± 0.14 picomoles of uptake to RBCs in the absence of zinc, and 2.29 ± 0.49 picomoles uptake to RBCs in the presence of zinc (Figure 3.5). This data agrees with previously reported data, which was carried out by previous students in the laboratory. Additionally, there is no statistically significant difference between C-peptide binding in the presence or absence of zinc. Zinc binding in the presence of albumin and the absence of C-peptide is essentially zero, while binding in the presence of C-peptide is reported at 2.65 ± 0.43 picomoles, agreeing with previously published data (Figure

3.6). In the presence of CFTRinh-172, RBCs bind significantly more C-peptide than the control in both the presence and absence of zinc, although there is not a statistically significant difference between the two inhibited samples (Figure 3.7). In the absence of zinc, CFTR-inhibited RBCs bind 3.23 ± 0.33 picomoles, while in the presence of zinc, they bind 2.99 ± 0.09 picomoles. Zinc binding is greater than the control in CFTR-inhibited RBCs when they are incubated with C-peptide (Figure 3.8). In the absence of C-peptide, binding is still essentially zero, however, in the presence of C-peptide binding is reported here at 2.88 ± 0.25 picomoles.

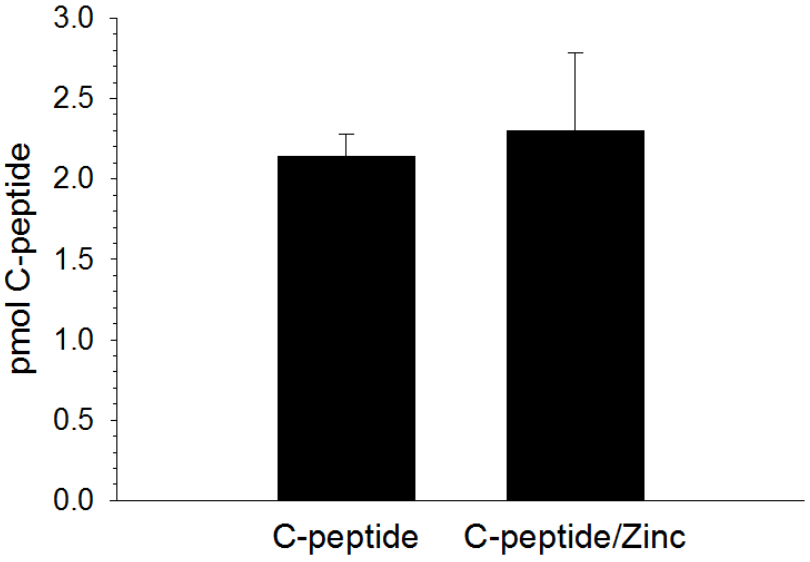

Figure 3.5: C-peptide binding in 7% RBCs. In the absence of zinc, approximately 2 picomoles of C-peptide bind to RBCs, which agrees with previously published data in the Spence group. In the presence of zinc, there is not a statistically significant difference from RBCs incubated with C-peptide alone. n=5, error=SEM

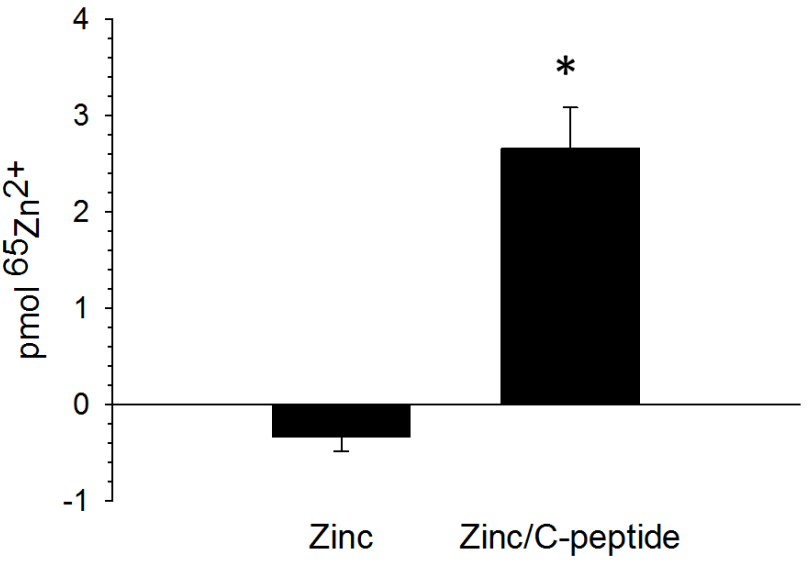

Figure 3.6: Zinc binding in 7% RBCs. In the absence of C-peptide, zinc does not bind to RBCs, which agrees with previously published data in the Spence group. In the presence of C-peptide, there is a statistically significant difference from RBCs incubated with zinc alone (approximately 2.5 pmol). n=5, error=SEM, *p<0.05

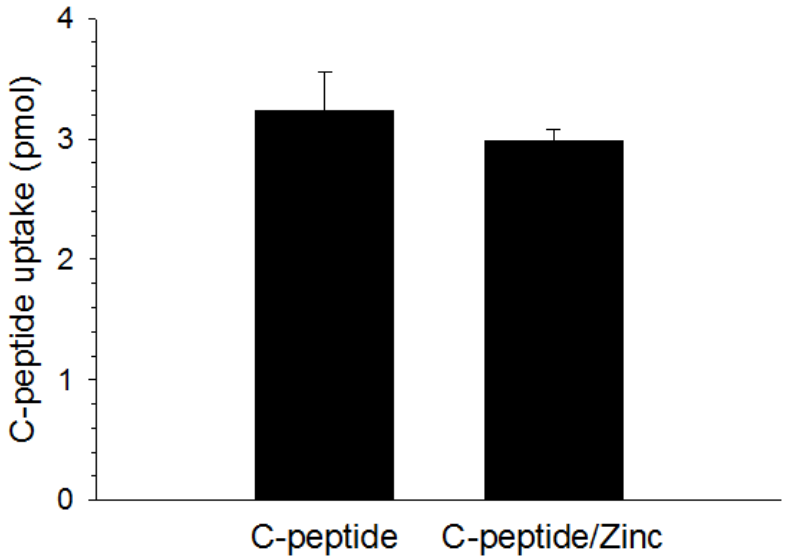

Figure 3.7: C-peptide binding in 7% RBCs incubated with CFTRinh-172. In the absence of zinc, approximately 3 picomoles of C-peptide bind to RBCs, which is significantly higher than in control RBCs. In the presence of zinc, there is not a statistically significant difference from RBCs incubated with C-peptide alone. n=5, error=SEM

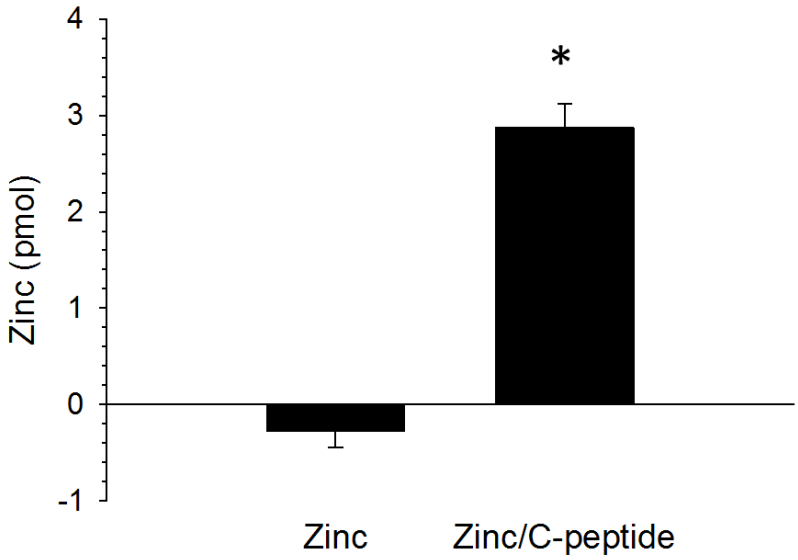

Figure 3.8: Zinc binding in 7% RBCs incubated with CFTRinh-172. In the absence of C-peptide, zinc does not bind to RBCs, which agrees with previously published data in the Spence group. In the presence of C-peptide, there is a statistically significant difference from RBCs incubated with zinc alone (approximately 3 pmol), which is significantly higher than control RBCs. n=5, error=SEM, *p<0.05

Static ATP release from a 7% solution of RBCs is at a basal level of 52.12 ± 5.60 nM in the control sample (Figure 3.9). Samples treated with C-peptide or zinc alone do not show a significant increase from the control, while samples treated with C-peptide and zinc together in albumin show a significant increase to 99.40 ± 7.83 nM. Similarly, glucose uptake in untreated RBCs is not significantly different between samples treated with C-peptide or zinc alone in albumin (Figure 3.10). Only in the presence of C-peptide and zinc in albumin is there a significant increase in glucose uptake. Static ATP release from RBCs incubated with CFTRinh-172 show a significant difference from the control, only releasing 34.75 ± 3.86 nM ATP (Figure 3.11). Incubation of CFTR-inhibited RBCs with C-peptide alone, zinc alone, or C-peptide and zinc together in albumin significantly

increases the ATP release, but only to the basal level of the control at about 50 nM. Glucose uptake in CFTR-inhibited cells (Figure 3.12) is not significantly different from the control, and only increases significantly when incubated with C-peptide and zinc together.

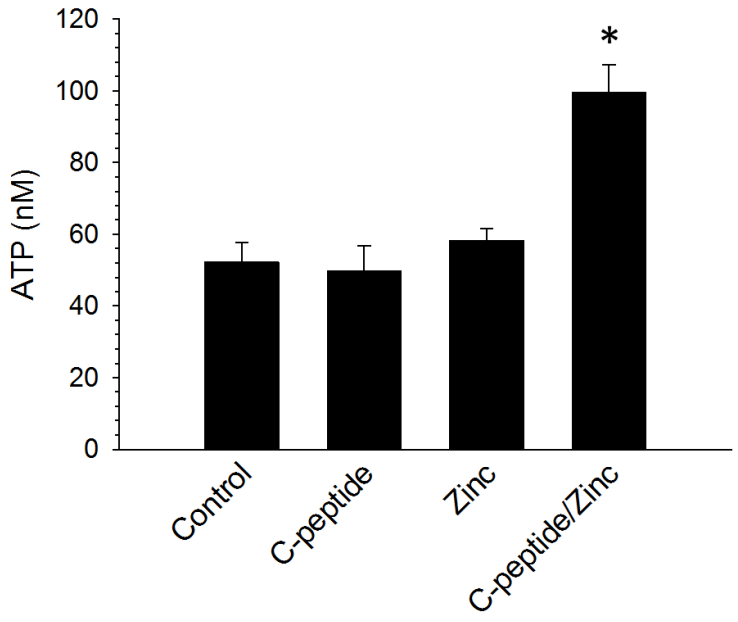

Figure 3.9: Static ATP release from 7% RBCs. Static ATP release from 7% RBCs incubated with nothing (control), C-peptide alone, zinc alone, or C-peptide and zinc together in PSS. Static ATP increases significantly in the presence of both C-peptide and zinc. n=5, error=SEM, *p<0.05

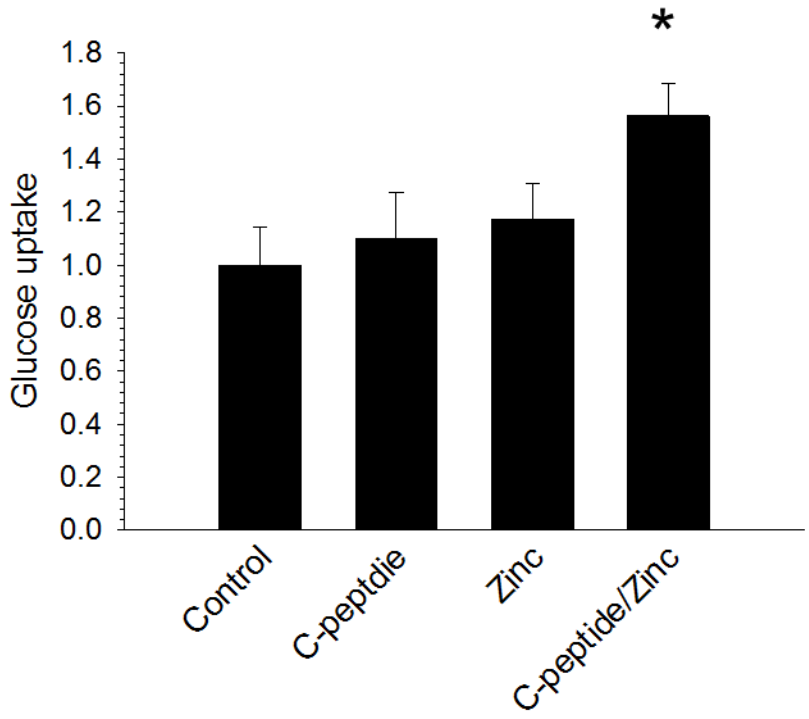

Figure 3.10: Glucose uptake by 7% RBCs. Glucose uptake by 7% RBCs incubated with nothing (control), C-peptide alone, zinc alone, or C-peptide and zinc together in PSS. Glucose uptake increases significantly in the presence of both C-peptide and zinc. n=4, error=SEM, *p<0.05

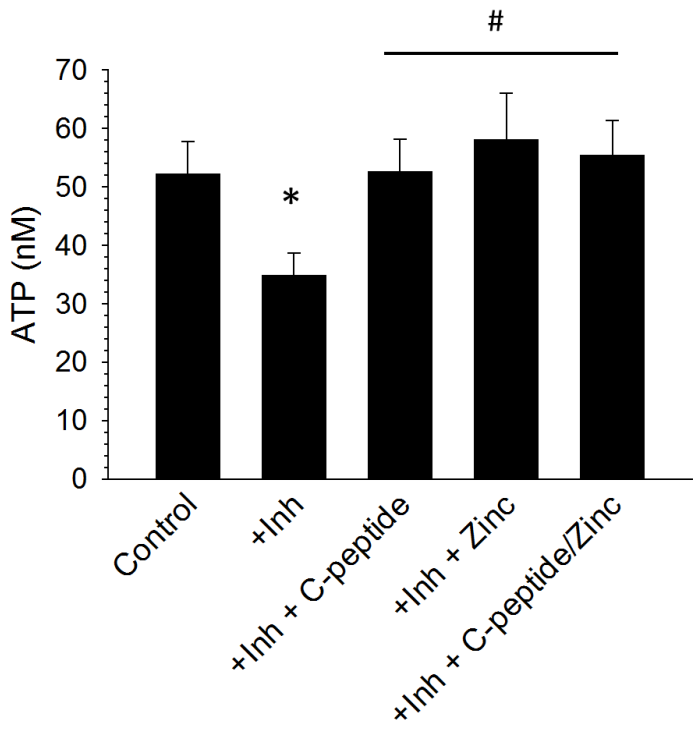

Figure 3.11: Static ATP release from 7% RBCs incubated with CFTRinh-172. Static ATP release from 7% RBCs incubated with CFTRinh-172 and nothing (control), C-peptide alone, zinc alone, or C-peptide and zinc together in PSS. Static ATP increases significantly in the presence of both C-peptide and zinc alone as well as together. n=5, error=SEM, *p<0.05 from control, #p<0.05 from the inhibitor

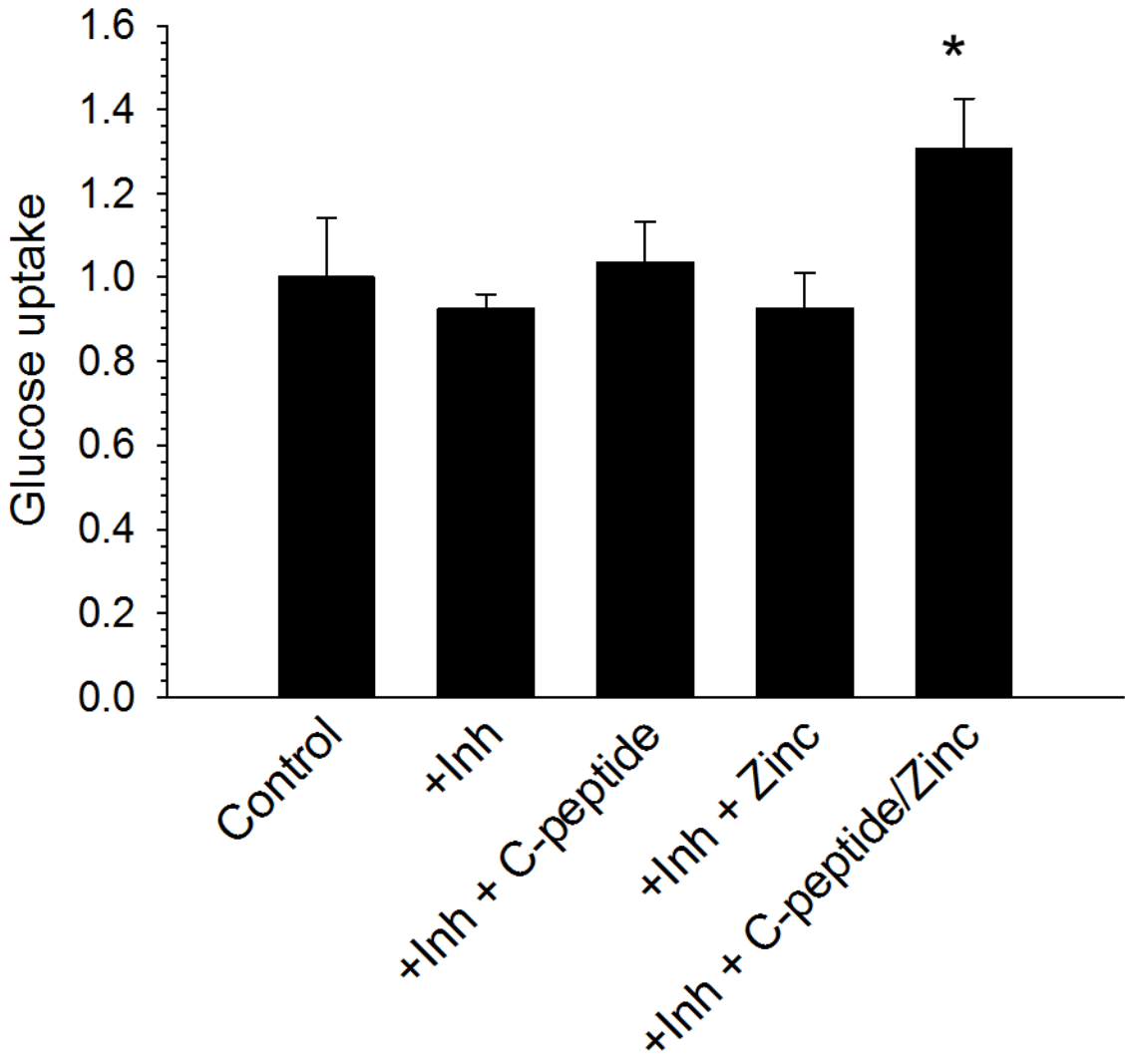

Figure 3.12: Glucose uptake by 7% RBCs incubated with CFTRinh-172. Glucose uptake by 7% RBCs incubated with CFTRinh-172 and nothing (control), C-peptide alone, zinc alone, or C-peptide and zinc together in PSS. Glucose uptake increases significantly in the presence of both C-peptide and zinc. n=4, error=SEM, *p<0.05

GLUT1 protein expression on the plasma membrane of RBC ghosts was measured by two different methods, as outlined previously. For SDS-PAGE and Western blot results, data is presented as the normalized value of the GLUT1 band to the β-actin band (Figure 3.13). RBC ghosts only show a significant increase in RBC GLUT1 protein expression in the presence of C-peptide and zinc together (Control: 0.99 ± 0.14; C-peptide and zinc: 1.77 ± 0.26). The second method of measuring plasma membrane GLUT1 used the FITC-labeled antibody against human GLUT1, and results are presented as a normalized value to the control (Figure 3.14). Results from this method agree with the SDS-PAGE data (Control: 1.0 ± 0.079; C-peptide and zinc: 1.76 ± 0.033). Additionally, this data shows that GLUT1 is not upregulated in the presence of C-peptide, zinc or insulin alone, C-peptide and insulin together, zinc and insulin together, or insulin stripped of zinc (insulin and EDTA). C-peptide and zinc (with or without insulin) is the only combination that significantly increases the amount of GLUT1 on the plasma membrane of RBC ghosts.

RBCs incubated with CFTRinh-172 exhibit significantly more GLUT1 on their plasma membranes (1.56 ± 0.16 by SDS-PAGE and Western blot; 1.34 ± 0.033 by FITC-labeled antibody, Figures 3.15 and 3.16). CFTR-inhibited RBCs treated with C-peptide or zinc alone do not show a significant difference from those incubated with the inhibitor alone by SDS-PAGE and Western blot or the FITC-labeled antibody against GLUT1. Only in the presence of C-peptide and zinc together (with or without insulin) is there a significant increase in the amount of GLUT1 on the plasma membrane of CFTR-inhibited RBCs.

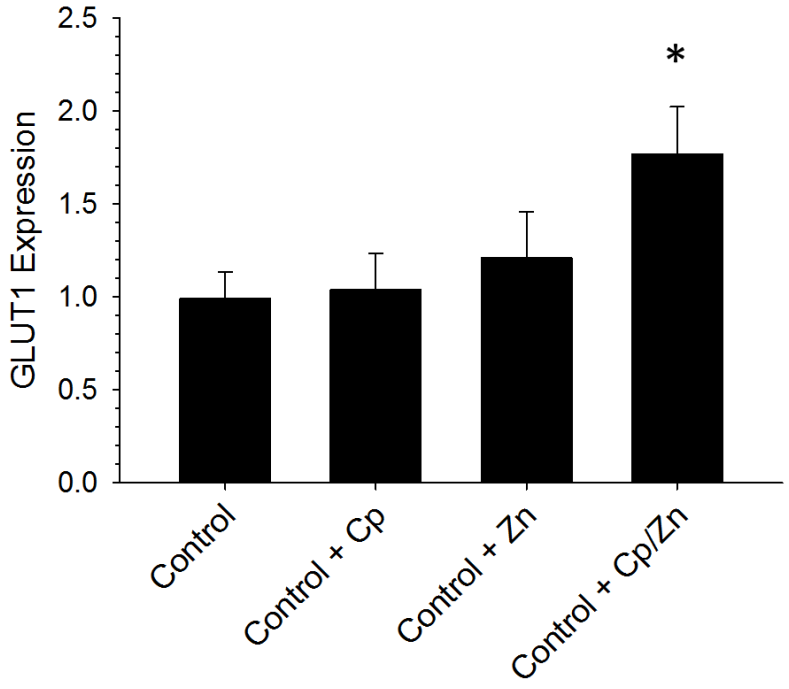

Figure 3.13: GLUT1 protein expression on the plasma membrane of RBC ghosts as determined by SDS-PAGE and Western blot. There is no significant difference between the control, C-peptide alone, or zinc alone; however, when C-peptide and zinc together are incubated with RBCs, GLUT1 protein expression is significantly upregulated. n=7, error=SEM, *p<0.05

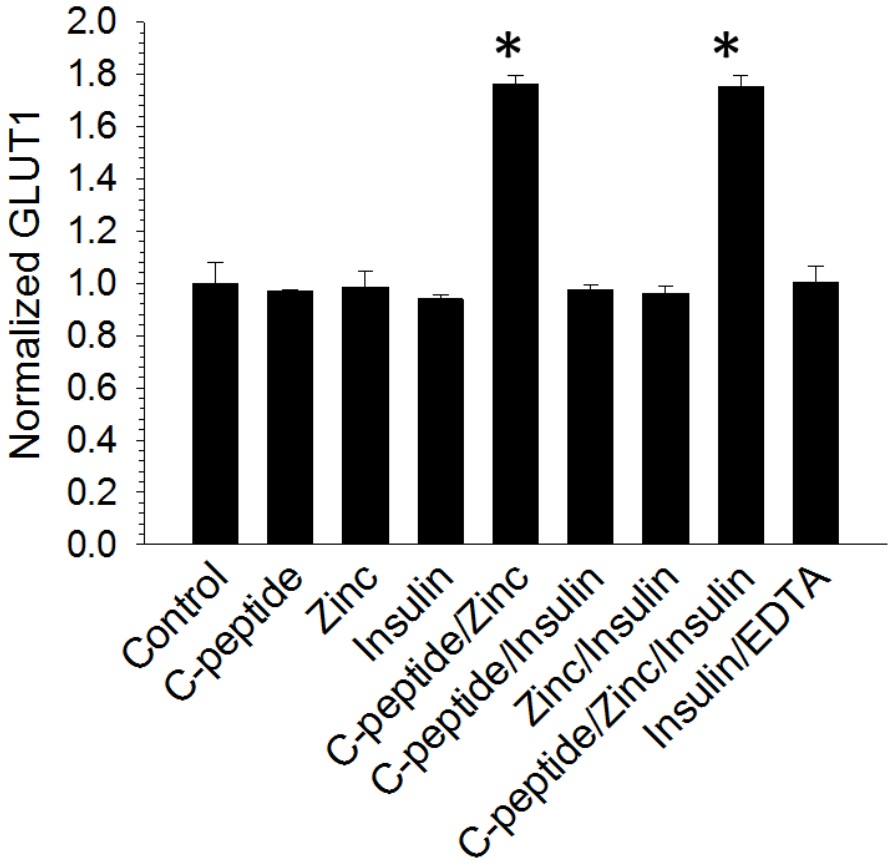

Figure 3.14: GLUT1 protein expression on the plasma membrane of RBC ghosts as determined by FITC-labeled antibody against GLUT1. There is no significant difference between the control, C-peptide alone, zinc alone, insulin alone, C-peptide/insulin, zinc/insulin, or insulin/EDTA; however, when C-peptide and zinc together are incubated with RBCs (with or without insulin), GLUT1 protein expression is significantly upregulated. n=3, error=SEM, *p<0.05

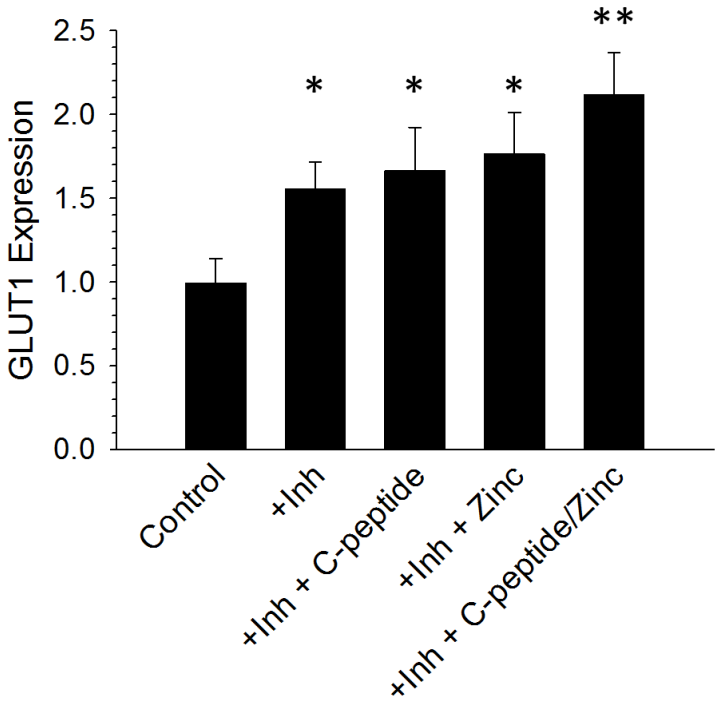

Figure 3.15: GLUT1 protein expression on the plasma membrane of RBC ghosts incubated with CFTRinh-172 as determined by SDS-PAGE and Western blot. There is no significant difference between the control, C-peptide alone, or zinc alone; however, when C-peptide and zinc together are incubated with RBCs, GLUT1 protein expression is significantly upregulated. n=7, error=SEM, *p<0.05 from the control, **p<0.05 from the inhibitor

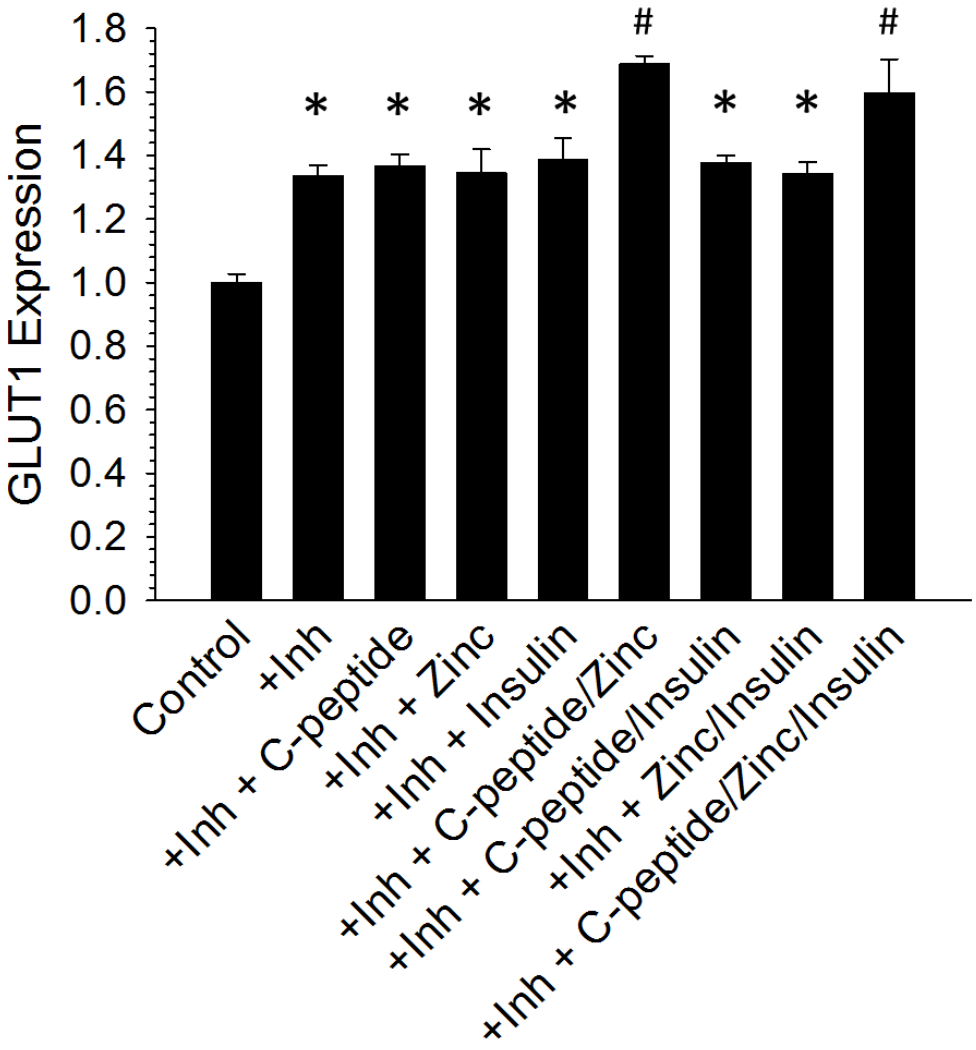

Figure 3.16: GLUT1 protein expression on the plasma membrane of RBC ghosts incubated with CFTRinh-172 as determined by FITC-labeled antibody against GLUT1. There is no significant difference between the control, C-peptide alone, zinc alone, insulin alone, C-peptide/insulin, zinc/insulin, or insulin/EDTA; however, when C-peptide and zinc together are incubated with RBCs (with or without insulin), GLUT1 protein expression is significantly upregulated. n=3, error=SEM, *p<0.05 compared to the control, # p<0.05 compared to inhibitor.

In stored RBCs, RBC ghosts show upregulation of plasma membrane GLUT1 in both AS-1 and AS-5 (Figure 3.17). In AS-1, RBCs show significantly upregulated GLUT1 protein expression as compared to those stored in AS-1N at and after week 3. In AS-3, there is no statistically significant difference from those cells stored in AS-3 or AS-3N. In AS-5, RBCs stored in hyperglycemic storage solutions show a significant increase from those stored in AS-5N at and after 2 weeks of storage.

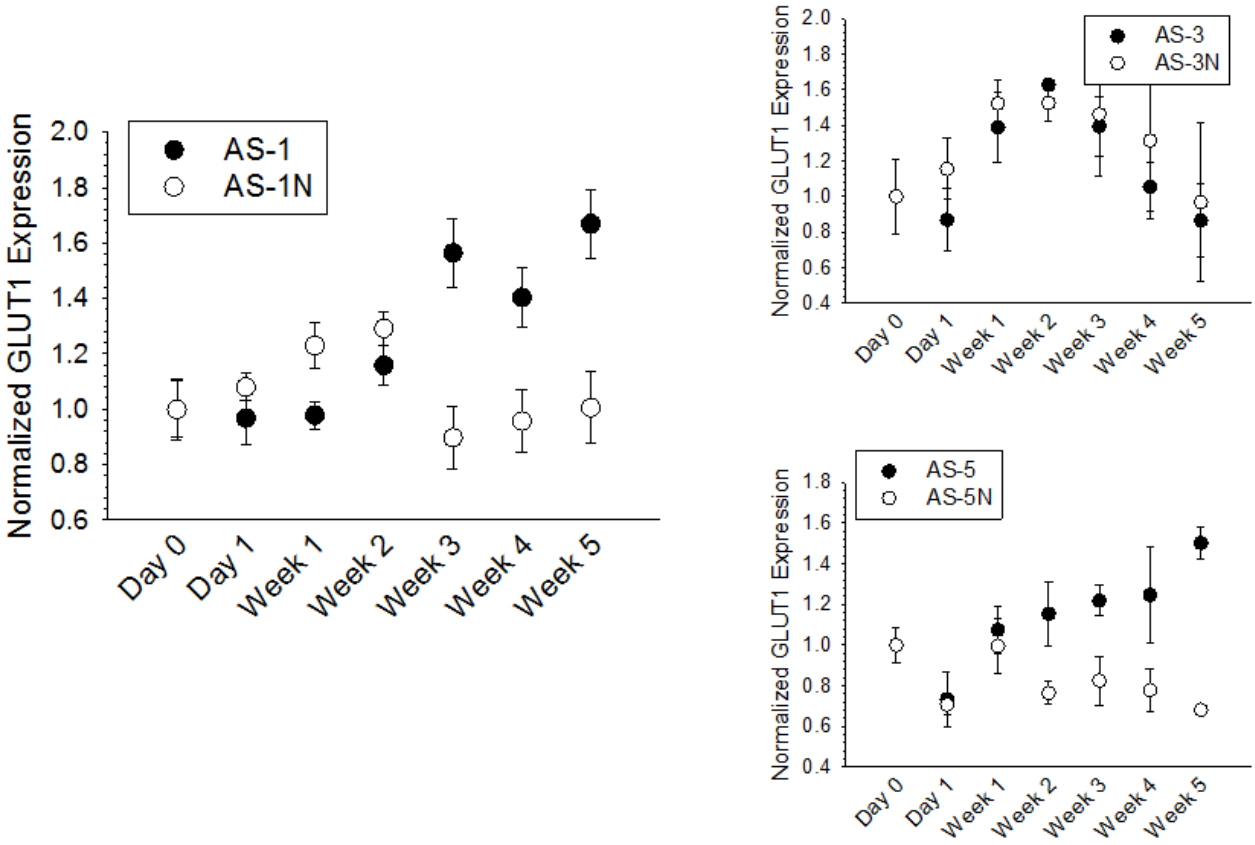

Figure 3.17: Stored RBC GLUT1 protein expression. GLUT1 protein expression on the plasma membrane of stored RBC ghosts as determined by SDS-PAGE and Western blot. n=7 (AS1), 3 (AS3), 5 (AS5)

GLUT1 membrane protein expression from control patients give GLUT1 normalized values of 1.00 ± 0.12 (SDS-PAGE and Western blot analysis), while GLUT1 membrane protein expression from patients with other neurological disorders, such as blindness, leukodystrophy, myasthenia gravis, and Parkinson's disease, show 1.08 ± 0.047 GLUT1 levels. MS patients have higher levels of plasma membrane GLUT1 at 1.34 ± 0.045 (Figure 3.18).

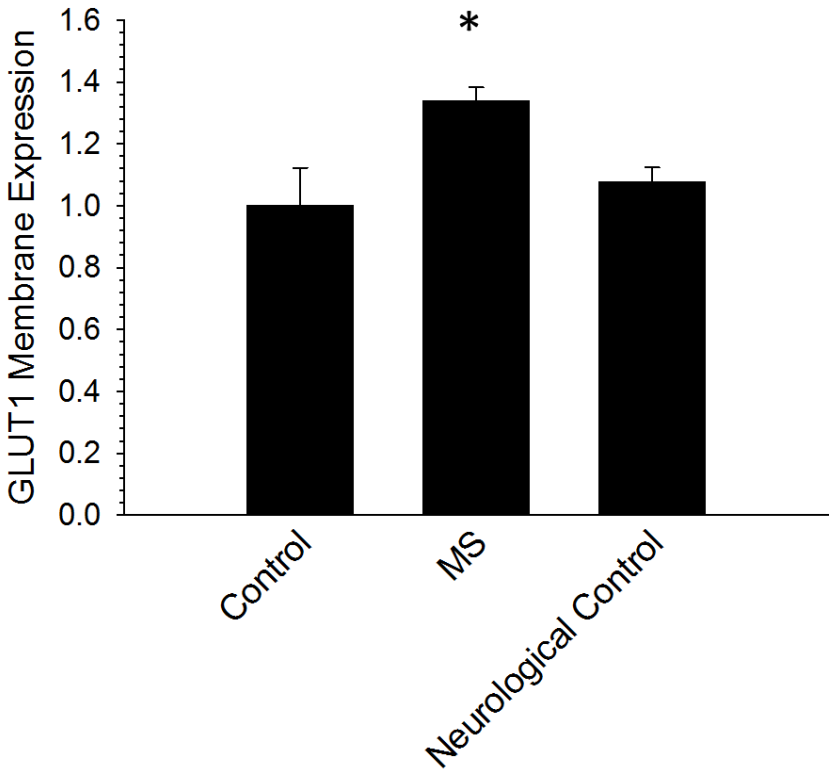

Figure 3.18: GLUT1 protein expression on the plasma membrane of RBC ghosts. Control patients (n=44), patients with MS (n=55) and patients with other neurological disorders (n=67). MS patients have significantly more GLUT1 on the plasma membrane of their RBC ghosts than controls and neurological controls. error=SEM, *p<0.05

3.6 Discussion

Previously reported uptake of C-peptide to a 7% RBC solution is about 2 picomoles in the absence of zinc, and 2.2 picomoles in the presence of zinc, which is statistically equal when taking into account the associated error.[41] Here, we report values that agree with these reports at about 2 picomoles in the absence of zinc and about 2.3 picomoles in the presence of zinc (Figure 3.5 and 3.6). Zinc uptake in the presence of albumin but the absence of C-peptide is essentially zero, while uptake in the presence of C-peptide is reported at about 2.5 picomoles, agreeing with previously published data.[41] Previously reported C-peptide and zinc uptake data were performed by two previous students in the laboratory, Wathsala Medawala (C-peptide, 2007-2010) and Suzanne Summers (zinc, 2008-2012). The author repeated these studies for consistency and accuracy with new reagents (2012-2015). In the presence of CFTRinh-172, C-peptide uptake and zinc uptake are increased as compared to control cells, but uphold the 1:1 molar ratio seen in control cells. This could be due to a larger amount of C-peptide receptors on the CFTR-inhibited cell surface, or due to a faster turnover of C-peptide and zinc in the CFTR-inhibited cell.

Static ATP release from RBCs alone is not significantly different from RBCs incubated with C-peptide or zinc alone, which is consistent with previously reported results in both static and circulating systems.[41] Additionally, glucose uptake in control RBCs corresponds with an increase in ATP release. Approximately 70% more glucose is utilized by RBCs that have been treated with C-peptide and zinc, which results in an 80% increase of ATP release as compared to the control ATP release from CFTR-inhibited cells exhibited significantly less ATP release than controls, which agrees with

previous data from Sprague *et al*.[5] Sprague's results showed a 65% decrease in ATP release in RBCs isolated form CF patients, a 50% decrease in those RBCs incubated with niflumic acid, and a 45% decrease in those RBCs incubated with glibenclamide. Here, we show approximately a 40% decrease in ATP release from RBCs incubated with CFTRinh-172. In the presence of C-peptide alone, zinc alone, or C-peptide and zinc together, CFTR-inhibited cells release statistically the same amount of ATP as control cells. Glucose uptake again corresponds to the increase in ATP release from CFTR-inhibited cells (about 30% increase in glucose uptake corresponds to a 30% increase in ATP release); however, this correlation only occurs when C-peptide and zinc are added together to the cells. One would expect that ATP release would increase significantly from the control when the inhibitor is present, as occurs in the control cells. However, this is not the case. ATP release increases significantly from the control in CFTR-inhibited RBCs that have been treated with only C-peptide or only zinc, which may indicate that the mechanism of ATP release from CFTR-inhibited RBCs is rescued upon only the binding of one of the two necessary players. However, the increase in the amount of glucose uptaken does correspond to the ATP release from these cells, which suggests that result is accurate.

In control cells, only the binding of C-peptide and zinc together induces an increase in ATP release, however, in CFTR-inhibited cells, C-peptide and zinc alone increase ATP release from these cells. One might expect that the increase in ATP release would be even greater in the presence of both C-peptide and zinc, but this is not the case. Additionally, glucose uptake is only increased in CFTR-inhibited cells in the presence of both C-peptide and zinc. Due to the fact that CFTR is involved in the mechanism of

ATP release, this data would suggest that it is not that C-peptide and zinc alone have a different mechanism of action in CFTR-inhibited cells; rather, that only in the presence of C-peptide and zinc together are these cells able to correctly utilize glucose, and that some part of the ATP release process is still impaired, even in the presence of C-peptide and zinc.

In addition to glucose uptake and ATP release, we show evidence here by two different methods that C-peptide and zinc together with albumin are able to increase the GLUT1 protein expression on the plasma membrane of RBCs. In control cells, C-peptide alone and zinc alone have no significant effect on GLUT1 protein expression, however, in the presence of C-peptide and zinc together, GLUT1 protein expression is increased by approximately 75%. Insulin has no effect on the translocation of GLUT1, and both methods indicate that it is not the zinc that may be contaminating the insulin that is responsible for this increase in protein expression.

In CFTR-inhibited RBCs, we show evidence that basal levels of GLUT1 are approximately 50% higher than in control patients, and that this protein expression is significantly increased when cells are treated with C-peptide and zinc together. Although CFTR-inhibited cells exhibit higher basal levels of GLUT1, protein expression levels only increase to the same level as the control cells (approximately 1.75 times the control) treated with C-peptide and zinc. Higher basal expression of GLUT1 has been shown in neutrophils from patients with CF,[71] but not in RBCs to the author's knowledge.

Here we report for the first time a mechanism by which C-peptide and zinc may participate in a signaling pathway in RBCs to promote glucose utilization and ATP release. It has previously been shown that intracellular C-peptide signaling leads to an elevation of intracellular calcium concentrations,[37, 72] phosphorylation of phospholipase C (PLC) and protein kinase C (PKC) isoforms,[73-75] phosphoinositide 3-kinase (PI3K) activation,[75, 76] and mitogen activated protein kinase (MAPK) signaling.[73, 74] These signals, in conjunction with the data presented here for the translocation of GLUT1, provide a compelling argument that C-peptide and zinc together may be acting in a manner similar to the way that insulin acts in GLUT4-containing cells, such as adipose and muscle cells.

As shown in Figure 3.19, the probable C-peptide receptor, GPR146, signals through $G_{\alpha i}$ to activate PLC and PI3-K. PI3-K goes on to activate transcription factors, while PLC catalyzes the conversion of PIP2 to DAG and IP3. DAG activates PKC, which, in insulin signaling, causes the translocation of GLUT1 to the plamsa membrane.

The increased level of GLUT1 in CFTR-inhibited RBCs, and their increase in C-peptide and zinc binding led us to explore if other diseased states exhibited the possibility of a similar mechanism. Previously, we had discovered that RBCs from MS patients had increased ATP release from RBCs, and subsequently discovered that they also bound significantly more C-peptide than control cells. Here we report that RBCs from MS patients have a basal level of GLUT1 that is approximately 30% higher than control cells.

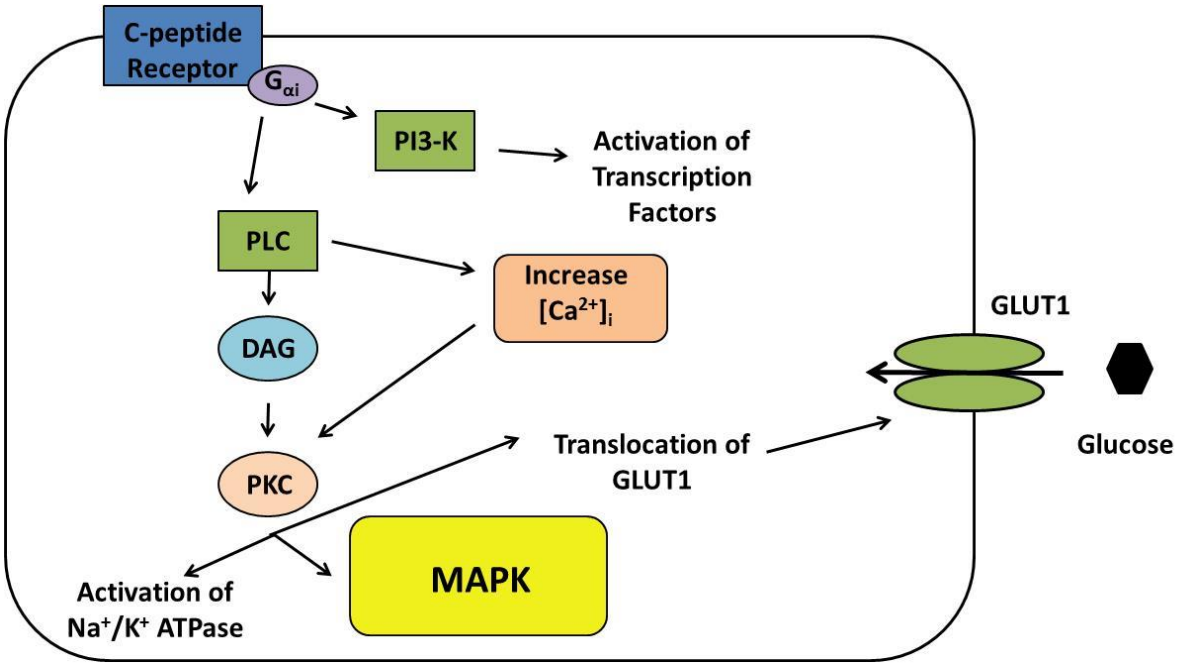

Figure 3.19: Proposed mechanism for C-peptide and zinc signaling in RBCs. The probable C-peptide receptor, GPR146, signals through Gαi to activate PLC and PI3-K. PI3-K goes on to activate transcription factors, while PLC catalyzes the conversion of PIP2 to DAG and IP3. DAG activates PKC, which, in insulin signaling, causes the translocation of GLUT1 to the plamsa membrane.

This increase in the basal level of GLUT1 in these cells is slightly lower than that of CFTR-inhibited cells; however, one would expect that GLUT1 translocation should only increase to approximately 1.75 times the amount present in control cells when treated with C-peptide and zinc. Although C-peptide and zinc are not likely to be used to treat MS symptoms or disease progression, there are treatments that affect GLUT1 levels that are utilized today: steroids. Steroids, such as prednisone, while generally effective in reducing inflammation, have serious side effects, including hyperglycemia.[47] Steroids have been shown to inhibit glucose transport in RBCs,[77] which would facilitate a hyperglycemic environment in the bloodstream. This would likely increase

the amount of GLUT1 at the plasma membrane of RBCs, facilitating an increase in glucose utilization and therefore an increase in ATP release.

Although it is unknown why MS and CF patients have higher basal levels of RBC GLUT1 protein expression, it is clear to us that this is in some way related to the study of C-peptide binding as a biomarker for MS[49] as well as insight into a mechanism by which glucose intolerance may play a role in both MS[78] and CF.[79]

An ideal environment for studying the effect of prolonged hyperglycemia on RBCs is through stored blood. As previously mentioned, the current FDA-approved storage solutions for RBCs are significantly higher in glucose levels (~40 mM) than in healthy humans (4-6 mM) or in patients with diabetes (7-9 mM). In the present research, we studied three different storage solution combinations and their normoglycemic counterparts: CPD/AS-1, CP2D/AS-3, and CPD/AS-5. RBCs stored in CPD/AS-1 exhibited higher levels of GLUT1 after three weeks of storage, while RBCs store in CPD/AS-5 exhibited higher levels of GLUT1 after only two weeks of storage. RBCs stored in CP2D/AS-3 were not significantly different from those stored in normoglycemic solutions at any time. A possible hypothesis for this difference is the addition of sodium citrate, citric acid, and monosodium phosphate, which are typically buffer and anticoagulant compounds, and the absence of mannitol. However, even though GLUT1 levels are similar to those stored in normoglycemic conditions, these stored RBCs still lose their ability to release the same amount of ATP as those stored in normoglycemic solutions.[80]

Taken together, the data presented here suggest a novel mechanism for the signaling of C-peptide and zinc that promotes GLUT1 translocation in cells unaffected by insulin. Additionally, we have shown that C-peptide and zinc together are able to positively affect CFTR-inhibited RBCs. This is essential in the treatment of CF and CFRD due to the deficiencies in RBC ATP release that is most likely compounded in the hyperglycemic environment that is present in CFRD. This research and other research on NO release resulting from RBC-derived ATP give a potential therapeutic for microvascular complications in CFRD and in T1D.

Additionally, we show abnormal levels of GLUT1 present in CFTR-inhibited RBCs and RBCs from MS patients, as well as RBCs stored in hyperglycemic environments, indicating that this transporter and its response to pancreatic secretions is essential in maintaining correct communication between the pancreatic system and the circulatory system. In CF, this is particularly important because of the fibrosis of the pancreas already present, and the development of CFRD.

It is clear that C-peptide and zinc are necessary secretions from the pancreas, and that they act on cells that do not respond to insulin. This discovery, along with the proposed signaling mechanism for translocation of GLUT1 in RBCs, similar to that seen in adipose and muscle cells with insulin signaling and GLUT4, provides a platform for further study, including how this combination may be utilized as a supplemental therapy in diabetes treatment.

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CHAPTER 4

THE EFFECT OF PANCREATIC SECRETIONS ON PERIPHERAL NEUTROPHILS

4.1 Neutrophils

Peripheral neutrophils are the most common white blood cell in the bloodstream, and are the first to respond to inflammatory signals within the body. Upon recognition of an inflammatory signal within a tissue, neutrophils adhere and roll along the endothelium prior to transmigration across endothelial cells toward the tissue, a process known as chemotaxis. Transient, rapid increases of intracellular calcium levels ($[Ca^{2+}]_i$) are required for migration of neutrophils to the site of inflammation.[1-4] Neutrophil function requires energy,[5] which is generated mainly by metabolism of glucose to lactate,[6, 7] while only 2-3% of glucose is oxidized through the citric acid cycle in neutrophils[8, 9]. Neutrophils also utilize glutamine at high rates, which is made into glutamate, aspartate, lactate, and CO_2.[10]

Neutrophils are a component of the innate immune system that are activated in response to inflammatory signals such as chemokines, complement factors, or chemoattractants. While in the blood vessels, neutrophils sense these chemotactic gradients and then begin their journey by attachment to the endothelial cells (Figure 4.1). Adherence of the neutrophils to the endothelium requires neutrophil interaction with P-selectin, a cell adhesion molecule on the surface of activated endothelial cells.

Increase in adhesion causes the neutrophil to flatten out and then begin the process of diapedesis, where they squeeze through the gaps in the endothelial cells.[11] This

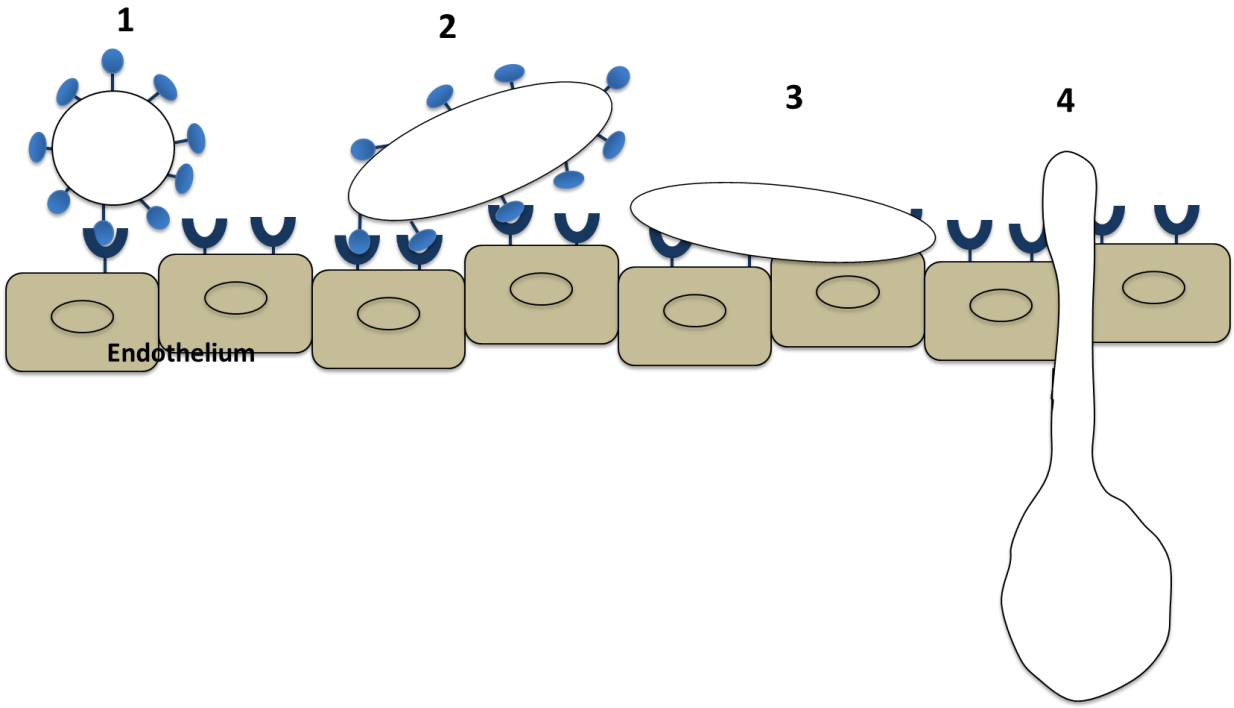


Figure 4.1: Mechanisms of neutrophil action. Mechanism of neutrophil adhesion and rolling to P-selectin (1), multiple adheisons that cause the neutrophil to begin flattening out (2), loss of selectins and flattening out along the endothelium (3), and diapedesis (4).

process can be paracellular (between endothelial cells) or transcellular (through endothelial cells).[12] After passing through the endothelium, chemoattractants draw the neutrophils to the site of inflammation or infection via chemotaxis.[11]

When neutrophils sense a chemoattractant, they lose their spherical shape and develop a polarity with an elongated shape with a pseudopod at the front and a bulbous uropod at the rear (Figure 4.2). At the front, an actin network aids in pushing out the pseudopod, where Cdc42 and Rac are active, while the uropod has a contraction/pulling

network of actin/myosin II, where Rho is active. The microtubules are aligned in the direction of movement and stabilize the cell polarity through a microtubule organizing center. Migrating neutrophils have a standing gradient of Ca^{2+}, where levels are high at the back and low at the front.[11]

One of the major chemoattractants is n-formylmethionylleucinylphenylalanine (fMLP), which is released during bacterial lysis. G protein-coupled receptors (acting through heterotrimeric G proteins) for fMLP are distributed equally over the surface of the cell, and when the pseudopod senses just 1-2% higher concentrations than the uropod, neutrophils are able to move towards the source of the gradient. Neutrophils also develop a localized gradient of ATP at the front of the cell. Hemichannels in the pseudopod release ATP, some of which is converted into adenosine by CD39 and CD73. Both ATP and adenosine feed back in an autocrine manner (P2Y2 and A3 receptors)[13] to contribute to chemotaxis by amplifying the polarity signaling. ATP acts through P2Y2 receptors to contribute to gradient sensing, while adenosine acts through A3 receptors to enhance cell motility.[11] Calprotectin is found in the cytosol of neutrophils and is a regulatory factor for the inflammatory process.[14]

Two types of granules exist in neutrophils: specific and azurophilic. Azurophilic granules contain microbicidal enzymes, proteases, acid hydrolases and bactericidal cationic proteins, while specific granules are produced during neutrophil maturation.[15] Specific granules are less dense and contain lysozyme, collagenase, lactoferrin, and histaminase,[16-18] as well as a flavoprotein important in the formation of NADPH oxidase enzyme that produces the toxic oxygen byproducts that are important in cytotoxic killing.

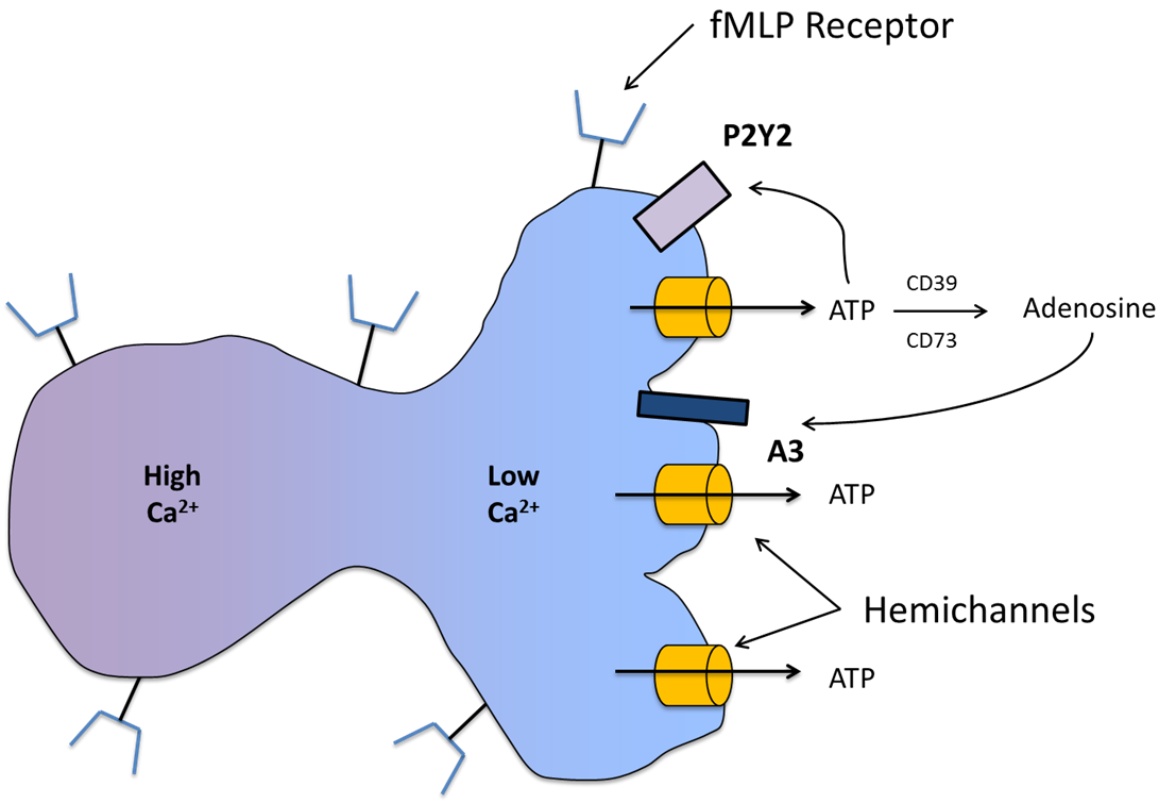


Figure 4.2: The polarization of neutrophils in the presence of a chemoattractant. At the back, the uropod has high calcium levels and an active actin/myosin II network. At the front, the pseudopod has a low calcium level and an active actin network. At the front of the cell, hemichannels release ATP, which can be converted to adenosine by CD39 and CD73. Adenosine and ATP both feed back in an autocrine manner to contribute to chemotaxis by amplifying the polarity signaling.

The exocytosis of granules is prompted by chemoattractants, where at the site of inflammation, phagocytosis and internal digestion of microorganisms occurs. The contents of granules assist in cytotoxic killing. For example, lactoferrin has antimicrobial functions through iron chelation[21] and hydroxyl radical formation.[22] Lysozyme digests glycopeptide debris[23] and hydrolyzes bacterial cell walls.[24]

Bactericidal activity of neutrophils depends on the release of these granule contents as well as the generation of ROS by assembly and activation of NADPH-dependent oxidase (Figure 4.3). The respiratory burst, or the activation of oxidative metabolism, involves the assembly of NADPH oxidase at the membrane, which generates the superoxide anion, which dismutates into hydrogen peroxide through three intermediates: O^{2-}, H_2O_2, and the hydroxyl radical. The superoxide anion can also be metabolized via the myeloperoxidase (MPO) pathway. MPO protein is contained in the azurophil granules, and amplifies the toxic potential of hydrogen peroxide by producing reactive intermediates including hypochlorous acid (HOCl). Additionally, neutrophils exhibit nitric oxide synthase activity, producing NO, which also has bactericidal activity.[25] Zinc is also an activator of NADPH oxidase.[26]

The bactericidal function of neutrophils is essential, as is the chemotaxis and migration process. When these functions do not occur properly, as in type 1 diabetes (T1D), type 2 diabetes (T2D), and cystic fibrosis related diabetes (CFRD), patients are at higher risk for developing infections as well as increased severity of infections.[6] Importantly, insulin does not change these immune complications, while some studies suggest that C-peptide might.

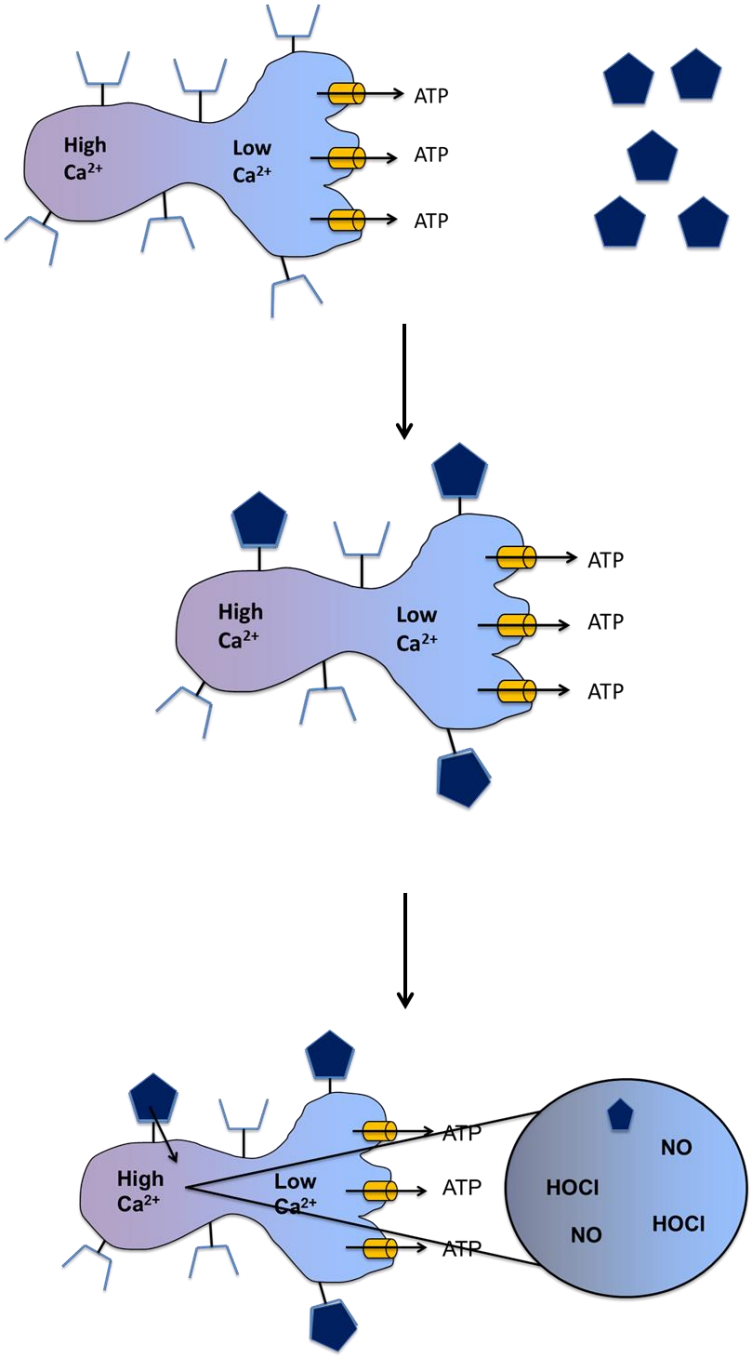


Figure 4.3: The mechanism of cytotoxic killing by neutrophils. The chemoattractant (blue hexagons) causes polarization of the neutrophil. GPCRs for fMLP become occupied and activate the release of azurophilic and specific granules, and the activation of NADPH-dependent oxidase at the membrane, which releases O^{2-}, H_2O_2, and the hydroxyl radical. MPO makes HOCl and NO from O^{2-}, which have bactericidal activity.

4.2 Anti-inflammatory effects of C-peptide

Although C-peptide treatment has been shown to significantly increase the number of leukocytes adhering to a wound in diabetic mice,[27] no studies have been published showing the interaction of C-peptide with peripheral neutrophils. However, C-peptide has been shown to have some anti-inflammatory properties (Figure 4.4), including the reduction of reactive oxygen species (ROS) through inhibition of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, inhibition of caspase-3, and slowing of apoptosis,[28-30] downregulation of NF-κB activity, decreased secretion of pro-inflammatory cytokines and chemokines,[31] and reduction of cellular adhesion molecules such as vascular endothelial growth factor (VEGF),[32] transforming growth factor β (TGF-β)[33, 34] and plasminogen activator inhibitor 1 (PAI-1).[35]

4.3 Inflammatory Dysfunction in Disease

Patients with diabetes (T1D, T2D, and CFRD) are at high risk for developing microvascular complications, such as neuropathy, nephropathy, and retinopathy. Initiation and progression of microvascular complications in these patients is thought to be influenced by chronic inflammatory responses, including high levels of ROS production in peripheral blood monocytes, activation of the NF-κB and mitogen-activated protein kinases pathways in blood mononuclear cells, and increased levels of inflammatory biomarkers in plasma and urine. [36-38]

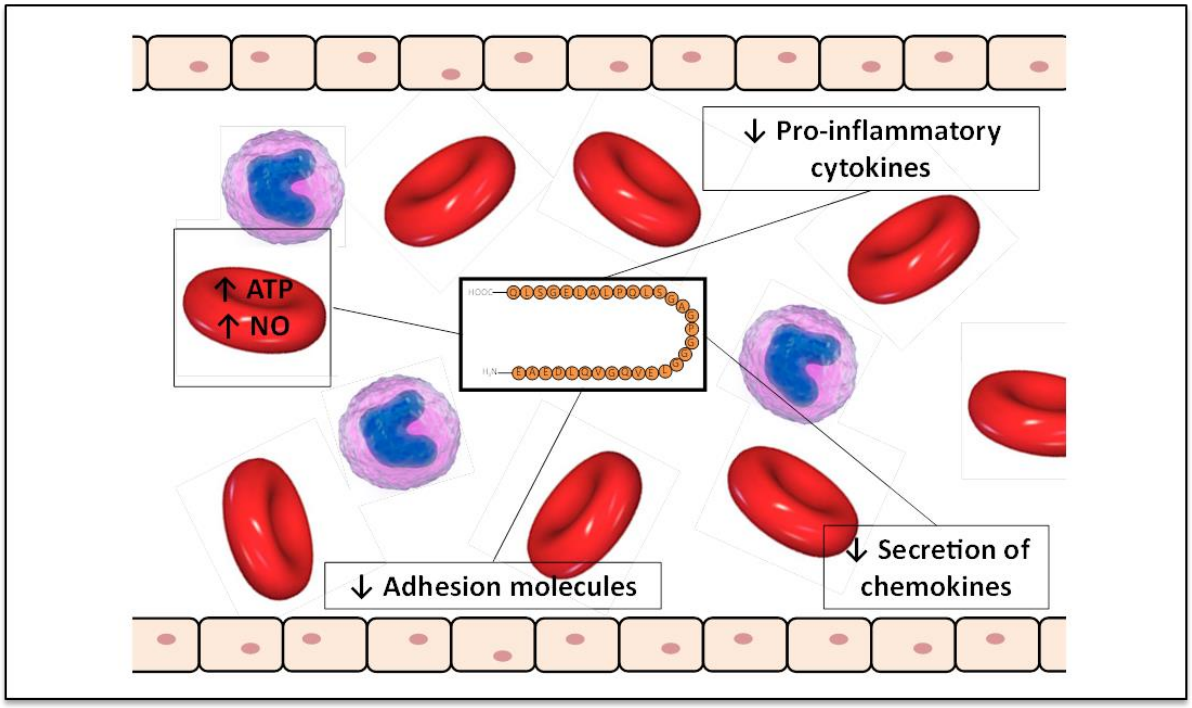


Figure 4.4: An overview of the mechamisms by which C-peptide has been shown to have anti-inflammatory characteristics. In red blood cells, C-peptide increases the release of ATP, which consequently increases the amount of NO released from endothelial cells, contributing to vasodilation. C-peptide has also been shown to decrease pro-inflammatory cytokines, adhesion molecules on endothelial cells, and secretion of chemokines.

Insulin alone does not decrease these inflammatory responses in T1D patients, but *in vitro* studies have shown anti-inflammatory properties of C-peptide, the 31-amino acid peptide co-secreted in equimolar amounts with insulin from the pancreatic beta cells.[29, 39-47] For many years after its discovery in 1967, C-peptide was thought to be a biologically inert molecule that assisted only in the folding of insulin in the secretory vesicles.[48] Since then, reports have shown that C-peptide alone is a bioactive peptide,[49] positively benefiting those with T1D, including improvement of nephropathy

complications, neuropathy complications, and better utilization of glucose by skeletal muscle.[50-53]

Though C-peptide has been shown to have positive effects in T1D patients, this peptide in pure form has no biological activity.[54] Rather, a metal has been found to be necessary for biological activity of pure C-peptide, zinc being the most biologically relevant.[54, 55] The mechanism of action of C-peptide is still not fully elucidated and no receptor has been identified; however, its action seems to be linked to GLUT1, [55] a glucose transporter present on human RBCs, neutrophils, and endothelial cells that is not affected by insulin. Additionally, reports show that albumin is necessary for C-peptide and zinc to induce biological activity in human RBCs.[56] C-peptide has been shown to bind to human skin fibroblasts [57], renal tubular cells and saphenous vein endothelial cells [58], and human RBCs.[56] Neutrophil recruitment and function are crucial in both chronic and acute inflammation, and patients with T1D have been shown to have fewer peripheral neutrophils than healthy controls.[59-61]

Patients with diabetes also show decreased adhesion and migration,[62] slower chemotaxis,[7] lower bactericidal activity,[63] lower rates of phagocytosis,[64] and lower production of ROS.[65] Some studies have suggested that insulin affects neutrophil function,[66] however this study was done in healthy control patients, who release insulin as well as C-peptide, so this result cannot be tied directly to insulin.

CFTR activity has been reported in neutrophils, [67-69] and neutrophils are the primary inflammatory response in the immune complications associated with CF. Neutrophils primarily infiltrate the CF lung, which suggests that CF has a prolonged primary

inflammatory response, as is typically seen in acute infection.[70] The primary chemoattractant in the CF lung is IL-8, which is produced at significantly higher levels by airway tissues and epithelial cells in the CF lung compared to controls.[71] Neutrophils are not only present in increased amounts in CF patients, but they are also dysfunctional as compared to control neutrophils. In CF patients, neutrophils show an increase in oxidative burst[72] and an increased propensity to release granule contents,[73] as well as abnormal pH regulation, which contributes to their dysfunction.[74] This data suggests that neutrophils are already primed for activation before they arrive at the site of infection. Neutrophils of CF patients show reduced chemotactic response to some[75-77] but not all[78] chemoattractants as well as a decreased number of receptors on CF neutrophils.[79] Cytokine production by neutrophils is also markedly different in CF patients.[80, 81]

Some antibiotics may act as immunomodulatory agents. In a study of systemic inflammatory markers in CF patients not infected with *Psuedomonas aeruginosa*, oral azithromycin significantly reduced neutrophil counts.[82] Tetracyclines also inhibit the function of neutrophils, possibly through binding Ca^{2+} and Zn^{2+}, both of which are required for collagenase function, which helps to break down extracellular structures.[83]

In order to study neutrophil functions and the effect that pancreatic secretions have on them, we turned to previously described assays, such as ELISAs and utilization of radiolabeled zinc, as well as fluorescent probes to study intracellular calcium, extracellular NO, and intracellular NO. To study neutrophil chemotaxis, we turned to a staple in our laboratory, 3D printing.

4.4 3D Printing

The concept of 3D printing began with Charles Hull in the 1980s, [84] and in 1993 the first 3D printer was made available.[85] Today, desktop 3D printers are available from commercial retailers.[86] Biomedical research has benefited greatly from the advent of 3D printing, including tissue scaffolding, including in bone, teeth, vascular and organ scaffolding.[87]

3D printing begins with the design of a model generated by computer aided design (CAD) software (AutoDesk). This file is then converted to .STL format, which stores information for the 3D model in triangulated sections.[88] As the amount of triangles that define a surface increases, the resolution of the printed device increases. The 3D printer converts the .STL file to a G-file, which divides the .STL file into two dimensional cross sections for printing. Multiple methods exist for the mechanism by which the printer creates the 3D model. These include sterolithography, inkjet printing, selective laser sintering, fused deposition modeling, and laminated object manufacturing. The 3D printing technique used in this research is a combinatorial method of stereolithography and inkjet printing known as PolyJet technology.[87]

Briefly, PolyJet technology is an additive manufacturing technique, where the 3D printer prints a single two dimensional layer of photocurable polymer and then initiates polymerization of the material by the use of a UV light. In void spaces, the printer lays down a removable layer of support material.

Commercial devices for studying neutrophil chemotaxis exist (Boyden chamber, micropipette-based assay, Dunn chamber),[89-93] however, these assays do not allow

for the real-time quantification of cell migration. For this reason, many groups have turned to creating their own microfluidic devices for chemotaxis applications,[94-97] as we report here.

Here, we present evidence that C-peptide binds to human peripheral neutrophils, but only when present with albumin and zinc does it increase the intracellular calcium levels, intracellular NO levels, and extracellular NO levels in response to a chemoattractant, and that insulin alone does not have this affect. We also provide evidence that the rate of chemotaxis is increased when albumin, C-peptide, and zinc are present, and that C-peptide's action is linked to GLUT1.

4.5 Materials and Methods

4.5.1 Buffer Preparation

All buffers were prepared in purified water (DDW, 18.2 MΩ). All reagents were purchased from Sigma Aldrich (St Louis, MO, USA) unless otherwise noted.

4.5.1.1 Physiological Salt Solution (PSS)

Physiological Salt Solution (PSS) contained 4.7 mM KCl (Fisher Scientific, Waltham, MA, USA), 2.0 mM $CaCl_2$ (Fisher Scientific), 140.5 mM NaCl, 12 mM $MgSO_4$ (Fisher Scientific), 21.0 mM tris(hydroxymethyl) aminomethane (Invitrogen, Carlsbad, CA, USA), 5.5 glucose, and 0.5% bovine serum albumin adjusted to a final pH of 7.4. For experiments in albumin-free PSS, all components remained the same without the addition of albumin. For experiments in glucose-free PSS, all components remained the same without the addition of glucose.

4.5.1.2 Phosphate Buffered Saline (PBS)

Phosphate Buffered Saline (PBS) contained 137 mM NaCl, 2.7 mM KCl (Fisher Scientific), 10.1 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 (Spectrum Chemical, New Brunswick, NJ, USA).

4.5.1.3 Sodium Chloride Solutions

A 3% dextran in 0.9% NaCl (0.9% = 9 g/L) solution and three NaCl solutions (0.9% NaCl, 0.2% NaCl, and 1.6% NaCl) were made and autoclaved before use.

4.5.2 Isolation of Human Neutrophils

Whole blood was drawn from healthy, consenting human donors by venipuncture into heparinized tubes. Whole blood was mixed with an equal volume of 3% dextran in 0.9% NaCl and allowed to sediment for 30 minutes. The resulting plasma rich layer was centrifuged at 250 *g* and the resulting cell pellet was resuspended in 0.9% NaCl to the original volume of whole blood. This suspension was separated by a density gradient using Hisopaque-1077 and centrifuged for 15 minutes at 800 *g*. The resulting cell pellet contained the desired neutrophils and contaminating RBCs. RBCs were lysed using 0.2% NaCl for 30 seconds and then isotonicity was restored with an equal volume of 1.6% NaCl. The suspension was centrifuged and the neutrophils collected in the cell pellet. Any remaining RBCs were lysed by repeating the aforementioned process a second time. Neutrophils were resuspended in PBS at approximately 15 million cells per mL (total of 5 mL) until use and were used within six hours of isolation.

4.2.3 Purification of C-peptide

Synthesized human C-peptide and the single amino acid mutant E27A (~80 % pure) were purchased from Peptide 2.0 (Chantilly, VA, USA), and purified using reverse phase high performance liquid chromatography (RP-HPLC) with a Shimadzu (Columbia, MD, USA) LC-20AB solvent system and a SPD-20AV UV-Vis absorbance detection system with a C18 preparative HPLC column (Grace, Deerfield, IL, USA) (25 cm column length, 10 µm particle size and 10 mm i.d.). C-peptide and E27A were purified separately by gradient elution (Table 4.1). Collected C-peptide and E27A peaks were lyophilized overnight and then stored at -20 ºC for future use. Purity of C-peptide and E27A were verified by MS/MS analysis (Thermo Scientific LTQ Orbitrap (Thermo, SanJose, CA, USA) equipped with an NanoMate electrospray source (Advion, Ithaca, NY, USA). E27A was purified by Yueli Liu and frozen at -20 °C for further use. C-peptide was purified by the author and frozen at -20 °C.

Table 4.1: Gradients and conditions for HPLC purification of C-peptide and its single amino acid mutant E27A. Solvent A contained 0.1% trifluoroacetic acid (TFA) in HPLC-grade water, Solvent B contained 0.089% TFA in 60% acetonitrile in HPLC-grade water. The flow rate was set at 5 mL/min.

C-peptide		E27A	
Time (min)	%B	Time (min)	%B
0	0	0	0
5	40	20	34
30	50	100	67
40	100	120	100
45	100	125	100
50	0	130	0
55	stop	135	stop

4.5.4 Experimental Setup

After isolation, neutrophils were resuspended in PBS in order to keep them inactive until use. Working stocks of C-peptide, zinc, insulin and E27A were made in DDW and warmed to room temperature prior to use. In microcentrifuge tubes, combinations of C-peptide, zinc, insulin and E27A (5% of the total volume of the sample) were allowed to incubate at room temperature for 3 minutes. Addition of PSS was immediately followed by addition of 100 μL of the neutrophil suspension. The total volume for all experiments was 500 μL. Samples were incubated at 37 °C for one hour after addition of neutrophils. Immediately after beginning incubation, an aliquot of neutrophils from the purified stock was counted on the hemacytometer to determine the cell count in each sample. After incubation, cells were pelleted by centrifugation at 250 *g* for 10 minutes. The cell supernatant and pellet were then separated by pipetting for further analysis.

4.5.5 C-peptide binding studies

Determination of C-peptide binding to neutrophils was performed using a commercially available enzyme linked immunosorbent assay (ELISA) kit (Millipore, Billerica, MA, USA). All samples were prepared in PSS. Studies of C-peptide binding were performed in the presence (PSS) and absence of albumin (albumin-free PSS). The cell supernatant was assayed for the presence of C-peptide using an ELISA-based determination. An enzymatically-tagged secondary antibody catalyzes a colorimetric reaction that is measured using a standard plate reader at 450 nm (Molecular Devices LLC, Sunnyvale, CA, USA), which is directly proportional to the amount of C-peptide present in the sample. The number of picomoles of C-peptide per million neutrophils

was determined by subtracting the number of moles remaining in the supernatant from the original number of moles added to the neutrophils.

4.5.6 Zinc binding studies

Zinc binding to neutrophils was performed using radioisotopic zinc ($^{65}Zn^{2+}$; 5.7 Bq/molar weight, PerkinElmer Inc., Waltham, MA, USA). All samples were prepared in either PSS or albumin-free PSS. The cell supernatant was used for this assay. After addition of 100 µL of scintillation cocktail (PerkinElmer Inc.), the amount of $^{65}Zn^{2+}$ remaining in the supernatant was measured using a scintillation counter (1450 MicroBeta PLUS, Wallac Inc., Finland). The amount of $^{65}Zn^{2+}$ uptake by the neutrophils was determined by subtracting the amount of $^{65}Zn^{2+}$ left in the supernatant from the amount originally added.

4.5.7 Intracellular Calcium Measurements

Neutrophils were isolated as previously described and incubated with 50 µM Fura-2 (Molecular Probes, Grand Island, NY, USA). Cells were washed in PBS to remove excess Fura-2 before incubation with C-peptide, zinc, the C-peptide mutant E27A, insulin, or a combination thereof in a 96-well plate in PSS, albumin-free PSS or glucose-free PSS. After incubation at 37 °C for one hour, the neutrophils were stimulated with 2 µM fMLP. Intracellular calcium levels were measured using the ratio of bound to free calcium (excitation 340 nm (bound); excitation 380 nm (free); emission 510 nm). These ratios were then correlated to the initial intracellular calcium levels to give a percent increase of intracellular calcium after fMLP stimulation.

4.5.8 Intracellular NO Measurements

Neutrophils were isolated as previously described and incubated with 5 μM DAF-FM DA (4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate, Molecular Probes). Cells were washed in PBS to remove excess DAF-FM DA before incubation with C-peptide, zinc, insulin, E27A, or combinations thereof in a 96-well plate in PSS. After incubation at 37 °C for one hour, the neutrophils were stimulated with 2 μM fMLP, whereupon intracellular NO levels were measured at excitation and emission wavelengths of 485 nm and 535 nm, respectively. Results are presented as the increase of intracellular NO compared to the control.

4.5.9 Extracellular NO Measurements

All measurements were the same as in Section 4.5.10, but the probe did not contain diacetate. The probe used was 5 μM DAF-FM (4-Amino-5-Methylamino-2',7'-Difluorofluorescein, Cayman Chemical, Ann Arbor, MI, USA). Results are presented as the increase of extracellular NO compared to the control.

4.5.10 Chemotaxis Measurements

A 3D printed device (Figure 4.5) was designed in Autodesk Inventor 2015 Student Edition. Files were exported as .STL files to an Objet350 Connex printer in the Department of Electrical and Computer Engineering at Michigan State University and printed in transparent Vero Clear material (Stratasys, Eden Prarie, MN, USA; composition: isobornyl acrylate 915-50%), acrylic monomer (15-30%), urethane acrylate (10-30%), acrylic monomer (5-15%), epoxy acrylate (5-15%), acrylate oligomer (5-15%), and photoinitiator (0.1-2%).[98]

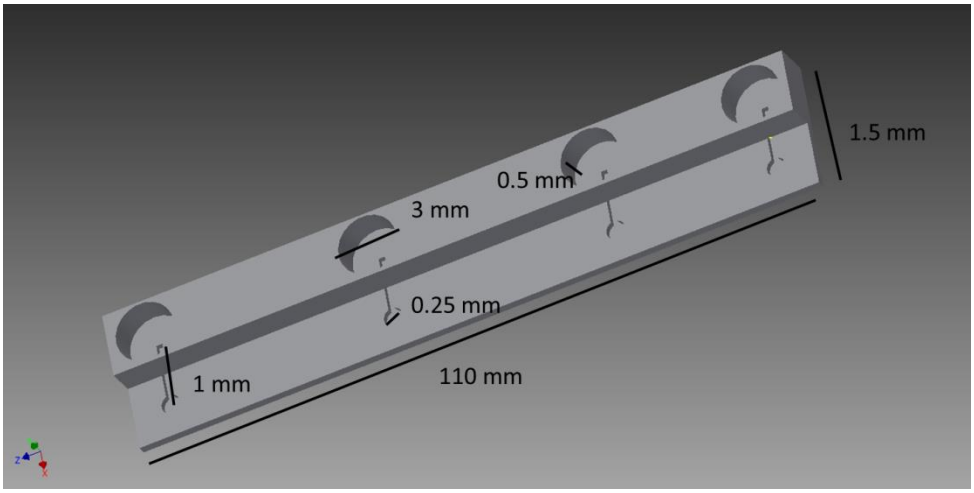


Figure 4.5: CAD file of the device. The device contains four spaces for Transwell inserts. The thin edge of the device is 0.5 mm thick and is polished before use under the microscope.

The device is 110 mm long and 3.5 mm wide with spaces for four Transwell inserts (Corning Inc., Corning, NY, USA). Channels are 1 mm long and have a 0.25 mm diameter opening where neutrophils are placed. Chemoattractant is placed in the Transwell inserts. Prior to use, the device was cleared of all support material and the device was sanded and polished to create the best possible visibility under the microscope.

Channels were coated twice with polystyrene (1 g polystyrene powder in 4 mL isopherone) and dried with nitrogen. Prior to the addition of purified neutrophils to the device, the channel was coated with fibronectin (2.5 µg/mL) to enhance cell adherence.

Neutrophils were incubated with a final concentration of 5 µM cell tracker red (Invitrogen) for enhanced visualization. Neutrophils were then incubated at 37 °C untreated or treated with C-peptide, zinc, or C-peptide and zinc combined. Cells were

then placed in the well and allowed to adhere to the fibronectin-coated device for 20 minutes before addition of 2 μM fMLP to the transwell insert. Neutrophil movement was tracked for 5 minutes under a TRITC filter and the rate of chemotaxis was calculated.

4.5.11 Glucose Utilization

Neutrophils were incubated with PSS containing 11 mM glucose. After pelleting by centrifugation, the supernatant was collected and analyzed for the amount of glucose present using an indirect fluorescent glucose assay. The working reagent (Figure 4.6) contains glucose 6-phosphate dehydrogenase (0.9 U/mL), hexokinase (1.7 U/mL), $NADP^+$ (1.25 mM), $MgCl_2$ (2 mM), and ATP (2 mM) in TEA buffer (triethanolamine, 0.3 M). An equal volume of the working reagent and the sample were incubated in a black 96-well plate for 15 minutes before reading the fluorescence at an excitation of 340 nm and emission of 460 nm and comparing the result against a standard curve.

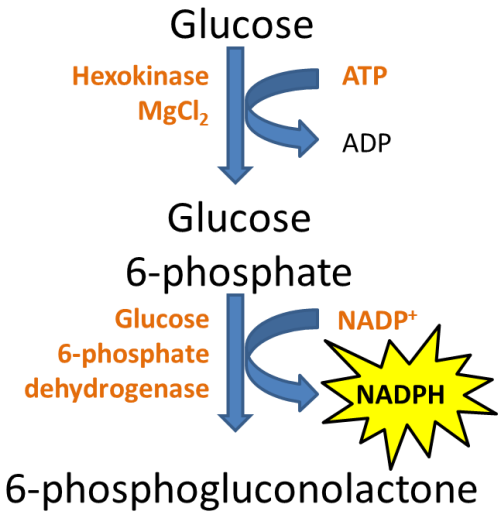


Figure 4.6: Reaction scheme of the glucose assay. The reaction scheme of the glucose assay is an indirect measure of glucose in a sample by measuring the fluorescence of NADPH.

4.5.12 Inhibition of GLUT1 with phloretin

For experiments with inhibition of GLUT1, incubation with phloretin (final concentration of 25 mM) for 30 minutes prior to placing cells in the 96-well plate.

4.5.13 Inhibition of CFTR with CFTRinh-172

CFTRinh-172 was purchased from Tocris and diluted in DMSO and then diluted in buffer to a working concentration. Neutrophils were incubated with a final concentration of 1 μM CFTRinh-172 for 30 minutes and then washed to remove excess inhibitor. Cells were then incubated with the appropriate reagents for analysis as previously described.

4.5.14 FITC-labeled GLUT1 Antibody

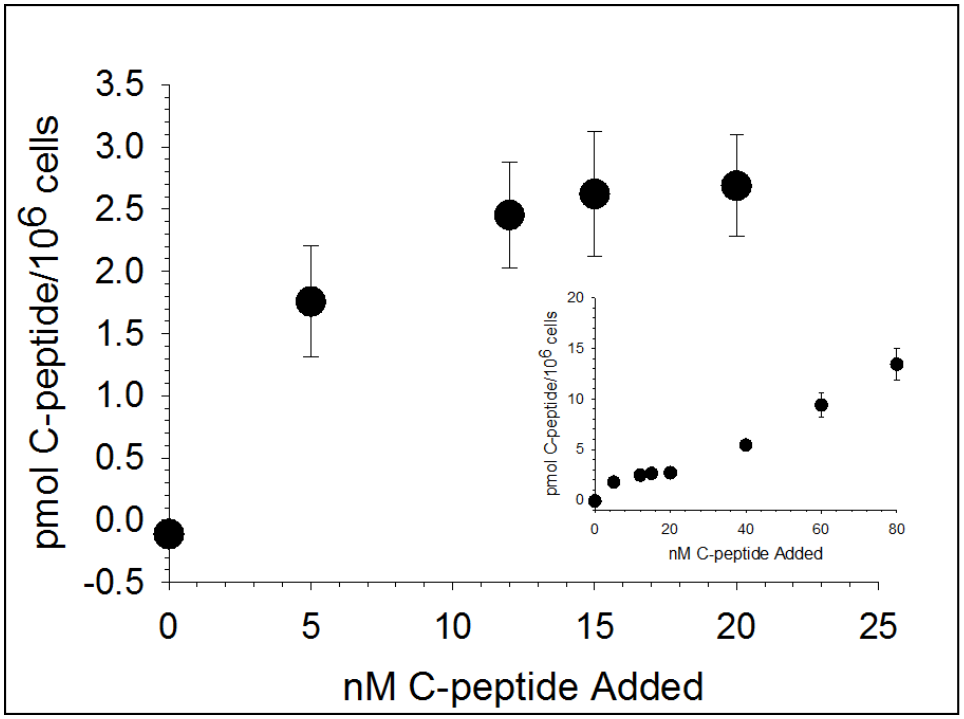
A FITC-labeled antibody against human GLUT1 was purchased from R&D Systems (Minneapolis, MN, USA). Neutrophils were isolated as previously described and treated with appropriate combinations of C-peptide, zinc, and insulin. Cells were pelleted and then incubated with 5 μL of the antibody (stock 0.5 g/mL in PBS) for 30 minutes on ice. Cells were washed three times in ice cold PBS prior to reading the fluorescence of the FITC-labeled antibody (excitation: 485, emission: 515). All samples were compared to the control for a relative amount of GLUT1 on the plasma membrane of the neutrophils.

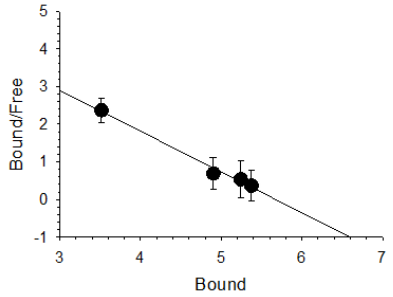
4.5.15 Statistical Analysis

All data was compared by Student's t-test and expressed as mean ± standard error of the mean (S.E.M.), and $p < 0.05$ was considered statistically significant.

4.3 Results

C-peptide binds to human peripheral neutrophils in a specific manner in the presence of albumin up to 20 nM C-peptide added before nonspecific binding is observed (Figure 4.7). Scatchard analysis of the specific binding region yields a K_a of 1.08 x 10^9 M^{-1} and a B_{max} of 2.84 pmol/10^6 cells. In the presence of equimolar zinc, there is no statistically significant difference in the specific binding region (Figure 4.8A). All subsequent secondary response experiments were performed at 20 nM (20 nM C-peptide added, 20 nM zinc added, 20 nM zinc and C-peptide added), where there is no significant difference between the amount of C-peptide bound to the neutrophils whether or not zinc is present (Figure 4.8B). Zinc binding to neutrophils is also specific in the presence of albumin, but in a greater range than C-peptide binding, up to 35 nM, before binding is nonspecific (Figure 4.9). Scatchard analysis of the specific binding region yields a K_a of 0.109 x 10^9 M^{-1} and a B_{max} of 6.52 pmol/10^6 cells. Zinc binds to neutrophils in the presence and absence of C-peptide, but is significantly higher in the presence of C-peptide (Figure 4.10A). At the concentration where all secondary response experiments are performed, there is a significant difference in the amount of zinc bound to the neutrophils when C-peptide is present (Figure 4.10B).





K_a (10^9 M^{-1})	B_{max} (10^{12} mol)
1.08	2.84

Figure 4.7: C-peptide binding to human peripheral neutrophils in the presence of albumin. Nonspecific binding occurs after 20 nM C-peptide added (inset). Scatchard analysis of the specific binding region yields an association constant (K_a) of 1.08 x 10^9 M^{-1} and B_{max} of 2.84 pmol/10^6 cells. (n≥3, error=SEM)

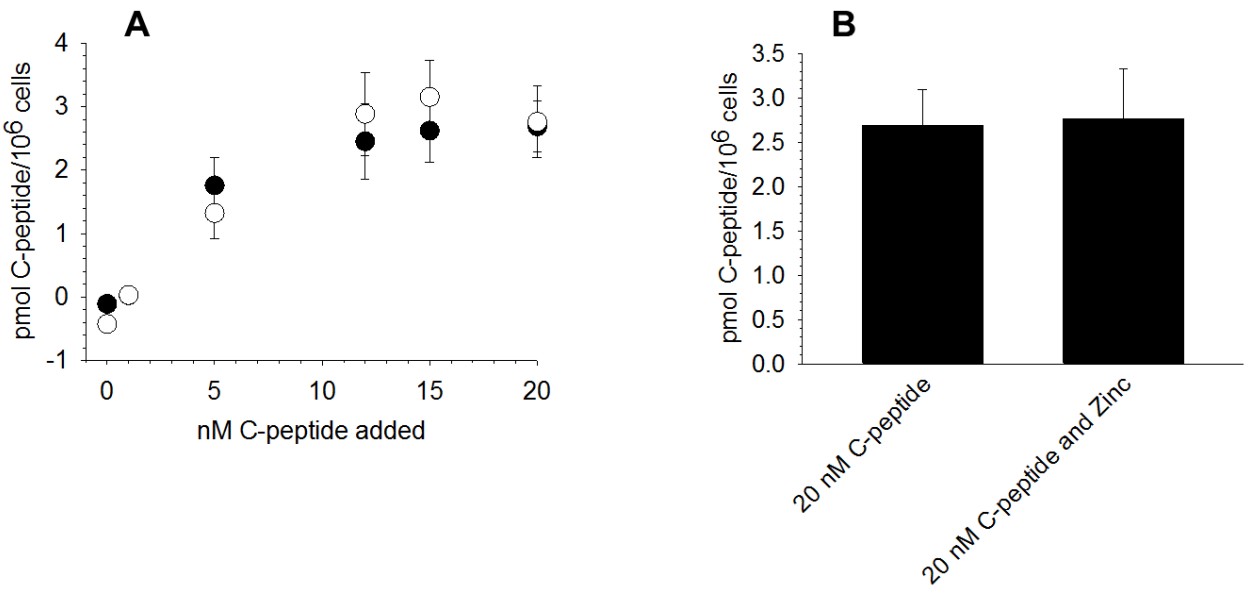
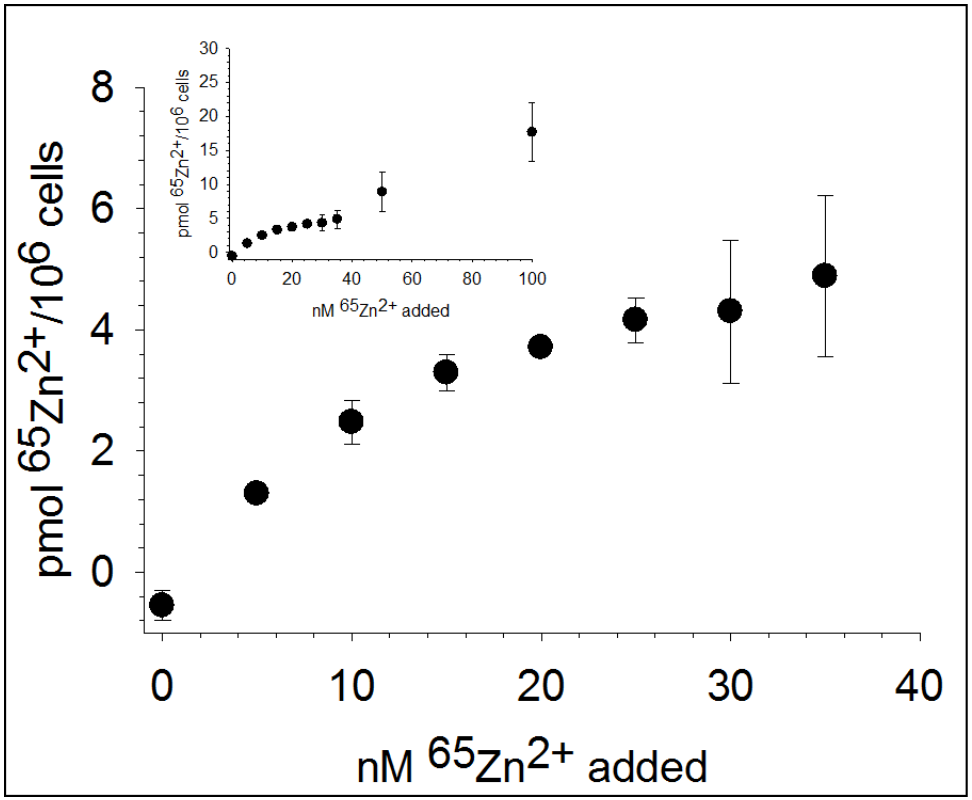
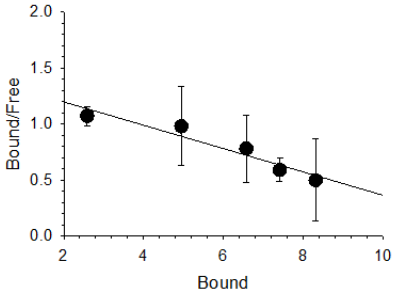


Figure 4.8: C-peptide binding in PSS in the presence and absence of equimolar zinc. (A) C-peptide binding in PSS in the presence (open circles) and absence (closed circles) of equimolar zinc. There is no statistically significant difference between the two conditions. (n≥3, error=SEM) (B) C-peptide binding to neutrophils in the presence and absence of 20 nM zinc. There is no statistical difference between the two conditions. (n≥9, error=SEM)





K_a (10^9 M^{-1})	B_{max} (10^{12} mol)
0.109	6.52

Figure 4.9: Zinc binding to human peripheral neutrophils in the presence of albumin. Binding is nonspecific above 35 nM (inset). Scatchard analysis of the specific binding region yields an association constant (K_a) of 0.109 x 10^9 M^{-1} and B_{max} of 6.52 pmol/10^6 cells. (n≥3, error=SEM)

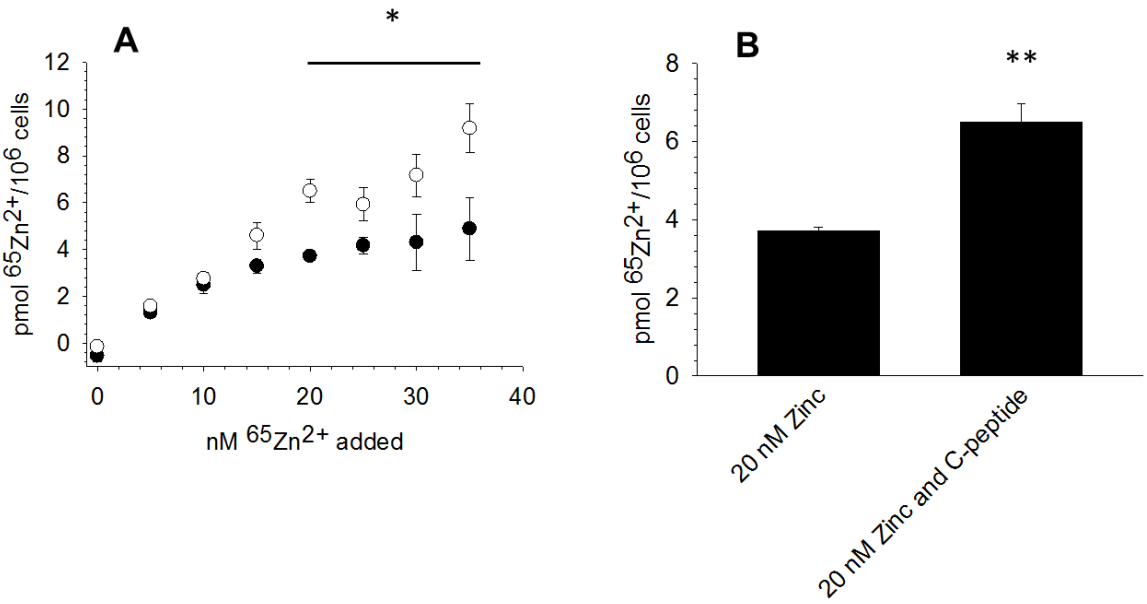


Figure 4.10: Zinc binding in the presence and absence of equimolar C-peptide. (A) Zinc binding to neutrophils in the presence (open circles) and absence (closed circles) of equimolar C-peptide. Zinc binding is significantly different than zinc binding in the absence of C-peptide above 20 nM. (n≥3, error=SEM, *p<0.05 from 20 nM to 35 nM) (B) Zinc binding to neutrophils in the presence and absence of 20 nM C-peptide. There is a significant difference between the two conditions. (n≥4, error=SEM, **p<0.03)

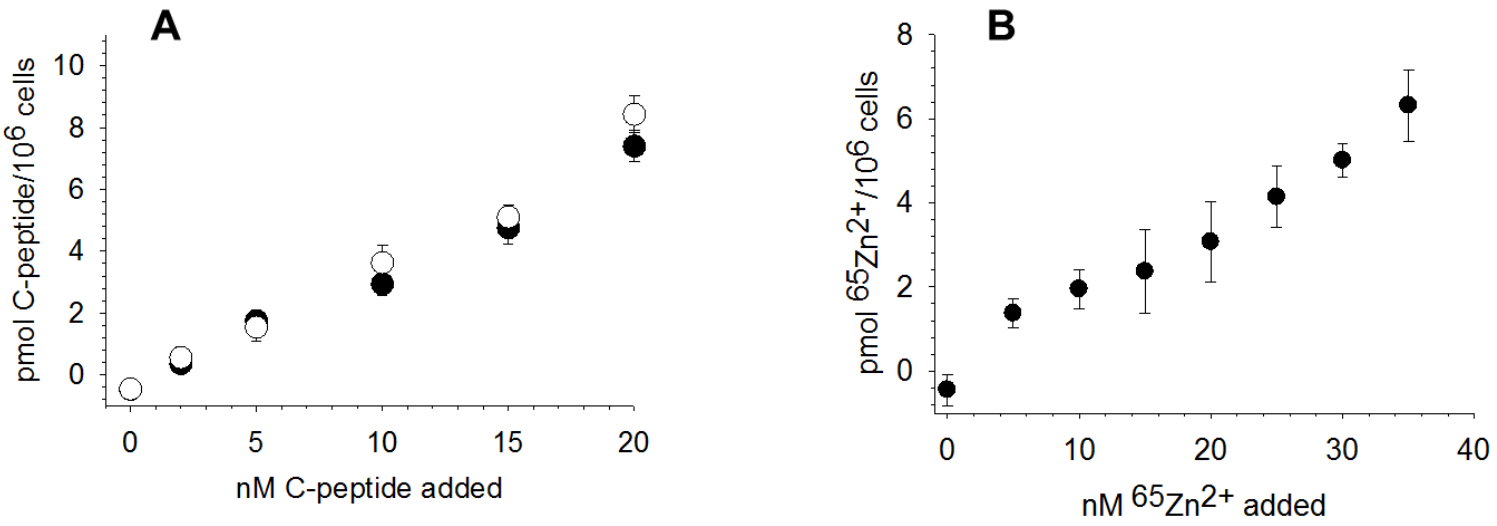


Figure 4.11: C-peptide and zinc binding in the absence of albumin. (A) C-peptide binding in the absence of albumin. Binding is nonspecific in the absence of zinc (closed circles) and the presence of zinc (open circles). There is no significant difference between the two conditions. (n≥3, error=SEM) for both conditions. (B) Zinc binds to the neutrophils in a non-specific manner when in the presence of C-peptide but the absence of albumin. (n≥4, error=S.E.M.)

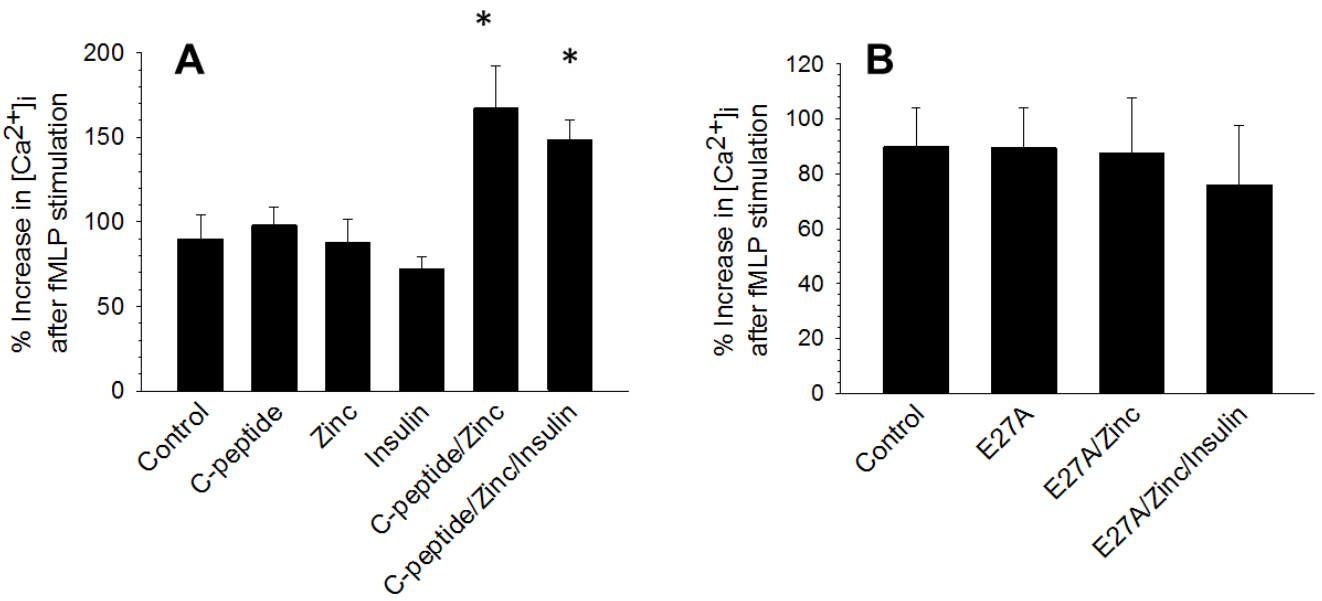


Figure 4.12: Intracellular Calcium Measurements. (A) The percent increase in intracellular calcium after fMLP stimulation in neutrophils in the presence of albumin when treated with each component separately and in conjunction with other components. Only when neutrophils are treated with zinc and C-peptide (with and without insulin) is the difference in intracellular calcium significantly different from the control. ($n \geq 4$, error=SEM, $^*p < 0.005$) (B) The percent increase in intracellular calcium after fMLP stimulation in neutrophils in the presence of albumin in the presence of the mutant C-peptide, E27A. Even in conjunction with zinc and/or insulin, no significant increase is observed from the control. ($n \geq 4$, error=SEM)

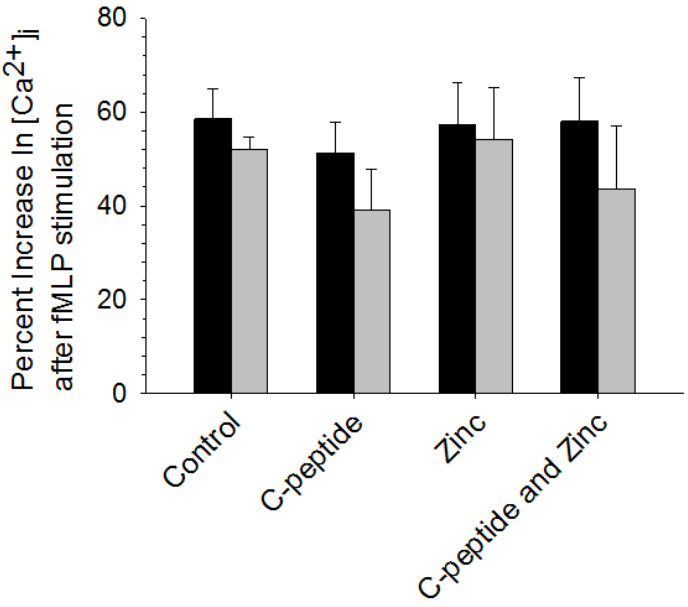


Figure 4.13: Intracellular calcium in the absence of glucose and in the presence of phloretin. The percent increase in intracellular calcium after fMLP stimulation in neutrophils in glucose-free PSS (black bars) and in PSS with phloretin inhibition (grey bars). There is no significant difference between any of the four conditions. (n ≥ 4, error=S.E.M.)

In the absence of albumin, C-peptide binding is nonspecific whether or not equimolar zinc is present (Figure 4.11A). Zinc does not bind to the neutrophils in the absence of

albumin (data not shown) but nonspecific binding is observed when equimolar C-peptide is present (Figure 4.11B).

Neutrophils alone show an increase in intracellular calcium by approximately 100% upon stimulation with fMLP, and there is no significant difference in this increase when neutrophils are incubated with C-peptide, zinc or insulin alone. Only when C-peptide and zinc are together with albumin is there a significant increase from the control. Insulin does not change this effect (Figure 4.12A). The single amino acid mutant of C-peptide E27A shows no change in intracellular calcium levels from the control (Figure 4.12B). When glucose is removed from the buffer, there is no change from the control, nor when GLUT1 is inhibited with phloretin (Figure 4.13).

Intracellular (Figure 4.14A) and extracellular NO (Figure 4.14B) measurements show the same trend as the intracellular calcium measurements: Only when together with zinc does C-peptide have a significant effect on the neutrophil response, while insulin alone does not have this effect. E27A also shows no significant change from the control. Additionally, the rate of chemotaxis is only significantly increased from the control when C-peptide and zinc are together with albumin (Figure 4.15). Neutrophils incubated with C-peptide or zinc alone do not utilize significantly more glucose than control cells, however, cells incubated with both zinc and C-peptide utilize significantly more glucose than control cells (Figure 4.17). Finally, surface GLUT1 expression is significantly increased in the presence of C-peptide and zinc with or without insulin (Figure 4.16).

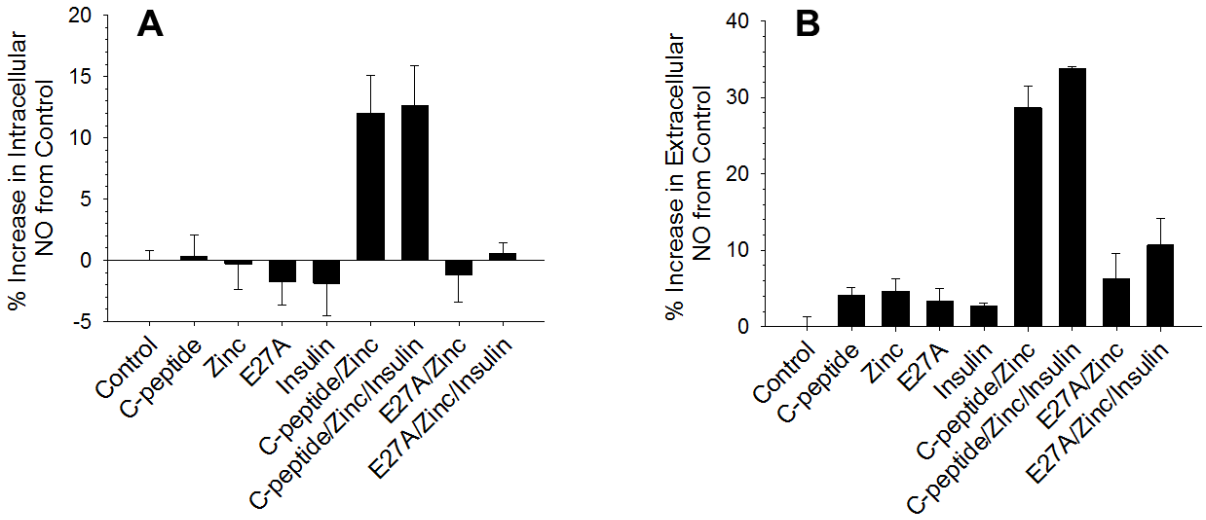


Figure 4.14: Intracellular and Extracellular NO Measurements. (A) The percent increase from control in intracellular NO upon treatment with each component alone and in conjunction with other components. (n=3, error=SEM) (B) The percent increase from control in extracellular NO upon treatment with each component alone and in conjunction with other components. (n=3, error=SEM)

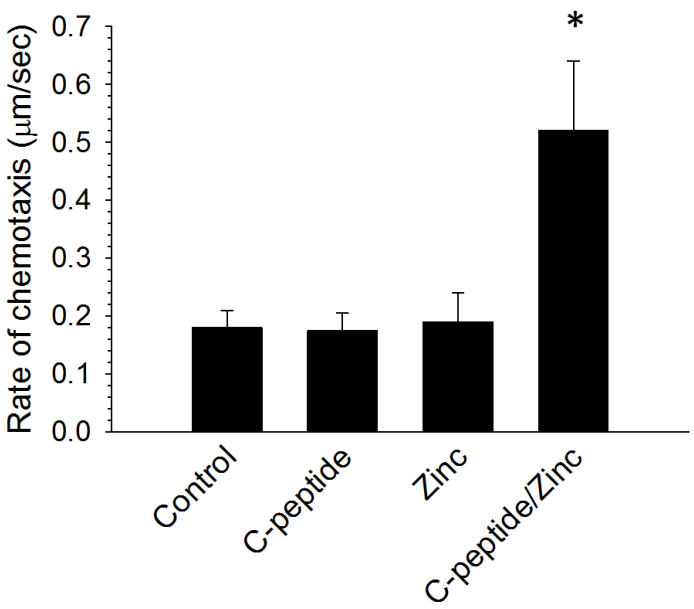


Figure 4.15: The rate of chemotaxis of neutrophils as measured via fluorescence spectroscopy on a 3D printed device. Only when treated with 20 nM C-peptide and zinc in the presence of albumin is there a significant increase in the rate of chemotaxis as compared to the control. (n=3, error=SEM, *p<0.002)

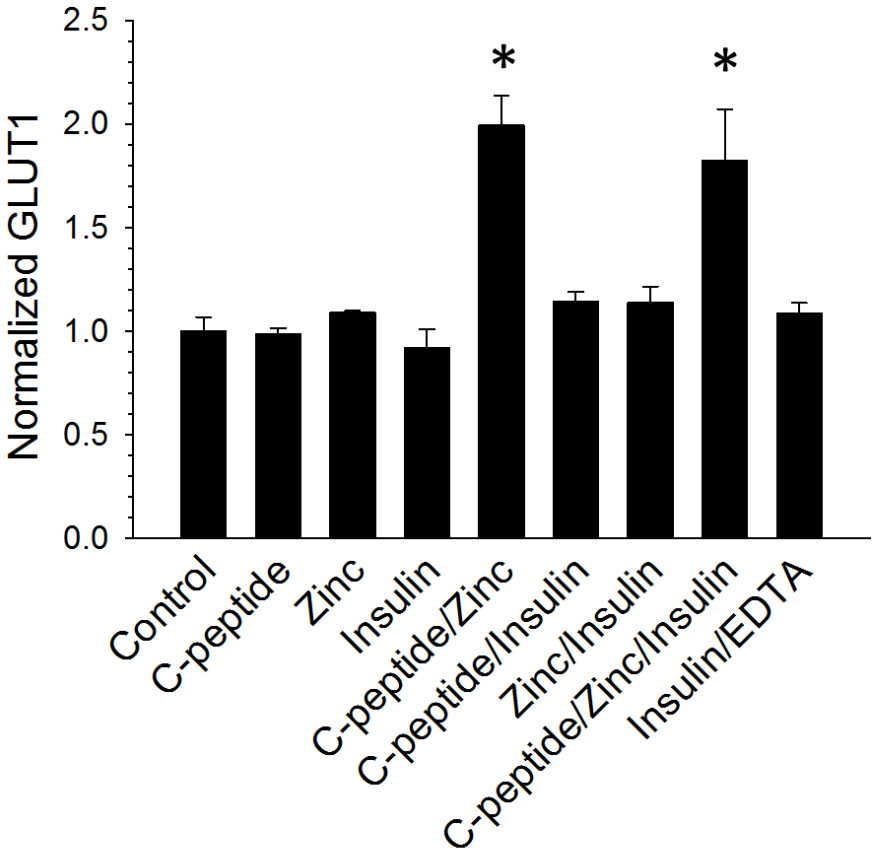


Figure 4.16: Normalized fluorescence of the FITC-tagged GLUT1 antibody. Only in the presence of C-peptide and Zinc (with or without insulin) is the surface GLUT1 level increased significantly. n=3, error=SEM, *p<0.01

Neutrophils in which CFTR was inhibited with CFTRinh-172 show no significant difference from control cells in their C-peptide uptake (Figure 4.17A) or zinc uptake (Figure 4.17B). CFTR-inhibited neutrophils utilize significantly less glucose than control cells, but still show a statistically significant increase in glucose utilization when treated with C-peptide and zinc in PSS (Figure 4.20). Intracellular NO levels increase in CFTR-inhibited cells when treated with zinc alone and with C-peptide and zinc together (Figure

4.18A), however, extracellular NO levels do not increase (Figure 4.18B). Intracellular calcium levels increase significantly after fMLP stimulation only in the presence of C-peptide and zinc (Figure 4.19). CFTR-inhibited cells exhibit a higher protein expression of GLUT1 on the plasma membrane than control cells (Figure 4.20). In the presence of C-peptide, zinc, or insulin alone, there is no difference from cells that are inhibited with CFTRinh-172 alone. Additionally, insulin stripped of any metal contamination (using EDTA) does not increase the protein expression of GLUT1. Only in the presence of C-peptide and zinc (with or without insulin) is the protein expression of GLUT1 increased on the plasma membrane of neutrophils. Neutrophils inhibited with CFTRinh-172 have a slower rate of chemotaxis than control cells (Figure 4.21), and only in the presence of C-peptide and zinc is the rate of chemotaxis significantly increased.

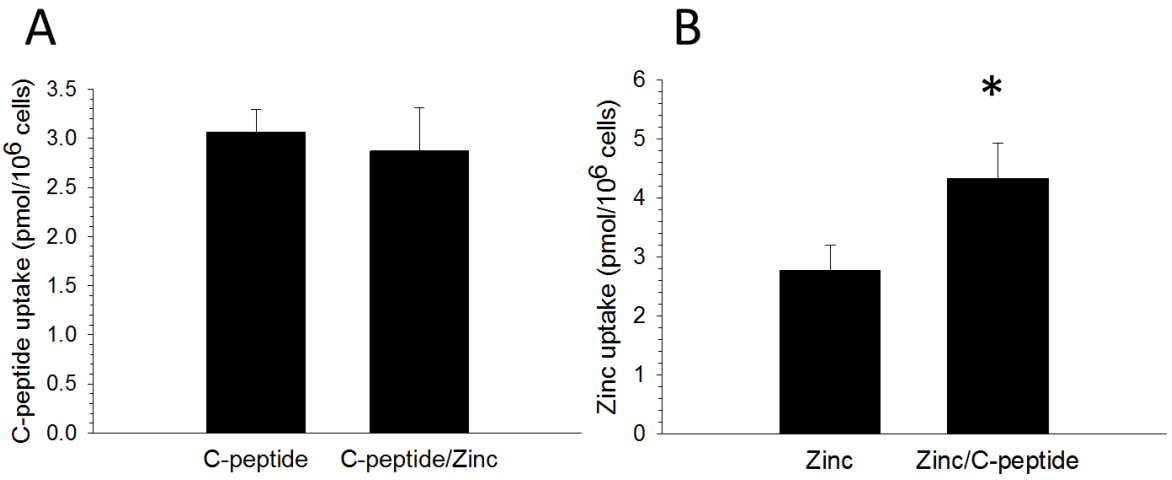


Figure 4.17: C-peptide and zinc uptake by neutrophils that have been treated with CFTRinh-172. (A) C-peptide uptake does not change significantly in the presence of C-peptide only or in the presence of zinc. (B) Zinc uptake, as in control cells, is significantly higher when both C-peptide and zinc are present than when zinc alone is present. n=5, error=SEM, *p<0.05

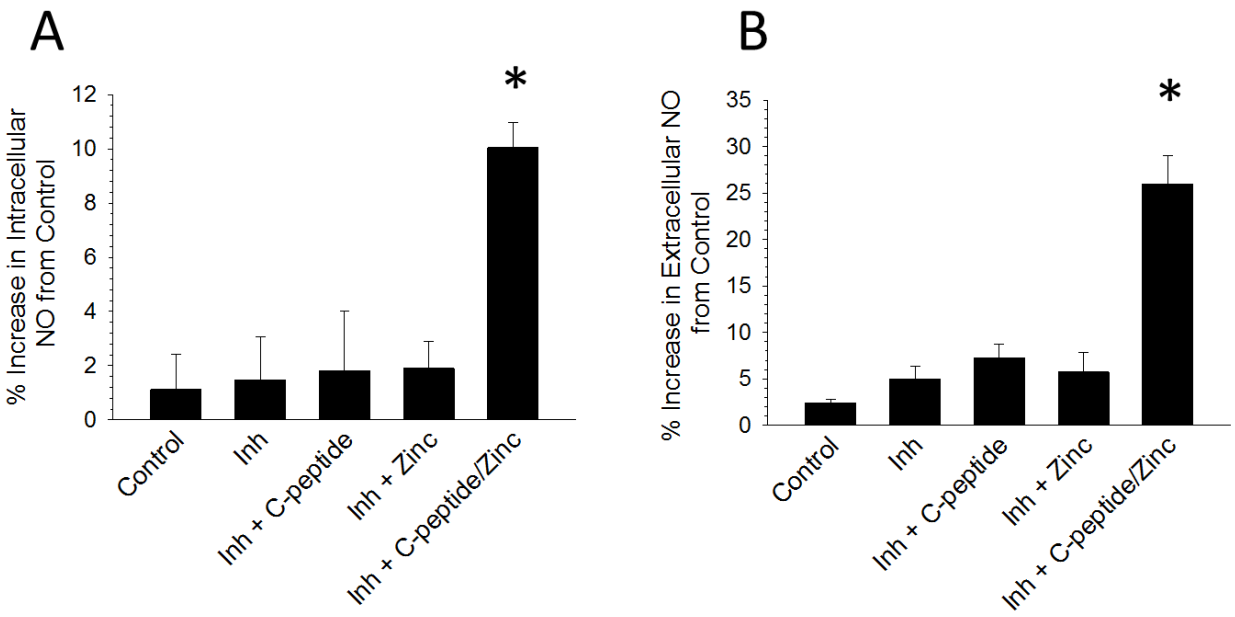


Figure 4.18: Intracellular and extracellular levels of NO in neutrophils incubated with CFTRinh-172. Intracellular NO levels are significantly different from the control in the presence of zinc only (*$p<0.05$) and in the presence of C-peptide and zinc (*$p<0.01$). Extracellular NO levels are statistically different from the control only when treated with C-peptide and zinc.

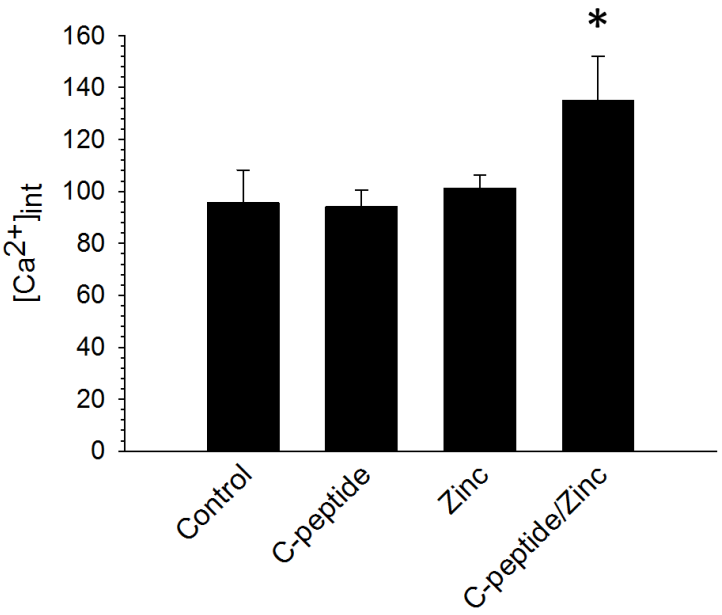


Figure 4.19: Intracellular calcium levels in neutrophils incubated with CFTRinh-172 after stimulation with fMLP. Only in the presence of C-peptide and zinc is there a significant increase in the intracellular calcium level. n=5, error=SEM, *p<0.05

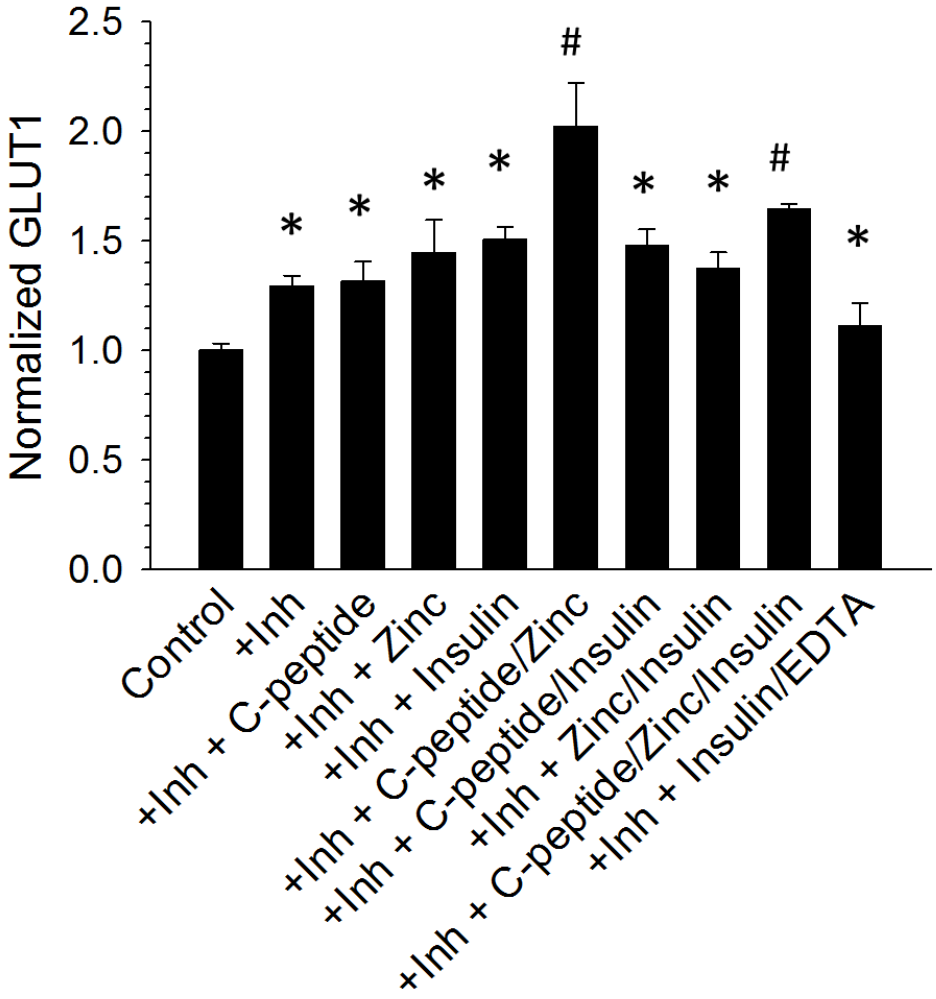


Figure 4.20: GLUT1 protein expression in neutrophils inhibited with CFTRinh-172. Neutrophil protein expression in CFTR-inhibited cells is significantly higher than in control cells. There is only a significant difference from the CFTR-inhibited cells when C-peptide and zinc together are added to the cells (with or without insulin). n=3, error=SEM, * $p<0.05$ compared to control, # $p<0.05$ compared to inhibitor.

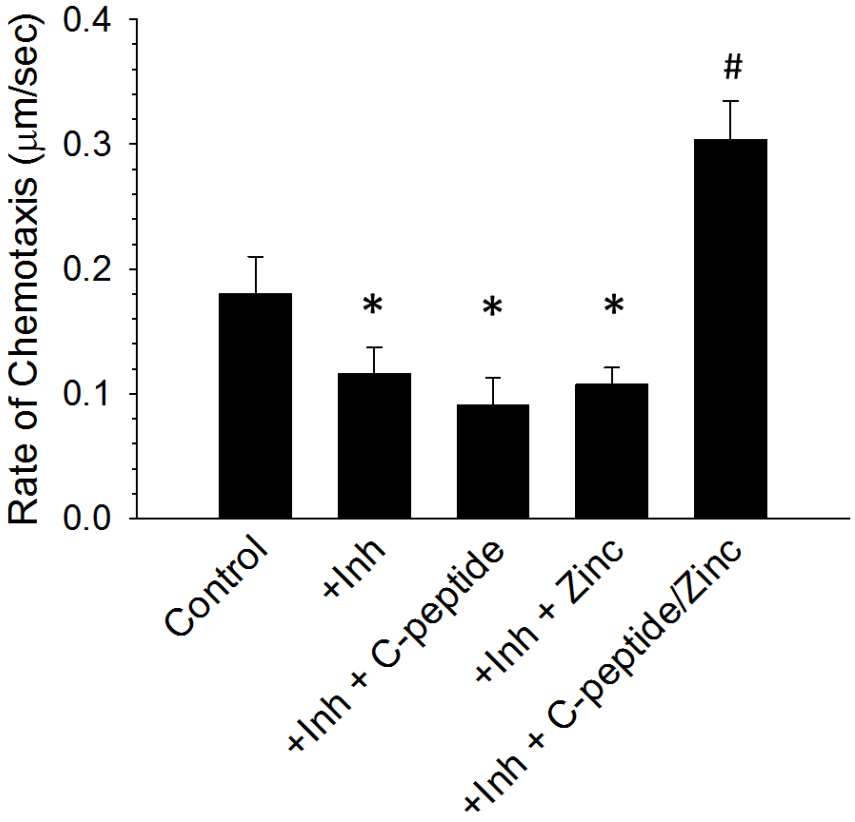


Figure 4.21: The rate of chemotaxis of neutrophils inhibited with CFTRinh-172. The rate of chemotaxis of cells incubated with the inhibitor have a slower rate of chemotaxis than the control, and there is no significant increase in the rate of chemotaxis when cells are incubated with C-peptide or zinc alone. Only in the presence of C-peptide and zinc together in albumin is the rate of chemotaxis increased. n=3, error=SEM, * $p<0.05$ compared to the control, # $p<0.05$ compared to the inhibitor.

4.4 Discussion

Specific binding of C-peptide to human RBCs in albumin-containing buffer has been reported to be statistically the same in the presence and absence of zinc, while specific binding of zinc only occurs in the presence of C-peptide.[56] These authors also reported that C-peptide and zinc bind to human RBCs in a 1:1 molar ratio. Neutrophils

show the same pattern as human RBCs, binding C-peptide specifically in the presence of albumin, with no statistical difference between binding in the presence or absence of equimolar zinc (Figure 4.7). Scatchard analysis of the specific binding region gives an association constant that is on the same order of magnitude as previously reported binding of C-peptide to renal tubular cells, fibroblasts, and endothelial cells[58].

At 20 nM of zinc added to the neutrophils, unlike RBCs[56], neutrophils bind zinc in the presence of albumin in both the presence and absence of equimolar C-peptide (Figure 4.9). However, the difference between the amount of zinc bound in the absence of C-peptide (3.71 ± 0.10 pmol/10^6 cells) and the presence of C-peptide (6.49 ± 0.48 pmol/10^6 cells) is statistically different (Figure 4.10B). Interestingly, the difference between these two conditions is 2.78 ± 0.67 pmol/10^6 cells, which is statistically the same as the amount of C-peptide bound to neutrophils in the presence of zinc. Zinc is a known chemoattractant for neutrophils, so it follows that some zinc would bind to the neutrophil in the absence of C-peptide, and that the difference in the amount of zinc that is bound to the neutrophils in the presence of C-peptide is what is actually being facilitated by the C-peptide and albumin. This, then, confirms the 1:1 molar ratio of C-peptide and zinc specifically bound to neutrophils in the presence of albumin, as seen with human RBCs.

Data presented here also show that albumin is necessary for C-peptide to bind specifically to neutrophils (Figure 4.11A). In the absence of albumin, zinc alone does not bind to neutrophils, while in the presence of C-peptide, neutrophils do bind zinc, indicating that C-peptide may be acting as a less efficient carrier of zinc than albumin (Figure 4.11B).

All secondary responses of neutrophils in response to a chemoattractant are significantly increased in the presence of C-peptide and zinc together in the presence of albumin. Intracellular calcium levels increase in untreated neutrophils by 95.68 ± 16.83 % in the presence of albumin, while only the combination of C-peptide and zinc is able to significantly increase intracellular calcium to 181.78 ± 28.85 % (Figure 4.12A). Additionally, intracellular and extracellular NO levels are both increased only in the presence of zinc and C-peptide, which indicates that the neutrophils will be better able to phagocytose bacteria.

Insulin alone cannot accomplish this increase, further indicating that exogenous insulin treatment alone of diabetics, though it helps to control blood glucose levels, does not alleviate or ameliorate these immune cell complications that lead to secondary complications of the disease.

In the absence of albumin, C-peptide and zinc together are not able to significantly increase intracellular calcium (Figure 4.11A). The fact that 20 nM C-peptide and 20 nM zinc alone are unable to increase the intracellular calcium levels in neutrophils in the presence and absence of albumin indicates that, in order for C-peptide to have an effect on these immune cells or immune complications in diabetes, it must be present with equimolar zinc as well as albumin. Intracellular calcium levels are similarly unaffected when GLUT1 is inhibited with phloretin or when glucose is not present in the buffer (Figure 4.13), indicating that the action of C-peptide and zinc on intracellular calcium levels in neutrophils is in some way linked to this glucose transporter.

The data presented here is the first indication in the literature that C-peptide directly interacts and binds to neutrophils, and further confirms that albumin and zinc are necessary for C-peptide to have a physiological effect. Additionally, we present evidence that the rate of chemotaxis is increased significantly by the treatment of neutrophils with C-peptide and zinc (Figure 4.15). The fact that E27A has no significant effect in these cells confirms that the glutamic acid residue is important not only in the binding of C-peptide to cells, but also in its secondary effects. Additionally, glucose uptake is significantly increased by 26 ± 1% only in neutrophils treated with C-peptide and zinc. This led us to investigate whether or not GLUT1 was translocated to the membrane in neutrophils that were treated with each of these pancreatic secretions.

GLUT1 is the major glucose transporter on human neutrophils. GLUT1 has been shown to translocate from intracellular vesicles to the plasma membrane in the presence of IL-3 and activation of a tyrosine kinase promote in a haemopoietic cell line.[99] More recent evidence suggests that GLUT1 translocation is regulated by IL-3, PI3K activity and activation of Akt.[100, 101] Some report that GLUT1 is recruited to the membrane in response to insulin, but that less so than GLUT4 in fat cells.[102] The research presented here shows that insulin alone is unable to cause significant translocation of GLUT1 to the plasma membrane of neutrophils as compared to the control. Additionally, insulin combined with EDTA shows no effect, indicating that contaminating zinc is not responsible for this translocation. Only when neutrophils are treated with C-peptide and zinc is GLUT1 translocated to the membrane in a statistically significant amount. This mechanism of action is similar to that seen in fat and muscle cells treated with insulin, and may indicate a new molecular mechanism for the action of

C-peptide in cells that do not respond to insulin. In this case, C-peptide is linked to glucose uptake and function of neutrophils, which is important in the immune complications associated with diabetes.

Activation of neutrophils requires glucose,[103] and hyperglycemic levels have been shown to activate neutrophils via glucose uptake by mass action. However, after prolonged exposure to high glucose levels, neutrophils revert to an inactive phenotype and do not respond to immunologic stimulation.[104] These mechanisms both contribute to the initial increase of activation of neutrophils followed by the suppression of cell activity in diabetes.

Of particular importance is the function of neutrophils in CF and CFRD, due to the immune deficiencies present in CF that are amplified in CFRD. In patients with CFRD, decreased levels of insulin and C-peptide have been reported, and patients are therefore treated primarily with insulin to control glycemic levels. However, these patients are still at risk for developing secondary complications. In addition to these complications, CF patients have deficiencies in their immune reactions, which leads to prolonged infections and increased susceptibility to infections, particularly in the lungs. Deficiencies in peripheral neutrophils in CF include increases in oxidative burst[72] and an increased propensity to release granule contents,[73] as well as abnormal pH regulation.[74] Neutrophils of CF patients also show reduced chemotactic response to chemoattractants[75-77] as well as a decreased number of receptors on CF neutrophils.[79] Cytokine production by neutrophils is also markedly different in CF patients.[80, 81]

Knowing that pancreatic secretions positively affect peripheral neutrophils from control patients, we then investigated if these secretions would have the same effect on cells in which CFTR had been inhibited, mimicking neutrophils of CF patients. Neutrophils inhibited with CFTRinh-172 show no difference in their uptake of C-peptide as compared to control cells, and show no difference when zinc is present or absent. Similarly, zinc uptake in CFTR-inhibited neutrophils is not significantly different than the controls with and without C-peptide. Intracellular and extracellular NO are increased only in the presence of C-peptide and zinc together, as are intracellular calcium levels. These results indicate that C-peptide and zinc may be a potential therapeutic target for immune system complications in CF and CFRD. Furthermore, glucose utilization by CFTR-inhibited neutrophils is only increased in the presence of C-peptide and zinc in albumin.

In the literature, CF neutrophils have been shown to have higher basal levels of GLUT1 and increased glucose uptake.[105] Here we present evidence that neutrophils inhibited with CFTRinh-172 do indeed have more GLUT1 at the plasma membrane, and that this level is further increased with treatment of C-peptide and zinc together (with or without insulin), but not insulin alone. Furthermore, C-peptide and zinc significantly increase the rate of chemotaxis of CFTR-inhibited neutrophils. This data indicates that the mechanism of action of pancreatic secretions is the same in control neutrophils as it is in neutrophils that mimic CF.

These *in vitro* results indicate that C-peptide may act as an immune system mediator, acting to increase the action of neutrophils, the first line of defense in infections, which

can decrease the overall immune system response, and perhaps help to decrease the chronic response seen in T1D and CFRD patients.

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Purpose of This Research

The discovery of insulin was initially thought to be a cure for diabetes,[1] and although it is necessary for survival, it is not a cure. Exogenous insulin is sufficient to lower blood glucose levels in patients with diabetes, however, it does not preclude patients from developing secondary microvascular complications such as retinopathy, neuropathy, and nephropathy. C-peptide replacement therapy in animal models of diabetes has shown a reduction in neuropathy[2-5] and nephropathy.[6, 7] In clinical trials of patients with T1D, beneficial effects in neuropathy[8, 9] and nephropathy are also present. [3, 10, 11] However, a large scale clinical trial of C-peptide replacement therapy (Ersatta) in T1D patients was shut down in phase 2b due to indistinguishable results from patients taking Ersatta and those taking the placebo.[12, 13]

As previously mentioned, the Spence group has made two essential discoveries toward explaining why this trial failed, despite all of the research that indicated it should have succeeded. First, Meyer *et al.* published that crude C-peptide (~80% pure) was actually contaminated with a metal, and when the metal was removed from C-peptide, its biological activity in red blood cells (RBCs) was abolished. [14] Addition of a physiologically relevant metal, Zn^{2+}, restored this biological action. [15, 16] Furthermore, Liu *et al.* discovered that healthy albumin was necessary for the biological action of C-

peptide and zinc, and that albumin exhibits a two-phase binding event to one molecule of zinc and then two molecules of C-peptide.[17]

5.2 Present Research

The data presented in this dissertation builds upon these two discoveries in three major ways: first, the effects of C-peptide and zinc were explored in another relevant cell type in the immune system, neutrophils; second, the effects of C-peptide and zinc were investigated in a model of cystic fibrosis (CF) in both RBCs and neutrophils; third, the potential molecular mechanism by which C-peptide and zinc are affecting cells that do not respond to insulin was reported.

We first investigated the effect of two important proteins on secretions the pancreatic beta cell, namely C-peptide, zinc, and insulin. ZnT-8 has been identified as a potential genetic marker for T2D, with the genetic polymorphism R325W resulting in altered glucose homeostasis, beta cell dysfunction and T2D in many study populations.[18-23] In T1D, ZnT-8 has been identified as an autoantigen,[24] making it an important protein to understand. The cystic fibrosis transmembrane conductance regulator (CFTR) is the protein that is defective in CF, and its function in pancreatic beta cells has just begun to be understood.

ZnT-8 downregulation in a rat insulinoma cell line (INS-1) results in dysfunction of all secretions from pancreatic beta cells. Insulin, C-peptide, and zinc secretions are decreased from these cells, indicating that ZnT-8 is involved in the regulation of proper secretions from pancreatic beta cells. Importantly, in ZnT-8 downregulated cells, the molar ratio of insulin to C-peptide secretions is decreased, which likely indicates that

proper cleavage of insulin from C-peptide in the secretory vesicle is impaired, as intracellular calcium levels are unchanged in these cells. Furthermore, the intracellular pH of these cells is significantly increased, indicating that the downregulation of ZnT-8 affects not only the secretions of insulin, C-peptide, and zinc, but also that it is influencing intracellular functions that the cell is unable to overcome. Figure 5.1 shows the proposed mechanism by which this increase in intracellular pH might occur.

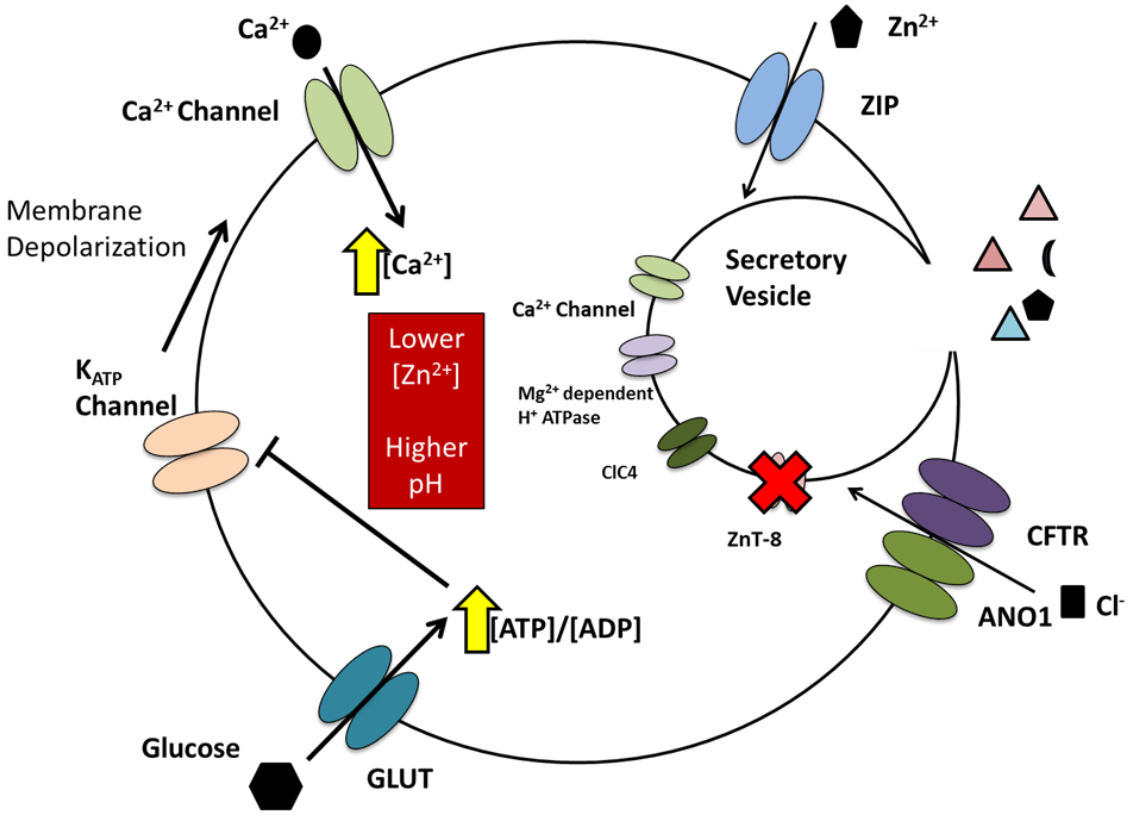

Figure 5.1: Proposed mechanism of the effect of downregulation of ZnT-8 in pancreatic beta cells. A decrease in the intracellular zinc level, a higher intracellular pH, and no change in the intracellular calcium levels as compared to controls may inhibit the activity of the proton pump on the secretory vesicle. Additionally, the necessary decrease in pH in the secretory vesicle is unlikely to occur, causing an increase of the proinsulin (insulin) to C-peptide ratio from the secretory vesicle.

T2D is characterized by insulin resistance, whereby insulin is secreted from the pancreas properly, but is not utilized by muscle and adipose cells correctly. In patients that exhibit this polymorphism in ZnT-8, these results may explain why resistance occurs. In fact, it may be that insulin is unable to be utilized by cells because it has not been properly cleaved from C-peptide, as we hypothesize here. Indeed, others have reported a disproportionate elevation of proinsulin is present in T2D patients,[25, 26] with a proinsulin: insulin ratio of 0.22 ±0.10 in T2D patients as compared to controls, who had a ratio of 0.097 ±0.03.[27] The normal amount of circulating proinsulin released from healthy human's beta cells is around 10%. In mild hyperglycemic conditions, this amount rises to 15-22%, and in extreme hyperglycemic conditions, as in diabetes, this rises to 40%.[28] With the data presented here, this resistance could be linked to defects in ZnT-8.

CFTR downregulation in INS-1 cells similarly showed a decrease in secretion patterns of insulin, C-peptide, and zinc, but the molar ratio of insulin: C-peptide was unchanged. Intracellular calcium levels remained the same, but intracellular pH levels decreased, which may contribute to decreased exocytosis, as CFTR has been linked to granule docking and exocytosis machinery in beta cells. [29-31] This data is critical in understanding how CF can lead to the development of cystic fibrosis related diabetes (CFRD). The prevailing theory is a combination of chronic pancreatitis and loss of the islet cells,[32-34] but recent research, including that presented here, indicates that the answer may be more complex. Figure 5.2 shows the proposed mechanism by which CFTR may affect C-peptide, zinc, and insulin secretions from pancreatic beta cells.

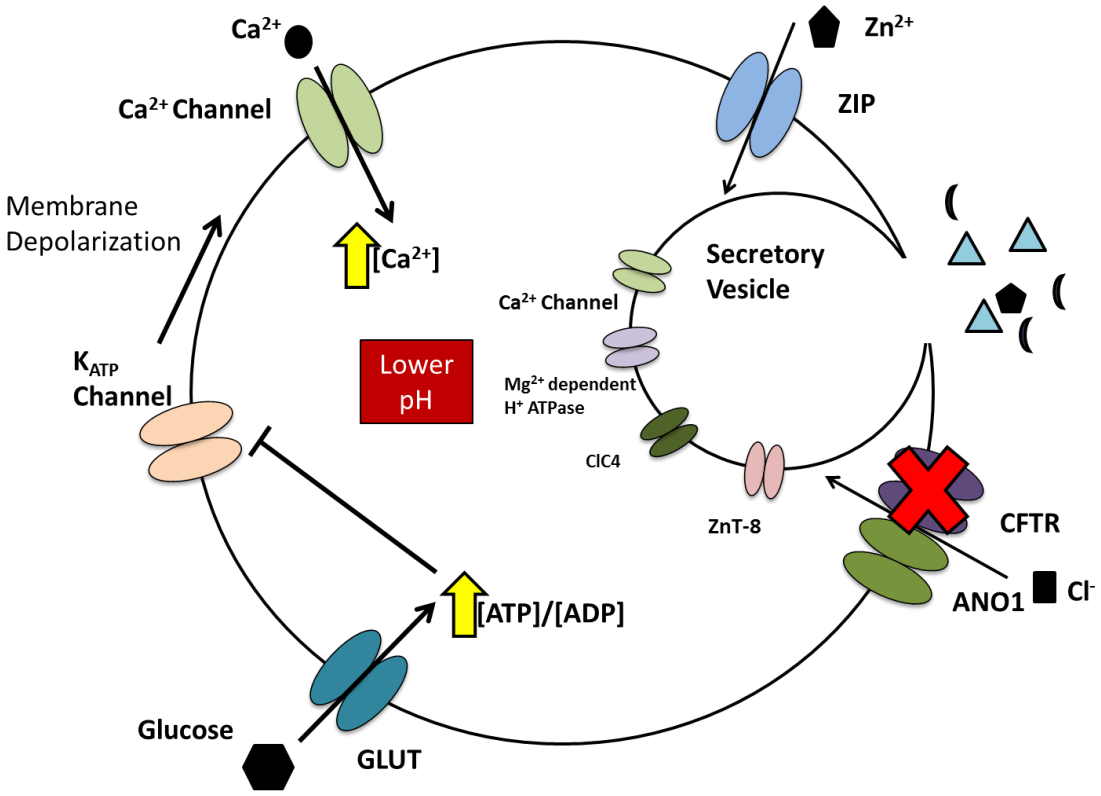

Figure 5.2: Proposed mechanism of the effect of downregulation of CFTR in pancreatic beta cells. A decrease in the intracellular pH, and no change in the intracellular calcium levels as compared to controls may point to the defect being in the exocytotic machinery, such as the SNARE proteins.

The understanding of these crucial proteins and their effect on pancreatic beta cell secretions is necessary to understand the etiology of T1D, T2D and CF/CFRD. It is also imperative to understand how these secretions are affecting other cell types in the body. It has long been accepted that insulin's action is through the insulin receptor, which is found primarily in muscle and adipose cells, to increase glucose uptake in these cells via translocation of GLUT4 (glucose transporter 4) to the plasma membrane. Insulin does not increase glucose uptake in other cell types, though we present

evidence here that other secretions from the pancreatic beta cells may act through a similar mechanism in cells that contain primarily GLUT1.

RBCs have previously been shown to release significantly more ATP upon deformation in the presence of C-peptide and zinc together with albumin.[14, 17] Additionally, RBCs have been shown to specifically bind C-peptide in both the presence and absence of zinc at about 2 picomoles per 7% RBC solution, though only bind zinc in the presence of C-peptide at approximately 2.5 picomoles per 7% RBC solution.[17] This 1:1 molar binding of C-peptide and zinc provided the basis to further investigate the molecular mechanism by which RBCs might be utilizing C-peptide and zinc to increase ATP release. Previously, Meyer *et al.* reported that RBCs treated with C-peptide and a metal utilized more glucose, which is the only source by which to produce ATP in the RBC.[14]

These results in control RBCs were confirmed in this dissertation, including C-peptide binding, zinc binding, ATP release (static), and glucose uptake. Furthermore, this research identified a proposed signaling mechanism by which C-peptide and zinc are acting in RBCs, through the translocation of GLUT1 to the plasma membrane. Additionally, this dissertation explored the effect of CFTR dysfunction on RBC function and their response to pancreatic secretions.

Sprague *et al.* reported that RBCs from CF patients and RBCs treated with CFTR inhibitors (niflumic acid and glibenclamide) released less ATP than control patients, but that this decrease was not due to decreased deformability.[35] Here we report the utilization of a potent, more recent CFTR inhibitor, CFTRinh-172, which also results in

significantly lower ATP release from RBCs as compared to controls. CFTR-inhibited RBCs exhibited increased C-peptide and zinc binding, though still at a 1:1 molar ratio. In the presence of C-peptide alone, zinc alone, or C-peptide and zinc together, CFTR-inhibited RBCs released statistically the same amount of ATP as control cells. Glucose uptake (30% increase) corresponds to the increase in ATP release from CFTR-inhibited cells (30% increase); however, this correlation only occurs when C-peptide and zinc are added together to the cells. These results suggest that ATP release deficiencies can be at least partially restored by C-peptide and zinc. Additionally, we show here for the first time that RBCs inhibited with CFTRinh-172 exhibit higher basal levels of GLUT1 by approximately 50% as compared to control RBCs. GLUT1 protein expression is, as in control cells, significantly increased in the presence of C-peptide and zinc together, with or without insulin. Insulin alone does not increase GLUT1 protein expression in either control or CFTR-inhibited RBCs.

These results in CFTR-inhibited RBCs are the first to show that pancreatic secretions can partially reverse the effect of dysfunctional CFTR. For the first time, we also show that GLUT1 translocation is linked to C-peptide and zinc's action in RBCs, and that CFTR-inhibited RBCs respond in the same manner as control cells to C-peptide and zinc.

Another important cell type that does not respond to insulin is neutrophils. Neutrophils are known to be dysfunctional in patients with T1D and T2D, including in chemotaxis,[36] phagocytosis,[37] and production of ROS;[38] and in patients with CF, with alterations in cytokine production, chemotaxis, ROS production, phagocytosis, and

apoptosis.[39, 40] As neutrophils are also a primarily GLUT1-containing cell type, we hypothesized that C-peptide and zinc might similarly affect RBCs and neutrophils.

Here we present evidence for the first time that neutrophils bind C-peptide and zinc in a 1:1 molar ratio similar to that exhibited in RBCs. The major difference in the binding between the two cell types is the binding of zinc. In RBCs, zinc binds only in the presence of C-peptide, while in neutrophils, due to the fact that zinc is a chemoattractant, zinc binds in the absence of C-peptide, but binds in significantly higher amounts in the presence of C-peptide. In addition, we present evidence that this binding and the subsequent cellular response is dependent on the presence of healthy albumin, as it is in RBCs.

The cellular response of neutrophils in response to C-peptide and zinc was here measured in the form of intracellular calcium levels, intracellular and extracellular NO levels, glucose uptake, GLUT1 protein expression, and chemotaxis. Intracellular calcium levels, which are critical for chemotaxis and phagocytosis, are increased in the presence of both C-peptide and zinc together, while either C-peptide or zinc alone do not increase intracellular calcium levels. Insulin is also insufficient to increase intracellular calcium levels. Intracellular and Extracellular NO are only significantly increased in neutrophils that have been treated with both C-peptide and zinc together with or without insulin, which is also important in phagocytosis. The rate of chemotaxis is significantly increased in the presence of C-peptide and zinc. As in RBCs, GLUT1 translocation to the membrane is significantly increased only in the presence of C-peptide and zinc with or without insulin.

Neutrophils inhibited with CFTRinh-172 bind statistically the same amount of C-peptide and zinc as control neutrophils, and only exhibit an increase in extracellular and intracellular NO and intracellular calcium in the presence of C-peptide and zinc together. GLUT1 levels are significantly higher in CFTR-inhibited cells than control cells, which agrees with previously published data.[41] CFTR-inhibited cells also exhibit slower rates of chemotaxis than control cells, agreeing with previously published data,[39, 42, 43] but chemotaxis is increased significantly upon treatment with C-peptide and zinc together.

These results are significant to the understanding of T1D and T2D immune complications and the treatment of CF/CFRD. As CF patients typically have altered neutrophil responses, this data indicates that pancreatic secretions positively affect neutrophils in both chemotactic and phagocytic capacities, and that these secretions could provide a potential therapeutic for patients. In diabetes (T1D, T2D, CFRD), this data may explain the increased propensity of diabetics to the development and duration of infections[44] and a potential treatment that would not only increase the utilization of excess glucose, but improve immune response and microvascular complications.

In insulin-responsive cells, insulin binds to the tyrosine kinase insulin receptor (IR), triggering a complex downstream cascade of signals. Phosphorylation and recruitment of adaptors initiates the activity of PI3K, which activates Akt/PKB and PKC cascades. The activation of the PI3K/Akt pathway leads to the translocation of GLUT4 vesicles to the plasma membrane, which allows glucose to enter the cell. This translocation of GLUT4 in response to insulin signaling is crucial to allow the cell to remove excess glucose from the bloodstream and metabolize it.

The data presented here suggest a novel molecular mechanism for C-peptide and zinc in cell types that are not responsive to insulin, but in a mechanism similar to that in insulin-responsive cells. The probable C-peptide receptor, GPR146, a G-protein couple receptor,[45, 46] signals a number of identified intracellular signaling mechanisms, including the elevation of intracellular calcium concentrations,[3, 47] phosphorylation of phospholipase C (PLC) and protein kinase C (PKC) isoforms,[48-50] phosphoinositide 3-kinase (PI3K) activation,[50, 51] and mitogen activated protein kinase (MAPK) signaling.[48, 49] In insulin responsive cells, PI3K and PKC activation lead to translocation of GLUT4. Assuming that these signaling pathways are similar in neutrophils and RBCs, Figure 5.3 shows the mechanisms described in the literature and the addition of the activation of translocation of GLUT1 that we present here.

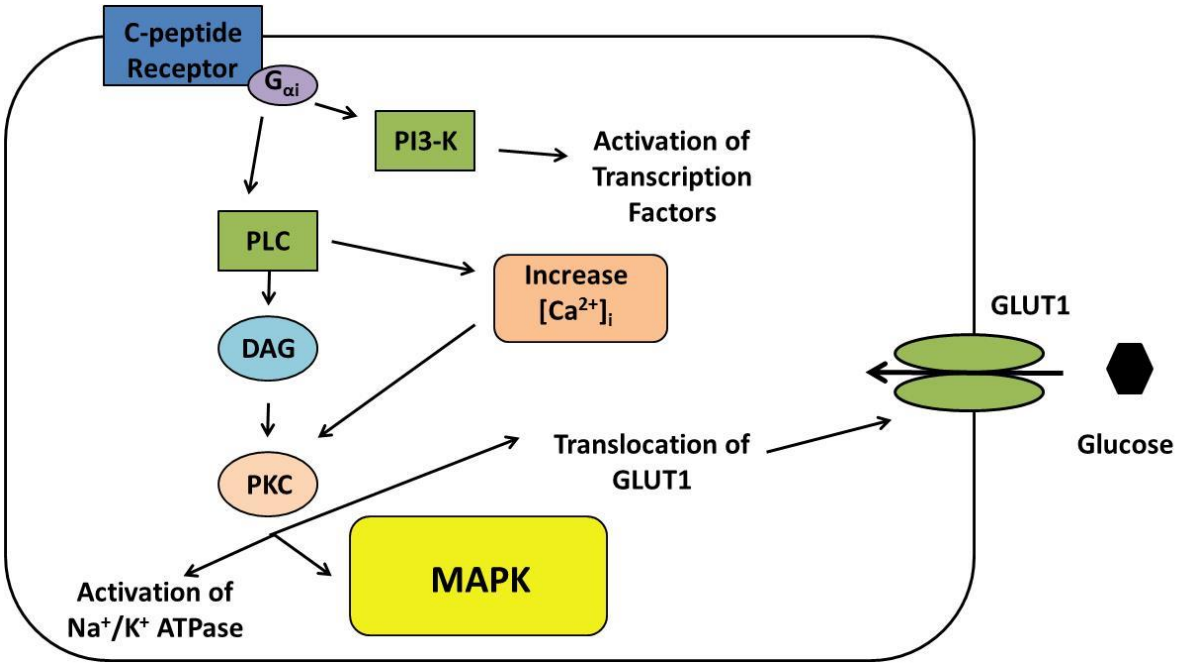

Figure 5.3: The proposed mechanism suggested here for C-peptide and signaling in RBCs and neutrophils. The probable C-peptide receptor, GPR146, signals through Gαi to activate PLC and PI3-K. PI3-K goes on to activate transcription factors, while PLC catalyzes the conversion of PIP2 to DAG and IP3. DAG activates PKC, which, in insulin signaling, causes the translocation of GLUT1 to the plamsa membrane.

5.3 Future Directions

The present research opens the door for three major future areas of research. First, it allows for *in vitro* studies to study inter-system and inter-tissue communications in disease models, including in T1D, T2D, and CFRD. Second, it allows for glucose flux experiments in RBCs and neutrophils to elucidate the way in which C-peptide and zinc are influencing glucose metabolism in these cell types. Finally, it opens the door for the investigation of GLUT1 translocation and signaling in these cell types via molecular biology techniques.

5.3.1 *In vitro* Inter-system Communication Studies

Previous work in the Spence group has focused on the utilization of 3D printed devices and the integration of cell culture Transwell inserts for inter-tissue and inter-system communication. Figure 5.4A shows the first generation of this device, designed by Chengpeng Chen. In this device, six parallel channels are able to be connected through commercial fingertight fittings and tygon tubing to form a closed loop. This tubing can be hooked up to a peristaltic pump to mimic circulation. Each channel has three spots for Transwell inserts, which can be used for cell culture or collection and detection of molecules of interest. Figure 5.4C shows a second iteration of this device, created by Cody Pinger, which replaces one cell culture well with a printed septum for use in introducing reagents or removal of a sample for offline analysis without disturbing the closed loop system.

The generation of stable cell lines downregulated in ZnT-8 and CFTR expression presented in this dissertation allow for the utilization of a better mimic of a disease on this platform. For example, one could culture ZnT-8 downregulated cells on one insert, flow RBCs from T2D patients in the circulation loop, and measure ATP release from RBCs in the second Transwell insert. Utilizing the printed septum, one could then introduce exogenous C-peptide and zinc together with albumin, or other drugs of interest into the circulation and obtain a much more realistic mimic of T2D. To mimic T1D, no INS-1 cells would be cultured on the device, as those cells are mostly destroyed in T1D. A mimic of CF/CFRD might include the growth of INS-1 cells downregulated in CFTR and RBCs from CF patients or RBCs incubated with a CFTR inhibitor. Alternatively, neutrophils could be flowed in the circulation rather than RBCs

to investigate the communication between the pancreatic and the immune systems in these diseases. Some additional considerations with these experiments that may be necessary include the coating of the bottom of the Transwell insert with endothelial cells or a selectin molecule for the neutrophils to adhere to and pass through to a chemoattractant in the well.

These mimics are much more realistic than current models for a number of reasons. First, pancreatic beta cells (here mimicked by INS-1 cells) release a number of other small molecules in addition to insulin, C-peptide and zinc that may be affecting RBCs and neutrophils.[52-54] Second, in a static system, cells may not act the same ways as they would *in vivo*, when they are circulating through the bloodstream. Third, this system allows for the utilization of whole blood instead of isolated RBCs or neutrophils that may be influencing the inter-system communication, such as the presence of glycated albumin in diabetic samples, which may impact the efficacy of C-peptide and zinc as well as drugs of interest.

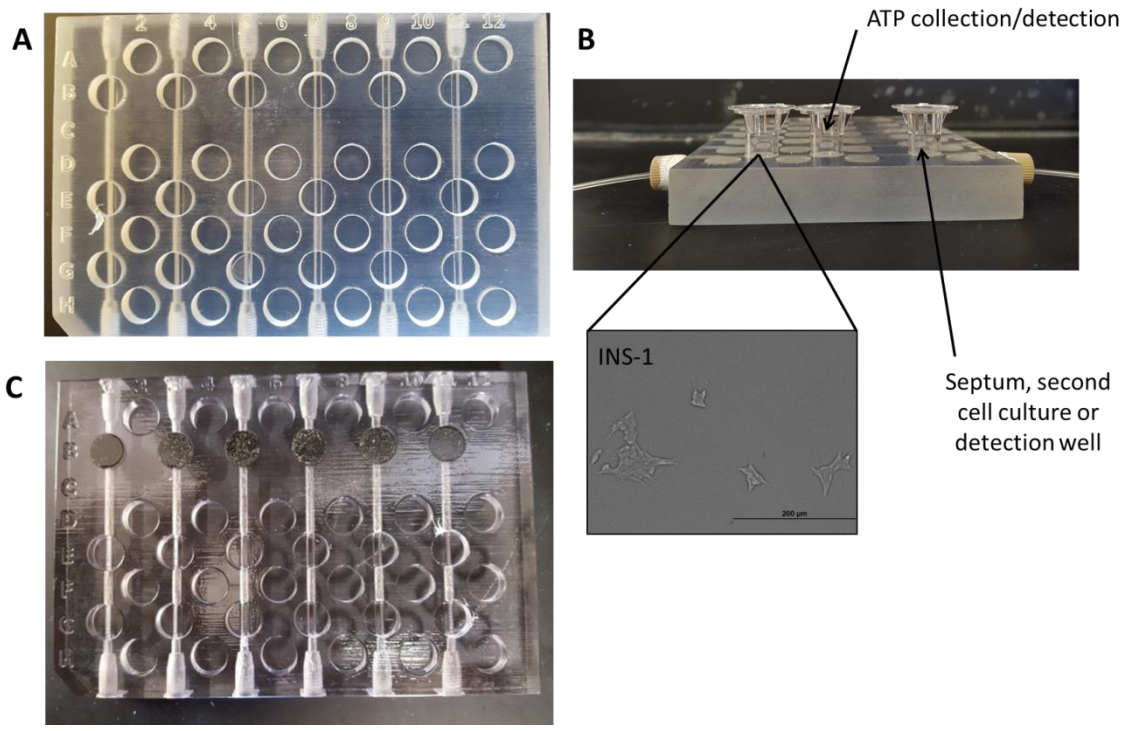

Figure 5.4 3D printed device for inter-system communication studies. Panel A shows the device designed by Chengpeng Chen, in which three cell culture inserts are placed over a channel in the device, which is then connected to tygon tubing and fingertight fittings for a closed loop. These wells can be used for many things, including the culture of INS-1 or downregulated cells, endothelial cells, or ATP collection and detection (Panel B). Panel C shows a second generation device design by Cody Pinger, in which the first cell culture well has been replaced with a 3D printed septum for introduction of reagents or removal of reagents for offline detection without disturbing the closed loop circulation.

A second method utilizing this same device that the Spence group is interested in is the utilization of coated antibodies on the Transwell inserts to selectively remove different components of pancreatic beta cell secretions. Although this is not a good disease model for T1D, T2D, or CF/CFRD, it is useful to selectively determine the effect of specific molecules on the circulating cells. For example, an antibody against C-peptide coated onto the Transwell insert would allow for all secretions from INS-1 cells through

the membrane except for C-peptide. In theory, one could completely remove all C-peptide from diffusing into the circulation, and measure a downstream signal to see if C-peptide alone is responsible for that effect. This method of selective determination of pancreatic secretions that affect ATP release from RBCs is currently under investigation in the Spence lab.

5.3.2 Glucose Flux Studies

It is clear from the evidence presented here in both RBCs and neutrophils that C-peptide and zinc are in some way influencing the metabolism of glucose in the cell. In RBCs, ATP release from the cell is increased when in the presence of C-peptide and zinc, however, it is unclear at this time if C-peptide and zinc are increasing the release of ATP already stored in the RBC or if it is increasing the metabolism of glucose to create ATP. Glucose utilization studies in RBCs treated with C-peptide and zinc indicate that the cell is likely increasing the amount of ATP being created in the cell because it is utilizing more glucose. Glucose flux studies would provide evidence to support this hypothesis.

Glucose flux studies are made possible by C13-labeled glucose and targeted metabolomics. By tracking the glucose that enters the cell and the metabolites that are released from that cell, one can determine the amount of C13 that is used to make these metabolites, such as ATP. Separation of the metabolites released from RBCs and the intracellular metabolites is simple, and separation of polar and nonpolar metabolites can be achieved using chlorophyll-methanol extraction. Both RBC and

neutrophil glucose flux is able to be studied in this manner, as well as cells that are incubated with inhibitors, such as CFTRinh-172.

5.3.3 Molecular Biology of GLUT1 Translocation

GLUT translocation has traditionally been studied by immunofluorescence,[55] flow cytometry,[56, 57] and fluorescently labeled antibodies.[58] As extensively as the signaling mechanisms and pathways have been described for insulin signaling and GLUT4 translocation, the same needs to be done for C-peptide and zinc in relation to GLUT1 translocation.

5.4 Implications in Disease

The research presented here has implications in the treatment of T1D, T2D, and CF/CFRD. In T1D, this research and others[14, 17] present motivation for the continued study of C-peptide and zinc a potential therapeutic for microvascular complications, and now in immune deficiencies as well. The failure of the Cebix trial has cast doubt over the *in vitro* and pilot *in vivo* studies that suggested that C-peptide was indeed a modulator of nephropathy and neuropathy symptoms in T1D. However, the addition of zinc and healthy albumin may not only increase the effectiveness of these studies, but also increase consistency between studies. This research also presents evidence that C-peptide does have a direct effect on modulating the immune system, namely neutrophils. This further warrants the continued study of C-peptide and zinc as a therapeutic in T1D.

In T2D, this research adds to the growing wealth of literature on the effect of ZnT-8 dysfunction in the etiology of the disease. Although it is unlikely that C-peptide and zinc

would be a useful therapeutic in T2D patients, this research provides a unique platform for *in vitro* studies of inter-tissue and inter-system communications that better mimic *in vivo* conditions.

In CF/CFRD, this research again provides motivation for continued study of C-peptide and zinc as a potential therapeutic, particularly in CFRD. In the past four years, two new drugs to help treat CF have been approved by the FDA: Ivacaftor[59] and Orkambi.[60] Both of these drugs are classified as genetic modifiers, and are specific to only two mutations in the CF population. Both Ivacaftor and Orkambi have been quickly approved by the FDA without much understanding of how they are working at a molecular level. As more research is done on the molecular mechanisms of these genetic modifiers, it will become crucial to understand the problems associated with CFTR defects in all biological systems, including in the pancreatic beta cells. Neither Ivacaftor nor Orkambi are cures for CF, although they are a huge scientific advance. It is likely that these drugs will be incredibly useful in alleviating some symptoms of the disease, however it is still likely that patients will develop complications such as CFRD, where therapies such as C-peptide and zinc will be useful.

The ultimate goal of this research, no matter how small, is to make the life of patients with these diseases better in some way. Whether in the understanding of the etiology of the disease, a new treatment, or in understanding the molecular mechanisms behind a particular complication, the goal is to help people, and it is my hope that this research has accomplished that goal in some small way.

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