ADIPOSE-DERIVED MOLECULAR AMPLIFICATION OF GLUCOSE TRAFFICKING IN THE BLOODSTREAM: A POTENTIAL NEW ROLE FOR LEPTIN

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Chemistry-Doctor of Philosophy

2018

ABSTRACT

ADIPOSE-DERIVED MOLECULAR AMPLIFICATION OF GLUCOSE TRAFFICKING IN THE BLOODSTREAM: A POTENTIAL NEW ROLE FOR LEPTIN

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Leptin, a hormone produced mainly by adipose tissue, is believed to balance energy levels *in vivo* by regulation of food intake and body weight. Higher concentrations of leptin are observed in obese people, who are thought to be "leptin resistant." Leptin resistance is common in patients with diabetes and studies show leptin can exhibit glucoregulatory effects on patients with type 1 and type 2 diabetes. In spite of this, obese diabetic patients do not exhibit a strong response to exogenous leptin. Thus, a detailed understanding of the glucose regulatory function of leptin is of crucial importance in order to overcome the shortcomings of leptin therapy and its use as a potential therapeutic for humans. We have provided a novel aspect on leptin function by investigating it in the blood stream. Previously, our group has reported that C-peptide, a 31 amino acid peptide secreted from pancreatic beta cells, binds to red blood cells (RBCs) and has cellular energetic effects. Here, we show that leptin actually amplifies the effects of C-peptide. ATP release from RBCs was measured in the presence of leptin, C-peptide, zinc, and combinations thereof, while also monitoring the translocation of GLUT-1 to the RBC membrane. In the presence of C-peptide and zinc, a 30% increase in RBC-derived ATP is measured; this signal is enhanced by another 20% in the presence of leptin. This effect was also amplified under high-glucose conditions, as seen in diabetes. however, leptin alone showed no effect on RBCs. Interestingly, leptin in the presence of zinc and C-

peptide increases GLUT-1 translocation by 20% compared to the control and samples including zinc and C-peptide only. ATP measurements were performed using Luciferin/ Luciferase chemiluminescence assay and GLUT-1 translocation was monitored by SDS-PAGE gel and western blot techniques. We have also measured an increase in the amount of C-peptide bound to RBCs in the presence of leptin, and a saturable dose dependent response was observed between C-peptide and RBCs. Binding studies were performed using C-peptide and leptin enzyme linked immunosorbent assay (ELISA) respectively. Our studies show that levels of leptin in the bloodstream may be correlated with glucose concentrations, and the ability of RBCs to use glucose. Therefore, leptin along with zinc and C-peptide can potentially be targeted as a therapeutic for diabetes.

To my lovely parents

ACKNOWLEDGMENTS

Numerous people helped me during the course of my graduate school and without their support, this process would be impossible. My advisor professor Dana Spence was one of those people that influence me not only in scientific way but also in personal perspective. His mentorship was led me to grow as a scientist and thought me how to think scientifically and guided me to grow trouble shooting skills. I am grateful to my committee members, Dr. Liangliang Sun, Dr. Jetze Tepe and Dr. Jian Hu for their advice and support. Tiffany has been fantastic senior for me; he kindly taught me to be a better chemist. I also want to send my thanks to Dr. Suzzanne Summers for her help and encouragement. I am thankful to my wonderful group members Cody Pinger, Andre Castiaux, Andre Heller, Becca Boyea, and Morgan Geiger.

Mostly, I express my gratitude to my parents, brother, sister and Bardia for their unconditional support and love. I wouldn't be able to finish this journey without their encouragement and support

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KEY TO SYMBOLS AND ABBREVIATIONS

AC	Adenylyl cyclase
ADA	American Diabetes Association
AGEs	Advanced glycation end products
APS	Adenosine 5'-phosphosulfate
ATP	Adenosine triphosphate
BCIP	5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt
BIRB	Biomedical and health institutional review board
BMI	Body mass index
CAD	Computer aided design
cAMP	3’5’-cyclic adenosine monophosphate (cAMP)
Cart	Cocaine amphetamine regulated transcript
cDNA	Complimantary DNA
CFTR	Cystic fibrosis trans-membrane conductance regulator
ChREBP	Carbohydrate-response element-binding protein
CNS	Central nervous system
ELISA	Enzyme Linked ImmunoSorbent Assay
eNOS	Endothelial nitric oxide synthase
GABA	γ amino butyric acid
GBM	Glomerular basement membrane
GDM	Gestational diabetes mellitus

Gi	Heterotrimeric G protein
GLUT	Glucose transporter
GLUT-1	Glucose transporter 1
GLUT-4	Glucose transporter 4
GPCR	G-protein coupled receptor
HbA1c	Glycated hemoglobin
HNC	Hyperosmolar non-ketotic coma
HRV	Heart rate variability
INS-1	Insulinoma cell lines
IRS	Insulin receptor substrate
ITC	Isothermal titration calorimeter
LA	Lactic acidosis
MC4R	Melanocortin-4 receptor
MFS	Major facilitator superfamily
NBT	Nitro-blue tetrazolium chloride
NCV	Nerve conduction velocity
NO	Nitric oxide
OGTT	Oral glucose tolerance test
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
POMC	Pro-opiomelanocortin

PP	Pancreatic polypeptide
PSS	Physiological salt solution
PVDF	Polyvinylidene difluoride
RBC	Red blood cell
SDS	Sodium dodecyl sulphate
TBS	Tris buffered saline
T1D	Type 1 diabetes
T2D	Type 2 diabetes
VEGF	Vascular endothelial growth factor
WAT	White adipose tissue
WHO	World health organization
ZIP	Zrt/Irt-like porteins

Chapter 1- Introduction

1.1 Diabetes Mellitus, Diagnosis and Causes

Diabetes Mellitus is a metabolic disorder associated primarily with the hormone insulin, which is secreted from pancreatic β-cells.[3] Insulin, a 58 kDa polypeptide hormone, facilitates glucose transport into certain cell types such as fat and muscle cells, which in turn may result in lower bloodstream glucose levels.[7] *Diabetes Mellitus,* commonly referred to as diabetes, is caused by impaired insulin deficiency or insulin efficacy.[3] Diabetes patients have a buildup of glucose in the bloodstream due to the defect in insulin function, which can lead to hyperglycemic conditions that result in different types of patient complications. Some of the short-term complications are thirst and hunger, while the more severe long-term complications include retinopathy (blindness), nephropathy (kidney failure), and neuropathy (nerve damage).[8]

Diabetes is rapidly becoming an epidemic in the United States. According to the 2017 Center for Disease Control (CDC) report, 9.4% of the total U.S. population (30.3 million people) is diabetic. This amounts to 23.1 million people that are already diagnosed with diabetes, and the remaining 7.2 million, or 23.8% of the people with diabetes are undiagnosed. Additionally, 84.1 million people are prediabetic, meaning that while they do not yet have diabetes, if they do not make changes in their lifestyle and diet, they will develop the disease. In total, diabetes affects one third of the total U.S. population. The prevalence of diabetes varies based on age, as well as ethnicity and sex. Diabetes affects all ages, but 30.2 million of the patients are aged 18 or older, which accounts for 12.2% of all adults in the U.S. Figure 1.1 illustrates the prevalence of diagnosed diabetes among adults older than 18 years by race and sex. Overall, American

Indian/Alaska Native women had the highest prevalence of diagnosed diabetes, followed by American Indian/Native American men and Black, non-Hispanic women.[2]

Diabetes was the seventh highest cause of mortality in the United States in 2015. The

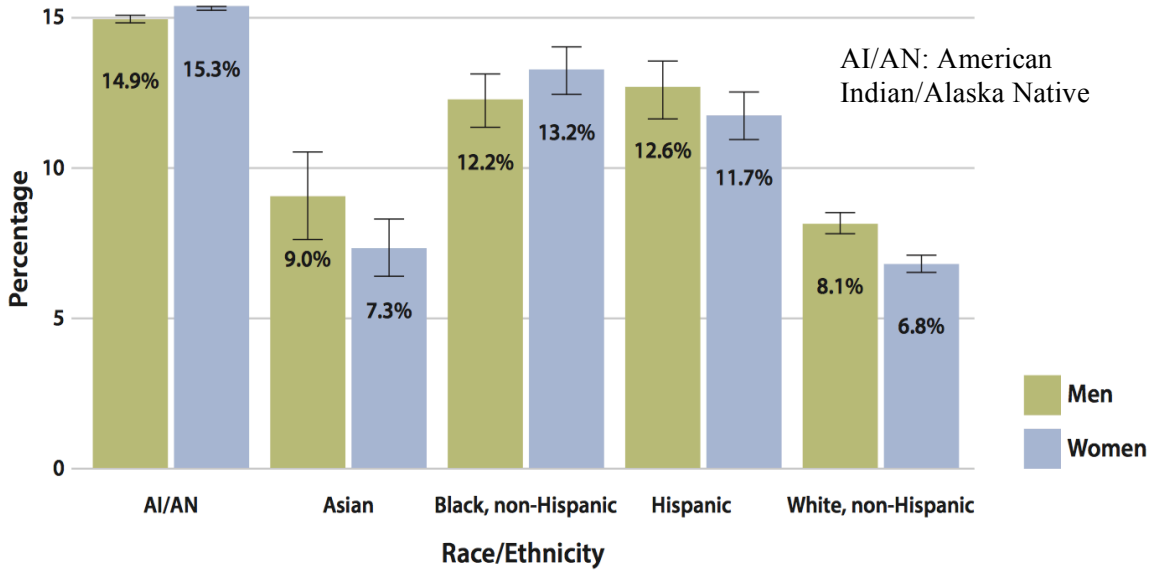

Figure 1.1 The Prevalence of Diagnosed Diabetes in US Population Aged Older than 18 based on Race and Sex.[2]

complications associated with diabetes are the major contributing factors leading diabetes to one of the top causes of death in United States.[2] The economic burden of diabetes on society is considerable. According to the 2012 National Diabetes Statistics Report, the total expenditure for diabetes in the U.S. alone is $245 billion for direct and indirect costs.[9] With the increasing incidence of diabetes, especially among younger people, the economic burden of diabetes will continue to increase unless necessary actions are taken to effectively control its onset.[10]

A hallmark feature of diabetes is elevated glucose concentration in the bloodstream. Therefore, blood glucose level is measured as an indicator of diabetes. There are two different methods employed for measuring blood glucose level according

to the American Diabetes Association (ADA)[11-12] and the World Health Organization (WHO).[13] The first method is a fasting plasma level, with a diagnostic cutoff point of above 126 mg/dL or 7.0 mM resulting in the diagnosis of diabetes. In this method, patients are advised not to eat or drink anything except water for 6-8 hours before the test. Blood is collected usually from the arm of the patients and plasma is separated from the cells to measure the blood glucose levels in it.[14] Retinopathy, one of the long-term complications of diabetes, becomes detectable at this level. The second way to measure blood glucose level is by using an oral glucose tolerance test (OGTT). This test is basically measuring how well the body absorbs glucose after administration of certain amount. Patients consume syrup that contains 75 mg of glucose and wait for two hours and then a blood sample will be collected.[15] The diagnostic cutoff point after an OGTT is 200 mg/dL (11.1 mM). The fasting plasma glucose test is the preferred test in the United States, due to the complexity and reduced precision associated with OGTT. However, a higher number of diabetes cases are identified using the OGTT.[16]

Another method used to diagnose diabetes is by measuring the percentage of glycated hemoglobin (HbA1c). Glucose attaches to hemoglobin A and as the levels of glucose in the blood stream increases, more glycated hemoglobin A will be formed. The healthy level of HbA1c is 5.7% and diabetic patients show levels of HbA1c greater than 6.5%. This test is most commonly used to examine how well current therapy is controlling the blood glucose level in the patient because it reflects the blood glucose over the course of 2-3 months.[17]

1.2 The Pancreas, a Multifunctional Organ

The human pancreas contains islets of Langerhans consisting of different cell types such as α-cells, β-cells, δ-cells and pancreatic polypeptide (PP) cells.[18-20] The pancreas functions as an exocrine gland, secreting digestive enzymes as well as endocrine function using the islet cells.[19, 21] The pancreas regulates the digestive system by secreting pancreatic fluid, which contains digestive enzymes and helps in the metabolism of carbohydrates, lipids, and proteins in the small intestine.[22] α-cells are primarily responsible for regulating gluconeogenesis, the conversion of amino acids to glucose via secretion of the hormone glucagon. α-cells secret glucagon when the levels of glucose in the bloodstream drops, such as during exercise and fasting[19] and they make up about 35-40% of the Langerhans islets.[18] δ-cells secret the hormone somatostatin to suppress the release of other hormones such as glucagon.[18] The release of somatostatin is stimulated by glucose. PP cells make up about 1% of the islets and are thought to be responsible for regulation of both functions of insulin as well as appetite.[23] β-cells make up the remainder of the islets (about 50%) and secrete a number of molecules including amylin, γ amino butyric acid (GABA), C-peptide, and insulin as well as zinc ions. Amylin is believed to act as a synergistic partner of insulin. GABA suppresses the secretion of glucagon via a receptor on α-cells.[24-26] The function of insulin, C-peptide, and zinc will be discussed in more detail in the following section.

1.2.1 Insulin

Insulin regulates the levels of glucose in the body by acting on the cells containing glucose transporter 4 (GLUT4); GLUT4 is commonly found in such cells as fat cells and muscle cells. Insulin binds to receptors, signaling the translocation of GLUT4 on the membrane of these cells and leading to an increase in glucose influx.

Insulin was discovered in 1921 by Dr. Frederick Banting and his medical student, Charles Best, while working in professor John Macleod's laboratory space at the University of Toronto. They discovered insulin by extracting a substance from the pancreas and testing it on a diabetic dog. Surprisingly, the substance kept the dog healthy and they decided to purify the extract to test on humans with the help of Bertram Collip. Banting tried insulin on himself for the first time in 1922 and a 14-year-old diabetic boy (Leonard Thompson) was the next to receive insulin. Frederick Banting and John Macleod share a 1923 Nobel Prize for the discovery of insulin with Charles Best and Bertram Collip.[27] The same year, Eli Lilly, a global pharmaceutical company, began producing insulin to supply the patients in need in North America. Fred Sanger determined the sequence of insulin in 1950 and was awarded the Nobel Prize in 1958, leading to inspirations to study insulin assembly.[28] The structure of the single chain insulin precursor, proinsulin, was discovered by Steiner in 1967.[29]

Preproinsulin that is proinsulin plus the signaling peptide is manufactured in ribosomes and consists of a 24-amino acid signal peptide, 21-amino acid A-chain, 30-amino acid B-chain, and a 31-amino acid linking peptide. Preproinsulin interacts with signal recognition particle in cytosol as shown in Figure 1.2.[30-32] Then, the signal peptide

is cleaved and degraded and proinsulin is translocated across the membrane of the rough endoplasmic reticulum into the lumen.[33] Next, proinsulin, which is composed of the A-chain, B-chain, and the connector peptide, is folded via formation of three disulfide bonds and transported to the Glogi apparatus via vesicular transfer and packaged into immature secretory vesicles as hexamers er.[34-35]

The amino acid sequence of proinsulin is shown in Figure 1.3. During the maturation process, proinsulin will be converted to insulin by endopeptidases and carboxypeptidase, cleaving off the connecting peptide, C-peptide.[36] Two acidic prohormone convertases, Type II secretory granule protease (PC2) and Type I secretory granule protease (PC 1/3) secreted from pancreatic β-cells are involved in the cleavage of C-peptide.[37-38] PC2 acts on the A-chain while PC 1/3 acts on the B-chain. In addition to C-peptide, zinc ions and a drop in the pH to 5.5 are also both involved in the maturation process of proinsulin and insulin biosynthesis. Next, residues will be removed by carboxypeptidase E and H,[39-40] which are active in pH 5.5 and have little to no activity at pH 7.4. The maturation process is depicted in Figure 1.4. and results in the formation of insulin, C-peptide, and ions such as zinc in the secretory vesicle.

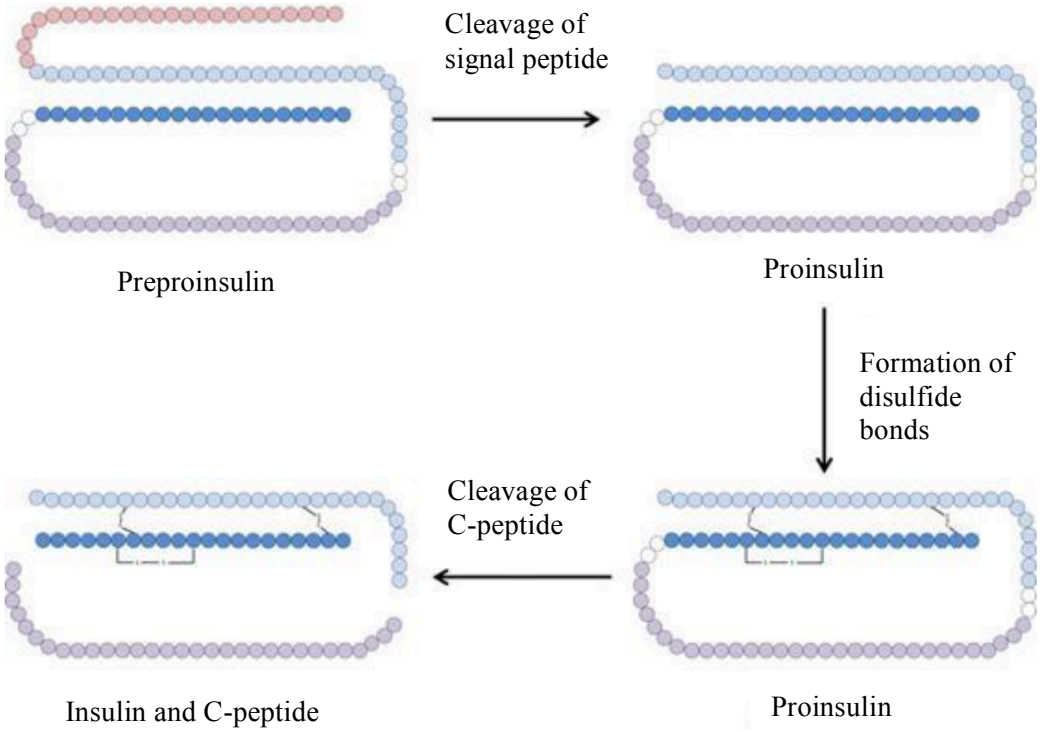

Figure 1.2 From Preproinsulin to Insulin. Signal peptide is shown in pink, A-chain in dark blue, B-chain in light blue and C-peptide in purple. Preproinsulin, containing signal peptide, is converted to proinsulin by cleavage of the signal peptide in lumen. Then, the product of this cleavage, proinsulin, will be transported to rough endoplasmic reticulum where disulfide bonds between A and B-chains are formed. Then, proinsulin is transported to immature vesicles and C-peptide is cleaved during the maturation process.

Insulin is a highly conserved peptide among different species[21] and its concentration in the blood stream rises and falls due to glucose stimulation in healthy humans. The concentration of insulin ranges between 50 pM to 200-500 pM.[41] The release of insulin is biphasic meaning that insulin will be released 5-6 minutes after glucose stimulation in the first phase of the release, and then the gradual release of insulin will begin and continues for more than 60 minutes in the second phase of release.[42]

Insulin exists in the secretory granules of pancreatic β-cells as stable crystalline hexamers,[43] complexed with zinc ions.[44-45] It's believed that there are two conformations

for insulin monomers in the crystalline hexamers depending on the first nine residues of the B-chain. In the R conformation, these residues are in an alpha helix, while in T conformation these residues are elongated.[46] The crystalline hexamers are floating in an aqueous halo of C-peptide, ions, and other small molecules.[43] These hexamers are stable in acidic pH of the secretory vesicles and exposure to the blood stream of pH 7.4 destabilizes them, leading to a 1:1 release of C-peptide and insulin.[47]

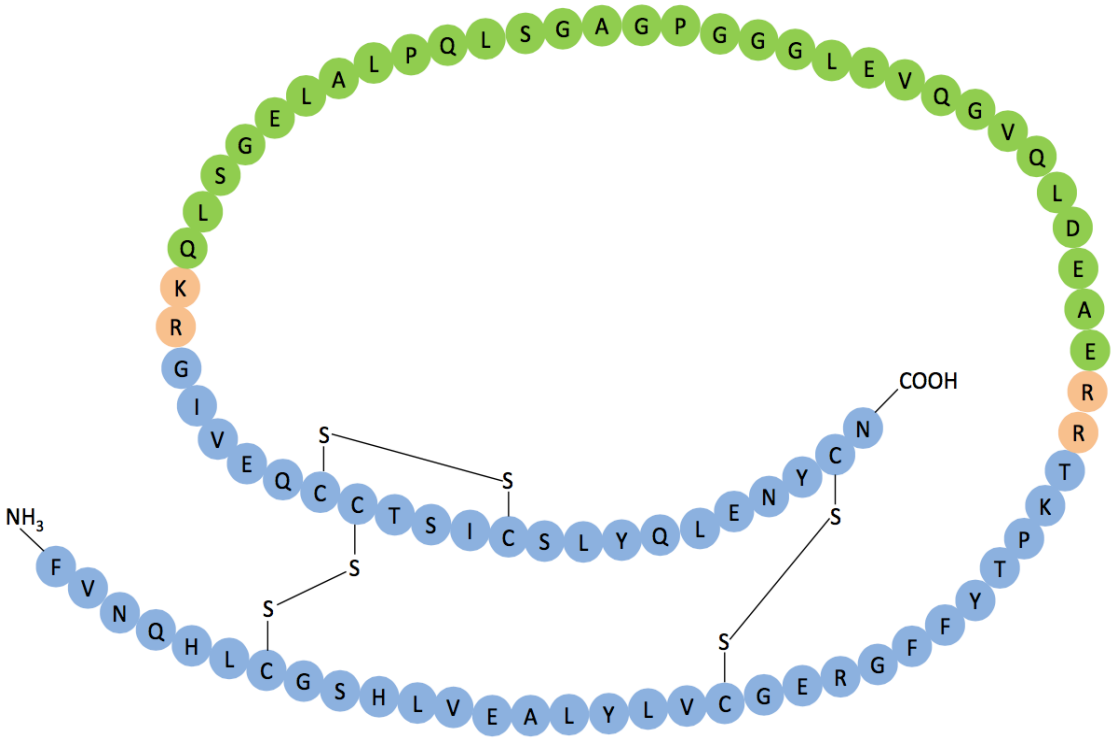

Figure 1.3. Amino Acid Sequence of Proinsulin. Proinsulin consists of A and B-chains of insulin connected via a 31 amino acid peptide, C-peptide (green).[5] There are three disulfide bonds in proinsulin, one of them is in A-chain and the other two connect A and B-chain, which make the conformation of proinsulin. When C-peptide is cleaved, the blue segments of A and B-chains for the insulin molecule. This results the production of C-peptide and insulin in equimolar ratio.

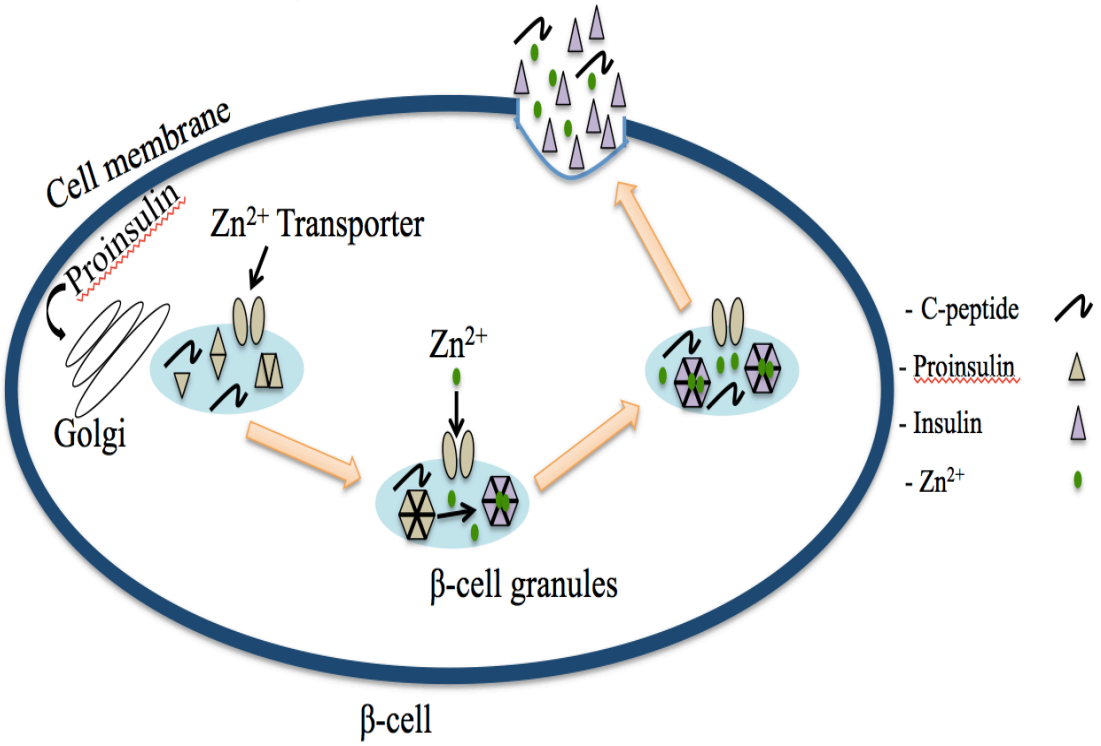

Figure 1.4. Maturation Process of Insulin in Pancreatic β-Cells. Proinsulin is transferred to Golgi after the signal peptide is cut from preproinsulin and packaged into hexamers in immature secretary vesicles. Then, proinsulin hexamers are converted to insulin hexamers by freeing the C-peptide via regulation of zinc. Insulin, C-peptide, and zinc are store in secretion granules until exocytosis upon stimulation by high glucose environment. Insulin, zinc, and C-peptide are released into blood stream in equimolar levels.

1.2.2 C-peptide

C-peptide was discovered in 1967,[48] although no biological activity was identified until the 1990s.[49] The 31-amino acid connector peptide becomes free after cleavage from proinsulin and is released to the blood stream in an equimolar concentration with insulin upon release.[21] This peptide is negatively charged with no ordered structure. Physiological C-peptide levels vary between 0.3-0.6 nM after overnight fasting and 1-3 nM after a meal.[50]

C-peptide has long been viewed as an inactive molecule serving as the linker between the two chains of insulin and stabilizing proinsulin.[49] However, C-peptide was an ideal indicator of pancreas function due to its longer half-life in the bloodstream compared to insulin. The half-life of insulin in the bloodstream is 5 minutes, making it difficult to measure directly in the bloodstream compared to C-peptide, which has a half-life of 30 minutes.[51-52]

The amino acid sequence of C-peptide is only 25% conserved between different species, and glutamic acid residues in positions 3 and 27 are the two most conserved residues.[21] However, the C-terminal pentapeptide sequence of C-peptide, EGSLQ, has been shown to have independent biological effects and could potentially serve as a binding site to cells or receptors.[53-54]

Initially, investigators hypothesized insulin-like function for C-peptide, but no glucose uptake was observed by C-peptide. However, interest in biological activity and mechanism of action of C-peptide persisted. Studies have shown that patients with little β-cell activity are less likely to develop diabetes complications compared to patients with

absolute C-peptide deficiency.[55-56] In addition, more recent studies have reported that C-peptide is biologically active and is effective in improving blood flow, kidney function and glucose uptake in skeletal muscle.[57-58]

1.2.3 Zinc

Zinc ions are essential in the body for the function of about 300 proteins, including synthesis and storage of insulin.[59] Two zinc ions are complexed to the insulin hexamer, making the presence of zinc necessary for insulin biosynthesis and storage. Pancreatic β-cells have one of the highest concentration of the zinc in the body, about 30 mM.[24, 60] About one third of the pancreatic β-cell zinc content is present in the secretory vesicles and large amounts[61] of zinc are produced from β-cells.

Zinc homeostasis is regulated via a complex network of transporters on the cell membrane, endoplasmic reticulum, Golgi, mitochondria, and secretory granules.[62] Zinc is transported from the cytosol to the extracellular space, or intracellularly into secretory vesicles via the ZnT family of the transporters. Zrt/Irt-like porteins (ZIP) family of transporters facilitates entrance of zinc into the cytoplasm from outside the cell or from intracellular compartments.[63]

1.3 Classification of Diabetes Mellitus and Current Treatments

There are four major types of diabetes: type 1 diabetes (T1D), type 2 diabetes (T2D), gestational diabetes mellitus (GDM) and other specific types of diabetes.[64] T1D accounts for 5–10% of all people with diabetes. T2D, or insulin resistance, is the most common type of diabetes and accounts for 90-95% of all cases.[65]

T1D, also known as juvenile diabetes or insulin-dependent diabetes, is characterized by the impaired function of pancreatic β-cells.[64] T1D is usually diagnosed early in life, in children and young adults.[65] There is no production of insulin in these patients as a result of the destruction of pancreatic β-cells, leading to insulin deficiency in the bloodstream and administration of exogenous insulin to control the blood glucose level.[66] The destruction of the pancreatic β-cells is thought to be immune-mediated, and is supported by lymphocyte infiltration into the islets and the presence of autoantibodies.[67-68] These factors, along with environmental factors, make the patients with T1D prone to other autoimmune diseases such as thyroid or gastrointestinal disease.[67] In addition, it is believed that the β-cells can be damaged by a combination of genetic, environmental, and immune factors. Pathogenesis of diabetes is associated with viral and microbial disease. For example, congenital rubella and childhood enterovirus are strongly associated with diabetes.[69-71]

T1D patients receive exogenous insulin due to insulin deficiency and the blood glucose level is monitored constantly via a sensor that is embedded in insulin pump and insulin is injected in response to high glucose levels. Insulin was considered as a cure for patients with T1D since its discovery in the early 1920s. However, insulin is not a cure, especially when diabetes-associated complications develop, which are the main cause of mortality in diabetic patients.[72-73] There are homologues of insulin available for patients to help insulin act faster such as Humalogue and Aspart insulin.[74-76] Exogenous insulin is usually administered with or after a meal. Alternative treatments are also available for T1D patients such as pancreas or islet transplantation.[77] In addition, recent studies are focused on the use of stem cells for β-cells regeneration therapy.[78]

T2D, previously known as non-insulin dependent diabetes, is the most prevalent form of diabetes. Patients with T2D are still able to produce insulin but they are insulin resistant.[79] The mechanism of insulin resistance is not yet clear, but studies have shown than insulin's action is impaired on skeletal muscles[80] that can result in accumulation of lipid metabolites in liver and skeletal muscle.[81] T2D usually occurs due to poor lifestyle habits such as unhealthy eating habits and lack of exercise. Poor lifestyle habits result in impaired glucose tolerance, subsequently leading to T2D d. Therefore, considering a healthy and active lifestyle might slow the progression to T2D.[82] Prolonged hyperglycemia can result in damage of pancreatic β-cells to produce insulin, leading to administration of exogenous insulin in T2D patients. Many studies believe that pancreatic β-cells are damaged due to the prolonged, high demands to produce insulin that result of being insulin resistant.[83-84]

Currently, T2D is treated by oral medications with a focus on either increasing insulin secretion from pancreatic β-cells or improving insulin sensitivity in peripheral tissues such as the liver, muscle cells, and adipose tissue.[85] Diet and exercise are strongly recommended to newly diagnosed T2D patients, along with oral medications such as metformin.[86] As the disease progress, other oral medications with different mechanisms of action are prescribed to the patients. For example, sulfonylureas act on β-cells to release more insulin. On the other hand, other medications such as thiazolidinediones may act on the muscle cells and fat cells to promote a more effective use of insulin. However, administration of exogenous insulin is required for patients with T2D due the decrease in insulin production by islets.[85]

GDM is a unique type of diabetes, usually diagnosed during pregnancy.[87] It occurs in

about 2%-5% of pregnant women and usually resolves upon delivery.[88] However, pregnant women with GDM might continue to be hyperglycemic after delivery. There is a 10% risk associated with GDM to be converted to T2D, especially during the first five years after pregnancy.[64, 88]

In addition to the three major types of diabetes discussed above, there are 56 other types of diabetes according to the American Diabetes Association (ADA).[12, 79] These specific types can be associated with non-immune mediated β-cell damage or excess secretion of hormones that oppose the action of insulin by increasing hepatic glucose production and decreasing insulin sensitivity, such as cortisol, growth hormone, glucagon, and epinephrine.[79, 89] Diseases such as cystic fibrosis, pancreatitis, pancreatic resection, and hemochromatosis are associated with non-immune mediated islet damage. Other rare types of diabetes can be cause by genetic defects, chemicals, and drugs.[79]

1.4 Diabetes Complications

1.4.1 Acute Complications

The most prevalent acute complications of diabetes are ketoacidosis[90], hyperosmolar non-ketotic coma (HNC), lactic acidosis (LA), and hypoglycemia. DKA usually occurs in patients with T1D, as it is defined by absolute insulin deficiency resulting in the release of fat by adipocytes; the liver will then convert the released fat to ketoacids resulting in ketoacidosis. This process can also occur in patients with T2D when the disease has progressed and islets are damaged. HNC is a severe acute complication that can result in death and mostly occurs in patients with T2D. This complication usually occurs when there is a relative insulin deficiency along with hyperglycemia, stupor, dehydration, and elevated serum osmolality. The presence of

circulating insulin prevents ketosis and acidosis, which can prevent HNC occurrence. LA is clinically defined by elevated levels of lactic acid with acidosis in the absence of ketoacidosis. This complication is relatively rare in diabetic patients. Hypoglycemia can occur due to the excess amount of insulin and usually happens in patients using exogenous insulin or insulin-releasing medications.[91]

1.4.2 Chronic Complications

Microvascular complications and macrovascular complications are the two major classifications of chronic complications. These complications greatly contribute to the cost of diabetes care and morbidity. Microvascular complications include nephropathy, neuropathy, and retinopathy while macrovascular complications include coronary artery disease, peripheral arterial disease, and stroke.[92]

Nephropathy occurs in about 35%-45% of patients with T1D and in about 20% in patients with T2D,[93-94] and is the major cause of renal failure in the United States.[95] Diabetic nephropathy has the greatest contribution in the rate of mortality in diabetic patients.[96] Nephropathy usually begins in patients with glomerular hyperfiltration with the development of microalbuminuria in the first five years of diabetes diagnosis.[97-98] Next,, the rate of glomerular filtration falls and patients start developing overt proteinuria in the next 5-10 years of diabetes progression, which will result in renal failure.[99] As nephropathy progresses, increase in the glomerular basement membrane (GBM) thickness, microaneurysm formation, mesangial nodule formation, and other changes occurs. These changes are depicted in Figure 1.5.[6] Elevated levels of vascular endothelial growth factor (VEGF) have been reported in diabetic nephropathy and inhibition of the

production of VEGF has been associated with a delay in the development of nephropathy.[92, 100] Administration of antihypertensive drugs is recommended to patients with nephropathy.[92]

Neuropathy damages the nerves in the extremities such as legs and feet and is also associated with the extent and duration of hyperglycemia. Neuropathy is the most common complication in T1D patients with 60%-70% prevalence.[101] Different

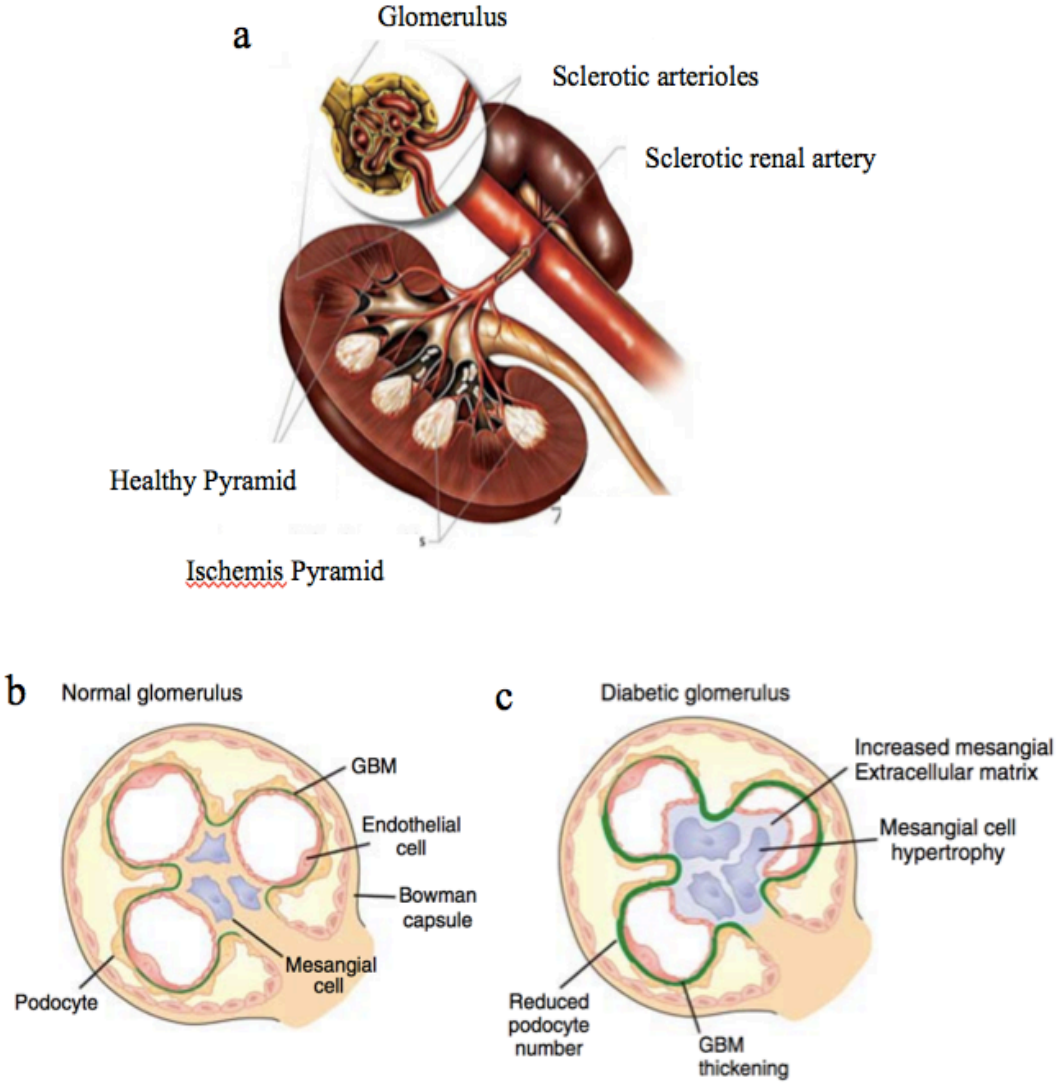

Figure 1.5. Changes in the Kidney as Nephropathy Progresses. a is showing different parts of the kidney**.** b is showing normal glomerulus and c is illustrating the changes in glomerulus in nephropathy.[6]

mechanisms such as increased polyol pathway flux, increased formation of advanced glycation end products (AGEs),[92] and oxidative stress have been used to link diabetes to neuropathy. Diabetic neuropathy has been categorized into different forms. Chronic sensorimotor distal symmetric polyneuropathy, the most common type, will result in burning, tingling, pain, and numbness in the patients.[102] One of the most aggressive forms of neuropathy is autonomic neuropathy, which can have the highest mortality rate due to impaired neurological function in most organs.[103] Peripheral neuropathy leads to non-traumatic amputation.[104] Patients with neuropathy can benefit from treatments that increase neuronal blood flow and improve blood supply to nerves such as vasodilatory agents.[105]

Retinopathy is the cause of blindness in diabetic patients and typically begins to develop 20 years after the onset of disease with T1D and 7 years after the onset of disease in T2D patients.[106-107] There are two forms of retinopathy; background, which is defined as the formation of a small hemorrhage in the middle layers of retina, and proliferative, which is caused by the formation of new blood vessels on the surface of the retina.[108] Increased polyol pathway flux results in elevated osmotic stress due accumulation of sorbitol and leads to loss of vision. In addition, cell injury due to AGEs[95, 109-110] or oxidative stress contribute to retinopathy.[111] Studies have reported that increasing the retinal blood flow via antioxidants can improve some of the vascular function but it is not beneficial in slowing down the progression of the disease.[112]

1.4.3 Mechanism Linking Diabetes and its Complications

There are four mechanisms used by researchers to explain the development of diabetes complications:

- Increased polyol pathway flux: This is the first mechanism proposed in 1966 to explain hyperglycemic complications.[113] Aldose reductase reduces glucose to sorbitol while consuming NADPH. Consumption of NADPH increases the intracellular oxidative stress, leading to osmotic vascular damage.[5, 114] NADPH is necessary to regenerate intracellular antioxidants.[5]
- Increased formation of advanced glycation end products (AGEs): This mechanism was proposed in late 1970s and it is based on glucose modifications on proteins and molecules.[115-116] In fact, according to this theory, glucose will irreversibly modify the proteins involved in gene transcription. In addition, AGE precursors can be transported out of the cells and modify the molecules in the extracellular matrix resulting in impaired function of the cells.[117-118] These precursors can modify the proteins in the blood stream as well. The modified proteins will then bind to AGE receptors resulting in vascular dysfunction due to the production of inflammatory cytokines and growth factors.[119-120] Animal studies have shown that inhibition of AGE production will result in the inhibition of structural changes in retinopathy.[121]
- Activation of protein kinase C (PKC) isoforms: This theory was proposed in the early 1990s, explaining the link between hyperglycemia and complications. Hyperglycemia will enhance the synthesis of diacylglycerol leading to activation of different isoforms of protein kinase C. These isoforms can alter gene expression.[122-124]

- Increased hexosamine pathway flux: This mechanism was proposed in the late 1990s.[125] Glucose is metabolized via glycolysis to glucose-6-phosphate and then fructose-6-phosphate. Excess intracellular glucose will result in excess production of fructose-6-phosphate, some of which will be converted to glucosamine-6-phophate and then to uridine diphosphate N-acetyl glucosamine, as depicted in Figure 1.6.[126-128] This will result in elevated expression of transforming growth factor-β1 and plasminogen activator inhibitor-1, leading to dysfunction of blood vessels.[129]

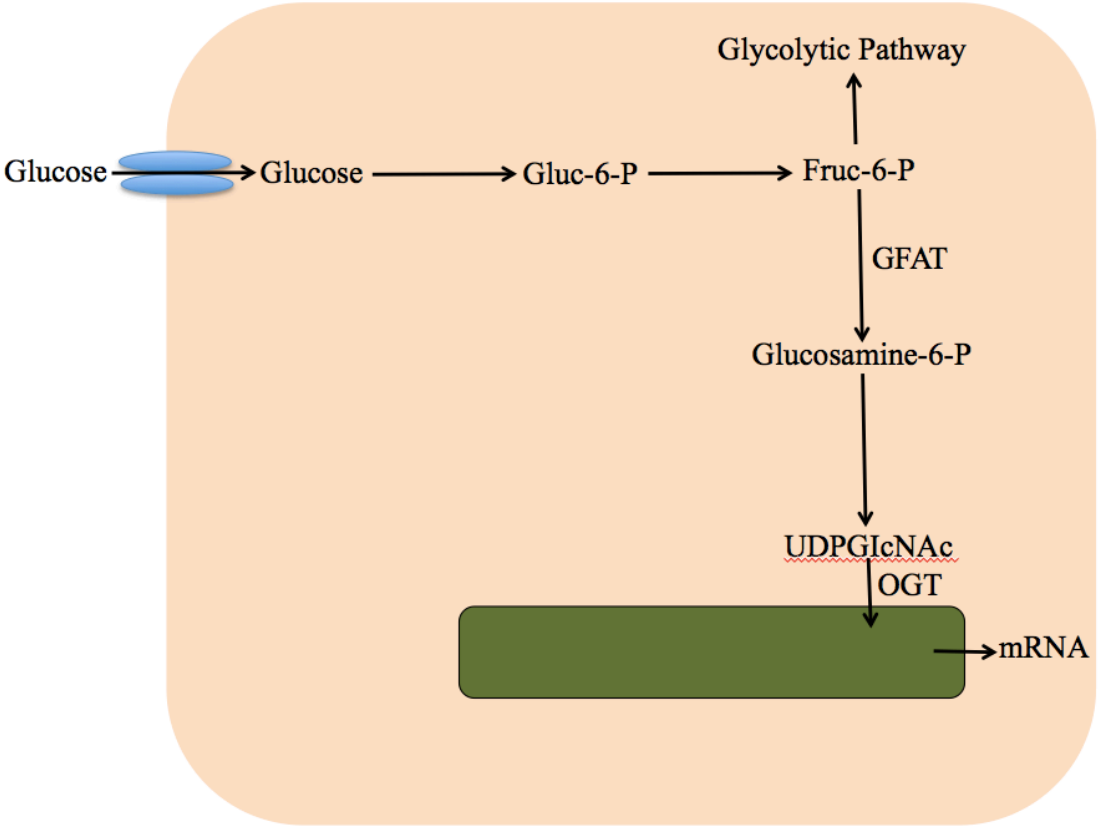

Figure 1.6. Increased Hexosamine Pathway Flux and Diabetes Complications.

These hypotheses about diabetes complications, along with new findings involving C-peptide[55-57] and its potential activity motivated researchers to investigate the biological role of C-peptide and its potential involvement in the treatment of diabetes complications.

1.5 Lack of C-peptide can Potentially Play a Role in Diabetic Complications

Short- and long-term treatments with C-peptide have shown improvements in autonomic and peripheral nerve function in T1D animals and humans. A low physiological dose of C-peptide was used in T1D rats for different periods of time. Prevention of acute nerve conduction velocity (NCV) defect was reported in T1D rats that consumed C-peptide for two months after the onset of disease. This resulted in improvement in the acute paranodal swelling[130] due to attenuated neural Na^+ and K^+-ATPase activity.[131] Dysfunction of NCV and Na^+ and K^+-ATPase activity are both affected[132] by impaired blood flow and increased polyol pathway activity, and C-peptide has been reported to increase the blood flow.[133] Hence, impaired NCV and Na^+, K^+-ATPase activity can potentially be associated with increased polyol pathway activity and impaired blood flow.[134]

In continuance, studies have shown C-peptide can prevent chronic nerve conduction defects such as axonal atrophy and degeneration. C-peptide administration for 8 months after the onset of diabetes corrected the axoglial dysjunction and paranodal demyelination completely. When C-peptide treatments began 5 months after the onset of T1D and continued for 3 months, paranodal dysjunction and axonal degenerations were corrected and nerve fiber regeneration increased as well. C-peptide treatment in a different form of T1D rat that began 6 weeks after onset of the disease and continued for two weeks

resulted in partial correction of loss of sciatic motor NCV, impaired sciatic blood flow, saphenous sensory NCV, and vascular conductance.[135] Interestingly, administration of NG-nitro L-arginine (L-NNA), a nitric oxide synthase inhibitor, along with C-peptide therapy in another group of rats abolished the nerve conductor effects of C-peptide. This data suggests that C-peptide effects are mediated through the NO synthase stimulatory vasodilation, information that will become important throughout the work presented and discussed in this dissertation.

There are two proposed mechanisms concerning the effect of C-peptide in NO-stimulated vasodilation – altering NO synthase expression[136] level directly, or increasing the endothelial flow-induced NO production.[135, 137-139] However, the amino acid sequence of C-peptide has been shown to be important for the effect on nerve conduction or perfusion, and no effect was observed when the sequence was altered.[135] Reduced nerve blood follow is reported in diabetic rats and C-peptide treatment significantly increased endoneurial blood flow in these animals. The effect of C-peptide on nerve function was also studied in humans, and 3 months of C-peptide therapy enhanced the sensory NCV in T1D patients. In addition, administration of C-peptide for 6 months in T1D patients with early stage neuropathy resulted in improvement of sensory NCV.[140] C-peptide was more significantly effective when used on the patients with the minimum degree of neuropathy, suggesting the importance of understanding diabetic complications in early stages.[141] C-peptide therapy also increased heart rate variability (HRV) due to improving the cardiac autonomic nerve function, which will result in decreased risk of cardiac arrhythmias and death.[141-143]

C-peptide can also increase endothelial NO production in a dose- and time-dependent

manner. When C-peptide was used in physiological concentrations, NO-production doubled and NOS inhibition resulted in no effect from C-peptide therapy.[144] The activity of Na^+ channels was diminished in T1D, leading to attenuated activity of Na^+, K^+ ATPase in peripheral nerve tissue. This resulted in accumulation of intra-axonal sodium and swelling of the paranodal region.[144-145] C-peptide treatments improved Na^+, K^+ ATPas activity by activating Na^+ channels and improved nerve function.[130, 145]

C-peptide can also increase transcription factors leading to elevated level of gene expression in neurotropic factors such as NGF, NT3, or IGF-I. Hence, thermal hyperalgesia (increased sensitivity to pain) and degeneration of unmyelinated fibers could be prevented, and pains caused by peripheral neuropathy could be eased. Figure 1.7. demonstrates different pathways that C-peptide can take to improve nerve function on T1D patients.[146-147]

C-peptide treatment is also capable of improving kidney function in T1D patients and diminishing inflammatory response in diabetes.[148]

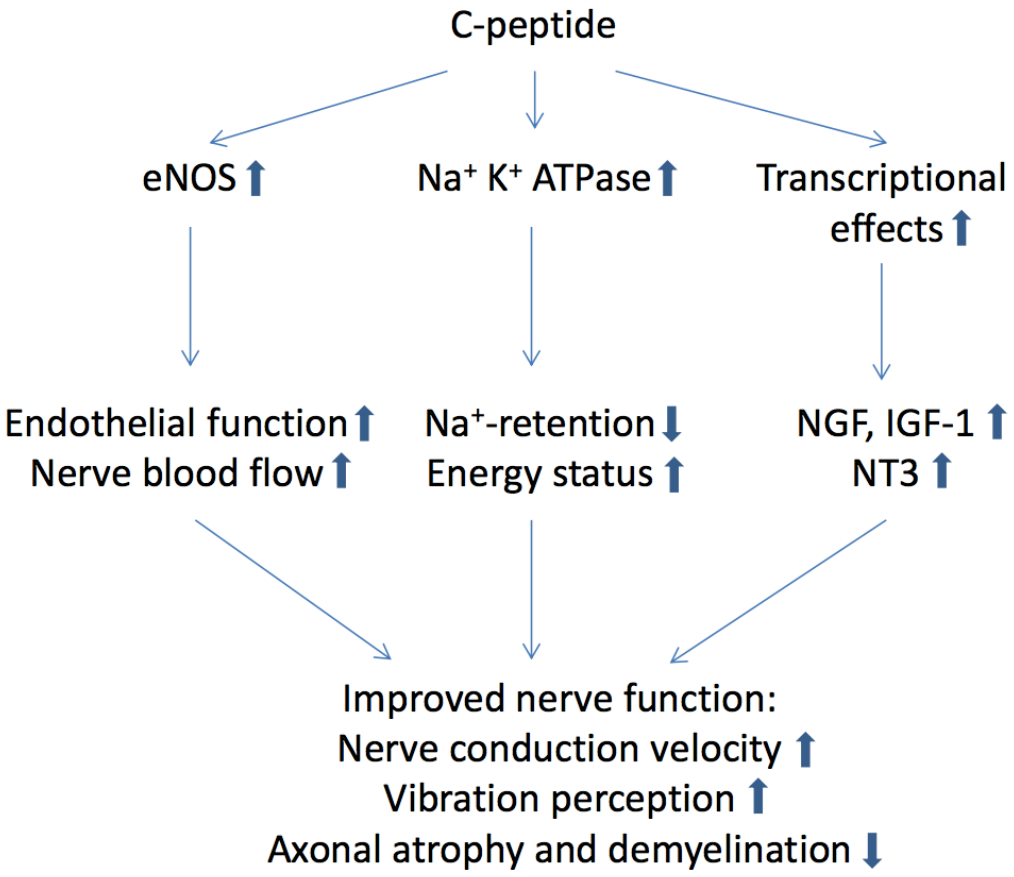

Figure 1.7. Nerve Function Improvement by C-peptide.

1.6 Mechanism of Vasodilation via NO Production by a Novel Formulation of Zn^{2+}, C-peptide, and Albumin

The Spence group proposed a mechanism by which the combination of Zn^{2+}, C-peptide and albumin can potentially improve blood flow by increasing endothelial NO production. Other groups proposed similar mechanisms, but the combination of the stimulatory molecules may not be completely understood.

It has been previously reported by the Spence group that C-peptide can increase ATP release from RBCs in the presence of a transition metal. ATP molecules released by RBCs increase NO production by binding to ATP receptors on endothelial cells and stimulating NO synthase. It is hypothesized by the Spence group that the increase in NO production can potentially help in blood flow regulation and correcting some of the complications in diabetes. This mechanism is illustrated in Figure 1.8.[149-150]

There is no bioactivity of C-peptide when only pure C-peptide is used, indicating that the presence of C-peptide alone does not explain C-peptide's biological role.[149-150] Meyer *et al*. reported that a commercially available C-peptide that contained an Fe^{2+} impurity increased the ATP release from RBCs and this increase reaches 2.9 fold of the basal levels after 8 hours of incubation. The same experiment was performed on T2D RBCs, which have lower basal ATP release from RBCs compared to the healthy controls. It was determined that RBC-derived ATP release was increased to those levels measured in healthy controls after 6 hours of incubation. In addition, C-peptide showed the same effect when used in the presence of Cr^{3+} instead of $Fe^{2+,}$ which was the impurity of C-peptide, except the bioactivity was maintained for 72 hours (about 24-48 hours longer than Fe^{2+}. Fe^{2+} has a faster ligand exchange rate compared to Cr^{3+}. The reason Cr^{3+} was chosen for this experiment was a study published by Haylock et. al. showing the

relationship between Cr^{3+} and glucose tolerance.[151] The experiment was also performed on healthy RBCs in the presence of phloretin, which is a GLUT-1 (glucose transporter 1 on the membrane of RBCs) inhibitor and RBC-derived ATP release remained the same as control cells with no metal-C-peptide complex. All of these experiments were performed in a physiological salt solution that contains albumin and the increase in ATP release was observed. Liu et. al. performed similar experiment in an albumin free buffer and no effect was observed from C-peptide and metal together in the absence of albumin.[152] This suggests that C-peptide in the presence of metal and albumin increases ATP release by RBCs, potentially via increasing glycolysis by increasing the cellular glucose transport using GLUT-1.[149] This hypothesis will be discussed in more details in the following chapters.

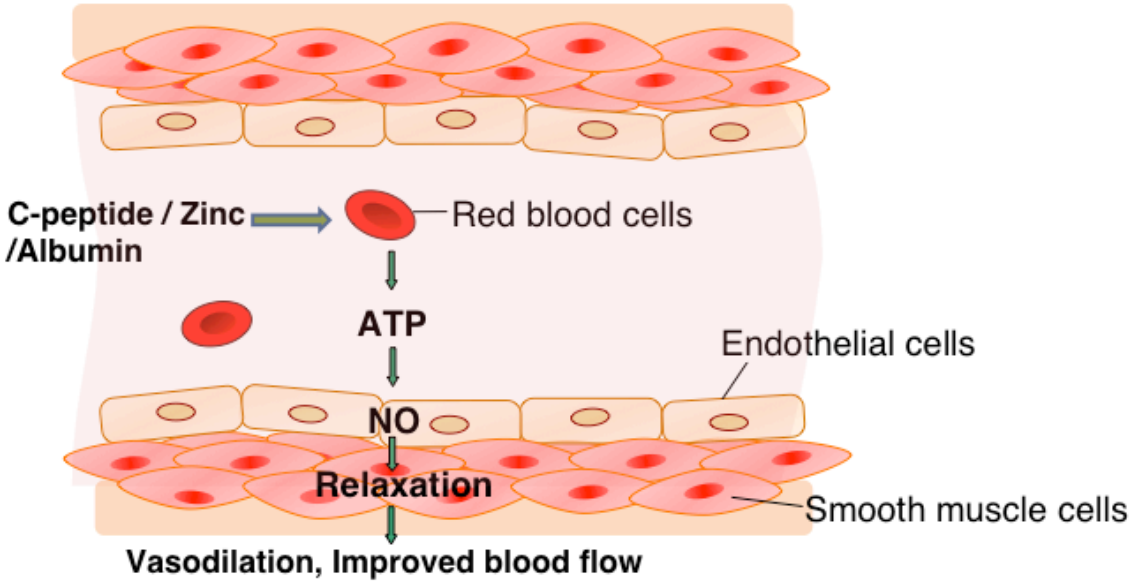

Figure 1.8. C-peptide in the Presence of Albumin and Zinccan Potentially Improve the Blood Flow via this Mechanism.

The concentrations of Cr^{3+} is not high enough *in vivo* for C-peptide to bind to it compare to mM levels of other metals such as sodium and magnesium. Another metal ion used to study the effect of C-peptide was Zn^{2+} because it is co-secreted from pancreatic β-cells and exist in mM levels, making it more likely to be involved in this pathway. A Zn^{2+}-C-peptide combination was used on healthy rat RBCs and the same effect was observed, resulting in ATP release being elevated by 80%. Again, the same experiment was performed using RBCs obtained from T2D rats and the RBC-derived ATP increased by about 30% suggesting that RBCs from T2D rats might be resistant to C-peptide, not unlike other cell's resistance to insulin. Additionally, glucose uptake by RBCs was studied in healthy and T2D RBCs in the presence of Zn^{2+} and C-peptide. The combination increased the glucose uptake by about 65% in healthy RBCs, while it increased glucose uptake in T2D RBCs by only 35%. This suggests the similar resistance in T2D patients in glucose uptake.

Taken all these findings together, Liu et al. showed that the presence of albumin is essential to observe the biological effect of C-peptide and no activity from the Zn^{2+}, C-peptide combination was observed when an albumin-free buffer was used. It is hypothesized that albumin can act as a carrier for C-peptide and Zn^{2+} since it is involved in the delivery of many molecules, proteins, and lipids in the bloodstream.[152]

Metformin is a medication used widely by T2D patients and it has been reported that metformin can increase blood flow by increasing glucose transport into RBCs. Meyer pretreated T2D RBCs with metformin for 2 hours and then incubated with Zn^{2+}, C-peptide for another 4 hours. The observed increase in ATP release was the same as that measured when using healthy RBCs. In addition, metformin was able to increase the

glucose uptake in T2D RBCs. Importantly, there wasn't any effect on RBCs when metformin was used by itself, or with Zn^{2+} or C-peptide only, suggesting that the presence of Zn^{2+}, C-peptide, and albumin (present in the buffer) trio is essential.[153-154]

It's worth noting that co-administration of insulin along with pure C-peptide did not show any biological effects. Previous studies have shown that the presence of insulin is beneficial for the biological role of C-peptide *in-vivo*.[155] However, the purity of C-peptide or insulin was not discussed, and it can be argued that the presence of insulin might have introduced a trace of metal, which is essential for the bioactivity of C-peptide, especially considering that insulin is often produced and/or sold with 0.5% by mass being zincTherefore, the observed effect of C-peptide might be due to the presence of the metal and not the insulin.

As mentioned earlier, Zn^{2+} is the most probable metal to be involved in the biological effect of C-peptide. It has been previously reported that Zn^{2+} is important in the prevention of diabetes complications.[156] More importantly, the concentration of the Zn^{+2} ion in pancreatic β-cells is very high[13] and it is also co-localized with C-peptide and insulin prior to secretion in the blood stream. Additionally, insulin forms a hexamer around Zn^{2+} with two Zn^{2+} ions in each hexamer.[60, 157-158] It has been reported that the effect of insulin will be maintained longer if Zn^{2+} is present and this was established before researchers understood the biosynthesis of insulin.[159] Zn^{2+} can regulate the cellular oxidative stress in diabetic patients because of its effect on the function of some antioxidant enzymes.[90]

It is hypothesized that Zn^{2+} activates C-peptide via electrostatic interactions while they reside in pancreatic β-cells before the secretion into the bloodstream. The Spence

group has investigated this hypothesis by mutating the acidic amino acids in C-peptide's sequence. There are five acidic amino acids at position 1, 3, 4, 11 and 27 and each of these amino acids where mutated to alanine to evaluate the electrostatic interaction between Zn^{2+} and C-peptide. These mutations were referred to as E1A, E3A, D4A, E11A, E27A and the interaction between Zn^{2+} and mutated C-peptide was studied using mass spectrometry. A decrease in the overall Zn^{2+} interaction was observed by each of the acidic residue substitutions. In addition, ATP release by RBCs was studied using the mutated C-peptide along with Zn^{2+} and a 50% reduction in RBC-derived ATP was observed, with E27A mutant having the most significant decrease. The ATP release was also examined using the C-terminal pentapeptide, EGSLQ, and the same biological activities was achieved, while the mutation E27A of the pentapeptide resulted in no biological effect.[160] These investigations suggest that all acidic amino acids are important in the interaction of Zn^{2+} and C-peptide with the intact C-terminal pentapeptide showing the same biological effect as C-peptide. To further understand the mechanism of C-peptide activity, the interaction of C-peptide and Zn^{2+} with RBCs will be discussed in chapter 3 of this dissertation.

1.7 Hypothesis of C-peptide Receptor Versus C-peptide-Carrier Molecule Receptor

The mechanism of interaction of C-peptide with RBCs is still unclear and researchers have proposed several theories on C-peptide receptors. The idea of a C-peptide receptor was first hypothesized when the binding between C-peptide and the human cell membrane was reported. The binding between C-peptide and human skin fibroblasts was studied using rhodamine-labeled C-peptide. The experiment demonstrated specific binding between C-peptide and human cells with a binding affinity of 3×10^9 M^{-1}. Additionally, it has been reported that C-peptide binds specifically to renal tubular cells and saphenous vein endothelial cells with a binding affinity of 3.3×10^9 M^{-1} and 2.0×10^9 M^{-1}, respectively. Specific binding of C-peptide in all these cases was in the nanomolar range, which is close to its physiological plasma concentration. Binding of C-peptide to saphenous vein endothelial cells suggest endothelial cell NOS stimulation by C-peptide. On the other hand, scrambled and D-enantio C-peptide was used to displaced bound Rh-labeled C-peptide and the experiments failed, which suggests the stereospecific nature of C-peptide binding.[54]

As mentioned earlier, the C-terminal pentapeptide of C-peptide, EGSLQ, is essential for bioactivity of C-peptide. Unlabeled C-terminal pentapeptide was used to experimentally displace bound Rd-labeled C-peptide and the experiment was successful, suggesting the same bioactivity for the pentapeptide as the full-length C-peptide. Also, it has been reported that C-terminal pentapeptide in proinsulin failed to displace Rh-labeled C-peptide suggesting that the C-terminus pentapeptide needs to be free to be active. Insulin and proinsulin failed to displace Rh-labeled C-peptide and unlabeled C-peptide was not able to displace Rh-labeled insulin, suggesting that C-peptide is not binding to an

insulin receptor and vice versa. However, a contradicting study has reported the low affinity specific binding between C-peptide and proinsulin receptor.[54]

One of the strongest theories for C-peptide receptor is a G-protein coupled receptor (GPCR) or a GPCR family, which are found in the cell membrane of eukaryotes. These proteins regulate various pathways in the body such as automated nervous system transmission, gustatory sense, immune system, and visual sense. GPCRs function by binding a molecule outside the cell and stimulating a signal transduction pathway. Interestingly, pertussis toxin suppresses GPCR activity And it has been reported that C-peptide bioactivity is mostly pertussis toxin sensitive.[54, 161] GPR146 has been studied as a potential receptor[162] for C-peptide as addition of C-peptide triggered GPR146 internalization in the gastric tumor cell line KATOIII. This study showed that the expression of GPR146 in KATOIII cells is essential for C-peptide stimulated cFos expression in these cells. However, this study does not contain enough evidence to consider GPR146 as C-peptide's receptor, because no physical interaction such as binding studies between C-peptide and GPR146 have been reported.[137] However, it can be concluded that GPCR is involved in the biological effect of C-peptide since pertussis toxin inhibited the specific binding between C-peptide and cell membrane.[54]

There are other studies reporting that C-peptide's bioactivity is not necessarily through the interaction with its specific receptors,[20] such as the interaction of C-peptide with tyrosine phosphatase 1B,[163] glycolytic enzyme,[12] and internalization of C-peptide to the cytosol of HEK 293 and Swiss 3T3 cells.[164] Additionally, it has been reported that retro and enantio C-peptide are as active as native C-peptide, suggesting that its function is mediated via non-chiral interaction other than stereospecific binding sites.[145]

C-peptide may be able to affect certain diabetic complications by improving blood flow. Unfortunately, prior studies by our group have also shown that C-peptide does not bind well to RBCs in hyperglycemic environments. Thus, finding molecules that help overcome this binding deficiency, or take the place of albumin, may be beneficial. Oral medications improve glycemic control, but they do not restore metabolic homeostasis or improve complications such as nephropathy, neuropathy and retinopathy. Hence, better therapeutics for T2D and its complications are urgently needed. Studies have demonstrated a role for the adiposity hormone, leptin, in glucose homeostasis and support that dysfunction of this hormone contributes to the pathogenesis of diabetes.

1.8 Leptin

Leptin is a hormone derived from adipose tissue and is believed to maintain the balance between food intake and energy expenditure. Parasymbiosis, studies on two species that are in close physical association, experiments were performed on genetically obese rodents by Jackson Laboratories and lipostatic hormone produced by white adipose tissue was identified in the 1950s.[165-166] Leptin's gene was first cloned by Zhang *et. al*. in 1994. Leptin is a protein with 167 amino acids and a 4-helix bundle tertiary structure, as shown in Figure 1.9. It is classified as an adipokine, which are hormones secreted by white adipose tissue (WAT) although they possess the typical tertiary structure of cytokines.[1] When functioning correctly, leptin regulates a number of physiological processes including appetite, body weight, and glycemic level. Leptin, after being secreted from adipose tissue, enters the central nervous system (CNS) and interacts with its receptor expressed in the brain located in the hypothalamus, and regulates food intake,

energy expenditure and other physiological processes through signal transduction pathways. Dynamics of leptin secretion were revealed by *in vitro* studies showing that leptin maintains a constant basal level due the continuous secretion by WAT in non-stimulated conditions. There is no sudden release of leptin upon stimulation, but the intracellular level of leptin significantly increases one hour after stimulation.[167]

Leptin secretion and action is a highly regulated process in the body, and is known to be altered in obese patients. Higher levels of leptin have been found in some obese rodents and humans. An increase in the concentration of plasma leptin has a linear correlation with body mass index (BMI, a measure of body fat based on weight and height).[168-169] Though obese humans have been found to have higher levels of leptin, some obese animals, such as rodents, lack leptin. Obese animals such as a variety of rodents were treated successfully by exogenous leptin intake, which resulted in a decrease in the food intake and body weight.[170-171] However, leptin treatment will not be effective in obese humans that have higher levels of leptin because they are not leptin deficient. Instead, it is believed that obese humans become "leptin resistant".[172-174] Investigations have shown that the hypothalamic arcuate nucleus (ARC) is an important site for the effect of leptin on glucose metabolism, while its receptors on the hypothalamus are involved in the control of energy balance. In a study performed by Coppari *et. al*., only a modest effect on food intake and body weight was observed after restoring leptin receptors to the ARC of leptin receptor-deficient mice, but the effect on improving hyperinsulinemia and normalizing the blood glucose levels was significant.[136] Most of the studies performed to understand the role of leptin in different diseases focus on the central nervous system. However, leptin is associated with diseases such as

cardiovascular disease or hypertension that have correlations to vessel dilation. The role of leptin in these types of disease and the potential correlation between glucose homeostasis by leptin will be discussed next.

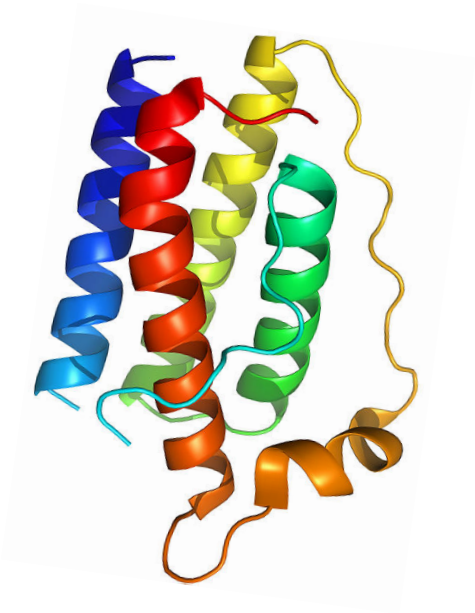

Figure1.9. Crystal Structure of Leptin.[1]

1.8.1 Vasodilatory Effect of Leptin on Hypertension and Heart Disease

Weight loss is promoted by leptin via sympathetic activation of brown adipose tissue and oxidation of fatty acids[175] and sympathetic outflow in renal, lumbar, hindlimb and adrenal gland sympathetic nerves..[176] Studies have shown that administration of exogenous leptin either through intracerebroventricular administration or chronic intravenous injections in rodents increase the mean arterial blood pressure and heart rate.[177] Leptin deficient ob/ob mice have lower arterial blood pressure compared to healthy littermates.[178] Leptin's ability to balance food intake is impaired in Agouti mice,

while renal sympathetic nerve activity is the same as the healthy littermates.[179-180] Additionally, leptin's ability to balance body weight and food intake was impaired in mice with a 10-week high fat diet, while the renal sympathetic nerve activity was maintained.[181] These findings suggest that leptin regulates blood pressure via sympathetic activation and higher concentrations of leptin seen in obesity results in hypertension. However, blood pressure regulation by leptin via activation of renal sympathetic nerve is remained intact while the effect of leptin to regulate body weight is abolished. This suggests selective central leptin resistance in obese patients, and may explain why some of the functions of leptin are maintained even when the body develops "resistance" to leptin. It is also possible that there are special receptors that are becoming resistant to leptin. To understand this, we need to understand the mechanism by which the body becomes "leptin resistant" or investigate more deeply to discover the actual reason for hyperleptinemia in obese patients.

Leptin acts on sympathetic nerve activity via α-MSH and melanocortin-4 receptor (MC4R) activation. SHU-9119, an MC4R receptor antagonist, blocks the leptin from stimulating renal sympathetic nerve activity and heart rate.[182-184] In contrast, SHU-9119 blocks the metabolic activity of leptin on food intake, suggesting that both sympathetic and metabolic actions are mediated via the same neural pathway.[184-185] This contravenes with the previous finding showing leptin resistance in metabolic pathways and leptin sensitivity in sympathetic pathways.

On the other hand, researchers have proposed development of leptin resistant vasculature as a cause of hypertension, which is in contrast with the hyperleptinemia caused by CNS leptin resistance. It has been previously reported that leptin has a direct

vasodilatory effect on the vasculature by stimulating NO production from endothelial cells and inducing aortic ring relaxation.[186-188] Additionally, administration of leptin increases serum nitrite/nitrate and decreases arterial blood pressure in the presence of sympathetic inhibitors.[189-190] This suggests that leptin is involved in vasodilation via direct interaction with endothelial cells to stimulate eNOS production of NO, which contrasts the increase in blood pressure by leptin due to the sympathetic pathway. Thus, hypertension could occur if the vasculature becomes resistant to leptin based on this theory.[189] However, intravenous administration of leptin in rodents has failed to demonstrate a direct vasodilatory effect. Also, the vasodilatory effect of leptin in regulating the blood pressure *in vivo* is yet to be studied and it has been demonstrated that leptin can induce vasodilation in both endothelial-dependent and endothelial-independent manner.[186, 188-190] Therefore, the mechanism of leptin-derived vasodilation is not completely understood.

Leptin may also affect renal blood pressure via sympathetic activation[191] and NO production.[187] The effect of leptin in the kidney depends on the duration of exposure to the hormone. Acute administration of leptin does not increase renal blood pressure in either animal or human subjects, whereas chronic administration of the hormone elevates the blood pressure in the kidney in animals. Traditionally, development of renal hypertension was associated with obesity-induced sympathetic activation via augmented sodium retention. Renal denervation decreases the antinatriuretic and hypertensive effect of obesity in dog models.[192] In contrast, recent studies have proposed that leptin stimulates NO production[193-194] and leptin-induced NO production in endothelial cells and blood vessels was demonstrated by Vecchione *et al*.[187] In addition, a hypotensive

effect of leptin has been reported when the sympathetic nervous system output was blocked, suggesting the vasodilatory effect of leptin.[189-190] Chronic hyperleptinemia results in elevated blood pressure in animal models and, as shown in Figure 1.10, studies have proposed different pathways to link leptin to hypertension in humans. Also, leptin has been positively correlated to systolic and diastolic blood pressure in obese and healthy subject.[195-200] Conversely, higher levels of leptin were found in hypertensive African Americans, but no correlation between leptin and blood pressure is reported when subjects were adjusted for obesity.[201] Collectively, it seems that levels of leptin are higher in hypertensive obese patients compared to normotensive obese patients, but leptin may not be the direct cause of hypertension.

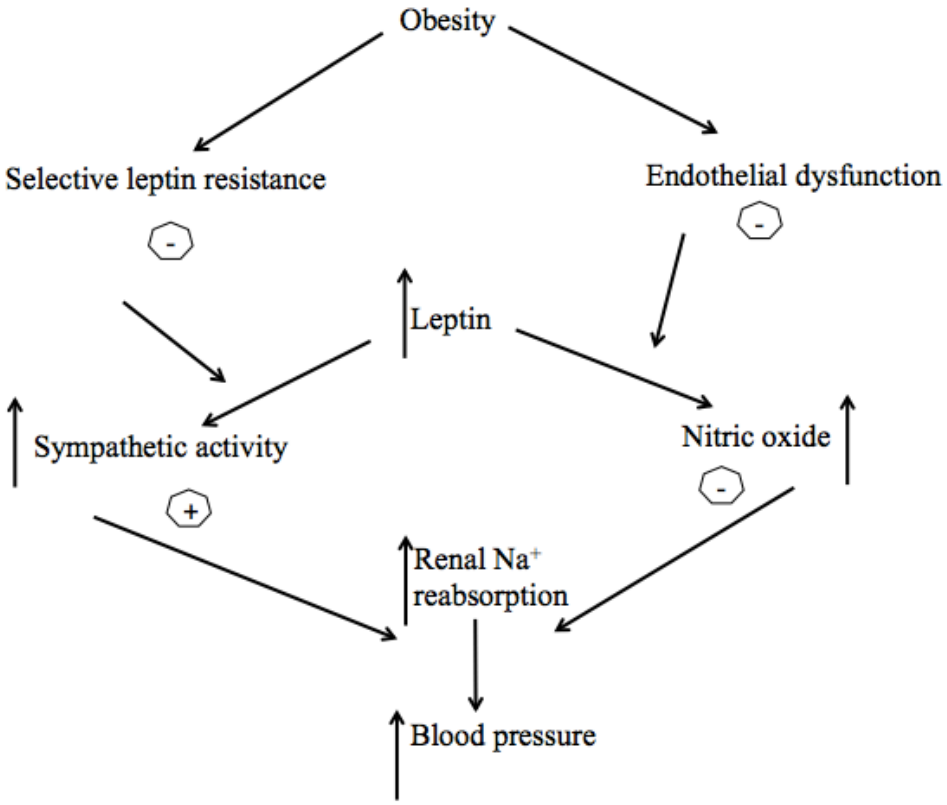

Figure 1.10. Renal Blood Pressure Effect of Leptin.

The effect of leptin in the coronary circulation was first investigated by Matsuda *et al*. and a 40% increase in coronary blood flow was observed in patients suffering from cardiac catheterization. This vasodilatory effect is independent of NO production blockades.[202] It has been reported that high plasma leptin concentrations have an inverse correlation to coronary vasodilation induced by adenosine in obese and healthy people.[203] In addition, the coronary vasodilatory effect of leptin is studied in isolated coronary arterioles and in anesthetized open-chest dogs.[204-205] In this study, leptin-induced vasodilation is associated with the expression of leptin receptors in human coronary endothelial cells and canine coronary arteries, suggesting endothelial-dependent vasodilation. This finding was further examined by inhibition of NO synthase, which abolished the vasodilatory effect of leptin. However, these effects by leptin are observed at concentrations much higher than the concentrations found in obese patients. No vasodilatory effect is reported at physiological concentrations. Even though leptin-induced endothelial dependent vasodilation is a pharmacological phenomenon, the underlying pathway is still unclear.

Atherosclerosis pathogenesis is highly correlated to endothelial dysfunction and it is crucial to understand the mechanisms that affect the endothelial function leading to the development of coronary disease.[206-207] Leptin has been studied as one of the factors affecting endothelial function, with contradictory results. For example, it has been reported that higher concentrations of leptin at the levels observed in obese patients will result in defective paracrine regulation of coronary circulation.[53] Administration of solution containing 10-90 ng/mL leptin decreased the vasodilatory effect of acetylcholine both *in vivo* and *in vitro*, while physiological concentrations of leptin did not alter the

acetylcholine-mediated vasodilation.[205] Therefore, the concentration of leptin is highly associated with potential coronary disease via endothelial dysfunction. It is believed that leptin can potentially mediate endothelial dysfunction via inhibition of NO synthase by PKC,[208-209] augmented superoxide production[210-211] and/or increases in release of endothelin-1.[212] Thus, leptin can both activate and inhibit NO-mediated vasodilation, although this paradox has yet to be resolved.

In contrast with the studies reporting correlation of leptin to the initiation of coronary endothelial dysfunction,[205, 210-211] other studies have reported leptin as an important factor in mediation of atherogenesis. O'Roukre *et al*. reported an increase in the levels of cholesterol esters in foam cells by leptin.[213] In addition, leptin can be inversely correlated with plasma HDL-cholesterol.[214-215] Leptin can be involved in the pro-inflammatory state observed in obese patients and it is strongly believed to correlate with C-reactive protein and serum amyloid A phase reactors.[216-217] In addition, the oxidative modification of plasma lipoproteins can be augmented by leptin.[218] Therefore, leptin may be strongly involved in mediating the oxidative stress and inflammatory environment of obese patients. Other studies have shown no effect of leptin on inflammation[219], oxidative stress[220], intima-media thickness[221-222] or development of atherosclerotic disease.[223-224] Also, it has been shown that insulin-resistant dogs with a high fat diet become resistant to the vascular effect of leptin.[204] Taking together, these findings suggest a controversial effect of leptin in coronary atherogenesis and the direct effect of leptin in pathogenesis of atherosclerotic disease has not been reported yet.

Several studies have reported a direct effect of leptin on cardiomyocyte function. Leptin is correlated with hypertrophy of neonatal rat ventricular myocytes[225-226] and

proliferation of both primary pediatric human cardiomyocytes and murine cardiomyocyte HL-1 cells.[227] Studies have shown that leptin can inhibit the ventricular myocyte contractile via intracellular signaling pathways such as activating endothelial-1 receptor.[228-229] In addition, matrix metalloproteinase-1 expression is stimulated by leptin.[230] In contrast, higher concentrations of leptin may protect the heart from lipid deposition[98] and administration of leptin in ischemic mouse hearts and oxidative stressed rat cardiomyocytes resulted in attenuated infarct size and delayed opening of the mitochondrial permeability transition pore.[231] Therefore, the effect of leptin on cardiac function is controversial and needs to be studied *in vivo*.

1.8.2 Timeline for Glucoregulatory Effect of Leptin

The terms *ob* and *db* respectively refer to the obesity and diabetes phenotypes in mice homozygous for the autosomal recessive mutations and were first explained in 1950 and 1966.[232-233] A timeline for the anti-diabetic effect of leptin is depicted in Figure 1.11.[4] The Friedman group first identified the gene "leptin" in obese mouse in 1994.[234] Soon after, the discovery of the leptin receptor was reported in 1995[1] and was encoded by the *db* gene in 1996.[235] The first glucoregulatory effect for leptin was reported by Pelleymounter and *et al.* in 1995, showing that lower doses of leptin can correct severe hyperglycemic conditions in *ob/ob* mice.[236] Schwartz and colleagues supported this observation by reporting that decreasing the amount of food ingested by leptin-treated *ob/ob* mice is not sufficient to observe the same effect as seen in leptin therapy.[237] Shimomura *et al*. reported that leptin therapy corrects the hyperglycemic condition and insulin resistance in lipodystrophy rodents in 1999.[169] The first glucoregulatory effect of leptin in humans was reported in 2002 and reported beneficial results on insulin

sensitivity and hyperglycemic conditions when leptin was administered to lipodystrophic patients.[238-239] In contrast, a 2011 study reported that leptin therapy is ineffective in correcting insulin resistance and hyperglycemic conditions in obese and T2D patients.[240-241] On the other hand, leptin therapy in hypoinsulinemic T1D rodents was greatly beneficial to correct hyperglycemia.[242-243] Researchers believe that the insulin-independent glucoregulatory effect of leptin is mediated by central nervous system (CNS).[244]

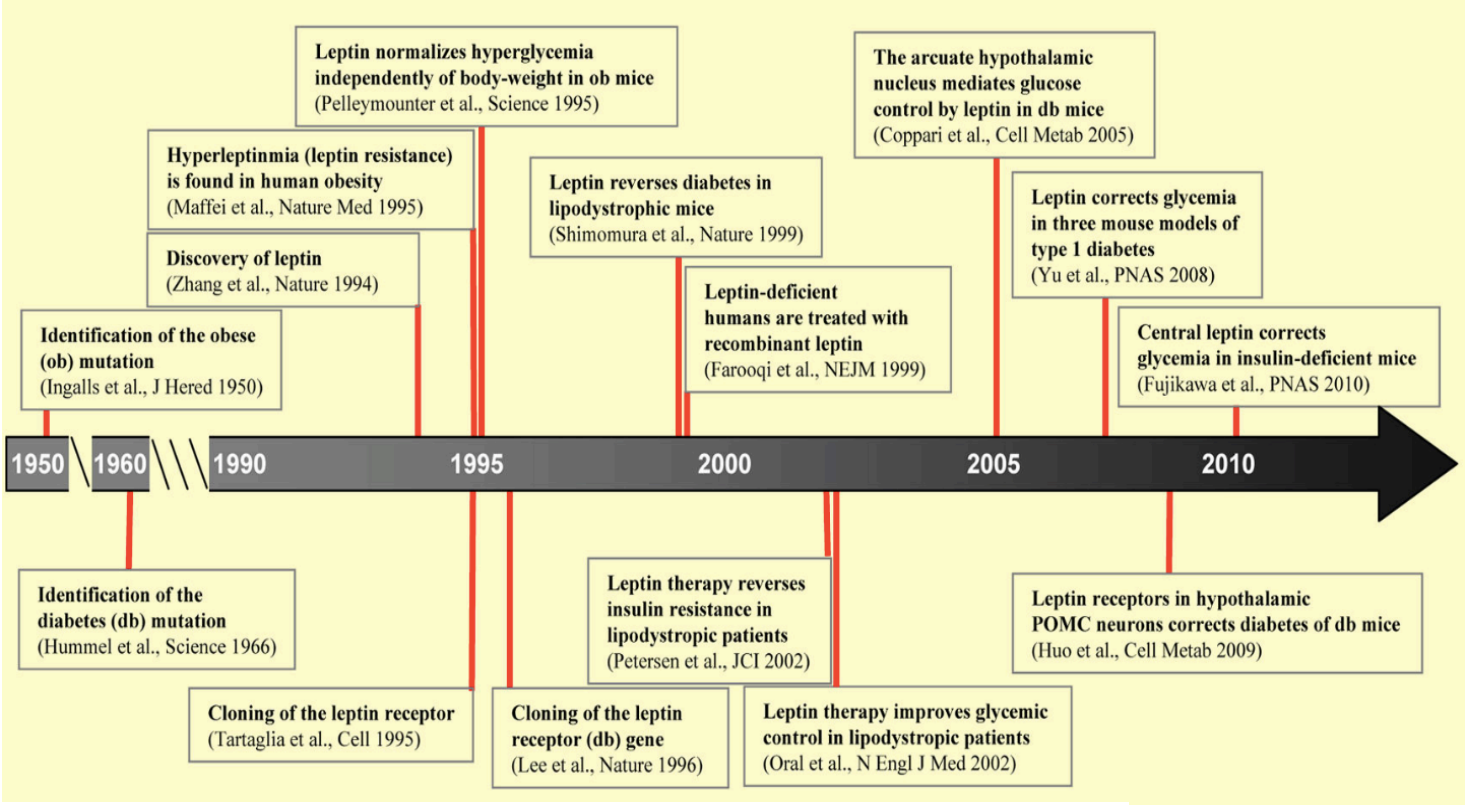

Figure1.11. Milestones in Glucoregulatory Actions of Leptin.[4]

1.8.3 Central Nervous System and Leptin

Coppari and colleagues first reported the anti-diabetic action of leptin mediated via the CNS in 2005 by showing that hyperglycemia conditions in obese, hyperinsulemia, and diabetic leptin receptor (*Lepr*)-null mice can be corrected only by restoration of *Lepr* expression in ARC neurons in a food intake, body weight independent manner.[136] Next, it was reported that selective expression of a long isoform of leptin receptor (LepRb) in the pro-opiomelanocortin (POMC)-expressing neurons within ARC sufficiently restores glycemic conditions in LepRb deficient *db/db* mice, suggesting ARC POMC neurons as the major candidate to mediate anti-diabetic actions of leptin.[245] This observation is valid only in young *db/db* mice and a partial effect was observed in older mice.[245] Additionally, deletion of LepRb in POMC did not create hyperglycemic conditions in lean non-*db/db* mice.[246] AgRP-expressing neurons in ARC, a key neuropeptide component of the central melanocortin system, also express LepRb and are antagonists of α-MSH at MC4R.[247-248] Obesity is thought to be caused by loss of MC4Rs in mice and humans,[247] and studies have suggested a role for central melanocortine system to control hyperglycemic conditions.[249-250] The study performed on *Lepr*-null mice to reactivate endogenous LepRs targeted both POMC and AgRP neurons and the hyperglycemia control was not age dependent.[136] Comparing this to the age-dependent hyperglycemia control obtained in *db/db* mice with only POMC-selective re-expression of LepRb suggest that AgRP may play a role in leptin-mediated glucose regulation as shown in Figure 1.11A. Bjorbaek *et al*. performed preliminary studies on selective expression of LepRp in AgRP neurons of *db/db* mice to assess the hyperglycemic condition and severe hyperglycemic condition was corrected.

Collectively, leptin is independently involved in peripheral glucose metabolism and energy homeostasis via POMC neurons. The question that arises here is how leptin can influence them both via one group of neurons. Different theories have been proposed to answer this question:

I. POMC cells are heterogeneous cells with different functions. Studies have demonstrated that POMC cells are highly heterogeneous and leptin can activate only 30%-90% of POMC neurons.[251] Also, insulin can inhibit only part of POMC neurons.[251-254] It is not clear whether leptin-activated neurons are insulin sensitive or not, making the subject more complicated.[251, 255]

II. Various neuropeptides and neurotransmitters are secreted by POMC-expressing neurons from axon terminals acting on different pathways. It has been reported that different neuropeptides are produced by POMC neurons such as α-MSH, β-MSH, γ-MSH, ACTH, β-endorphin[256], cocaine amphetamine regulated transcript (Cart)[257], and nesfatin[258]. POMC-expressing neurons produce heterogeneous neurotransmitters such as GABAergic, glutamatergic, and cholinergic as depicted in Figure 1.11B. All these neurons, neuropeptides, and neurotransmitters are candidates to regulate leptin-mediated glycemic control.[259-261] Excessive investigation is needed to fully elucidate the role of POMC neuronal population and POMC secreted molecules on glucose metabolism versus energy balance.

III. Energy balance and glucose homeostasis are regulated via different intracellular signaling pathways. Explaining different signaling pathways is beyond the goal of this dissertation.

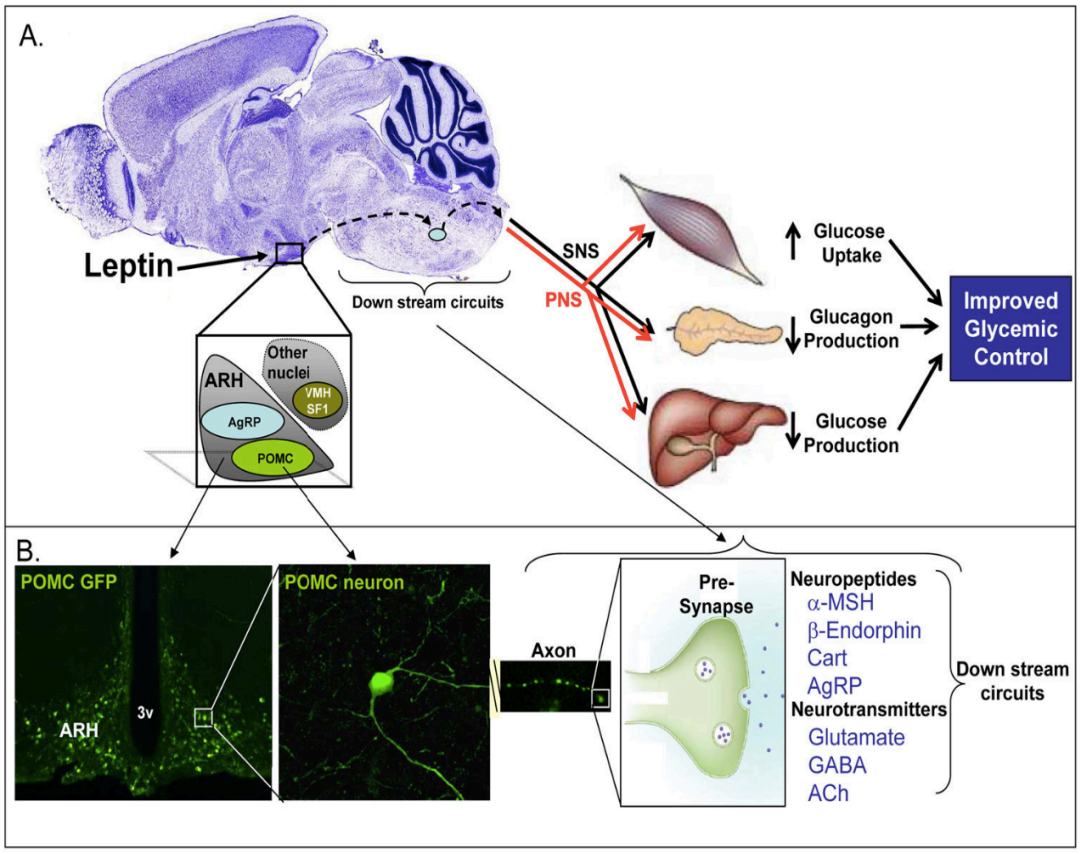

Figure 1.12. Schematic Model of Anti-diabetic Actions of Leptin via CNS Neuronal Mediators, Efferent Pathways, and Peripheral Mechanisms. A. represents the anti-diabetic actions of leptin by efferent and central neuronal pathways. ARC POMC and AgRP expressing neurons are the most likely candidates to regulate leptin-mediated glucose control, which happens by engaging efferent pathways such as regulation of sympathetic (SNS) and parasympathetic (PNS) branches of the autonomic nervous system. This may eventually be beneficial in muscle glucose uptake, pancreatic glucagon production and/or hepatic glucose production. B. POMC neurons produce different neuropeptide and neurotransmitters. POMC-derived neuropeptides are alpha-melanocortin stimulating hormone (α-MSH), β-Endorphin, Cocaine and amphetamine-regulated transcript (Cart) and Nesfatin-1. POMC neurons produce heterogeneous neurotransmitters suggesting that each subpopulation produce glutamate, GABA and acetylcholine (ACh).[4]

1.8.4 Effects of Leptin on T1D

Leptin mono-therapy, in which no exogenous insulin is administered, in mouse models of T1D resulted in improvements of diabetic conditions and correction of lethal consequences of insulin deficiency.[244, 262] Administration of leptin along with insulin in two patients with T1D significantly improved glucose profile and insulin sensitivity in a way that insulin dose was cut back by 30-50% compared to pre-leptin treatment dosage. These findings motivate the researcher to study leptin as a potential therapeutic for T1D, and a clinical trial on the safety and efficacy of the hormone is ongoing.[263]

1.8.5 Effects of Leptin on T2D

Studies demonstrated that leptin therapy in T2D mouse models improved insulin sensitivity and glucose and lipid imbalances.[136, 171, 264-265] Controversially, recent studies on two T2D patients have shown marginal effect to improve insulin resistance.[240-241] However, the small number of patients who volunteered to participate in the clinical studies with available results makes the conclusion about leptin therapy almost impossible. In order to target leptin as a potential therapeutic, we need to understand the mechanism or cause of the so called "leptin resistance" before this problem can be resolved.

Therefore, there are many studies strongly suggesting leptin as a potential candidate to correct hyperglycemic conditions. In addition, various studies have investigated a role for leptin in vessel dilation. It was discussed earlier in this chapter that vessel dilatory-mediated improved blood flow can potentially be beneficial for diabetic complications. Thus, it is worth to study the role of leptin in the blood stream since it is

being transported to the brain through blood stream. The effect of leptin on C-peptide interaction with red blood cells (RBCs) and its actions on them will be investigated throughout this dissertation.

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Chapter 2- A Potential Role for Leptin in Regulating Blood Flow

2.1 Introduction

The principle of blood moving throughout the body to carry vital needs was first introduced by Greek philosophers in the 6th century BC.[1] William Harvey was the first scientist to systematically describe blood circulation in 1628 and report that the heart is responsible for pumping blood.[2] RBCs were first visualized as discs in a milky medium by Swammerdam and Leeuwenhoek in the late 1910s.[4] Hunefeld discovered the O_2 carrying protein, hemoglobin, in 1840,[5] and RBCs were then considered to be the hemoglobin-containing cells responsible for carrying O_2 from the lungs to tissues and organs. During the early part of the 20th century, Krogh became interested in gas exchange in living organisms, and he reported an increased use of the O_2 of the blood during exercise. His investigations revealed that oxygen delivery occurs due to newly opened capillaries in skeletal muscle resulting in an increase in the diffusion surface,[6] but Krogh was unable to explain the alteration in perfusion distribution to satisfy the O_2 demand in tissue. This theory was based on capillaries being the sole supplier of O_2. Since then, investigations continued, and scientists reported that capillaries are not the sole source of O_2 transfer.[3] Most of the studies on this subject are focused on other means of O_2 transfer, such as the mechanism by which the resistance vessels alter their diameter via changes in the autonomic nervous system or vasoactive mediator levels. These mechanisms potentially contribute to tissue perfusion, but they cannot explain the regulations of O_2 transfer based on the needs of skeletal muscle.

Barcroft published a book in 1914 entitled The Respiratory Function of the Blood in which he stated, “the cell takes what it needs and leaves the rest.” He explained that

the O_2 delivery occurs in the following three steps. The first step is a call for O_2 in the tissue in demand. Next, a response to the call for O_2 should be generated, followed by the delivery of O_2 to the tissue and the transfer of O_2 from the blood to the tissue in demand. The last step involves a mechanism by which blood obtains its O_2. Barcroft investigated different mechanisms using these three components to explain the tissues "call for O_2" but none of his scenarios could comply with the complexity of the response.[7]

A study performed by Stein and Ellsworth in 1993 has potential to answer Barcroft's "call for O_2" question by proposing that RBCs release ATP in response to a demand for O_2 in skeletal muscle.[8] Bergfeld and Forrester had reported that RBCs of healthy humans release ATP as a response to hypoxia (lack of O_2) and hypercapnia (excessive carbon dioxide) in an earlier paper in 1992.[9] Since then, it has been observed that RBCs of humans, rats, hamsters, and rabbits all release ATP due to reduced O_2 tension (~ 35 Torr) suggesting that the ATP release is due to the decrease in O_2.[10-12]

After being released from the RBCs, ATP can bind to purinergic receptors on endothelial cells, leading to the initiation of downstream vasodilatory actions[13-14] and improves the blood flow to the tissue in demand.[15] Therefore, RBCs optimize O_2 delivery in regions with low O_2 tension via ATP and O_2 release.

RBCs release ATP in response to mechanical deformation[16-18] and receptor-mediated activation of erythrocyte membrane-bound β-adrenergic receptors or prostacyclin receptors in a concentration-dependent manner.[19-20] Mechanical deformation occurs when the cells pass through the microcirculation or are exposed to low O_2 tension.[9-11, 21-22] The amount of RBC-derived ATP released due to mechanical

deformation depends on the magnitude of the stimulus. The ATP release occurs via the activation of a rapid and well-regulated signaling pathway[10, 23] within RBCs initiated by conformational changes in hemoglobin.[21, 23-25] Heterotrimeric G protein (Gi)[22, 26], adenylyl cyclase (AC)[16, 20, 27], protein kinase A (PKA)[27], the cystic fibrosis trans-membrane conductance regulator (CFTR),[16] and pannexin 1[28] are the components involved in this signaling pathway.

G proteins are a family of proteins that act as the signal transmitter for the cell by transmitting the signal upon external stimulation to the interior components of the cell. There are two classes of G proteins, namely monomeric small GTPase and heterotrimeric G protein complexes. The latter is composed of three subunits: α, β, and γ. Activation of G proteins occurs by the dissociation of its subunits.[29] Membrane-bound β-adrenergic receptors or prostacyclin receptors activate the G_s form of the G protein leading to an increase in 3'5'-cyclic adenosine monophosphate (cAMP) and ATP release by RBCs.[20, 22, 30] However, it has also been reported that the G_i protein is activated when RBCs are exposed to mechanical deformation or reduced O_2 tension.[22, 31] It is observed that the α subunit of the G_i protein clearly inhibits the activity of some AC sub units. However, the β subunit of protein G_i activates the AC isoforms, which will initiate the rest of signaling pathway.[26] Interestingly, when pertussis toxin, which inhibits dissociation of G_i protein into its subunits, is incubated with RBCs, ATP release is prevented upon stimulation.[31] It is well established that G_i is involved in the process of ATP release by RBCs upon low O_2 pressure stimulus, but the mechanism by which low O_2 tension activates the G_i protein needs to be further elucidated. It has been proposed that the hemoglobin molecules on the

membrane of RBCs go through conformational changes leading to activation of G_i protein of some other components of the signaling pathway.[21]

If ATP release by RBCs is accepted as an effective O_2 delivery source, it possibly initiates a vasodilatory effect not only in the region in need of O_2, but also in the sites beyond that region.[32] The mechanism of ATP release by RBCs is depicted in Figure 2.1 and explained more thoroughly here. Mechanical deformation activates G_i protein on RBCs leading to the activation of AC and an increase in cAMP.[3] The elevated level of cAMP activates PKA, and as a result, the CFTR protein. This results in the release of ATP via pannexin1 from RBCs. ATP then binds to an endothelial purinergic receptor (P2Y) on endothelial cells and stimulates endothelial nitric oxide synthase (eNOS) to convert L-arginine to L-citrulline with NO as a byproduct. Once produced in the endothelium, NO participates in smooth muscle cell relaxation, vasodilation, and improved blood flow.[26-27, 31].

The regulation of blood flow by the process of ATP release from RBCs is vital to the health of many systems in the body. Interestingly, impaired blood flow is thought to be responsible for many complications seen in patients with diabetes, including retinopathy, nephropathy, and neuropathy.[33] Patients with diabetes are either unable to produce insulin, as in the case of type 1 diabetes (T1D), or are resistant to insulin, as in the case of type 2 diabetes (T2D).[34] In either situation, the end result is increased blood glucose levels. T1D patients administer exogenous insulin because pancreatic β-cells are not able to produce and other molecules that are co-secreted with it such as C-peptide and zinc. *In vivo*, in healthy persons, C-peptide is released in equimolar amounts with

insulin,[35] and C-peptide, along with zinc and albumin, has been reported to stimulate RBCs to release more ATP than untreated RBCs.[36] Albumin may serve as the carrier for zinc and C-peptide. Unfortunately, in a high glucose environment, C-peptide is not as effective, and albumin may become glycated. In addition, C-peptide and zinc does exist in T2D patients but it is not functioning effectively. Therefore, it is important to find molecules that can overcome this binding deficiency or replace albumin. To fulfill this role, we have begun to look at leptin.

One of the proposed mechanisms for obesity is resistance to the hormone leptin. T2D and obesity are closely related. It has been reported that 30% of obese people will develop T2D, and 85% of patients with T2D are obese.[37] One of the complications of diabetes is neuropathy, associated with impaired blood flow. Studies have illustrated that leptin can lead to vessel dilation by stimulating NO production. Kimura *et al.* found that leptin relaxes arterial rings in a dose dependent manner, and that the vessel dilation by leptin was abolished when the endothelial cells were removed or when eNOS was inhibited.[13-14, 38] In addition, leptin-stimulated NO production and vessel dilation has been reported in rat and dog coronary arterioles.[38-39] RBC-derived ATP stimulates eNOS to produce NO, which results in vessel dilation and improved blood flow as discussed earlier in this chapter. Collectively, leptin may be involved in the mechanism by which zinc and C-peptide bind to RBCs to stimulate ATP release, especially in people who are obese or have increased levels of fat tissue, the producer of leptin. To test this hypothesis, RBCs were treated with various combinations of zinc, C-peptide, and leptin, with and without the presence of albumin, before measuring the amount of ATP released from the RBCs using a luciferin/luciferase chemiluminescence assay.

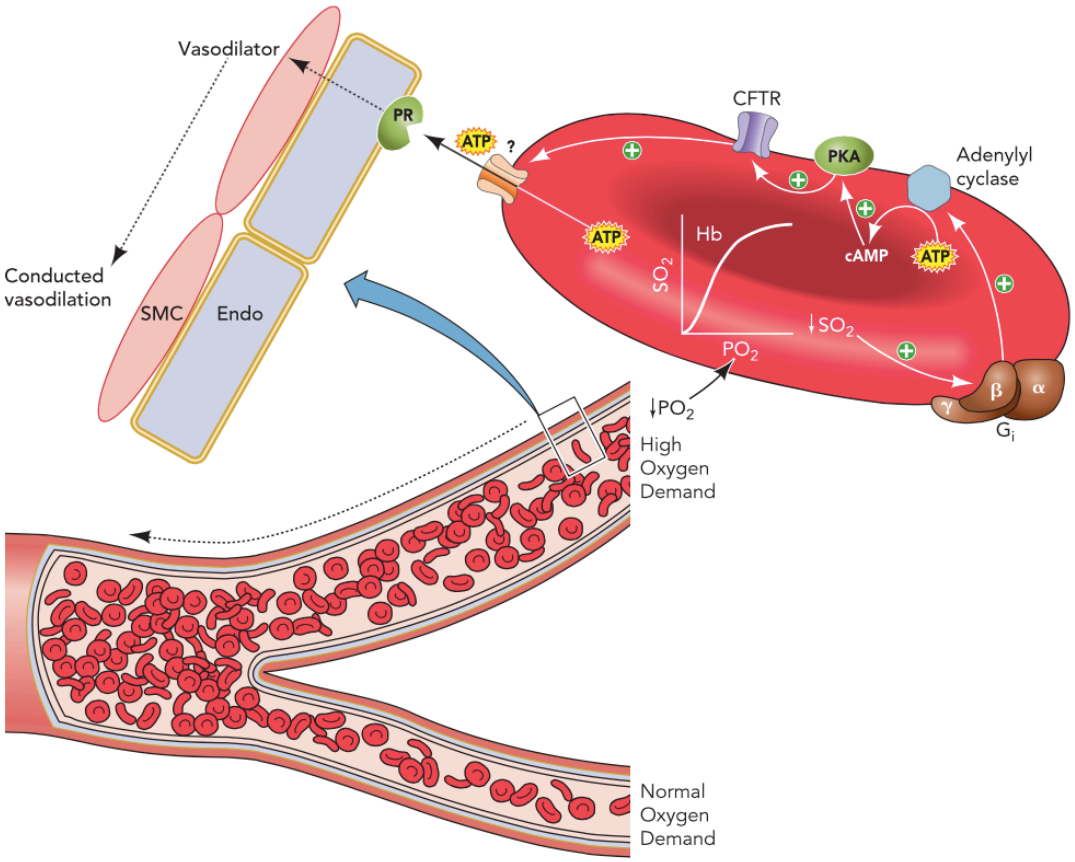

Figure 2.1. The Mechanism of ATP Release by RBCs and Vessel Dilation via Endothelial NO Production. RBCs travel to the regions with low oxygen tension (PO_2) leading to the diffusion of oxygen to the tissues and a decrease in oxygen saturation (SO_2) of RBCs in microcirculation. Decreased oxygen tension and mechanical deformation activate the G_i protein and stimulate ATP release by RBCs. ATP then binds to endothelial purinergic receptor P_2Y and stimulates NO production. NO is a well-known mediator to initiate vessel dilation and improve blood flow. Gi, heterotrimeric G protein; ATP, adenosine triphosphate; cAMP, 3'5'cyclick adenosine monophosphate; PKA, protein kinase A; CFTR, cystic fibrosis transmembrane conductance regulator; ?, potentially pannexin1, a conduit for ATP release; PR, purinergic receptors; +, stimulation; endo, endothelium; SMC, smooth muscle cell.[3]

2.2 Experimental Methods

2.2.1 Preparation of RBCs in Regular and Albumin-Free Physiological Salt Solution (PSS)

For all experiments involving human donors, informed consent was obtained using consent forms and procedures that were approved by the Biomedical and Health Institutional Review Board (BIRB) at Michigan State University. Whole blood was collected from healthy and consenting donors into heparinized tubes (Fisher Scientific, Waltham, MA) by venipuncture on the day of the experiment. The collected whole blood was then centrifuged at 500*g* for 10 minutes, and the buffy coat and plasma were removed by aspiration. The remaining RBCs were washed 3 times in physiological salt solution (PSS) containing, in mM, 4.7 KCl, 2.0 $CaCl_2$, 140.5 NaCl,12 $MgSO_4$, 21.0 tris (hydroxymethyl) aminomethane, 5.5 glucose, and 0.5% bovine serum albumin at a pH of 7.40. All the chemicals are purchased from Sigma Aldrich, St. Louis, MO unless it is stated otherwise. The hematocrit of the purified RBCs was then determined using a hematocrit centrifuge (CritSpin, Iris Sample Processing, Westwood, MA). RBCs that were used in the studies with albumin-free PSS were prepared in the same manner, but the RBCs were washed in albumin-free PSS. The process of the isolation of RBCs from the whole blood is shown in Figure 2.2.

2.2.2 Preparation of Samples in Regular and Albumin-free PSS

Recombinant human leptin (R&D Systems, Minneapolis, MN) was prepared by dissolving 1 mg of leptin in 1 mL of 20 mM Tris-HCl at pH 8.00. To incubate the RBCs with leptin for experimentation, the appropriate volume of leptin (76 μL) stock was added to the sample vials to obtain a final concentration of 3.8 nM. Then, the appropriate volumes of purified RBCs and PSS were added to the sample tubes to create a 7%

hematocrit and a total volume of 950 μL, followed by incubation of the samples at 37°C for 1 hour. After incubating for 1 hour, samples were centrifuged at 500*g* for 5 mins and 25 μL of 800 nM zinc and C-peptide was added to the vials to have the final concentration of 20 nM for zinc and C-peptide. Then, samples were incubated for another 2 hours at 37°C. The working solutions of zinc (800 nM) were prepared by by adding 10 μl of 80 μM of zinc stock to 990 μL of distilled and deionized water (DDW). Similarly, a solution of 800 nM C-peptide was prepared by adding 100 μl of 8 μM C-peptide stock to 900 μL of DDW . After the second incubation, the ATP release from RBCs are now ready for measurement To perform measurements in the absence of albumin, samples were prepared in the same manner, except albumin-free PSS was used to isolate RBCs and prepare samples. Sample preparation is shown in Figure 2.2.

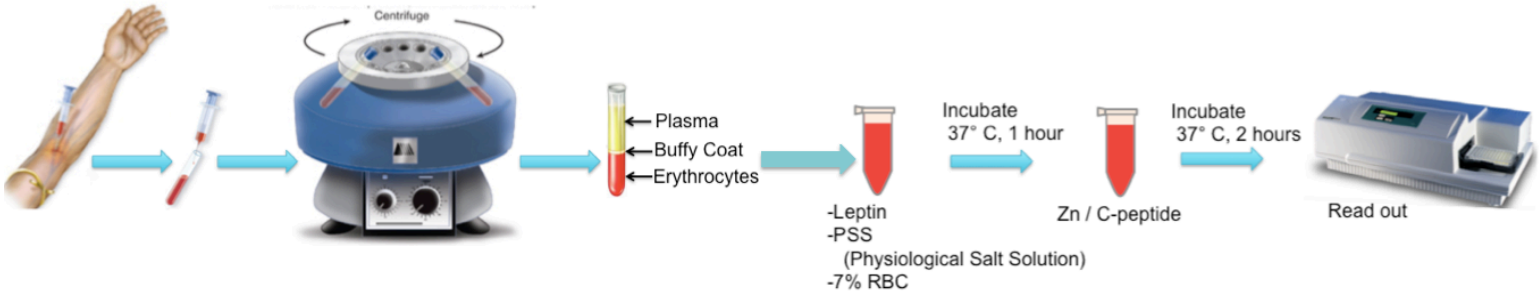

Figure 2.2. Isolation of RBCs and Sample Preparation Using Leptin.

2.2.3 Leptin Treated RBC Sample Preparation when Leptin is Washed from the Sample

Isolation of RBCs was completed as described in section 2.2.1 in albumin-containing PSS. To learn more about the interaction of leptin with RBCs, samples were prepared as in section 2.2.2, but leptin was washed out of the samples after the first hour of incubation at 37°C by centrifuging the samples at 500*g* for 10 minutes and removing the supernatant by aspiration. This step was repeated two more times and a known amount of the supernatant was removed each time. At the end of third wash, fresh PSS was added to the sample, along with 20 nM zinc and C-peptide. Samples were incubated for another 2 hours at 37°C.

2.2.4 Sample Preparation in High Glucose PSS

Isolation and purification of RBCs was performed as in section 2.2.1, except 10 mM or 15 mM glucose PSS was used in place of 5.5 mM glucose. Sample preparation is also explained in section 2.2.2, and again 10 or 15 mM glucose PSS was used as the buffer.

2.2.5 Sample Preparation using T1D RBCs

Whole blood was collected by venipuncture into heparinized tubes (Fisher Scientific) from consenting patients with type 1 diabetes (T1D) on the day of the experiment. Regular and albumin-free buffers were used for this experiment. Sample preparation is explained in detail in sections 2.2.1 and 2.2.2.

2.2.6 Sample Preparation using 7% Whole Blood

Whole blood was collected into heparinized tubes (Fisher Scientific, Waltham, MA) by venipuncture from healthy and consenting donors on the day of the experiment. The hematocrit of the whole blood was measured using a hematocrit centrifuge (CritSpin,

Iris Sample Processing). In this case, the RBCs were not purified from the whole blood, and 7% hematocrit whole blood was used for this study. The rest of the sample preparation continued as described in section 2.2.2.

2.2.7 Measurement and Quantification of ATP Release

The amount of ATP released from RBCs was measured after 3 hours of incubation. The standard addition method was used for quantification of ATP release by preparing varying concentrations of ATP standards in the corresponding buffer (PSS, albumin-free PSS, or high glucose PSS). ATP stock was prepared by adding 1.32 g of albumin to 10 mL of water followed by serial dilutions to prepare 20 μM and 2 μM solutions. The last two dilutions were made in PSS to a total volume of 10 mL to make 20 μM and 2 μM ATP. Next, 1 mL of the final concentrations of ATP standards were prepared using the 2 μM stock solution in PSS. Albumin-free PSS was used to prepare standards for the experiments performed in the absence of albumin. Four aliquots (120 μL) of each RBC sample were added to the 96 well plate, and 30 μL of ATP standard solutions with varying concentrations were then added to RBC aliquots. Finally, 10 μL of luciferin/luciferase (prepared by dissolving 5 mg of luciferin and 100 mg firefly lantern extract in 5 mL deionized water) were added to each sample, and the chemiluminescence signal of the samples was measured using a plate reader (Molecular Devices LCC, Sunnyvale, CA). The signal intensities were plotted against the concentrations of ATP standard solutions as depicted in Figure 2.2.3. This plot is extrapolated to the x-axis intercept, and the ATP concentration in the sample is quantified as the absolute value of the x-axis intercept.

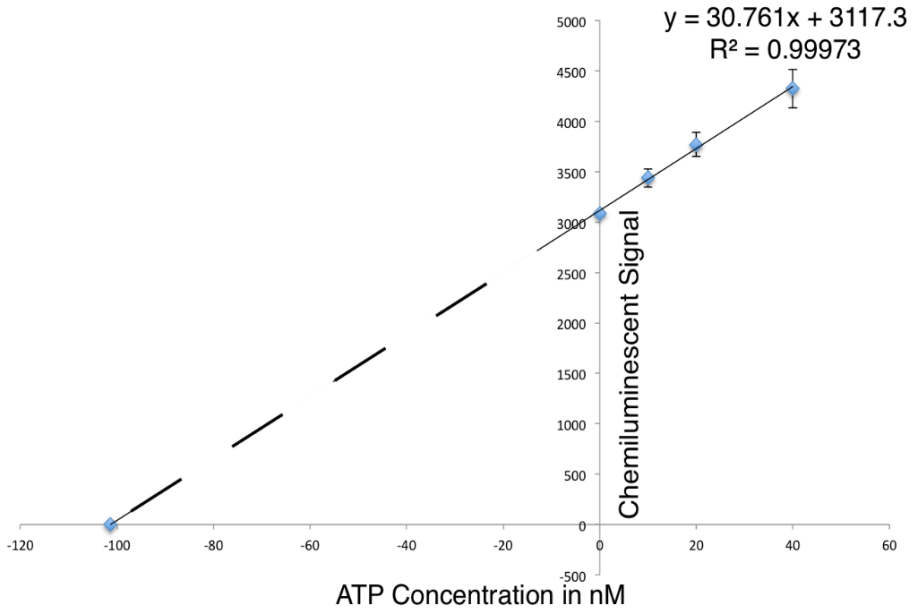

Figure 2.3. Quantification of ATP Release using the Standard Addition Method. Varying concentrations of ATP standard are added to RBCs followed by addition of luciferin/luciferase, and the ATP released from the RBCs was quantified by extrapolating to the x-axis intercept.

2.3 Results

2.3.1 Leptin can Increase RBCs-Derived ATP in the Presence and Absence of Albumin

RBCs from healthy control donors were incubated with various combinations of zinc, C-peptide, and leptin. RBCs with zinc and C-peptide showed significantly higher ATP release (195.7 ± 8.5 nM, $p<0.001$) when compared to healthy control samples that did not include zinc and C-peptide (163.4 ± 5.4 nM). Interestingly, when zinc and C-peptide were added to the samples of RBCs containing leptin, ATP release increased by another 25% (244.2 ± 9.0 nM, $p<0.005$) compared to the samples containing only zinc

and C-peptide. Samples containing only zinc, only C-peptide, or only leptin, and all other combinations showed no significant increase in ATP release, as shown in Figure 2.4.

This study was performed in albumin-free PSS as well. Figure 2.5 illustrates that samples containing only zinc and C-peptide did not show any significant increase in ATP release when albumin-free PSS was utilized, as compared to RBCs alone. Samples containing zinc, C-peptide, and leptin showed a significant increase in ATP release (103.3 ± 4.3 nM, $p<0.001$) compared to the samples containing zinc and C-peptide only (61.4 ± 2.4 nM) in the absence of albumin.

2.3.2 Leptin must be Incubated with RBCs in the Presence of Zinc and C-peptide to Elucidate Cellular Effect

To study the effect of leptin mechanistically, ATP release was measured when leptin was removed from the sample after a 1 hour incubation, before the addition of zinc and C-peptide. As depicted in Figure 2.6, no increase in ATP release was observed when leptin, zinc, and C-peptide were present, but leptin had been removed by washing the samples (110.8 ± 5.6 nM) compare to RBCs treated with zinc and C-peptide only (111.7 ± 4.9 nM).

2.3.3 Leptin Enhanced the Levels of ATP Release by RBCs in High Glucose PSS

To mimic the high glucose environment in patients with diabetes, RBCs were washed in 10 mM or 15 mM glucose buffer and incubated in the corresponding buffer. Surprisingly, samples containing zinc, C-peptide, and leptin showed an increase in ATP release by RBCs (96.3 ± 1.17 nM, $p<0.05$) in 10 mM glucose PSS to a level where the percent increase was statistically the same as that observed using leptin, zinc, and C-peptide treated RBCs (146.2 ± 12.3 nM) in regular PSS (5.5 mM glucose). ATP release by RBCs in 15 mM glucose PSS increased when leptin, zinc, and C-peptide were present

compare to samples containing zinc and C-peptide (87.66 ± 6.27 nM, $p<0.04$ vs 73.0 ± 5.0 nM). The results for 10 mM and 15 mM glucose buffer are shown in Figure 2.7 and 2.8, respectively.

2.3.4 Leptin Increases ATP Release by T1D RBCs in Regular and Albumin-Free PSS

Whole blood from T1D patients and healthy controls was collected, and the RBCs washed in either regular or albumin-free PSS. Regardless of the sample type or buffer, all samples containing zinc, C-peptide, and leptin showed an increase in ATP release. Leptin-treated healthy RBCs in PSS had the largest increase (66.6 ± 2.59 nM, $p<0.01$) compared to the control healthy RBCs (33.0 ± 1.4 nM). Leptin treated T1D RBCs in regular PSS also showed an increase (39.7 ± 2.89 nM, $p<0.05$) compared to control T1D cells (23.7 ± 0.97). However, the amount of ATP released by T1D RBCs was lower compared to healthy cells. The same result was observed using albumin-free PSS and samples containing zinc, C-peptide, and leptin showed an increase in ATP release. Healthy cells containing leptin, zinc, and C-peptide elevated the levels of ATP release (28.1 ± 1.46, $p<0.05$) compared to the corresponding control (23.2 ± 1.5 nM) and T1D RBCs containing all three substances had an increase in ATP release (21.4 ± 1.40, $p<0.05$) compare to T1D control samples (13.3 ± 0.5 nM). The results are depicted in Figure 2.9.

2.3.5 The Effect of Leptin on ATP Release was also Confirmed in 7% Whole Blood

7% whole blood samples were treated with zinc, C-peptide, leptin, and different combinations thereof. Zinc and C-peptide treated samples resulted in an increase in the amount of ATP released (26.0 ± 2.3 nM, $p<0.05$) compared to the control samples (17.5 ± 1.6 nM). Interestingly, samples treated with zinc, C-peptide, and leptin showed an even

larger increase (33.6 ± 3.0, $p<0.05$) compared to the samples containing zinc and C-peptide only (26.0 ± 2.3 nM) and confirmed the trend observed in treated RBCs. This study was performed in albumin-containing buffer. The results are shown in Figure 2.10.

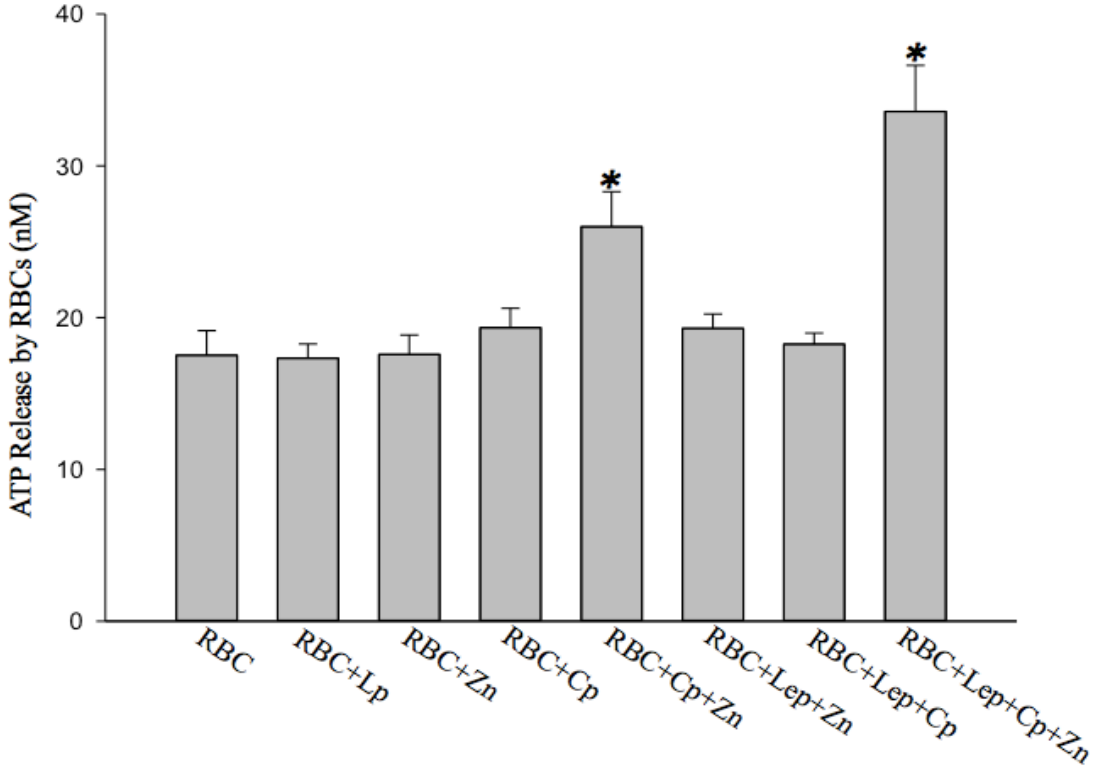

Figure 2.4. Determination of ATP Release by RBCs in the Presence of Leptin using Albumin Containing PSS. Zinc and C-peptide increase the amount of ATP released by RBCs significantly, compare to the control cells. Leptin is able to significantly enhance this effect. Leptin alone or other combinations with zinc and C-peptide did not show any effect. [Zn] = 20 nM, [Cp] = 20 nM, [Leptin] = 3.8 nM, 7% RBC, error bars: SEM, $n \geq 4$, $p < 0.05$.

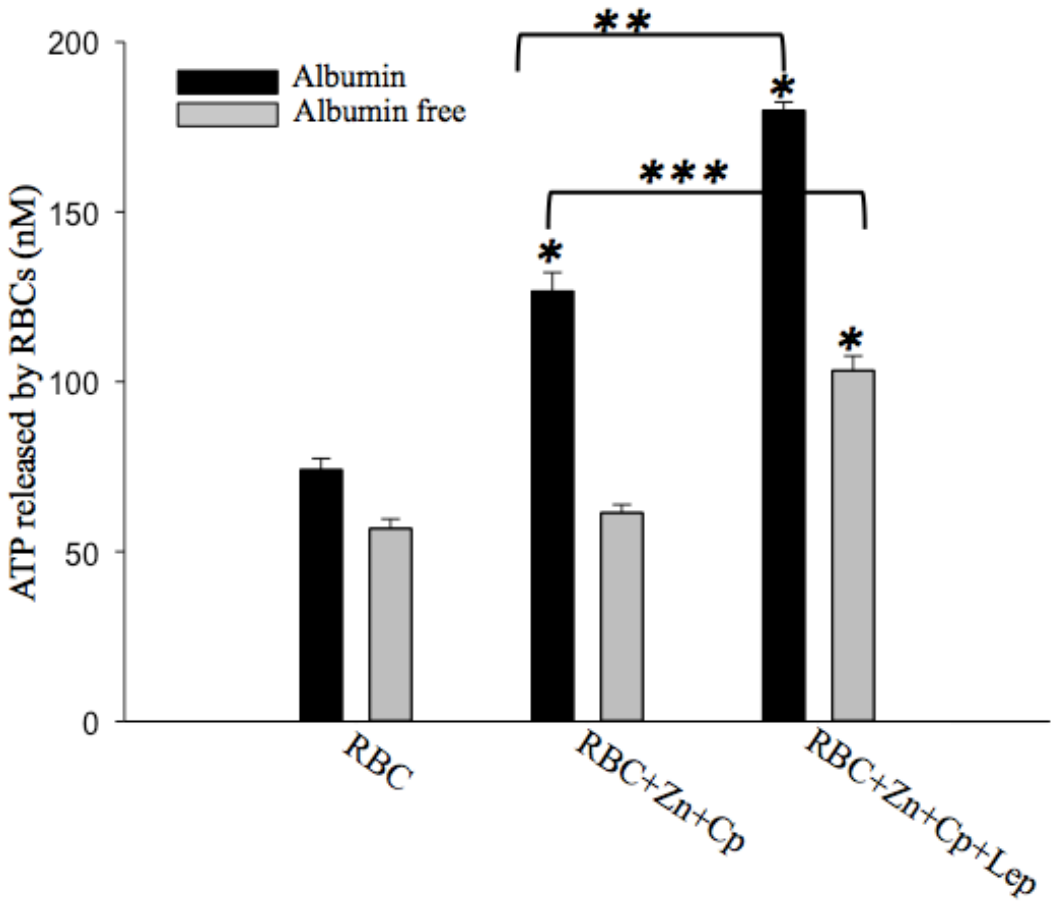

Figure 2.5. Leptin Enhances RBC-Derived ATP in the Presence and Absence of Albumin. Black bars show RBCs in albumin containing PSS and grey bars show samples in albumin-free PSS. As depicted here, samples containing zinc and C-peptide in albumin containing PSS increase ATP release by RBCs and leptin significantly enhances this effect. However, no effect on ATP release was observed by RBCs treated with zinc and C-peptide in albumin-free PSS. Interestingly, leptin increase ATP release by RBCs in albumin-free PSS and the increase was statistically the same as the increase observed by zinc and C-peptide in regular PSS. [Zn] = 20 nM, [Cp] = 20 nM, [Leptin] = 3.8 nM, 7% RBC, error bars: SEM, n=5, $p < 0.001$.

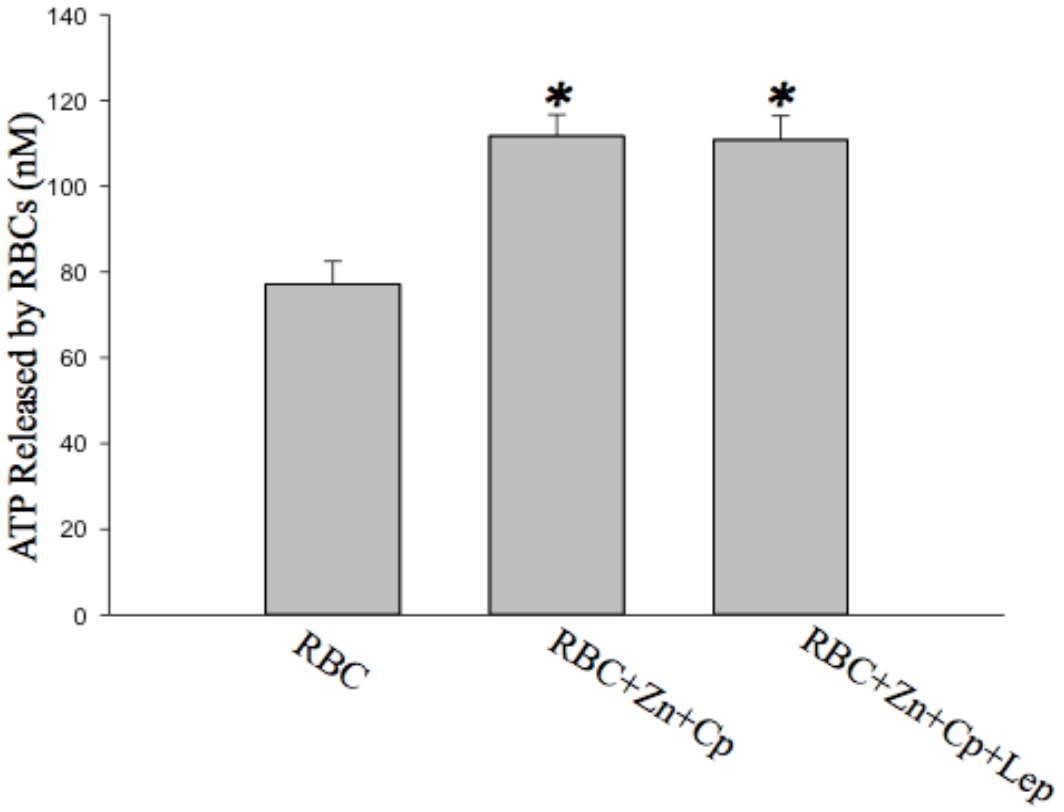

Figure 2.6. Leptin Cellular Effect only occurs in the Presence of Zinc and C-peptide. Leptin did not show any effect on RBCs, compared to the samples containing only zinc and C-peptide, when it was removed from the sample before the addition of zinc and C-peptide. [Zn] = 20 nM, [Cp] = 20 nM, [Leptin] = 3.8 nM, 7% RBC, error bars: SEM, n=6, $p < 0.001$.

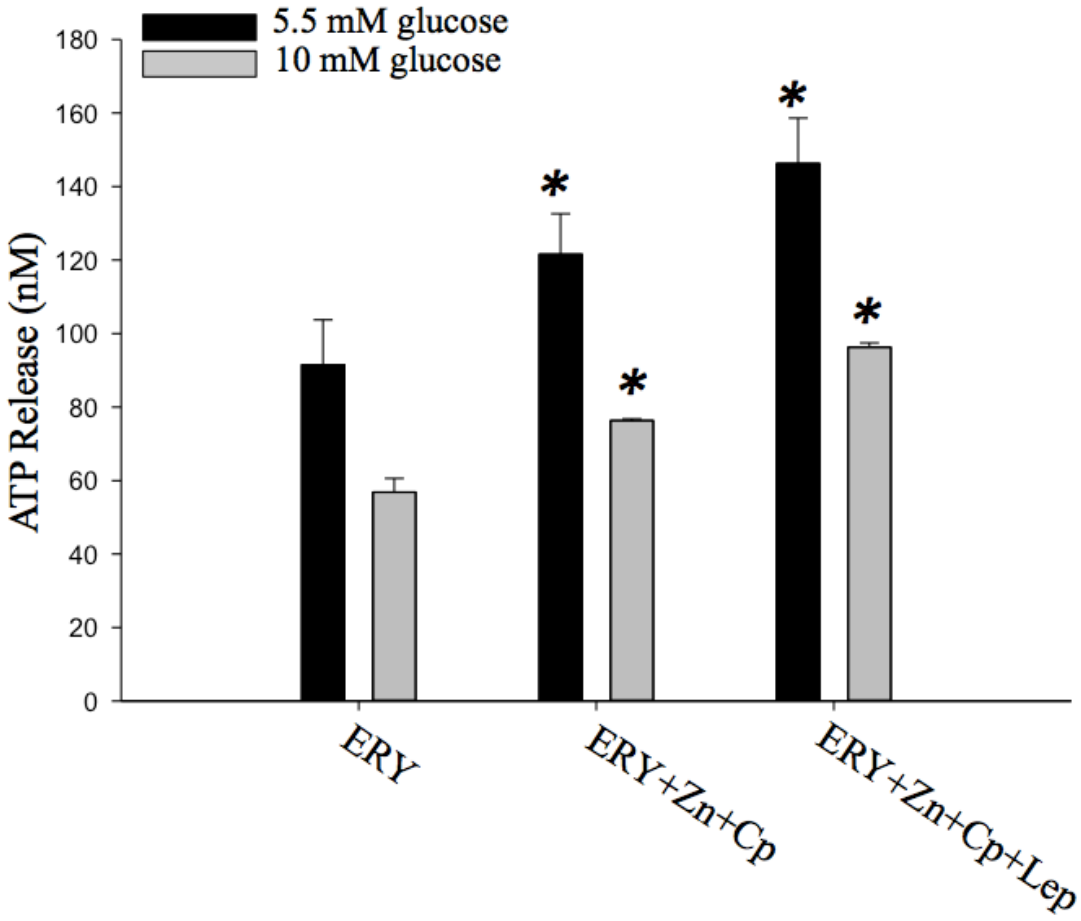

Figure 2.7. Leptin Elevates the levels of ATP Release by RBCs in 10 mM Glucose PSS. Black bars show RBCs samples in regular PSS and grey bars show samples in 10 mM glucose PSS. RBCs release less ATP in high glucose conditions. Leptin enhances zinc and C-peptide effect in high glucose PSS, but the increase is less significant. [Zn] = 20 nM, [Cp] = 20 nM, [Leptin] = 3.8 nM, 7% RBC, error bars: SEM, n=4, $p < 0.005$.

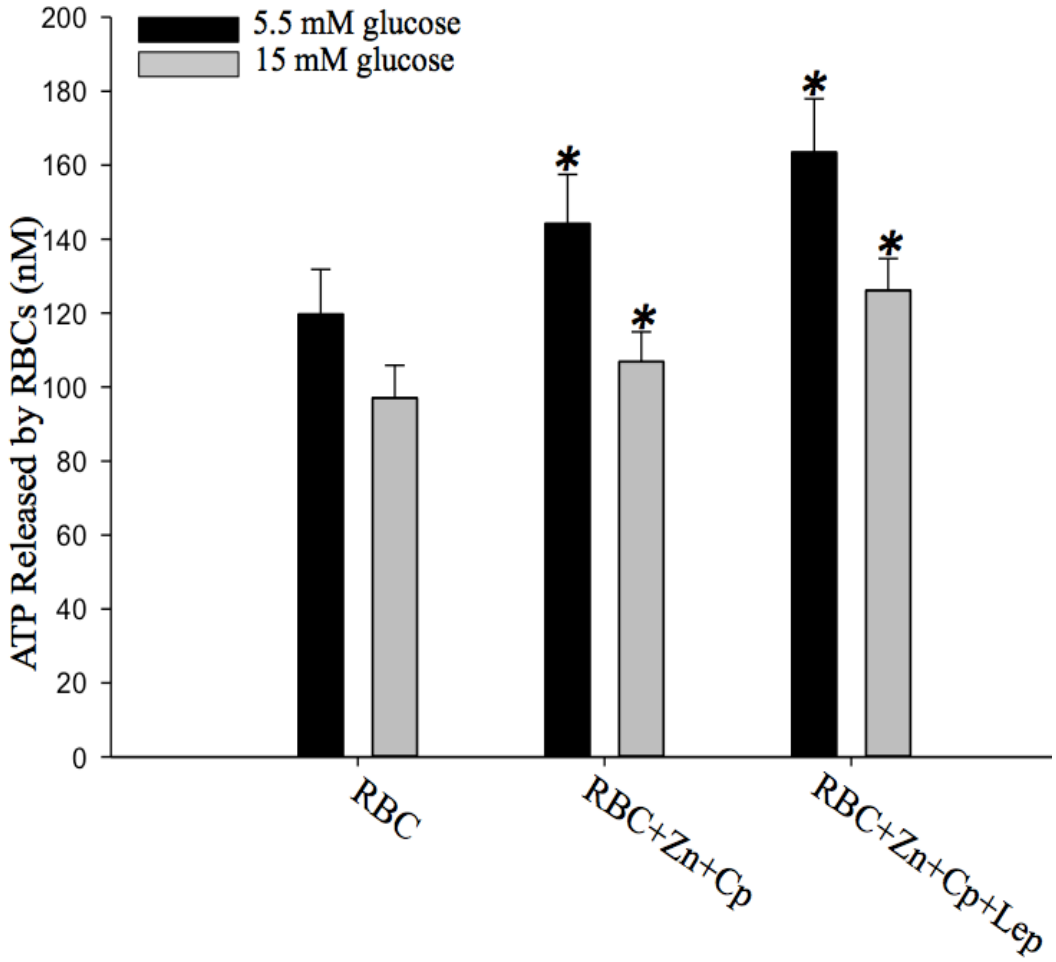

Figure 2.8. Leptin Elevates the levels of ATP Release by RBCs in 15 mM Glucose PSS. Black bars show RBCs samples in regular PSS and grey bars show samples in 15 mM glucose PSS. RBCs release less ATP in high glucose conditions. Leptin enhances zinc and C-peptide effect in high glucose PSS, but the increase is less significant. [Zn] = 20 nM, [Cp] = 20 nM, [Leptin] = 3.8 nM, 7% RBC, error bars: SEM, n=6, $p < 0.005$.

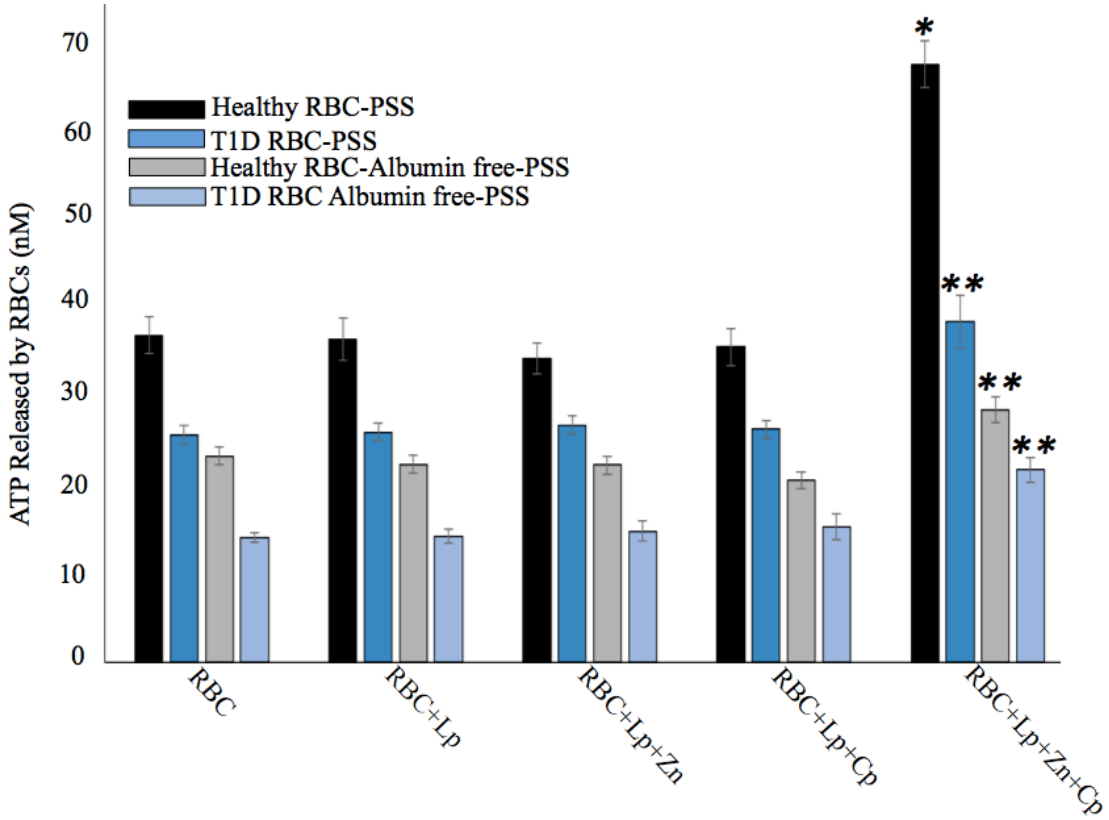

Figure 2.9. ATP Release Increase from T1D and Healthy RBCs upon Stimulation by Leptin, Zinc, and C-peptide. Black bars show healthy RBCs in regular PSS, medium grey bars show T1D cells in regular PSS. Dark grey bars show healthy RBCs in albumin-free PSS and light grey bars show T1D RBCs in albumin-free PSS. RBCs stimulated with zinc, C-peptide, and leptin elevated the levels of ATP released in both cell types and buffers. [Zn]= 20 nM, [Cp] = 20 nM, [Leptin] = 3.8 nM, 7% RBC, error bars: SEM, n=5, $p < 0.005$.

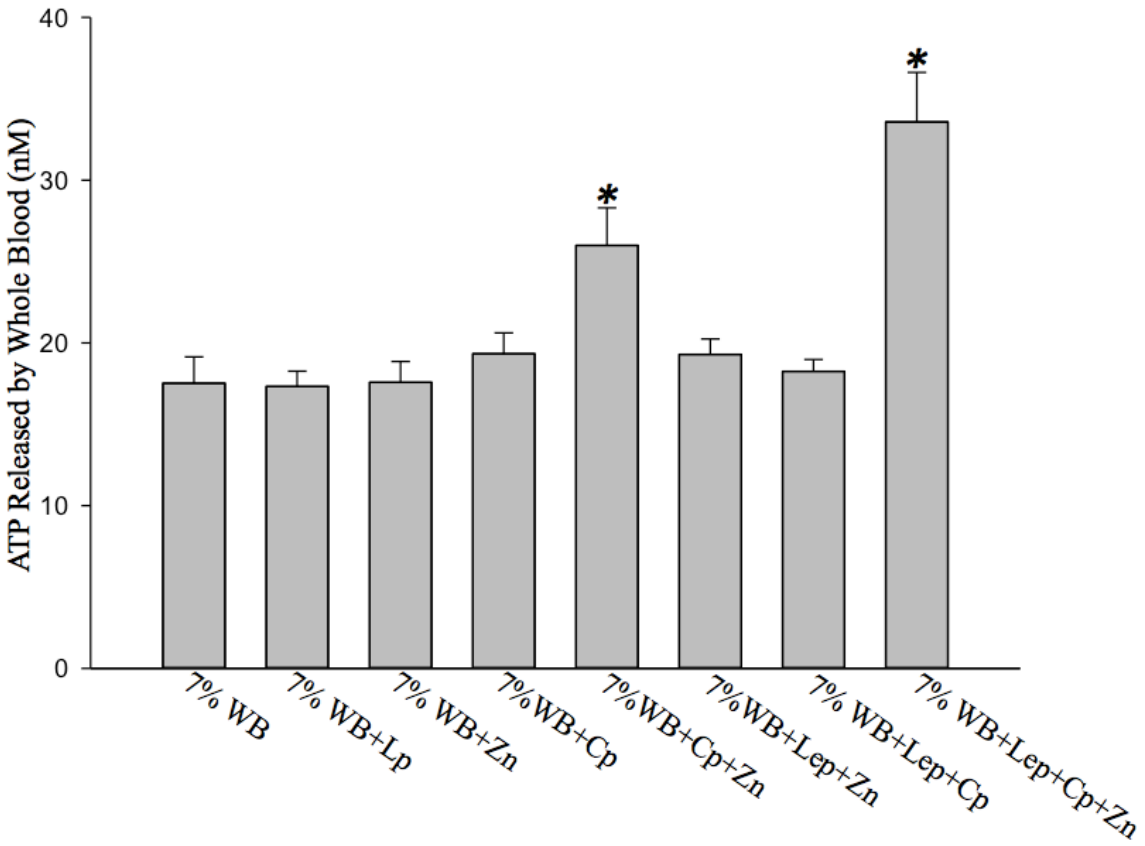

Figure 2.10. ATP Release by RBCs was Elevated in the Presence of Leptin, Zinc, and C-peptide in 7% Whole Blood Samples. Samples treated by leptin, zinc, and C-peptide had the greatest increase in ATP release using 7% whole blood instead of RBCs only. [Zn]= 20 nM, [Cp] = 20 nM, [Leptin] = 3.8 nM, 7% whole blood, error bars: SEM, $n \geq 4$, $p < 0.005$.

2.4 Discussion

Leptin is secreted by adipose tissue into the bloodstream and travels to the brain where it crosses the blood brain barrier and is thought to interact with its receptors in the central nervous system to balance energy, food intake, and many other actions. The delivery of leptin to the central nervous system seems to have an important role in the regulation of appetite and energy homeostasis. In addition, obese people are referred as "leptin resistant" due to higher levels of leptin in their plasma, but the factors that play a role in leptin resistance are still unknown.

Most of the studies performed to understand the effect of leptin on glucose homeostasis are focused on the central nervous system.[40-41] Recent studies have also investigated the role of leptin on vessel dilation, which could lead to a better understanding and management strategy of diabetic complications. Kimura, *et al*. found that leptin relaxes arterial rings in a dose dependent manner, and the vessel dilation by leptin was abolished when the endothelial cells were removed or when nitric oxide synthase was inhibited.[13-14, 38] Here, we propose a new theory on the role of leptin by studying its interactions with blood cells.

Our group has previously shown that RBC samples treated with zinc and C-peptide in albumin-containing PSS increase ATP release,[36] which will potentially result in downstream vessel dilation. The results are depicted in Figure 2.11. Here, we investigated the effect of leptin on ATP release from RBCs. Interestingly, leptin increases ATP release by RBCs in the presence of zinc, C-peptide, and albumin. Importantly, leptin alone has no effect on RBCs. As demonstrated in Figure 2.11, our group has shown that albumin is essential to observe metabolic effects on RBCs in the presence of zinc and C-

peptide. It was demonstrated that C-peptide can bind to RBCs in the presence of albumin and ATP release from these cells occurs in the presence of the zinc, C-peptide, and albumin trio.[36] Here, we demonstrate that leptin can increase the ATP release from RBCs containing zinc and C-peptide in the absence of albumin. The increase was not as significant as the increase with leptin in the presence of albumin, but it is statistically the same increase as observed by zinc and C-peptide in the presence of albumin (without leptin).

Albumin is the carrier for many proteins, lipids, and other molecules in the blood stream. Thus, it can be proposed that the presence of albumin is necessary as the carrier of zinc and C-peptide to deliver them to RBCs. Observing C-peptide's metabolic effects in the presence of leptin in albumin-free buffer suggests that leptin can potentially substitute for albumin in the blood stream. Therefore, leptin may take over albumin's responsibility to carry zinc and C-peptide to RBCs when it is not functioning properly, such as when it is glycated due to continued exposure to a high glucose environment. To understand this better, we need to investigate the binding and/or interaction between C-peptide and leptin.

To study the effect of leptin mechanistically and know whether its effect is additive to zinc and C-peptide or dependent on the presence of them, all of the leptin added to the sample originally was removed from the sample by centrifugation and removal of the supernatant before the addition of zinc and C-peptide. Interestingly, samples containing zinc, C-peptide, and leptin did not show any increase in ATP release compared to the samples containing zinc and C-peptide only (no leptin was added to the sample). This means that the effect of leptin on RBCs is dependent on the presence of

zinc and C-peptide even though it is not dependent on the presence of albumin. Therefore, the increase in ATP release is likely due to leptin facilitating the delivery of zinc and C-peptide to RBCs in a similar manner to albumin. This experiment also supports the idea that leptin possibly binds to/interacts with C-peptide in order to carry it to RBCs.

Diabetes and obesity are closely associated, and leptin is the hormone involved in obesity. In addition, C-peptide's cellular effects are potentially beneficial in improving blood flow and preventing/delaying diabetic complications, and leptin is enhancing these effects. Therefore, it is important to study the effect of leptin in high glucose buffer to see how effective leptin is in a high glucose environment, such as one that would be seen in diabetic patients. To do this, RBCs were washed and prepared in 10 mM glucose PSS to mimic diabetic blood glucose levels, and the ATP released by the RBCs was studied. Leptin, surprisingly, increased ATP release from the RBCs, and the percent increase was statistically the same as the percent increase in regular PSS (5.5 mM glucose). The amount of ATP released by the RBCs that were purified and incubated in high glucose buffer was lower than that released by healthy RBCs. This is in agreement with previous studies performed in the Spence lab on T2D RBCs.[42] The results observed by Meyer *et. al.* are depicted in figure 2.12. Thus, leptin can potentially be effective in a high glucose environment if C-peptide is functioning properly. However, this study needs to be performed on diabetic RBCs to be able to mimic diabetic conditions *in vitro*. Healthy RBCs were used for this study and will potentially respond differently than diabetic RBCs because of the extent of damage they are subjected to exposure to high glucose environment for long periods of time.

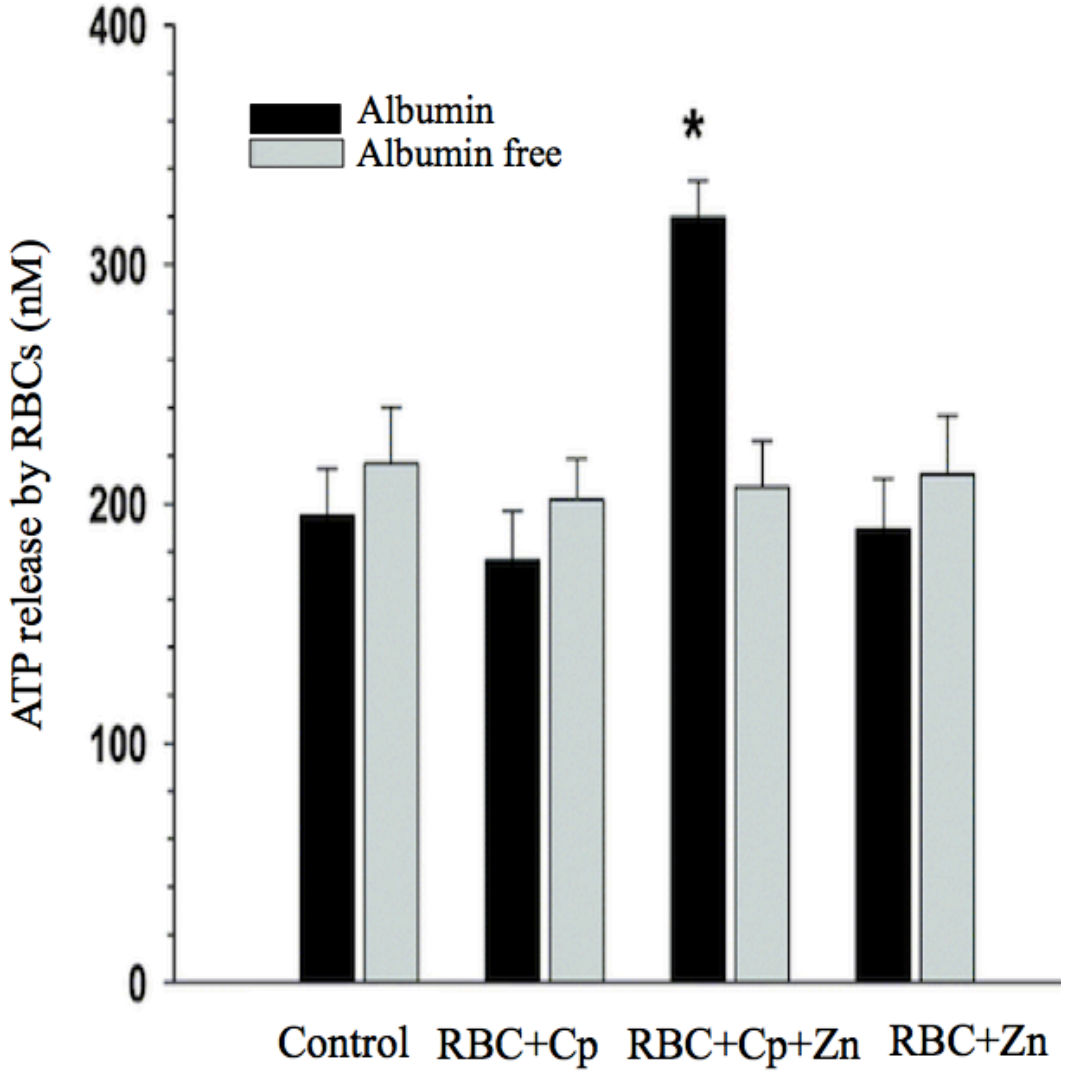

Figure 2.11. The Presence of Albumin is necessary for Cellular Effects of C-peptide. Black bars are showing 7% RBCs in regular PSS that contains albumin. Grey bars are showing 7% RBCs in albumin-free PSS. This figure demonstrates that C-peptide enhances RBC-derived ATP in the presence of zinc and albumin. Taking out any of the substances in this trio will result in no effect from C-peptide. [Zn]= 20 nM, [Cp] = 20 nM, 7% RBCs, error bars: SEM, n=5, $p < 0.005$.

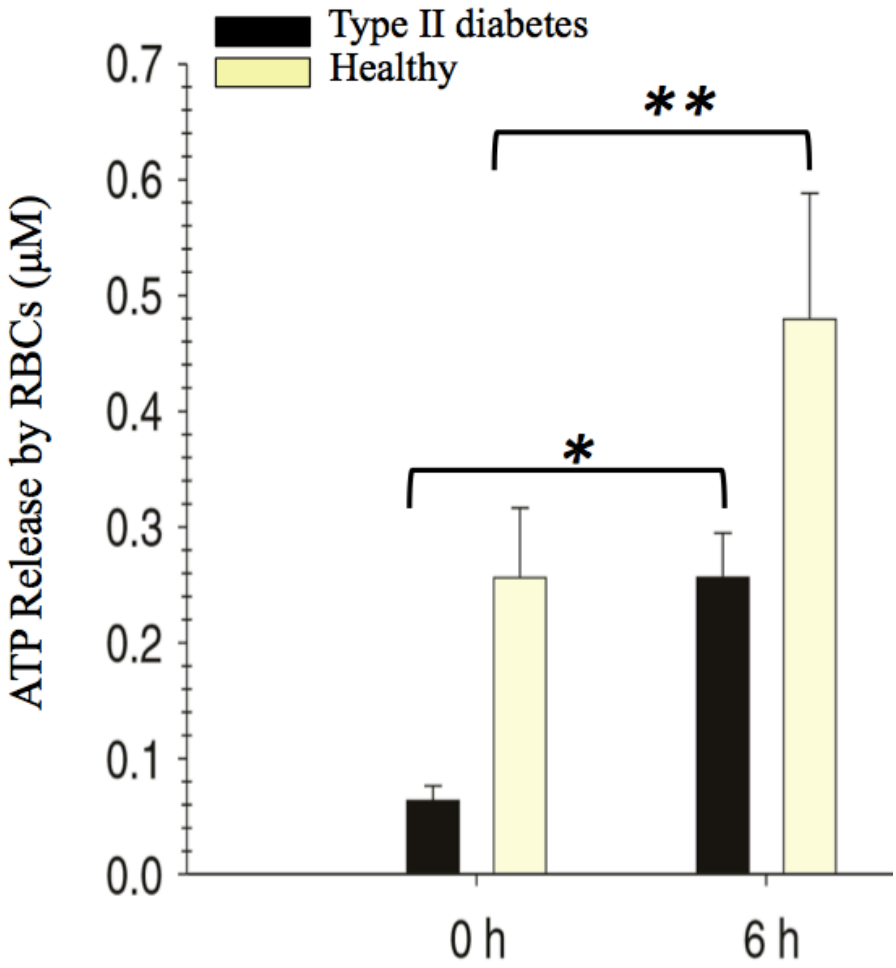

Figure 2.12. ATP Released by T2D RBCs is less than Healthy Cells, but C-peptide Restores the Levels of ATP in T2D RBCs to Healthy Levels. Black bars show T2D RBCs and white bars show healthy RBCs. The level of ATP released by T2D RBCs is lower than that of healthy cells. Healthy RBCs are incubated with C-peptide for 6 hours and ATP levels increased from 260 ± 60 nM to 480 ± 109 nM. T2D RBCs basal ATP level was 64 ± 13 nM and 6 hours incubation with C-peptide restored ATP levels to basal healthy levels (260 ± 39 nM). [Cp] = 20 nM, 7% RBCs, error bars: SEM, n=7, $p < 0.005$.

Pancreatic β-cells are damaged in patients with T1D and they cannot secret insulin and C-peptide. Insulin is administered in these patients exogenously to balance the glucose homeostasis, but studies have shown that insulin alone is not enough to prevent or delay the diabetes complications.[43] Administration of C-peptide seems to be important in these patients to elicit cellular effects from RBCs, which may result in vessel dilation. Also, finding molecules that can enhance the cellular effects of C-peptide may be beneficial in high glucose conditions. Here, leptin has been administered along with zinc and C-peptide to T1D RBCs as a candidate molecule to enhance C-peptide's cellular effect in high glucose condition. The effect of leptin on RBCs was examined on T1D RBCs in both albumin-free and albumin-containing PSS, and healthy RBCs were used as the control. The presence of leptin, C-peptide, and zinc was necessary to observe an increase in ATP release in all four conditions. The levels of ATP released by healthy and T1D cells in regular PSS were higher than that in albumin-free PSS. The increase in ATP release by healthy RBCs in the presence of zinc, C-peptide, and leptin was more significant than the increase observed in T1D RBCs treated with zinc, C-peptide, and leptin. However, treated T1D RBCs ATP release was statistically the same as the ATP release of healthy control RBCs, suggesting that leptin-treated diabetic RBCs can possibly act as healthy RBCs in terms of ATP release and eventually NO production. As mentioned before, the amount of ATP released by RBCs in albumin-free buffer was lower than the cells in samples containing albumin, and T1D cells release less ATP compared to healthy RBCs in albumin-free PSS, as expected. However, leptin, zinc, and C-peptide treated RBCs demonstrated an increase in ATP release, and the increase in T1D cells elevated the levels of ATP release to healthy basal levels, as expected.

All the studies previously performed on the effects of C-peptide were performed using isolated, purified RBCs. Therefore, no other molecules and or proteins were present other than the molecules and proteins that were added exogenously. It is of crucial importance to perform the same studies using whole blood to mimic the *in vivo* conditions. This experiment will help us to understand whether or not other molecules present in blood will interfere with the effect of C-peptide and leptin. Thus, 7% whole blood was incubated with zinc, C-peptide, leptin, and combinations thereof. The amount of ATP released by 7% whole blood was lower than that observed using 7% RBCs. This may be due to the presence of other molecule such as pyruvate phosphatase that can chew up ATP. Interestingly, the trend observed using 7% whole blood was the same as that seen with 7% RBCs. ATP release was elevated in the presence of zinc and C-peptide which confirms the results observed previously by the Spence group using RBCs. In addition, samples treated with zinc, C-peptide, and leptin demonstrated a more significant increase compared to the samples containing zinc and C-peptide alone. These results seem promising in the path of finding complementary therapeutics to insulin that may be beneficial in preventing diabetic complications.

At this point, it is imperative to understand the mechanism of the increase in ATP release by RBCS when treated by leptin, zinc, and C-peptide. ATP is produced via glycolysis and glucose needs to be transported into the cells in order for glycolysis to occur. Glucose is transported via glucose transporters, and therefore, it seems worthy to investigate the changes in the glucose transporters on the RBC in the presence of zinc, C-peptide, and leptin.

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Chapter 3- Elevated Level of RBC-Derived ATP by Leptin can be Correlated to Increase in GLUT-1 Translocation

3.1 Introduction

The major source of energy for all eukaryotes is glucose. In humans, all cells use glucose for their energy needs, and breakdown of endogenous glycogen stores (mainly in the liver) provide this energy. The energy stores are refueled via the glucose in the diet, which is distributed across the body after being digested and absorbed through the gut wall.[1] Glucose can also mediate gene transcription, glucoregulatory neuron activity, enzyme activity, and hormone secretion. The effect of glucose on gene expression is mediated by the transcription factor carbohydrate-response element-binding protein (ChREBP).[2] The activity of transcription factors is regulated via glucose flux in the glucosamine pathway by promoting O-GlcNAcylation.[3] An important regulator of gene transcription has been identified as Sirt1 deacetylase, which is modulated by NAD^+ production through glycolysis.[4] In addition, gene expression is controlled by glucose metabolism via the production of acetyl-CoA that induces histone modifications.[5]

Glucose is the major stimulus for insulin production in pancreatic β-cells, and changes in glucose concentration activates/inhibits groups of glucose-sensitive neurons in the brain. These neurons modulate food intake, energy expenditure, and glucose homeostasis.[6] Glucose uptake by tissues is the first step that should occur in order for glucose to perform glucoregulatory functions and is controlled by the level of glucose transporter translocation at the cell surface. Multiple glucose transporter isoforms exist with various kinetic properties and cell surface expression levels leading to regulation of glucose uptake, metabolism, and signal generation to preserve the metabolic integrity of cells in the body.

3.1.1 Glucose Transporters

In 1948, LeFever was the first scientist who proposed that a specific component in the cellular membrane is required for glucose transport across the cell membrane when studying glucose uptake into human red blood cells (RBCs).[7] It was not reported until the 1970s that glucose transport across the plasma membrane of RBCs was mediated via membrane embedded proteins,[8-9] even though Widdas postulated a mobile carrier mechanism to explain the kinetics of glucose transport in sheep placenta.[10] In 1985, Complimentary DNA (cDNA) encoding glucose transporter protein on the RBCs was cloned[11] and 13 other members of the SLC2A protein family have been identified as glucose transporters (GLUT) in humans since then (GLUT-1-14). SLC2-facilitator glucose transporter families are shown in table 3.1.[12] The role of these glucose transporters in maintaining glucose homeostasis is depicted in Figure 3.1.[13] The GLUT protein family is a member of the Major Facilitator Superfamily (MFS) of membrane transporters and are composed of ~ 500 amino acids. The structure, shown in figure 3.2, is predicted to have 12 transmembrane α-helices and one N-linked oligosaccharide. The GLUT family members have been classified into three subclasses based on sequence homology and structural similarities: class I (GLUTs-1-4) are glucose transporters, class II (GLUTs-5, 7, 9, and 11) are fructose transporters, and class III (GLUT-6, 8, 10, 12) are structurally members of the GLUT family but poorly understood.[14]

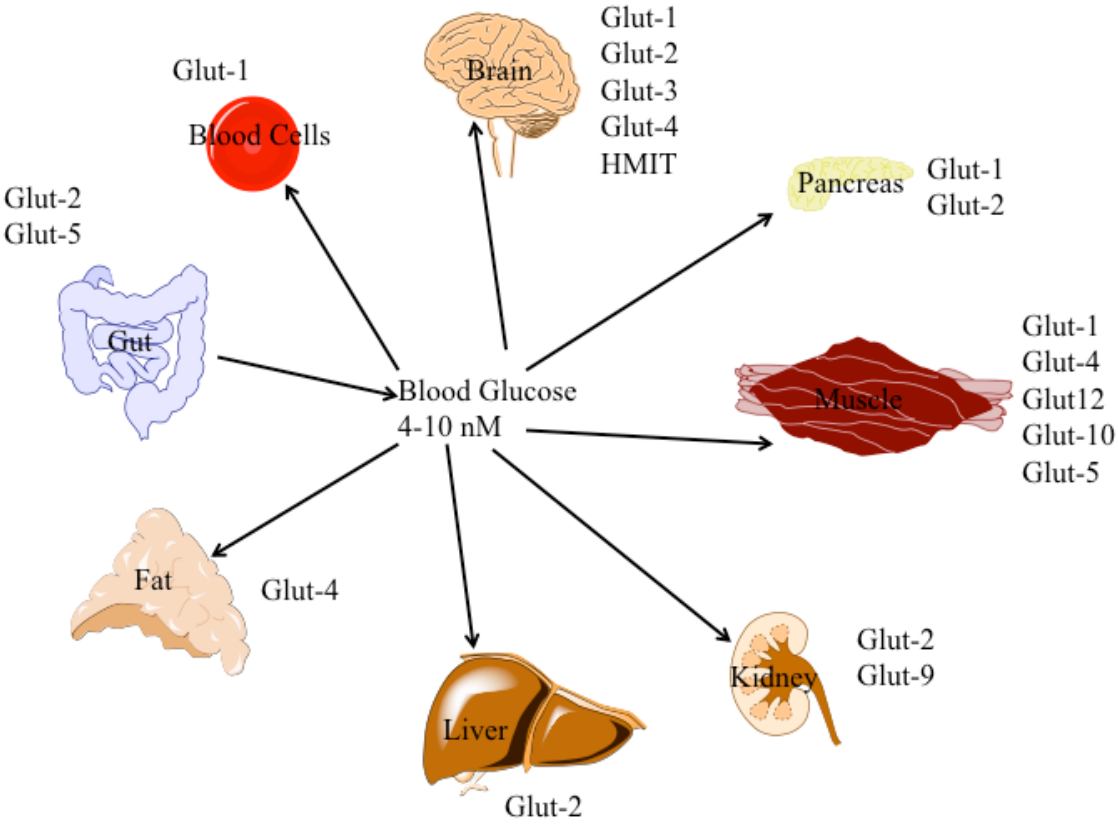

Figure 3.1. Role of GLUT Proteins in Glucose Hemostasis.

Table 3.1. SLC2 – Facilitative GLUT transporter family.

Human gene name	Protein name	Predominant substrates	Tissue/cellulat distribution	Link to disease
SLC2A1	GLUT-1	glucose, galactose, mannose, glucosamine	RBCs, brain, blood-brain barrier, blood-tissue barrier	paroxysmal exertion-induced dyskinesia, dystonia-18, Glut1 deficiency syndrome
SLC2A2	GLUT-2	glucose, galactose, fructose, mannose, glucosamine	liver, islet of Langerhans, intestine, kidney, brain	Fanconi-Bickel syndrome, (type 2 diabetes)
SLC2A3	GLUT-3	glucose, galactose, mannose, xylose	brain (neurons), testis	
SLC2A4	GLUT-4	glucose, glucosamine	adipose tissue (white and brown), skeletal and cardiac muscle	T2D
SLC2A5	GLUT-5	fructose	small intestine, kidney	
SLC2A6	GLUT-6	glucose	brain, spleen, leucocytes	
SLC2A7	GLUT-7	glucose, fructose	small intestine, colon, testis, prostate	
SLC2A8	GLUT-8	glucose, fructose, galactose	testis, brain, adrenal gland, liver, spleen, brown adipose tissue, lung	
SLC2A9	GLUT-9	urate (glucose, fructose)	kidney, liver, small intestine, placenta, lung and leucocytes	renal hypouricemia
SLC2A10	GLUT-10	glucose, galactose	heart, lung, brain, liver, skeletal muscle, pancreas, placenta and kidney	arterial tortuosity syndrome
SLC2A11	GLUT-11	glucose, fructose	heart, muscle	
SLC2A12	GLUT-12	glucose	heart, prostate, skeletal muscle, placenta	
SLC2A13	HMIT	*myo*-inositol	brain, adipose tissue	
SLC2A14	GLUT-14		testis	

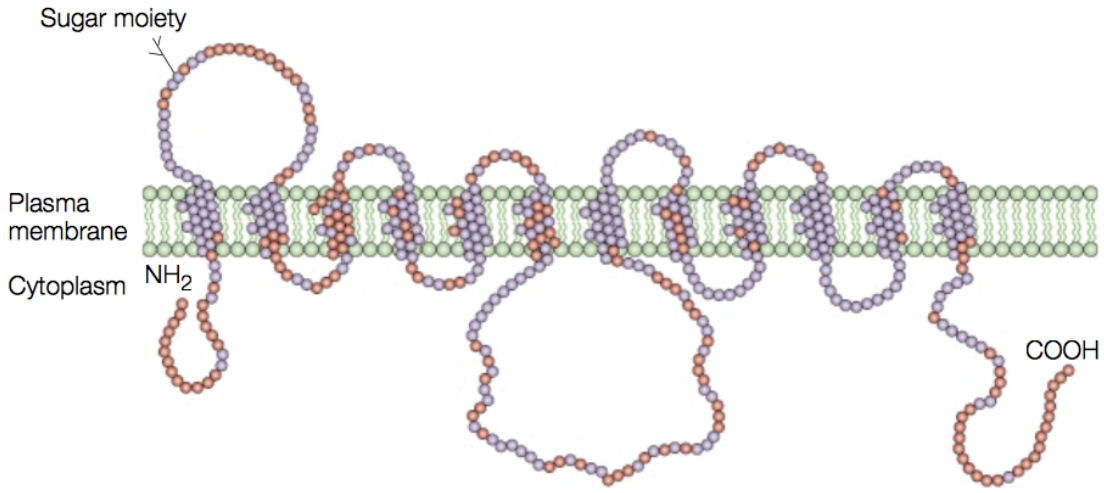

Figure 3.2. Schematic Demonstration of GLUT Proteins Structure. GLUT proteins are comprised of 12 transmembrane helices with both amino and carboxyl termini in cytosol.

GLUT-1, GLUT-2, and GLUT-4 will be discussed in more details due to the relevance to this dissertation.

3.1.2 GLUT-1

GLUT-1 is mainly responsible for transporting glucose, but it is also able to transport mannose, galactose, and glucosamine.[15] GLUT-1 is usually expressed along with another isoform of the GLUT family and is highly expressed in the membrane of RBCs, as it is responsible for the transport of glucose between plasma and the RBC's cytoplasm. In addition, GLUT-1 is the main glucose transporter expressed in brain endothelial cells and is involved in cerebral glucose uptake.[16-18]

GLUT-1 is encoded by the SLC2A1 gene and is one of the most extensively studied transport proteins and it was the first transport protein to be purified[9, 19] and cloned.[20] The mechanism of glucose transport has been studied by many scientists since the early 1950s, when radioiostopic substrates became available. This lead to several contradictory mechanisms being proposed, including alternating conformation mechanisms for glucose

transport, and fixed sides model in which multiple binding site are available on both sides of the membrane.[15, 21-25]

3.1.3 GLUT-2

GLUT-2 is expressed highly in pancreatic β-cells, in the basolateral membrane of intestinal cells, and in kidney epithelial cells and has a high k_m for glucose,[26] ensuring fast equilibrium for glucose between extracellular space and the cell cytosol at physiological glycemic levels. Altering the surface expression of GLUT-2 does not have metabolic regulatory effects because the rate-limiting step for glucose metabolism is glucose phosphorylation. However, the expression of GLUT-2 is sufficiently reduced in diabetic conditions, which limits glucose access to hexokinase.[27] Rises in glucose levels stimulates insulin secretion in pancreatic β-cells and inhibition/absence of GLUT-2 results in abolished glucose stimulated insulin secretion.[6]

It has been reported that transgenic mice that express fusion proteins, including EGFP with a cytoplasmic loop of GLUT-2 in the nuclease alter the liver, β-cells, and renal function.[28] However, the correlation between this model and the function of intact GLUT-2 in the plasma membrane is not clear. A study has been reported that a relationship exists between the preference for foods containing sugar and a mutation in GLUT-2, suggesting an association between GLUT-2 and glucose sensitive cells in the brain and periphery.[29]

3.1.4 GLUT-4

GLUT-4 was discovered as a distinct glucose transporter in the 1980s and more than 1800 publications are associated with the keyword GLUT-4,[30] suggesting the high importance of GLUT-4 in glucose hemostasis. GLUT-4 regulates glucose uptake via the complex mechanism mediated by insulin and the disruption of this regulation leads to diseases such as T2D and obesity. Pioneering scientists reported the insulin-mediated GLUT-4 translocation from intracellular space to the cell surface in muscle and fat cells in the early 1980s[31-33] leading to continued studies on this regulation for the next three decades.[34] Acute and chronic regulation of GLUT-4 in muscle cells, as the main disposal site for glucose needs to be fully understood.[35]

Insulin is involved in the regulation of glucose homeostasis at different parts of the body including attenuating gluconeogenesis and the break-down of glycogen to reduce hepatic glucose output and increasing glucose uptake into muscle and fat cells via facilitated diffusion.[36] A heterotetrameric transmembrane protein located on the membrane of fat and muscle cells serves as the insulin receptor and is comprised of two α-subunits as insulin binding domains and two β-subunits as signal transduction domains. α-subunits go through conformational changes once insulin is bound leading to the binding of ATP to intracellular domain of β-subunits, activating receptor autophosphorylation.[37-38] Next, insulin receptor substrates (IRS) 1 and 2, along with other protein substrates, are phosphorylated[36, 39-41] activating phosphatidylinositol 3-kinase (PI3K) resulting in activation of several kinases including PI-dependent protein kinase- 1 and -2,[42] Akt,[43] protein kinase C (PKC),[44] and wortmannin-sensitive and insulin-stimulated serine kinases.[45] Finally, glucose uptake occurs by translocation of an

intracellular pool of GLUT-4 to the cell membrane[31-32] and glucose is stored in the form of glycogen. The insulin signal transduction pathway is depicted in Figure 3.3.

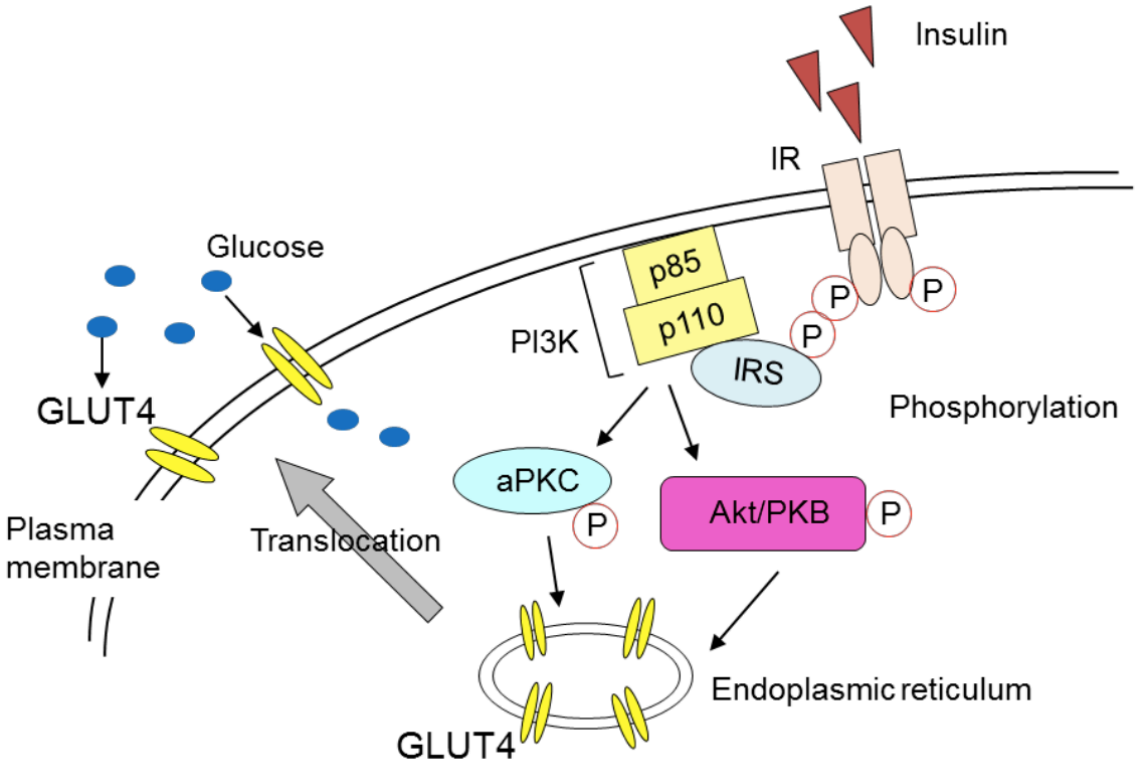

Figure 3.3. Insulin-Mediated Translocation of GLUT-4 and Insulin Signal Transduction Pathway. IR: insulin receptor, IRS: insulin receptor substrate, PI3K: phosphatidylinositol 3-kinase, PKB: protein kinase B, PKC: protein kinase C.

It is known that GLUT-4 translocation is mediated by insulin and exercise[1] but no known stimulus has been reported for GLUT-1 translocation of RBCs. In a collaboration with Tiffany Janes, we will show that the combination of zinc, C-peptide, and albumin can potentially be a stimulus for GLUT-1 translocation to the surface of RBCs. To test this hypothesis further, the trio has been used on T1D RBCs and the same results were observed. In addition, leptin may be involved in the regulation of blood glucose level by facilitating glucose transport into RBCs. Therefore, GLUT-1 translocation of RBCs is

monitored in the presence and absence of leptin to examine the role of leptin in glucose transport and potentially downstream energy homeostasis.

3.2 Experimental Methods

3.2.1 Preparation of RBCs in Regular and Albumin-free Physiological Salt Solution (PSS)

Fresh RBCs were collected and purified on the day of the experiment in PSS or an albumin–free form as described in chapter 2. RBC hematocrit was measured immediately after isolation from whole blood and the amount of RBCs required to make a 7% RBC solution in a total of 1 mL sample was calculated.

3.2.2 Sample Preparation at Varying Concentrations of C-peptide and Zinc for ATP Measurement and Monitoring GLUT-1 Translocation

Three sets of samples were prepared in PSS and one set was prepared in albumin-free PSS. The first set contained varying concentrations of C-peptide ranging from 0 to 50 nM. An 800 nM C-peptide stock solution was used and the appropriate amount was added to the vials to make final C-peptide concentrations of 0, 2.5, 5, 10, 20, and 50 nM in 1 mL. Then, sufficient amount of PSS were added to the sample tubes followed immediately by the addition of packed RBCs to make the total volume of 1 mL at a hematocrit of 7%; these samples were then incubated at 37°C for 2 hours. The second set of samples contained varying concentrations of zinc in PSS. An 800 nM stock solution of zinc was prepared and used to make working solutions with concentrations of 0, 2.5, 5, 10, 20, and 50 nM. Appropriate amounts of zinc and C-peptide were added to vials and incubated for 3 mins followed by the addition of sufficient amount of PSS and packed RBCs to the sample tubes to make the final hematocrit of the samples 7% in a total volume of 1 mL. The samples were then incubated at 37°C for 2 hours. The third set of

samples contained both zinc and C-peptide in the concentrations mentioned above. Then, one set of samples was prepared in albumin free PSS containing varying concentrations of zinc and C-peptide in the range mentioned above in the preparation of the third set of samples.

3.2.3 Preparation of T1D RBCs in Regular and Albumin Free PSS for ATP Release Measurements and Probing GLUT-1 Translocation

T1D Blood was collected from consenting patients on the day of the experiment and RBCs were isolated as described in chapter 2. Zinc only, C-peptide only, and zinc and C-peptide (20 nM each) were added to the vials and incubated for 3 mins. Then, sufficient amount of PSS were added to the sample tubes followed immediately by the addition of packed T1D RBCs to make the total volume of 1 mL at a hematocrit of 7%. Samples containing zinc and C-peptide (20 nM each) were prepared using T1D RBCs in albumin free PSS as explained. Healthy RBCs were also collected as control and zinc and C-peptide treated RBC samples were prepared in regular and albumin free PSS.

3.2.4 Preparation of Samples in Regular and Albumin-free PSS for Leptin Treated RBCs

Sample preparation was performed as described in section 2.2.2 in the corresponding PSS or albumin-free PSS. Healthy and T1D RBCs, and 7% whole blood were used for this study.

For the T1D study, two sets of healthy RBC and two sets of T1D RBC samples were prepared containing leptin, leptin and zinc, leptin and C-peptide, leptin and zinc and C-peptide in regular and albumin-free buffer. For the 7% whole blood study, the hematocrit was measured right after collecting the blood and the calculations performed to dilute it to 7% hematocrit in PSS.

3.2.5 Quantitative Determination of ATP Release from RBCs

The method to measure and quantify ATP was described in section 2.2.7.

3.2.6 Preparation of Ghost Samples for GLUT-1 Translocation Studies

Samples prepared in section 3.2.2 and 3.2.3, and 3.2.4 were centrifuged at 500*g* for 10 minutes. Then, the supernatant was removed and the sample vials were filled with lysis buffer (10 mM Tris HCl and 0.2 mM EDTA (J.T. Baker, Center Valley, PA) at a pH of 7.2). Next, samples were incubated at 4 °C for 30 mins. The samples were then centrifuged at 22000*g* at 4 °C for 15 mins. The supernatant was removed and the vials were again filled with lysis buffer. Samples were centrifuged at 22000*g* at 4 °C for 5 mins. Supernatant was removed and the last step was repeated two more times, or until the supernatant was clear and the pellet was white. The pellets were then stored at -20 °C.

3.2.7 10% Acrylamide Gel Preparation and SDS-PAGE Procedure

Glass spacers and plates were cleaned prior to use, as failure to do so will result in uneven gel preparation and leaking of gel before polymerization. The glass spacers were cleaned with 95% ethanol and wiped clean, then rinsed with distilled water and wiped clean again. A 10% acrylamide resolving gel was prepared by adding 5 mL of 1.5 M tris buffer at pH 8.8, 4 ml of 30% acrylamide (Bio-Rad, Hercules, CA), 5.9 ml of DDW, 75 µl of 20% sodium dodecyl sulphate (SDS) (Sigma Aldrich, St. Louis, MO), 75 µl of 10% Adenosine 5'-phosphosulfate (APS) (Sigma Aldrich, St), and 25 µl of TEMED (Bio-Rad). The cast is filled with the resolving gel up to 2 cm left to the top with resolving gel immediately after preparation. Isopropanol was added to the surface of the resolving gel to make the surface even. While the resolving gel polymerizes on the bench for 20-30 minutes, a 5% acrylamide stacking gel was prepared. To prepare the stacking gel, 0.62 ml

of 0.5 tris pH 6.8, 0.83 ml of 30% acrylamide, 3.8 ml of DDW, 25 µl of 20% SDS, 50 µl of 10% APS, and 5 µl TEMED are mixed in a tube and added on top of the resolving gel after discarding the IPA. The combs are inserted into the cast immediately after pouring the stacking gel, which polymerizes in 15-20 minutes. Next, sample buffer was prepared by adding 50 µl of β-mercaptoethanol (Sigma Aldrich) to 950 µl of commercially available sample buffer. Next, 5 µl of the ghost samples were added to 100 µl of the sample buffer (Bio-Rad, Hercules, CA) and incubated at room temperature for 15-20 minutes. Once the stacking gel was polymerized, the comb was removed and 3 µl of the samples were loaded into the well. 1X running buffer was prepared by a 1:10 dilution using a 10X running buffer (144 g glycine, 30.2 g tris, 10 g SDS in 1L) and was added to the tank. The gel was run at 100-120 V for 1-1.5 hour.

3.2.8 Buffer Preparation for Western Blot Analysis

10X transfer buffer was prepared by adding 3.028 g tris, 14.4 g glycine, and 200 ml methanol in total volume of 1L of DDW at pH 8.3 and 1X transfer buffer was prepared by mixing 100 ml of 10X transfer buffer and 200 ml of methanol and diluting to a total volume of 1L with DDW. Tris buffered saline (TBS) was made by adding 2.43 g of tris-HCl, 29.22 g of NaCl to the total volume of 1L of double distilled water at pH 7.5. TBST was a mixture of 0.05% of tween 20 (Sigma Aldrich, St. Louis, MO) in TBS (250 µl tween 20 in 500 ml of TBS). Last, bicarbonate buffer was prepared by adding 4.12 g of $NaHCO_3$ and 0.237 g of $MgCl_2$ in total volume of 500 ml of double distilled water.

3.2.9 Western Blot Analysis

Once the proteins were separated on the gel, all proteins were transferred to a Polyvinylidene difluoride (PVDF) transfer membrane (EMD Millipore, Burlington, MA) at 15 V overnight for further western blot analysis.

Prior to the addition of antibodies to the membrane, the membrane was blocked for 1 hour in 5% dry milk in Tris-buffered saline. After washing, primary antibodies for GLUT-1 (Abcam, Cambridge, MA) and spectrin (Novus Biological, Littleton, CO) (diluted 1:1000), were added to the PVDF membrane and allowed to incubate for 1 hour at room temperature. Once rinsed, secondary antibodies (Sigma Aldrich, St. Louis, MO) (diluted 1:3000) were added and allowed to incubate for one hour. Sequential incubation with nitro-blue tetrazolium chloride (NBT) (Sigma Aldrich, St. Louis, MO) and 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (BCIP) (Sigma Aldrich, St. Louis, MO) produced a colorimetric indication of the GLUT1 and spectrin present, and the data was analyzed by scanning the membrane to a computer and utilizing ImageJ software to quantify band thickness and intensity. GLUT1 band thickness is normalized to spectrin band thickness, and all samples are normalized to the control samples.

3.3 Results

3.3.1 RBC-Derived ATP Release at Varying Concentrations of Zinc and C-peptide in the Presence and Absence of Albumin

C-peptide and zinc in the presence of albumin stimulates RBCs to release ATP. As C-peptide and zinc were added to RBCs (along the x-axis), the ATP release statistically stays at the same level, except when the trio of zinc, C-peptide, and albumin are present together. Closed triangles in Figure 3.4 depict samples containing varying concentration of zinc and C-peptide in albumin-free PSS. Increasing the concentration of zinc and C-peptide does not increase RBC-derived ATP compare to the control RBCs. in albumin free PSS. Open triangles and closed circles show samples containing zinc only and C-peptide only, respectively, in PSS. Changing the concentration of zinc or C-peptide does not affect the ATP release from RBCs. Open circles show samples containing zinc and C-peptide in PSS. The ATP release increases from 42 ± 1.3 nM to about 76 ± 1.5 nM in a saturable dose dependent response before the release begins to level off. This project was in collaboration with Tiffany Janes and GLUT-1 experiments were performed by her. The results are shown in this chapter for clarity purposes.

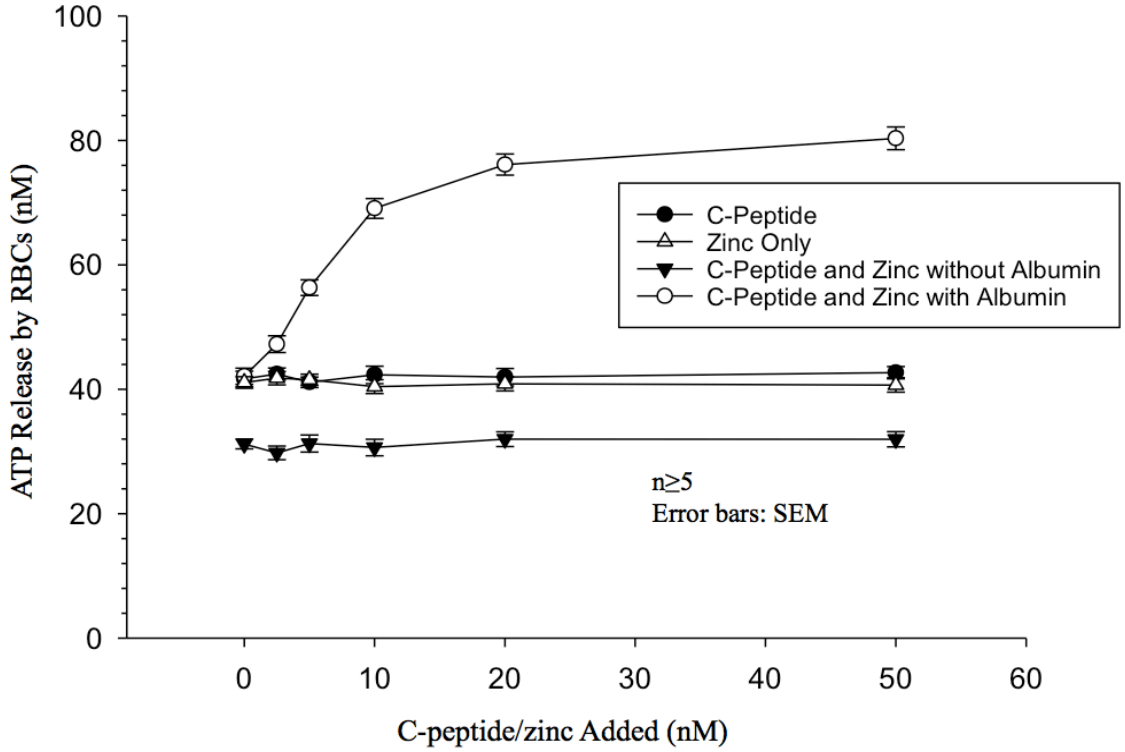

Figure 3.4. Changes in RBC-Derived ATP at Varying Concentrations of Zinc/C-peptide in Regular and Albumin Free PSS. Closed circles show samples containing C-peptide in PSS. Open triangles contain zinc in PSS. The closed triangles show samples containing varying concentrations of zinc and C-peptide in albumin-free PSS, while the open circles show samples containing zinc and C-peptide in PSS. No change in ATP release by RBCs is observed unless zinc, C-peptide, and albumin are present.

3.3.2 RBCs GLUT-1 Translocation Changes at Varying Concentrations of Zinc and C-peptide in the Presence and Absence of Albumin

Glucose is transported into RBCs via the glucose transporter, GLUT-1. The results in Figure 3.5 reveal that C-peptide and zinc alone, or when they are used together, do not statistically change the RBC GLUT-1 levels. When albumin is present with increasing concentrations of C-peptide and zinc, the GLUT-1 translocation increases in a saturable and dose-dependent manner (25.5%) compared to the control. This experiment was performed by Tiffany Janes.

3.3.3 T1D and Healthy RBC-Derived ATP in the Presence and Absence of Albumin

Figure 3.6 depicts the amount of ATP released by healthy and T1D RBCs in the presence and absence of albumin. A significant increase is observed when 20 nM zinc and C-peptide are added to healthy and T1D RBCs (51.7 ± 3.3 nM, $p< 0.05$ and 34.5 ± 2.9 nM, $p<0.05$ respectively) compared to the control healthy and T1D RBCs (34.2 ± 3.7 nM and 24.9 ± 2.8 nM respectively) in PSS. No significant increase in ATP release was observed in zinc and C-peptide-treated healthy and T1D samples (25.6 ± 3.1 nM and 17.3 ± 2.4 nM, respectively) compared to the control healthy and T1D cells (22.2 ± 1.8 nM and 16.0 ± 1.4 nM respectively) in albumin-free PSS as expected. It is worth noting that basal ATP release by T1D cells is lower than healthy cells in both buffers.

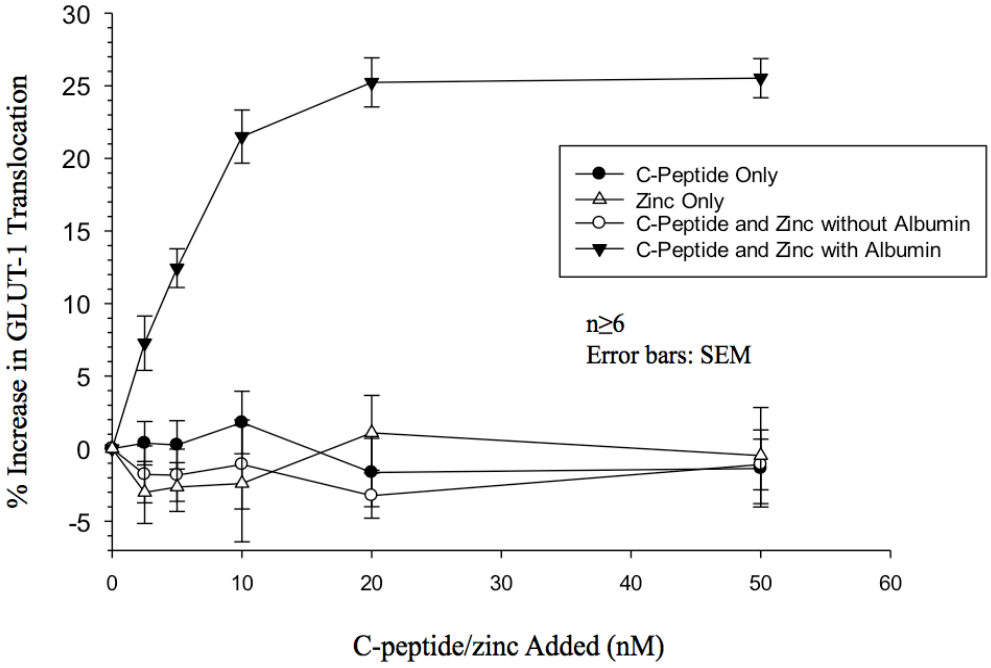

Figure 3.5. Monitoring GLUT-1 Translocation at Varying Concentrations of Zinc/C-peptide in Regular and Albumin Free PSS. Closed circles show samples containing C-peptide in PSS. Open triangles show samples containing zinc in PSS. Open circles show samples containing varying concentrations of zinc and C-peptide in albumin free PSS while closed triangles show samples containing varying concentration of zinc and C-peptide in regular PSS. No change in the RBC membrane GLUT-1 is observed unless zinc, C-peptide, albumin trio is present.

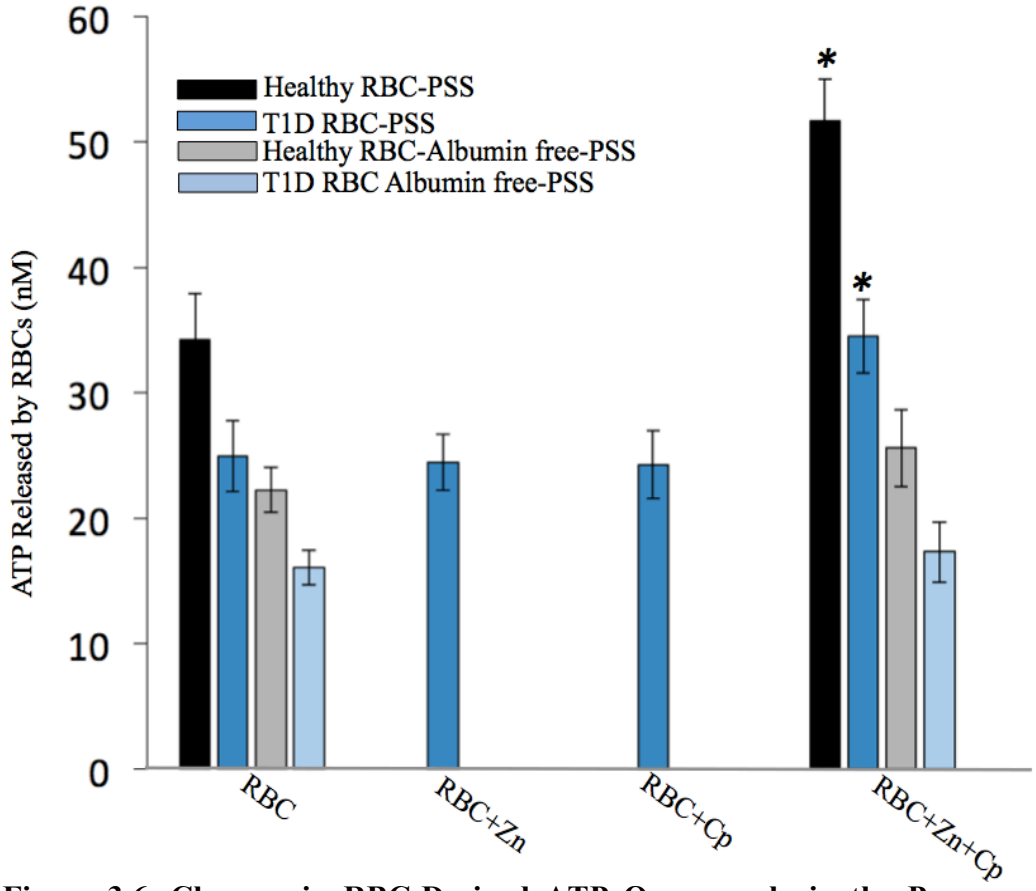

Figure 3.6. Changes in RBC-Derived ATP Occurs only in the Presence of Zinc, C-peptide, and Albumin. Black bars show healthy RBCs in regular PSS and dark blue bars show T1D RBCs in regular PSS. Grey bars show healthy RBC in albumin free PSS and light blue bars show T1D samples in albumin free PSS. As demonstrated here ATP release by both types of RBCs increased when zinc, C-peptide, and albumin are present. [Zn]= 20 nM, [Cp] = 20 nM, 7% RBCs, error bars: SEM, $n \geq 7$, $p < 0.005$.

3.3.4 Healthy Versus T1D RBC GLUT-1 Translocation

Figure 3.7a and 3.7b depict GLUT-1 translocation in the presence and absence of albumin respectively. All samples are normalized to control T1D RBCs in albumin containing buffer. Results show the basal GLUT1 content of T1D RBCs is 23.6% ± 4.2% lower than basal control levels, however, when T1D RBCs are combined with the complex of C-peptide, zinc, and albumin, the GLUT1 content increases to statistically the same value as untreated control RBCs. This increase in GLUT1 content is not observed when any component of the complex is absent.

3.3.5 Leptin Further Increases GLUT-1 Translocation to the Membrane of RBCs

Results in Figure 3.8 show that zinc and C-peptide increased GLUT-1 translocation (31.5% ± 6.1%, $p<0.05$) in PSS. However, there is no increase in the absence of albumin. Leptin increased GLUT-1 translocation by 52.5% ± 5.6%, $p<0.05$ in PSS. Interestingly, leptin also increased GLUT-1 translocation (32.0% ± 2.9%, $p<0.05$) in BSA-free PSS, and the increase was statically the same as the increase observed by zinc and C-peptide in PSS.

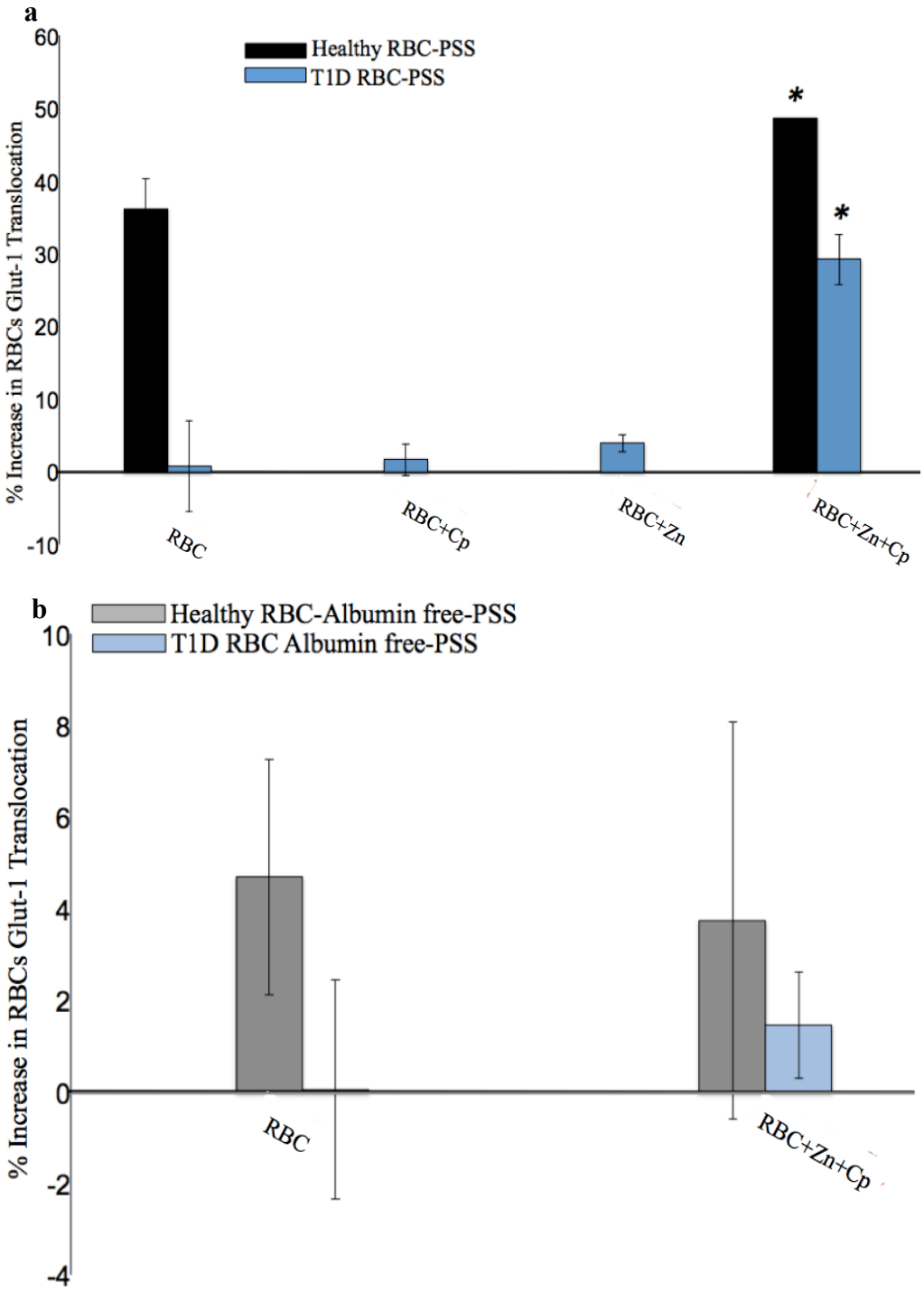

Figure 3.7. GLUT-1 Translocation in Healthy and T1D RBCs in the Presence and Absence of Albumin. (a) healthy and T1D samples in PSS treated with zinc and C-peptide showed an increased in GLUT-1 translocation in both types of RBCs. [Zn]= 20 nM, [Cp] = 20 nM, 7% RBCs, error bars: SEM, n≥6, p< 0.005. In (b) samples in albumin-free PSS resulted in no increase in GLUT-1 translocation. [Zn]= 20 nM, [Cp] = 20 nM, 7% RBCs, error bars: SEM, n≥7.

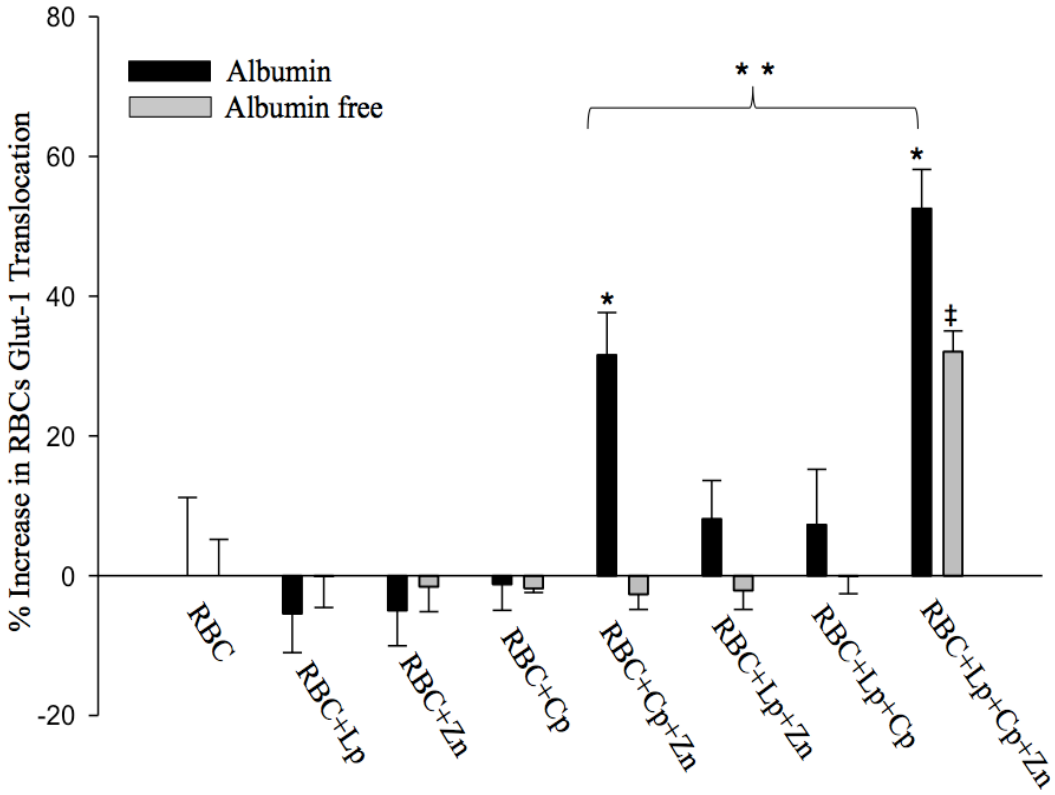

Figure 3.8. GLUT-1 Translocation to the Membrane was Enhanced in the Presence of Zinc, C-peptide, and Leptin in Regular and Albumin free PSS. Black bars show samples in regular PSS and grey bars show samples in albumin free PSS. Zinc and C-peptide treated RBCs showed elevated levels of GLUT-1 translocation in the presence of albumin and no effect was observed when albumin was absent. However, samples containing zinc, C-peptide, and leptin were able to enhance GLUT-1 translocation in the presence and absence of albumin. [Zn]= 20 nM, [Cp] = 20 nM, [Leptin] = 3.8 nM, 7% RBCs, error bars: SEM, n=6, $p < 0.005$.

3.3.6 GLUT-1 Translocation Monitoring in 7% Whole Blood in the Presence of Leptin

Samples containing whole blood at 7% hematocrit were used for this study and the results in Figure 3.9 show the percent increase in GLUT-1 translocation of RBC membranes. Any combination of zinc, C-peptide, or leptin did not show an effect on GLUT-1 translocation unless zinc and C-peptide were present or leptin, zinc, and C-peptide were present in PSS. A 7% whole blood sample incubated with zinc and C-peptide in the presence of albumin significantly increased GLUT-1 translocation by 27.9% ± 2.8%($p<0.05$). GLUT-1 translocation further significantly increased to 67.3% ± 4.9%, $p<0.05$ when leptin was added to the above combination,.

3.3.7 Monitoring GLUT-1 Translocation in T1D Versus Healthy RBCs in Regular and Albumin-Free PSS

All samples are normalized to control T1D samples as depicted in Figure 3.10. The basal level of T1D GLUT-1 translocation in PSS and albumin-free PSS is lower than healthy RBCs, which closely associated the ATP study performed previously in this chapter. Figure 3.10a shows that leptin, zinc, and C-peptide treated healthy RBCs in regular PSS doubled the GLUT-1 translocation (109% ± 12.4%, $p<0.05$) while T1D RBCs treated the same way also significantly enhanced (albeit to a lesser extent) GLUT-1 translocation (48.9% ± 8.5%, $p<0.05$). Figure 3.10b shows that leptin, zinc, and C-peptide treated healthy and T1D RBCs increases GLUT-1 translocation to 68.8% ± 9.6%, $p<0.05$ and 51.4% ± 9.6%, $p<0.05$, respectively, in albumin-free buffer. It is worth noting that basal levels of GLUT-1 of healthy RBCs in regular PSS was 38.8% ± 10.2% when all the samples are normalized to T1D control cells. The basal GLUT-1 translocation level in healthy RBCs in albumin free PSS was by 23.2% ± 2.9%.

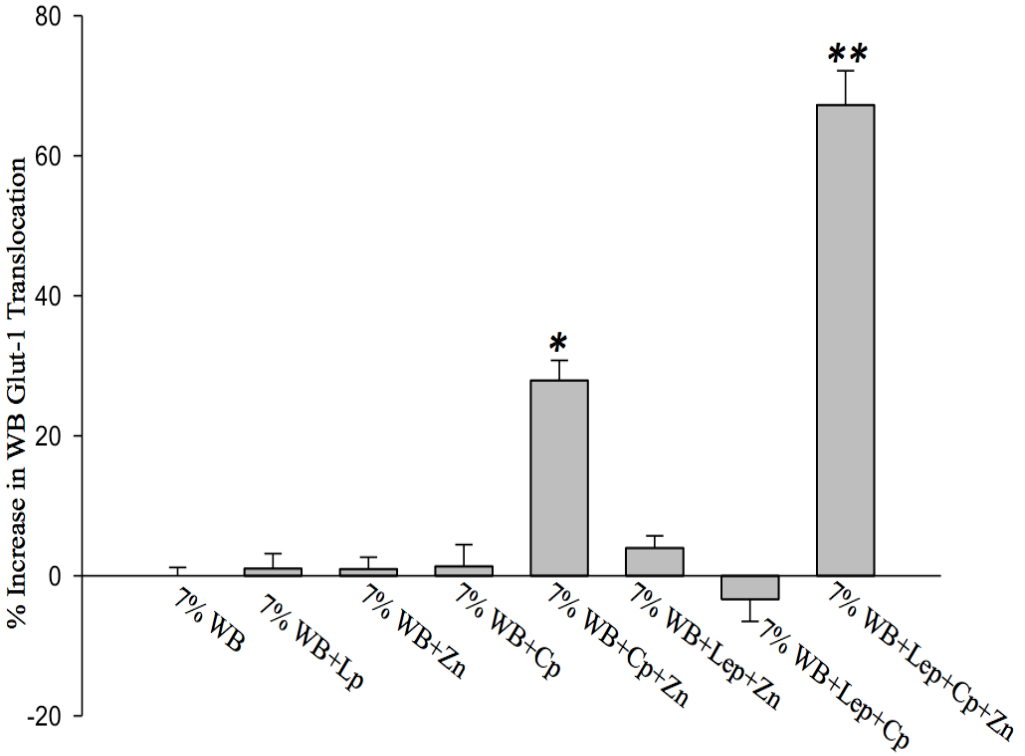

Figure 3.9. The Trend Stayed Consistent for GLUT-1 Translocation Between 7% Whole Blood (WB). Samples are prepared in regular PSS and zinc and C-peptide treated samples increased GLUT-1 translocation. Also, leptin, zinc, and C-peptide enhanced GLUT-1 translocation further more. This trend was observed monitoring GLUT-1 translocation using 7% WB. [Zn]= 20 nM, [Cp] = 20 nM, [Leptin] = 3.8 nM, 7% WB, error bars: SEM, n=6, $p < 0.005$.

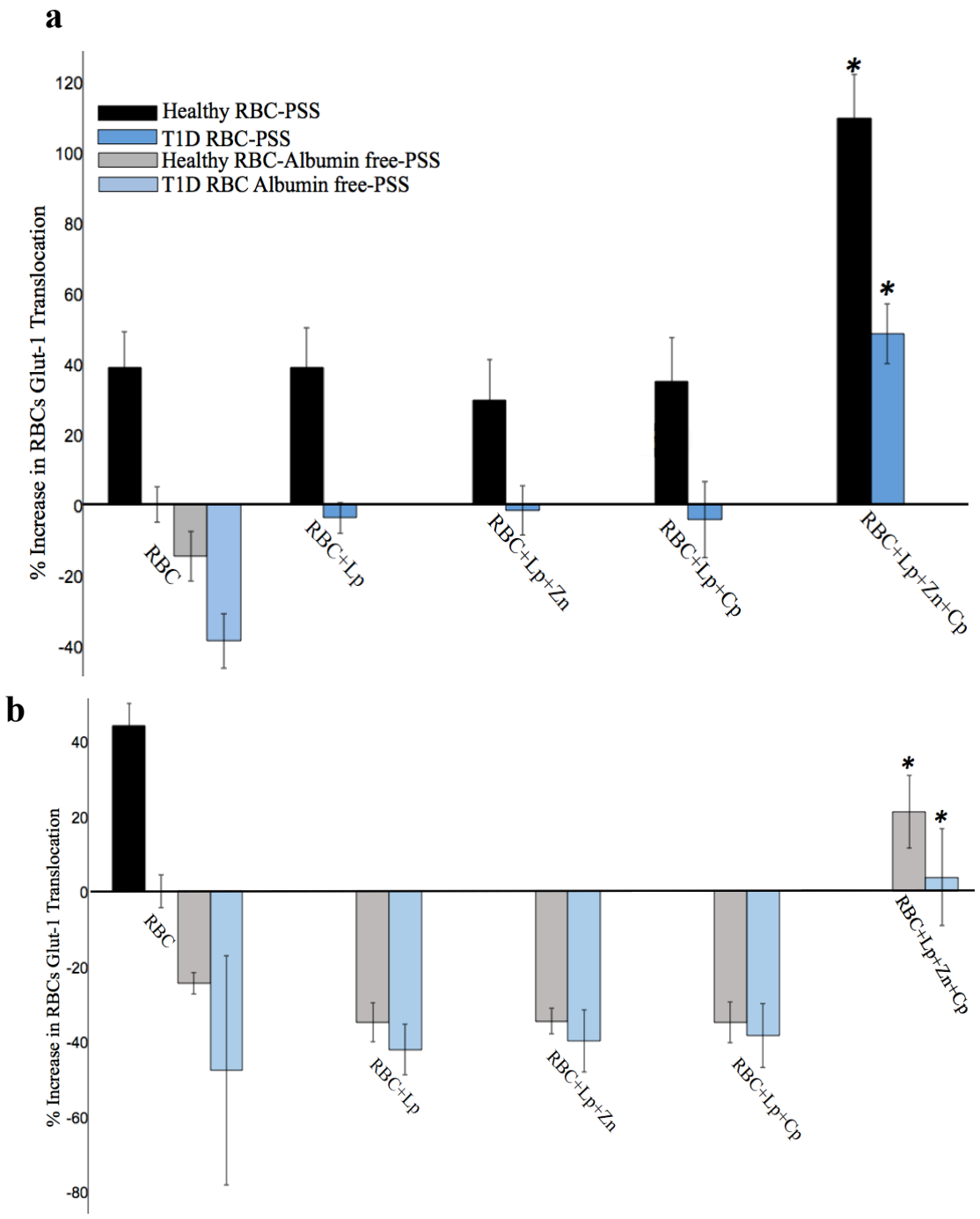

Figure 3.10. Leptin Enhances T1D and Healthy RBC Membrane GLUT-1 in Regular and Albumin Free PSS. Top figure (a) shows samples in regular PSS and black bars show healthy RBCs, dark blue bars show T1D samples. [Zn]= 20 nM, [Cp] = 20 nM, [Leptin] = 3.8 nM, 7% RBCs, error bars: SEM, n=6, $p < 0.005$. Bottom figure (b) shows samples in albumin free PSS and grey bars show healthy RBCs and light blue bars show T1D cells. [Zn]= 20 nM, [Cp] = 20 nM, [Leptin] = 3.8 nM, 7% RBCs, error bars: SEM, n=6, $p < 0.005$. Basal level of GLUT-1 content in T1D RBCs is lower than healthy RBCs in both buffers. Samples treated with leptin, zinc, and C-peptide are capable to enhance GLUT-1 translocation in both types of RBCs and in the presence and absence of albumin.

3.4 Discussion

Insulin therapy along with healthy diet and exercise has increased life expectancy in patients with diabetes. A study has reported that the average T1D patient's lifespan is shorter than the average healthy individuals by thirteen years, but there has been a fourteen-year increase in life expectancy since 1975.[46] However, diabetic patients stiff develop severe complications , motivating a search to find complementary therapeutics to prevent or delay these complications.

Our group has studied the effects of C-peptide on vessel dilation vigorously using static and flow based experiments. In a study performed in this chapter, the effect of C-peptide on T1D RBCs was investigated and compared to healthy RBCs. The first experiment performed was measurement of ATP released by treated RBCs in a C-peptide/zinc dose dependent manner in the presence or absence of albumin. Increasing the concentration of zinc and C-peptide increased ATP release by RBCs until the cells get saturated, but only in the presence of albumin. Taking one of the elements of this trio out of the samples resulted in no increase in ATP release. This experiment confirms the previous results in the Spence group. ATP is mainly generated via glycolysis in RBCs and more ATP release suggests more glycolysis occurring in RBCs. More glucose needs to be transported into RBCs in order to have higher rate of glycolysis, and glucose is being transported into RBCs via GLUT-1. Therefore, probing the translocation of GLUT-1 to the membrane of RBCs should be coupled with ATP release studies to be able to explain the reason behind increased ATP release by RBCs in the presence of zinc, C-peptide, and albumin.

Translocation of GLUT-1 was monitored at varying concentrations of zinc and C-peptide in the presence and absence of albumin by Tiffany Janes. Interestingly, the GLUT1 results confirmed the ATP release experiment results and the presence of zinc, C-peptide, and albumin was necessary in order to see any change in GLUT-1 translocation to the membrane when compared to the control samples. The experiment was performed using zinc only, C-peptide only, or in albumin free buffer and no effect was observed.

ATP release by T1D and healthy RBCs was monitored in the presence and absence of albumin. T1D cells basal ATP level was lower compared to the healthy cells in both buffers. Zinc and C-peptide treated RBCs showed an increase in ATP release in the presence of albumin but no increase was observed in albumin free buffer as expected. Zinc treated or C-peptide treated T1D RBCs showed no increase in ATP release. A similar experiment was performed by Tiffany Janes probing GLUT-1 translocation and the results confirmed ATP release experiment results. GLUT-1 translocation was enhanced in the presence of zinc, C-peptide, and albumin in both types of RBCs and no increase was observed in the albumin-free buffer.

Coupling GLUT-1 translocation studies with ATP release helps us to understand the mechanism underlying the effect of C-peptide on RBCs. C-peptide along with zinc and albumin may trigger RBCs to consume more glucose when subjected to high-glucose environments by facilitating GLUT-1 translocation, resulting in elevated levels of ATP release. Previous studies have reported that administration of C-peptide results in lower morbidity rates in T1D patients due to neuropathy, nephropathy, and retinopathy.[47-54]

However, currently C-peptide is not being used as a complimentary therapeutics in diabetic patients. A start-up company named Cebix made an effort to use a C-peptide analogue as a therapeutic for diabetic patients, but the peptide treatment failed in phase 2b clinical trials. Two factors that are important to observe reproducible effects from C-peptide are the purity and formulation.[55] The positive and reproducible effects of C-peptide are seen by administrating C-peptide along with zinc and albumin, and also purifying the C-peptide by high-performance liquid chromatography prior to using it. Therefore, it is worth noting that C-peptide will not be functional in the absence of zinc and albumin before considering it as a complimentary therapeutics for diabetes.

C-peptide does not bind to RBCs well in high glucose conditions, as discussed earlier. On the other hand, leptin and insulin have shown similar effects on the control of food intake and energy metabolism. Leptin and insulin have been shown to regulate each other, and both play important roles in the control of glucose homeostasis.[56] We reported that leptin enhances the effect of C-peptide on RBCs by further increasing their ATP release in chapter 2. In addition, the leptin-induced increase in ATP released by RBCs under high glucose conditions, or in T1D RBCs, was statistically the same as the increase observed in healthy RBCs or under normal glucose conditions. In this chapter, the effect of leptin on GLUT-1 translocation was investigated.

Healthy RBCs were treated with zinc, C-peptide, leptin, and combinations of them in the presence and absence of albumin. Zinc and C-peptide treated RBCs enhanced GLUT-1 translocation only in the presence of albumin as expected. However, samples treated with zinc, C-peptide, and leptin enhanced GLUT-1 translation in the presence and

absence of albumin. The increase in GLUT-1 translocation in the samples containing zinc, C-peptide, and leptin in the absence of albumin was statistically the same as the increase observed by zinc and C-peptide in the presence of albumin. This may suggest that leptin can potentially act as the carrier molecule for zinc and C-peptide. Therefore, the amount of C-peptide delivered by leptin only to the cells in the absence of albumin is similar to the amount delivered by albumin only in the absence of leptin. However, the amount of C-peptide delivered to the cells is higher when both leptin and albumin are present.

GLUT-1 translocation monitoring experiment was performed in 7% whole blood and the same trend as in 7% RBCs was observed. This experiment shows that the presence of other proteins and molecules are not interfering with the function of leptin and the increase observed was very similar to the increase observed using 7% RBCs. However, it would be interesting to perform this experiment in albumin free PSS to confirm the hypothesis proposed on the role of leptin as a carrier further.

GLUT-1 translocation in T1D RBCs was also studied in the presence of leptin. T1D RBCs showed lower basal GLUT-1 levels, as expected. All samples are normalized to T1D control RBCs. Healthy RBCs in regular PSS had the highest basal GLUT-1 levels, and samples in albumin-free PSS had lower levels than control T1D in regular PSS by ~ 20%. T1D RBCs in albumin-free PSS has the lowest basal GLUT-1 levels. However, leptin, zinc, and C-peptide treated RBCs showed increase in GLUT-1 translocation in all four conditions and interestingly, leptin restored GLUT-1 levels in T1D samples back to the basal levels observed in healthy control RBCs.

We discussed the studies focusing on glucoregulatory effects of leptin extensively in chapter 1 and we reported that scientists are trying to grasp this effect since the 1950s. One part that is missing in all of these studies is the effect/function of leptin in the blood stream. Leptin is transported to the brain via the blood stream and spends some time there after being secreted. Therefore, it is possible to act on RBCs and help them consume more glucose whenever it is needed. Here, we showed that leptin delivers more C-peptide to RBCs and is effective in the absence of albumin as well. However, the mechanism by which leptin enhances C-peptide cellular effect needs to be elucidated. The effect of leptin in the interaction between RBCs and zinc/C-peptide will be investigated in the next chapter and leptin binding to the cells will also be studied.

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Chapter 4- Mechanistic Studies of the Effect of Leptin on RBCs

4.1 Introduction

Extensive studies on diabetes over the past three decades resulted in improved antidiabetic medications, advanced glycemia monitoring systems, and easier patient to physician access.[2-3] However, diabetic patients are still at a greater risk to develop cardiovascular disease, cancer[5-6], and coronary artery disease.[7-9] The risk of coronary artery disease in patients with T1D is greater than 90% for those 55 years and older.[10-11] Insulin therapy helped T1D patients and transformed a lethal disease to a manageable condition with higher life expectancy, and medications for T2D patients improved glycemic conditions in those patients. However, these interventions did not restore metabolic hemostasis and administration of these medications over a long period of time may result in severe diabetes-associated comorbidities. Thus, alternative antidiabetic approaches are urgently needed.

Leptin has been investigated as an antidiabetic therapeutic for more than half a century. In addition to its role in energy homeostasis, evidence shows that leptin is involved in glucose metabolism.[14] Hypoleptinemic rodents develop insulin resistance and diabetes[15-16] and leptin treatments resulted in decreasing the blood glucose and insulin levels.[17] Additionally, leptin administration alleviated the severe insulin resistance conditions in both rodents[18-19] and humans.[20] Leptin has been investigated in clinical trials as a potential therapeutic for diabetes as well as other disease. A summary of these studies is shown in Table 4.1.[13] Many scientists made an effort to explain the involvement of leptin in different diseases via focusing on central nervous system and proposed contradictory theories.

Table 4.1. Different Clinical Trials of Leptin and the Contradictory Results.[13]

Condition	Basal Serum Leptin Levels	Intervention	Outcome
Obesity (Leptin deficiency)	<0.04 ng/ml	Leptin	Improves obesity and endocrinological imbalances[21-23]
Obesity	>15 ng/ml	Leptin	Does not improve obesity[24-25]
Obesity	>15 ng/ml	Leptin	Modestly improves obesity[26]
Lipodystrophy-induced T2D	<5ng/ml	Leptin	Improves lipid and glucose imbalance[20, 27]
HIV and Lipodystrophy-induced T2D	<5ng/ml	Leptin	Improves glucose but not lipid imbalance[28]
Lipodystrophy-induced T2D	~5 ng/ml	Leptin	Improves lipid but not glucose imbalance[29]
Hypothalamic Amenorrhea	<5ng/ml	Leptin	Recuperates menstruation and corrects gonadal abnormalities[30-32]
T2D and Obesity	>15 ng/ml	Leptin	Poorly improves glucose imbalance[33-34]
T1D and Lipodystrophy	<5ng/ml	Leptin	Improves lipid and glucose imbalance[35]

Scientists have also made attempts to investigate alternative approaches to explain the therapeutic effect of leptin in diabetes and other diseases. One approach is to understand the vessel dilatory effect of leptin and how this aspect of leptin's function can be beneficial to understand the mechanism of action of leptin more thoroughly.[1, 36-37] Preliminary studies in this area has shown that leptin relaxes the arterial ring in a dose dependent manner by stimulating NO production. Mice arterial rings were precontracted with 1×10^{-6} M of phenylephrine and the relaxation by leptin was monitored. The results are depicted in Figure 4.1. Relaxation by leptin was abolished when endothelial cells were removed.[1]

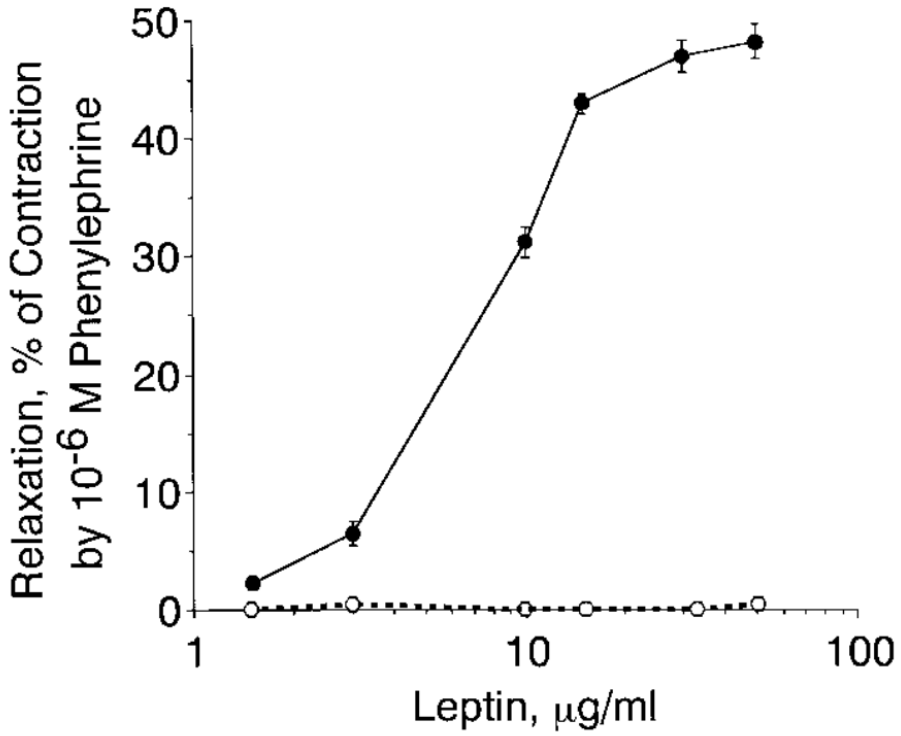

Figure 4.1. Mice Arterial Rings Relaxation by Leptin in a Dose Dependent Manner. Arterial rings were precontracted by phenylephrine and leptin induced relaxation was monitored in the presence (closed circles) and absence (open circles) of endothelial cells.[1]

Collectively, a better understanding of the mechanism of action of leptin is required. We have already demonstrated in this dissertation that leptin can affect RBCs and enhance the cellular effects of C-peptide, such as increased ATP release from the RBC, which may lead to downstream vessel dilation. These results may possibly explain the effect of leptin on NO production. Here, we report leptin's effect on C-peptide and zinc binding to RBCs and the interaction between leptin and RBCs.

4.2 Experimental Methods

4.2.1 Blood Collection and RBCs Isolation

Blood collection and purification of RBCs in the appropriate buffer was performed as described in section 2.2.1.

4.2.2 Preparation of Samples for ^{65}Zinc Uptake Studies by RBCs

RBCs were incubated for three hours with ^{65}zinc and C-peptide (20 nM each) in PSS to observe the effect of leptin on ^{65}zinc binding to RBCs. Sample preparation is explained in more detail in section 2.2.2. The same samples were prepared in and albumin-free PSS to study the effect of leptin on ^{65}zinc uptake by RBCs in the absence of albumin. Next, ^{65}zinc uptake by RBCs was investigated at the range of 0-12 nM leptin. Concentrations of ^{65}zinc and C-peptide were held constant at 20 nM each in regular PSS. The samples were then centrifuged at 500*g* for 5 min, and the supernatant was collected. A set of standards was prepared in the corresponding buffer at the concentrations ranging from 0 to 40 nM. The concentration of ^{65}zinc remaining in the supernatant was measured for 5 times using a scintillation counter after mixing 200 µl of the sample and 100 µl of scintillation cocktail (Perkin Elmer, Waltham, MA) in a clear-bottom 96 well plate. The uptake by RBCs was calculated by subtracting the concentration of ^{65}zinc in the

supernatant using the scintillation counter (Perkin Elmer, Waltham, MA) from the amount originally added to the samples. A set of standards was prepared in order to quantitatively determine the amount of ^{65}zinc in the samples and treated the same way as the samples.

4.2.3 Preparation of Sample for C-peptide Uptake Studies Using Enzyme Linked ImmunoSorbent Assay (ELISA)

Treated RBCs were incubated with zinc, C-peptide, leptin, and combination thereof for three hours in regular and albumin free buffer. In addition, C-peptide binding to RBCs was investigated at varying concentrations of leptin (0.4, 1.2, 2.4, 3.8, 4.8, 6.5 and 10 nM) while concentration of zinc and C-peptide were held constant (20 nM each). All samples were centrifuged after incubation at 500*g* for 5 min and the supernatant was collected and used as the sample for an ELISA (ALPCO, Salem, NH)-based determination of C-peptide. 50 μL of the supernatant was diluted in distilled and deionized water (DDW) to a total volume of 1 mL before being used for the ELISA experiment. A set of standards was prepared in water in the range of 0-50 nM. A standard plate reader (Molecular Devices LLC, Sunnyvale, CA) was used to measure the absorbance of the samples at 450 nm. The concentration of C-peptide binding to the cells was determined by subtracting the number of the moles of C-peptide in the supernatant as measured by ELISA from the original number of moles added to the RBCs, which was 20 picomoles for all samples here (20 nM in 1 mL of solution).

4.2.4 Preparation of Samples for Leptin ELISA

Varying amounts of leptin were added to the sample tubes to reach final concentrations ranging from 0-25 nM in a 7% solution of RBCs in PSS prior to incubation for three hours. No zinc or C-peptide was added to the samples. Leptin standards were prepared in the sample buffer in concentrations ranging from 0,-250 pM. After 3 hours of incubation, the samples were centrifuged at 500*g* for 5 min, and the supernatant was collected to study the interaction between leptin and RBCs using ELISA (Bosterbio, Pleasanton, CA) after diluting 6 µL of the supernatant with sample buffer in total volume 120 µL. The concentration of leptin binding to the RBCs was determined using the subtraction method explained in section 4.2.3. The experiment was repeated using the same concentrations of leptin to study the specificity of the binding between leptin and RBCs. After 3 hours of incubation, samples were centrifuged at 500*g* for 5 min, and a known amount of supernatant (~ 850 µl) was collected for subsequent ELISA after diluting 6 µL of the supernatant with sample buffer in total volume 120 µL. Next, 850 µL of fresh PSS was added to sample tubes containing RBCs and incubated for another three hours. At the end of six hours of incubation, samples were centrifuged at 500*g* for 5 min, and the supernatant was collected to study the interaction between leptin and RBCs using ELISA after diluting 50 times in sample buffer.

4.3 Results

4.3.1 The Effect of Leptin on Zinc Uptake by RBCs

The effect of leptin on ^{65}zinc uptake by RBCs was studied by treating samples with ^{65}zinc and C-peptide alone, resulting in a^{65}zinc uptake of (1.68 ± 0.3 nM, $p<0.001$) compared to control samples (-0.043 ± 0.31). Leptin, ^{65}zinc, and C-peptide treated RBCs showed a significant increase in ^{65}zinc uptake by RBCS (3.6 ± 0.6 nm, $p<0.001$) compared to the control sample and sample treated with ^{65}zinc and C-peptide alone. The results are shown in Figure 4.3.

The next experiment was performed to examine the effect of leptin in the absence of albumin. As shown in Figure 4.4 samples containing ^{65}zinc, C-peptide, and leptin in the presence of albumin showed an increase in ^{65}zinc uptake (2.6 ± 0.3 nM, $p<0.04$) compared to the samples containing ^{65}zinc and C–peptide (1.1 ± 0.2 nM, $p<0.04$). Samples in albumin-free PSS showed non-specific binding of ^{65}zinc when treated with ^{65}zinc and C-peptide only (16.5 ± 0.9 nM) or with ^{65}zinc, C-peptide, and leptin (16.9 ± 0.7 nM).

The effect of leptin on ^{65}zinc uptake by RBCs was also examined by varying the concentration of leptin. ^{65}Zinc uptake by the RBCs increased when the concentration of leptin increased, but the increase became smaller as the leptin concentration approached 5.0 nM and it started increasing at leptin concentrations higher than 5.0 nM as shown in Figure 4.5.

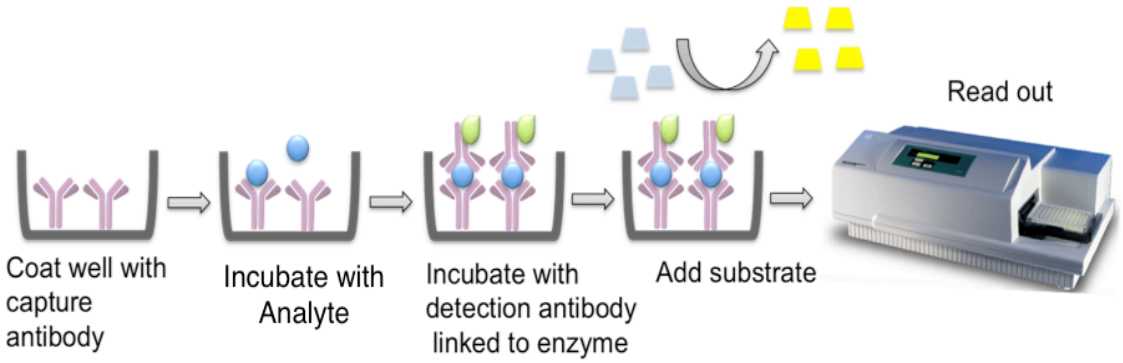

Figure 4.2. Steps of Enzyme Linked Immunosorbent Assay (ELISA). Sample containing C-peptide added to the well and captured by the primary antibody. Then, enzyme conjugated antibody will be added to the wells followed by addition of substrate. Enzyme converts the added substrate into a colored product.

4.3.2 The Effect of Leptin on C-peptide Uptake by RBCs

The effect of leptin on C-peptide uptake was examined in albumin-free and regular PSS (Figure 4.6). Samples containing zinc and C-peptide in PSS resulted in a C-peptide uptake of 1.7 ± 0.1 nM, $p<0.05$ and RBCs treated with zinc, C-peptide, and leptin increased the uptake to 3.1 ± 0.1 nM, $p<0.05$. Zinc and C-peptide treated RBCs in albumin-free PSS did not show C-peptide uptake and when leptin was added to the combination of zinc and C-peptide the uptake was increased to 1.9 ± 0.1 nM, $p<0.05$.

Finally, the effect of leptin concentration on C-peptide uptake by the RBCs was measured. As shown in Figure 4.7, increasing the concentration of leptin resulted in elevated C-peptide uptake to 3.2 ± 0.2 nM when 4.8 nM leptin was added and then, the curve reached a plateau and the uptake remained almost constant at higher concentrations of leptin.

4.3.3 The Binding of Leptin to RBCs

A titration curve of leptin was performed to investigate the binding between RBCs and leptin and the results are depicted in figure 4.8. The levels of leptin bound to RBCs increased by increasing the concentration of leptin, although the increments decreased at leptin concentrations greater than 15 nM. This may indicate that the cells are getting saturated as evident by the plateau. To investigate this further, the same experiment was performed and the leptin uptake was quantified after 3 hours and 6 hours of incubation. The results were similar to the results shown in Figure 4.9 after 3 hours of incubation. Then, fresh buffer was added to RBCs and incubated for another 3 hours and the amount of leptin in the supernatant was quantified. Interestingly, free leptin was observed in the supernatant after 6 hours of incubation, which may indicate that the binding between leptin and RBCs was non-specific. This will be discussed in more details in the discussion section. The results are shown in Figure 4.9.

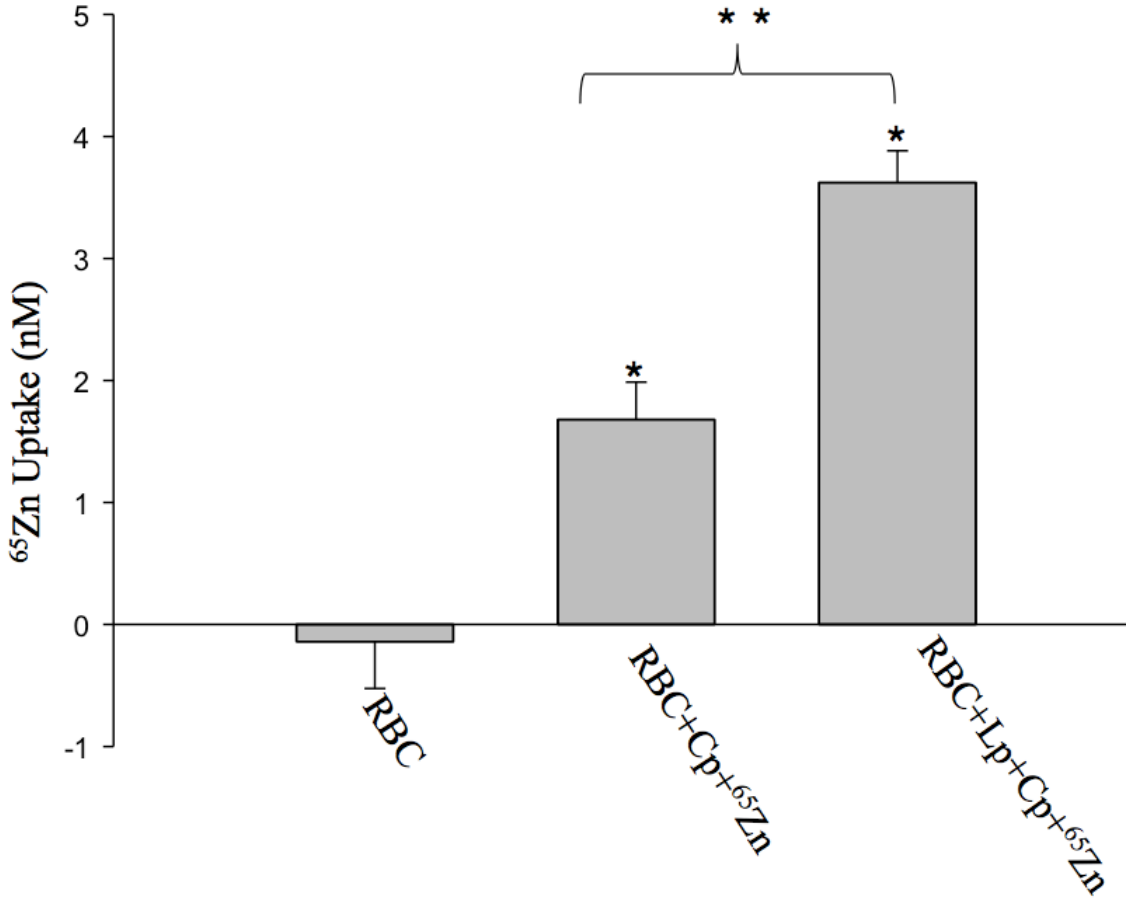

Figure 4.3. Zinc Uptake Increases in leptin treated RBCs. The experiment is performed in regular PSS containing albumin and radioisotope zinc was used. Samples treated with ^{65}zinc, C-peptide, and leptin increased ^{65}zinc compare to the samples treated with ^{65}zinc and C-peptide. * shows that the bars are significantly different than the control (RBC) bar and ** shows that ^{65}zinc and C-peptide bar is significantly different that ^{65}zinc, C-peptide, and leptin bar. [Zn]= 20 nM, [Cp] = 20 nM, [Leptin] = 3.8 nM, 7% RBCs, error bars: SEM, n=6, $p < 0.004$.

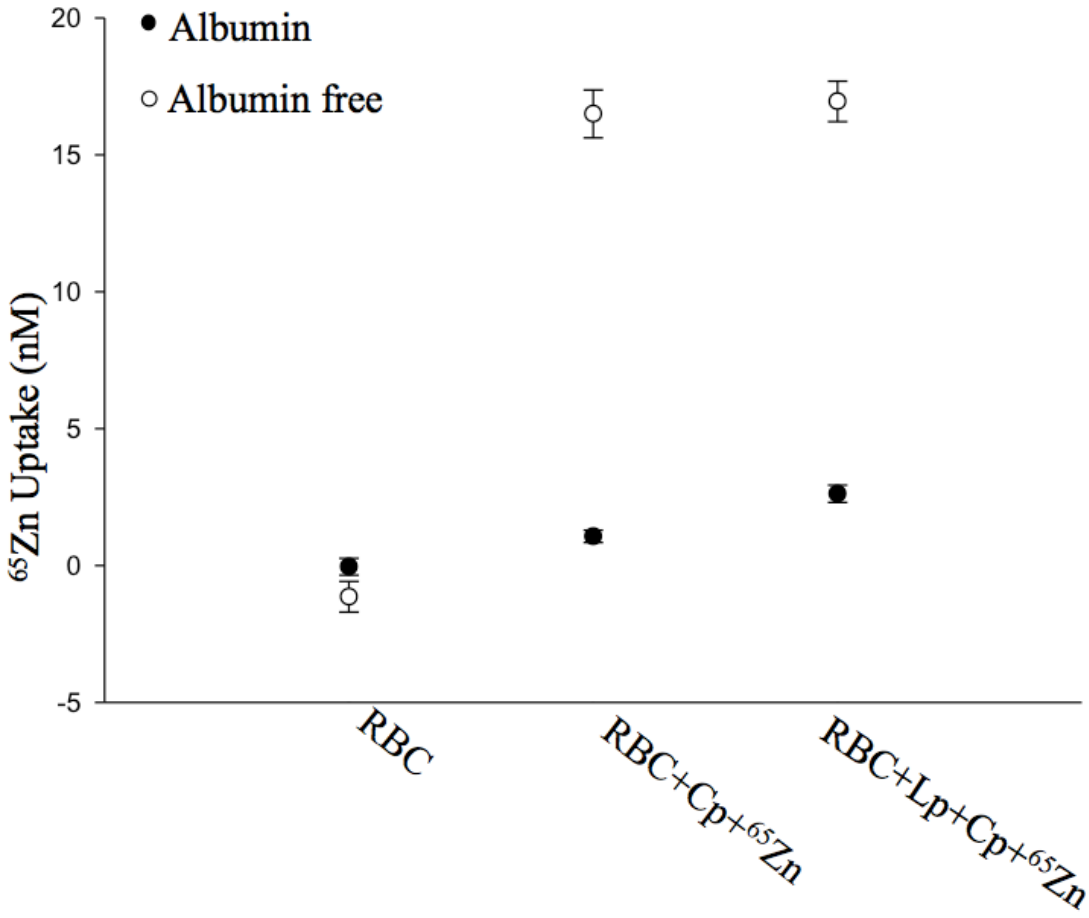

Figure 4.4. Non-specific Binding of ^{65}Zinc Was Observed in Albumin Free PSS in the Presence and Absence of Leptin. The closed circles are showing RBC samples in regular PSS nd open circles show samples in albumin free PSS. [Zn]= 20 nM, [Cp] = 20 nM, [Leptin] = 3.8 nM, 7% RBCs, error bars: SEM, n=6, $p < 0.004$.

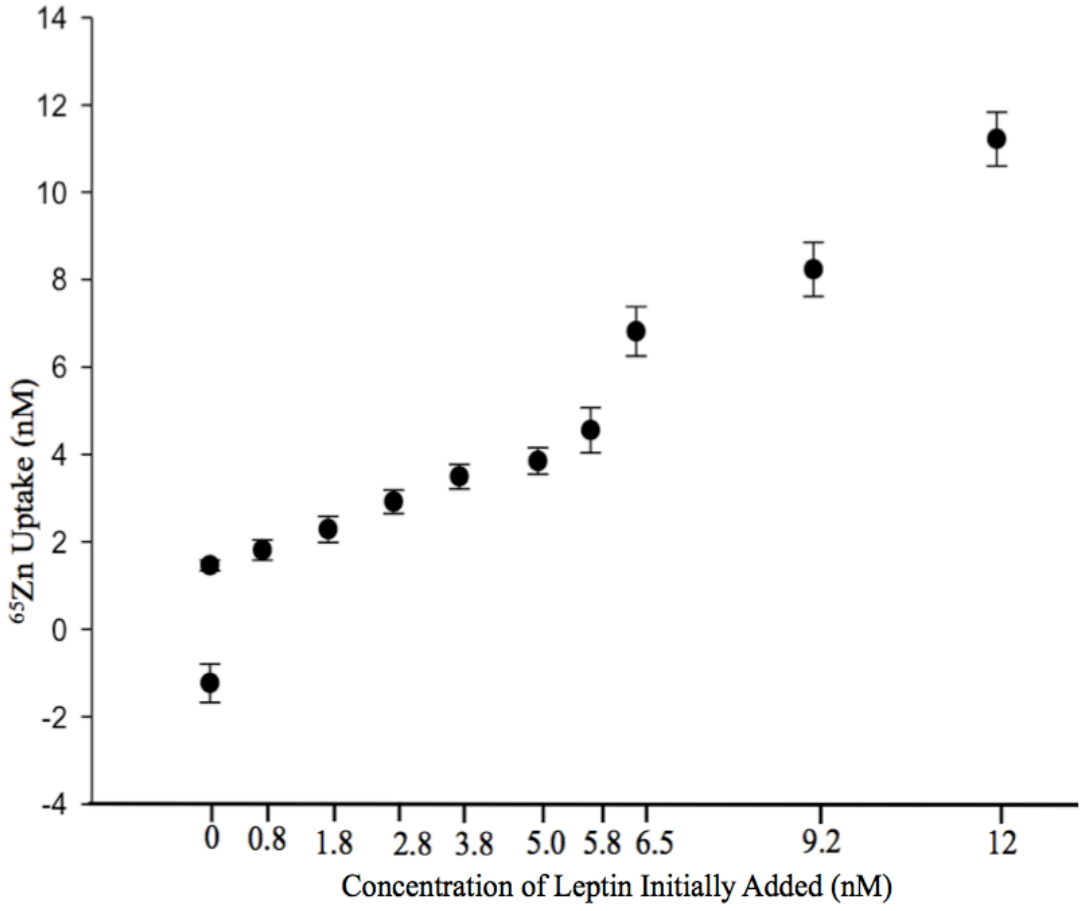

Figure 4.5. Increasing the Concentration of Leptin Increased ^{65}Zinc Uptake by RBCs. All the samples contain zinc and C-peptide except the control sample. The uptake increased by increasing the concentration of leptin but the increments became smaller as the concentration of leptin got closer to 5 nM. Then, the increments started getting bigger, which may indicate non-specific binding. [Zn]= 20 nM, [Cp] = 20 nM, 7% RBCs, error bars: SEM, n≥6.

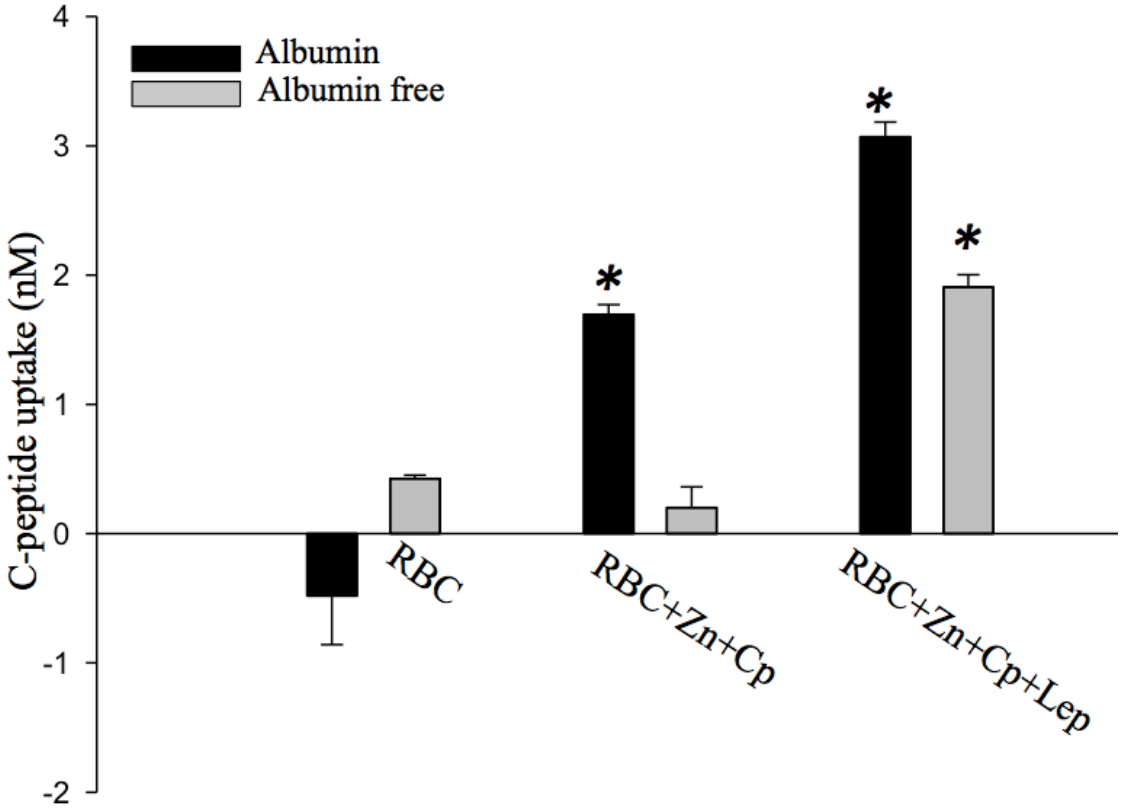

Figure 4.6. Treating RBCs with Leptin Resulted in Augmented C-peptide Uptake in the Presence and Absence of Leptin. Black bars show samples in regular PSS and grey bars show samples in albumin free PSS. [Zn]= 20 nM, [Cp] = 20 nM, [Leptin] = 3.8 nM, 7% RBCs, error bars: SEM, $n \geq 3$, $p < 0.001$.

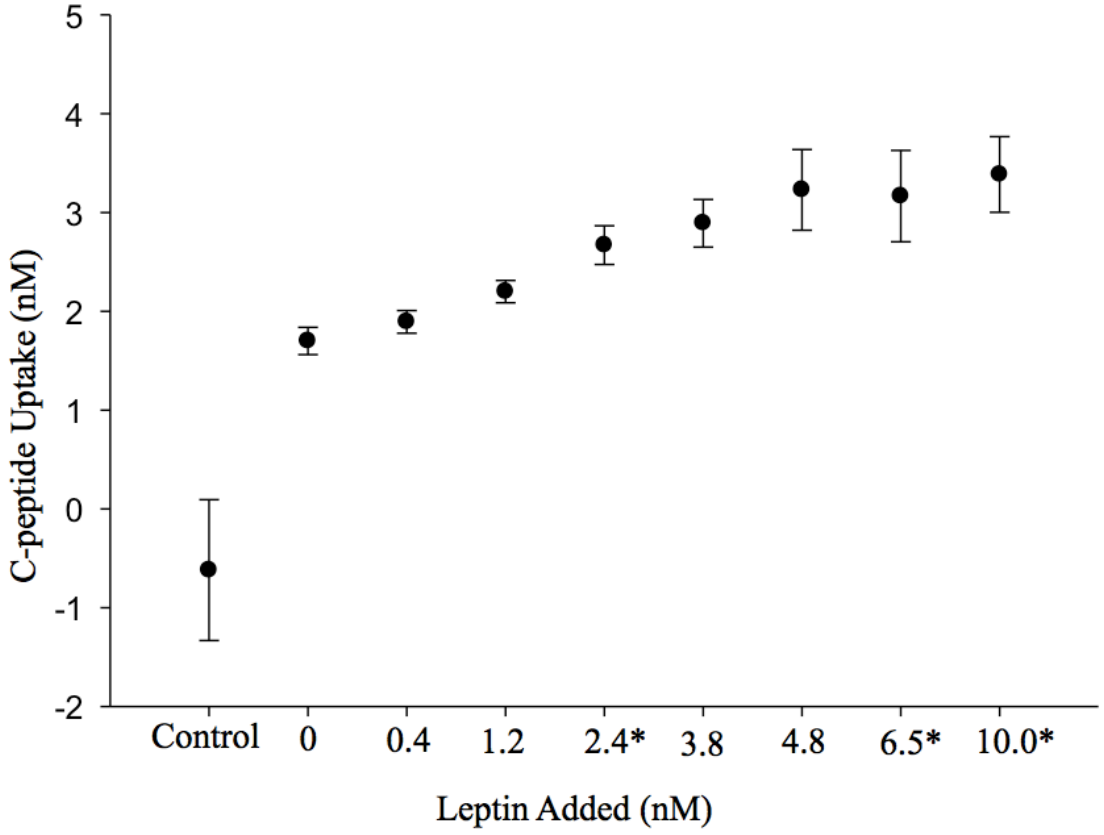

Figure 4.7. C-peptide Uptake by RBCs Increased by Increasing the Concentration of Leptin Added to the Samples. All the samples contain zinc and C-peptide except the control. C-peptide uptake by RBCs increased when the concentration of added leptin increased and it reached a plateau at concentrations higher than 4.8 nM leptin. [Zn]= 20 nM, [Cp] = 20 nM, 7% RBCs, error bars: SEM, n=6 and n=5 for *.

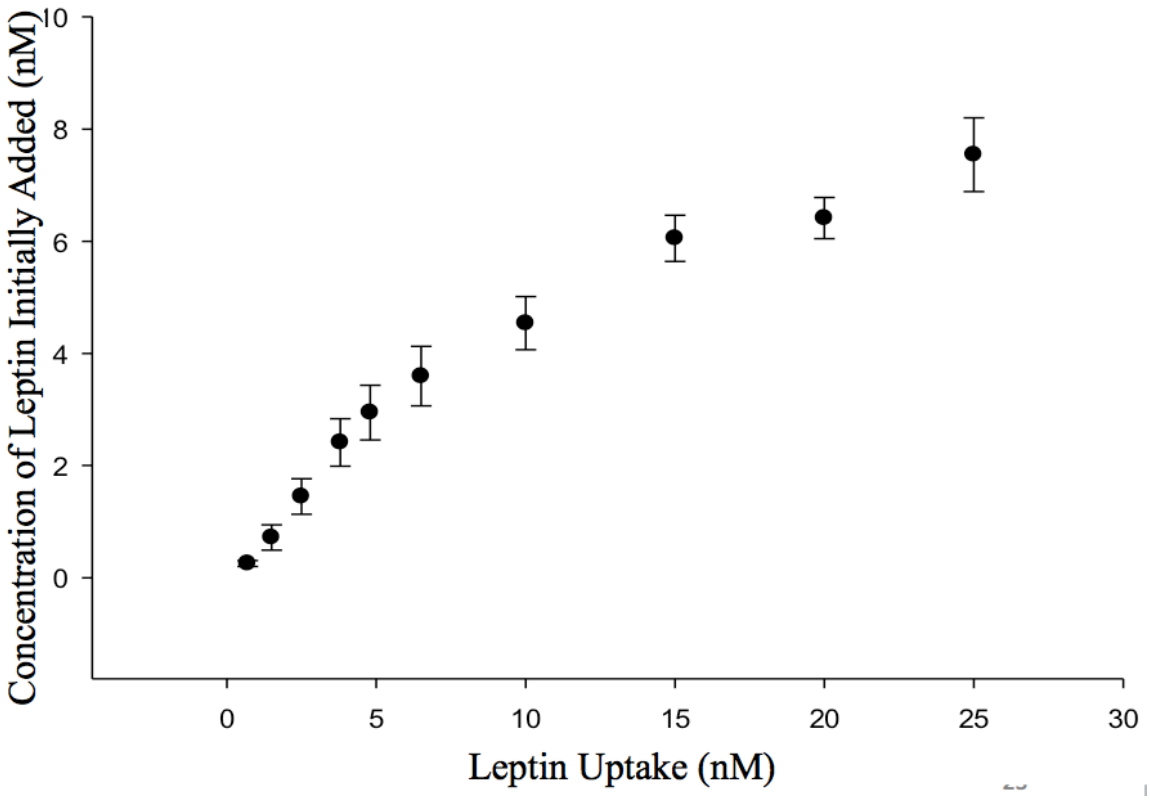

Figure 4.8. Increase in Leptin Uptake as the Original Concentration of Leptin Increases. Leptin uptake increased until it reached a plateau at concentrations of leptin higher than 15 nM. 7% RBCs, error bars: SEM, n=6.

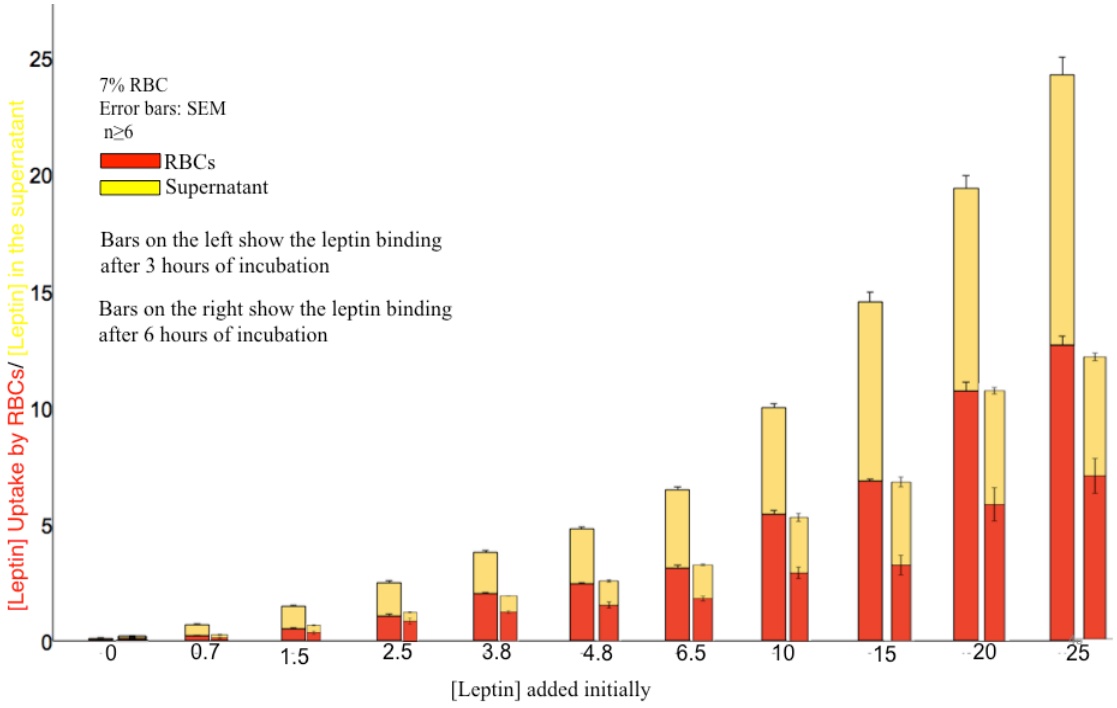

Figure 4.9. Leptin "Bound" to RBCs after 3 Hours of Incubation were Released to the Fresh Buffer Added Indicating Non-Specific Binding. Leptin uptake by RBCs was quantified by subtraction method after 3 hours and fresh buffer was added to the cells. Then, the concentration of leptin in the supernatant was measured after 6 hours of incubation and half of the leptin that was "bound" to RBCs was observed in the supernatant.

4.4 Discussion

Liu et. al previously investigated C-peptide uptake in the presence and absence of zinc and zinc uptake in the presence and absence of C-peptide at varying concentration of C-peptide and zinc. C-peptide uptake remained almost the same in the presence and absence of zinc as shown in Figure 4.10a. However, ^{65}zinc uptake was abolished in the absence of C-peptide while it increases by increasing the original concentration of ^{65}zinc in the presence of C-peptide as shown in Figure 4.10b.[4]

In addition, Yueli Liu studied C-peptide and ^{65}zinc uptake in the absence of albumin and no uptake of ^{65}zinc or C-peptide was observed when albumin was not present in the solution as shown in Figure 4.11.[12] On the other hand, Tiffany Janes showed that specific binding of ^{65}zinc occurs when albumin and C-peptide are present and samples containing ^{65}zinc and C-peptide showed non-specific binding in the absence of albumin as depicted in Figure 4.12. Therefore, the presence of C-peptide and albumin is necessary to observe specific ^{65}zinc uptake and the presence of albumin is necessary to observe specific C-peptide uptake. Here, ^{65}zinc and C-peptide uptake is investigated in the presence of leptin along with considering the experiments performed previously to design the experiment.

Leptin treated RBCs showed increase in ^{65}zinc uptake by RBCs compare to samples containing ^{65}zinc and C-peptide only and may suggest that leptin is able to deliver more zinc to RBCs. Samples containing ^{65}zinc and C-peptide showed non-specific binding of zinc to RBCs in the absence of albumin as we expected. Leptin, C-peptide, and ^{65}zinc treated RBCs showed non-specific binding as well and the non-specific uptake by RBCs was statistically the same as the uptake observed in samples containing ^{65}zinc

and C-peptide. Next, the effect of different concentrations of leptin on ^{65}zinc uptake was investigated since the presence of leptin increased ^{65}zinc uptake in regular PSS. ^{65}Zinc uptake increased gradually by increasing the concentration of leptin and the increments became smaller until 5.0 nM of leptin was added to the samples. Then, the increments started becoming larger, which may indicate non-specific binding to RBCs. It is hard to say that a binding curve was observed for ^{65}zinc binding to RBCs at varying concentration of leptin. The general trend of the curve looks like a binding curve due to reaching a plateau and then showing increasing at certain concentrations after the plateau. However, the increases after the plateau are not as large as it is expected in a traditional binding curve. ^{65}Zinc uptake of 3.8 ± 0.3 nM is observed when 5.0 nM leptin is added to the samples and if the uptake is subtracted from the uptake observed by the samples containing ^{65}zinc and C-peptide only, a 1:2 ration is observed for ^{65}zinc:leptin. This ratio is seen in all leptin concentrations smaller than 5.0 nM. However, this trend is not observed at concentrations higher than 5.0 nM and ^{65}zinc to leptin ration increases. This experiment needs further investigation to conclude that the curve observed is a binding curve for ^{65}zinc.

Next, C-peptide uptake in leptin treated samples was investigated in the presence and absence of albumin. The C-peptide ELISA assay is a sandwich-based assay in which a secondary antibody tagged enzymatically catalyzes a colorimetric reaction as shown in Figure 4.2. C-peptide uptake in the samples treated with zinc, C-peptide, and leptin was further increased compared to samples containing zinc and C-peptide only. As we discussed earlier, no C-peptide uptake was observed in the absence of albumin. Interestingly, leptin treated RBCs showed C-peptide uptake in the absence of albumin.

The latter increase was statistically the same as the increase observed by zinc and C-peptide containing RBCs in regular PSS. This may suggest that leptin may serve as the carrier protein for zinc and C-peptide and leptin and albumin work in parallel when albumin is present. However, leptin is potentially the only carrier molecule replacing albumin and the amount of C-peptide delivered to RBCs is statistically the same as C-peptide delivered by albumin only. Changes in C-peptide uptake at varying concentrations of leptin was investigated to further investigate this hypothesis. C-peptide uptake increases by increasing the concentration of leptin and the increments become smaller as it gets closer to leptin concentration≥4.8 nM and RBCs become saturated at concentrations higher that 4.8. Therefore, leptin enhances C-peptide uptake by RBCs until they get saturated. A 3:1 ratio of leptin:C-peptide is observed at concentrations higher than 3.8 nM of leptin.

Lastly, the binding between RBCs and leptin was investigated at varying concentrations of leptin. RBCs uptake increased by increasing the concentration of leptin and the increments became smaller as the original concentration of leptin became closer to 15 nM (300 pM used in the wells) leading to saturation at higher concentrations. The standard curve became saturated at concentrations greater than 250 pM and the question was whether the saturation occurs due to RBCs becoming saturated or the wells getting saturated. Therefore, another experiment was performed and leptin uptake was performed after 3 hours and 6 hours of incubation. Similar results to the first experiment were observed after 3 hours of incubation. However, bound leptin should stay bound to RBCs when fresh buffer was added to the samples if the binding was specific. Surprisingly, the amount of leptin that was quantified as bound to RBCs after 3 hors of incubation started

equilibrating with the fresh buffer and was almost distributed in half between RBCs and the buffer. This results can potentially suggest that leptin binds to RBCs non-specifically since it gets knocked off the cells by the addition of a fresh buffer and equilibrates in the new condition. However, an equilibrium would be established when fresh buffer is added even with specific binding because the equilibrium would be dictated by the binding constant. Therefore, determining the binding constant is necessary for this experiment.

In 1983, Ockner et. al. hypothesized a model for albumin receptor in which albumin and ligand complex bind specifically to the binding site of the cells. This binding favors a rapid dissociation of the ligand from albumin and the entrance of the ligand to the cells via its particular uptake mechanism. Then, albumin molecule dissociates from the complex receptor and return to the circulation.[38] Leptin may bind to RBCs as a complex with the ligand (C-peptide) specifically based on this experiment and no specific binding occurs when leptin interacts with RBCs by it self. Further investigations are needed to confirm this hypothesis.

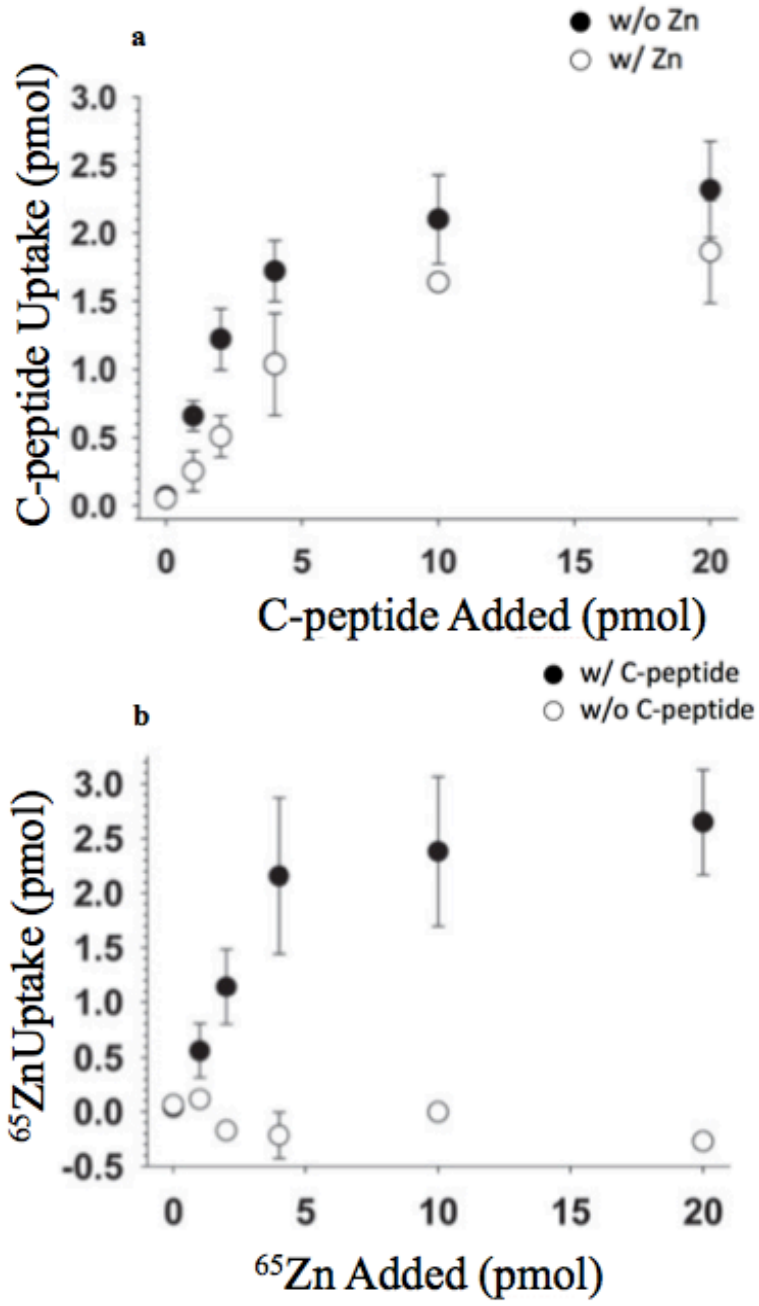

Figure 4.10. C-peptide Uptake Occurs in the Presence and Absence of Zinc but Zinc Uptake Only Occurs in the Presence of C-peptide. Closed circles show samples without zinc and open circles show samples with zinc in a. Closed circles show samples with C-peptide and open circles show samples without C-peptide in b.[4]

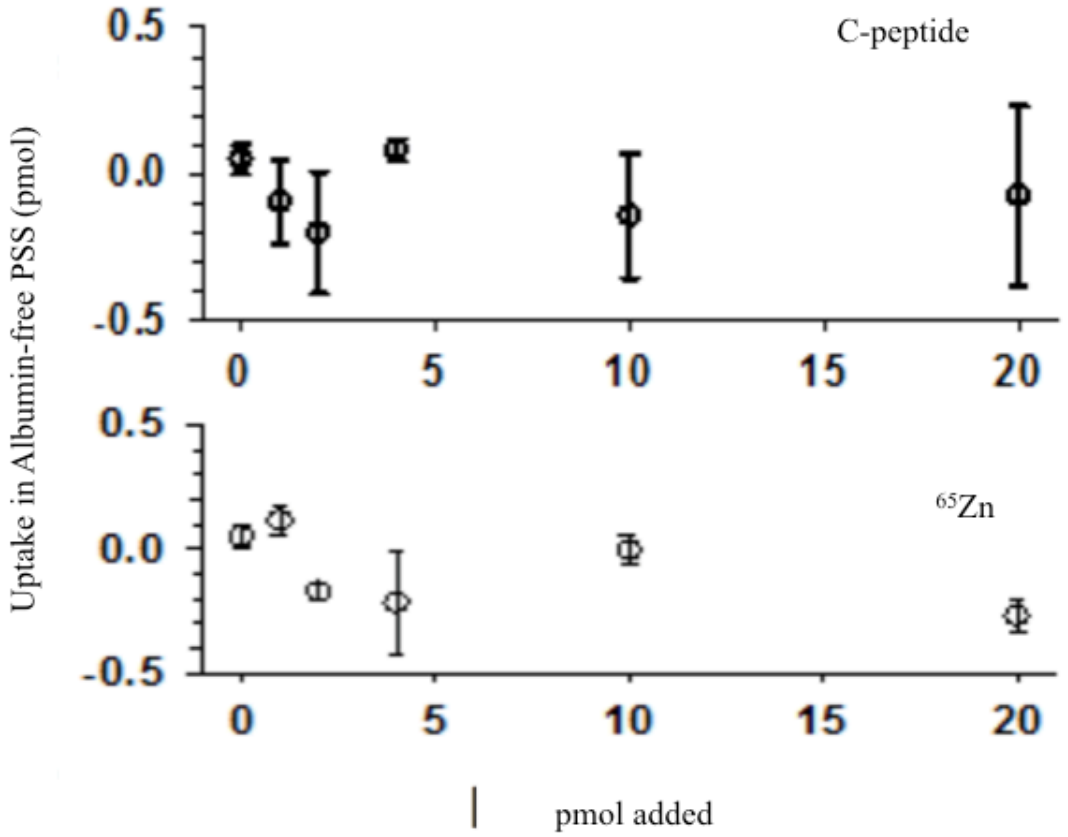

Figure 4.11. C-peptide and Zinc Uptake by RBCs was Abolished in the Absence of Albumin. Top figure shows C-peptide uptake by RBCs in albumin free buffer and the bottom figure shows zinc uptake by RBCs in albumin free PSS. These samples are treated with C-peptide only and zinc only Respectively.[12]

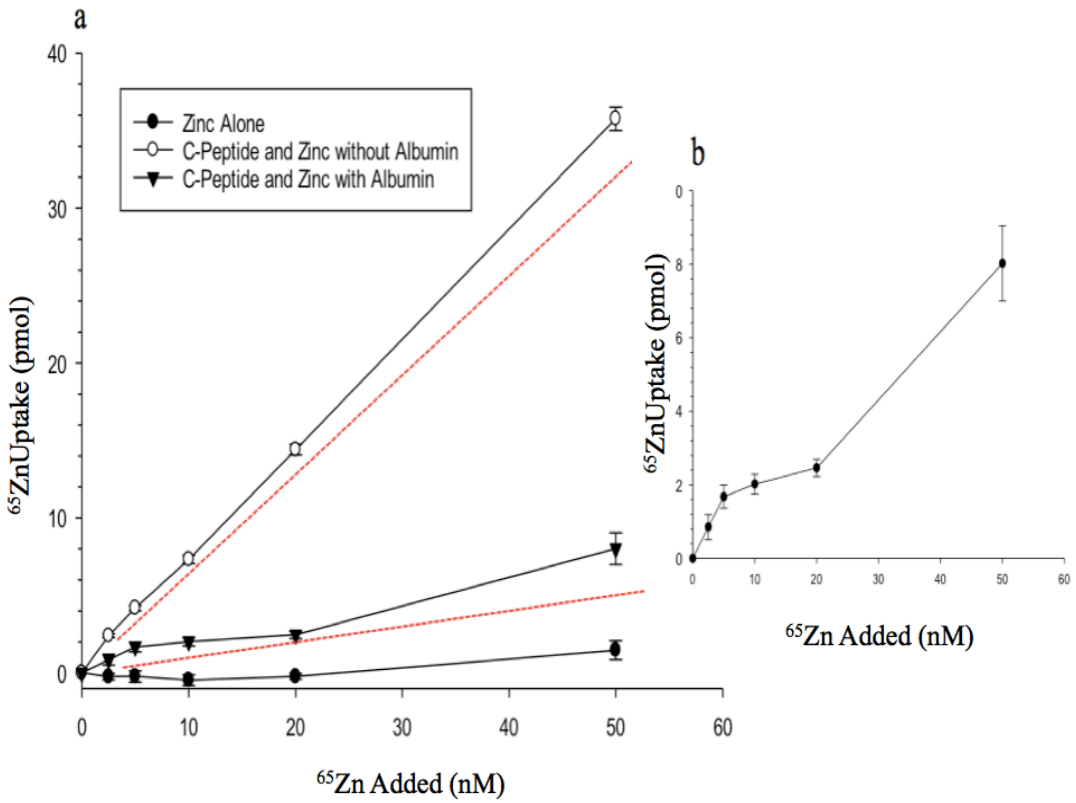

Figure 4.12. Samples Treated with Zinc and C-peptide Show Non-specific Binding in the absence of Albumin. a) Closed circles show samples containing zinc only and no binding with RBCs occurred. Closed triangles show samples containing zinc and C-peptide in the presence of albumin and a specific binding curve was observed. Open circles show samples containing zinc and C-peptide in the absence of albumin and non-specific binding was observed. b) zoom out of the specific binding between C-peptide and RBCs in the presence of albumin. These data is obtained by Tiffany Janes and is unpublished.

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Chapter 5- Conclusion and Future Directions

5.1 Conclusion

C-peptide, a 31-amino acid peptide discovered in the 1960s, is co-secreted with insulin from pancreatic β-cells. It was originally thought that C-peptide was only involved in the proper folding of insulin and for decades, no other biological role was known. C-peptide replacement therapy was reported as beneficial for diabetic complications in the 1990s.[1-3]

Insulin therapy transforms lethal diabetes to a manageable condition, but it is not a cure for diabetes. Insulin helps maintain normal blood glucose levels, but it does not prevent diabetic complications. Complementary C-peptide therapy reduced neuropathy[4-7] and nephropathy[8-9] in diabetic animal models. In addition, C-peptide therapy in clinical trials of T1D patients seemed beneficial in reducing neuropathy[10-11] and nephropathy.[7, 12-13] Despite this evidence, C-peptide is not an approved therapeutic for diabetes, and a clinical trial of C-peptide replacement therapy failed in phase 2b because the results of patients taking C-peptide as a therapeutic were not distinguishable from the patients taking a placebo.[14-15] The research presented here, and elsewhere provides strong evidence that C-peptide requires a carrier for delivery to the red blood cell (RBC). Studying C-peptide's mechanism of action may be advantageous in understanding the reason behind the failure of previous clinical trials.

Scientists have proposed that C-peptide therapy may improve blood flow by improving vessel dilation. Improved blood flow is thought to be a key determinant in the prevention or delay of diabetic complications.[16-18] One of the well-known vessel dilator molecules is endothelial cell-derived nitric oxide (NO).[19] RBCs generate mM levels of

ATP via glycolysis[20] and release it to the extracellular matrix upon stimulation, such as low pH,[21] hypoxia,[22] changes in osmotic pressure,[23] and mechanical deformation.[24-25] The released ATP binds to its receptors on endothelial cells and stimulates endothelial NO synthase (eNOS), producing NO and resulting in muscle cell relaxation and vessel dilation.[20]

The Spence group has investigated the reasons for C-peptide replacement therapy extensively and made two discoveries. Meyer *et al*. showed that crude C-peptide is contaminated with a metal and removing the metal abolished C-peptide's function. Addition of a metal such as zinc, iron, or chromium restored C-peptide's biological activity,[26-27] and among these metals, zinc is the most probable metal to interact with C-peptide due to its high concentrations in pancreatic β-cells.[28-30] Since then, C-peptide has always been co-incubated with zinc in all the experiments performed by the Spence group. Additionally, Liu *et al.* discovered that the presence of intact albumin is necessary for C-peptide to exert a biological effect, and they also reported that albumin binds to zinc and C-peptide in a two-phase binding event.[31] C-peptide used in the clinical trial was not administered along with a carrier molecule and the structure was also altered. Unfortunately, C-peptide is not as effective in a high glucose environment and finding molecules that enhance the biological effects of C-peptide and restore metabolic hemostasis is urgently needed.

Leptin is secreted by adipose tissue and is believed to maintain the balance between food intake and energy expenditure.[16, 32] The plasma leptin level is higher in obese human and rodents.[26] Leptin has been studied as an antidiabetic medication for more than 60 years, and it has been found to regulate glucose homeostasis as well as

energy balance.[33] Rodents that lack leptin develop insulin resistance and diabetes,[34-35] and leptin therapy decreased blood glucose and insulin levels.[36] Also, leptin therapy diminished insulin resistance in human and rodents.[37-38] In addition, scientists investigated leptin therapy in insulin deficient T1D patients and observed that leptin therapy alone or along with insulin therapy provided glycemic control without increasing body fat or up-regulating cholesterologenic and lipogenic transcription factors and enzymes observed with insulin monotherapy in T1D.[39] Leptin therapy was able to prevent dangerous fat storage in liver and muscle cells and insulin resistance caused by insulin monotherapy in T1D mice.[39-40] To date, leptin therapy has been limited to diabetic patients with lipodystrophy,[41-42] and scientists are trying to grasp the glucoregulatory function of leptin to determine leptin efficacy in diabetes, in general. Researchers are investigating alternative approaches to dissect the role of leptin in glucose metabolism and its mechanism of action. In this construct, they find that leptin is involved in vessel dilation by stimulating NO production.[18, 43-44] Taken together, a novel approach is proposed in this dissertation to examine the glucoregulatory role of leptin and its involvement in vessel dilation by studying the effect of leptin on RBCs and in conjunction with C-peptide combination.

Our group has previously shown that the combination of C-peptide, zinc, and albumin stimulate RBCs to release ATP, which may stimulate eNOS and result in NO production and vessel dilation. However, no effect was observed from C-peptide treated RBCs in the absence of albumin.[31] Here, we demonstrated that the addition of leptin to the C-peptide combination, C-peptide, zinc, and albumin, results in augmented ATP release from RBCs compare to samples containing zinc and C-peptide only. Also, leptin

alone does not have any effect on RBCs. Interestingly leptin enhances ATP release by RBCs in the absence of albumin. To examine this effect mechanistically, leptin was removed from the RBC samples before the addition of zinc and C-peptide and surprisingly, no change in ATP release was observed in these samples. This suggests that the effect of leptin on RBCs is dependent on and not additive to zinc and C-peptide, even though it is not dependent on albumin. The effect of leptin appears to be additive to albumin because the increase in ATP release observed after incubation of RBCs with leptin, zinc, and C-peptide in albumin-free buffer was statistically the same as the increase observed after incubation with zinc and C-peptide in PSS. It was proposed by the Spence group that albumin serves as the carrier for zinc and C-peptide.[31] Here, we hypothesize that leptin may also act as a carrier molecule and function separate from, but in parallel to, albumin. To further investigate this hypothesis, the effect of leptin was also examined in high glucose conditions to mimic the high glucose environment in diabetes, and the increase in RBC-derived ATP observed was statistically the same as the increase observed in normal glucose condition.

Leptin therapy has been investigated in T1D patients, and it is being used in T1D patients with lipodystrophy as a therapeutic, as mentioned earlier. Here, we tested the effect of leptin on T1D RBCs and compared it to healthy RBCs, and an increase in ATP release by T1D RBCs was observed in the presence and absence of albumin, but the increase was not as significant as with healthy RBCs, contrary to the results obtained from RBCs incubated in high glucose buffer in which ATP release was statistically the same as RBCs in PSS. This may be due the exposure of T1D RBCs to a high glucose environment for almost their entire lifetime, where as healthy RBCs were exposed to a

high glucose environment for only three hours. T1D RBCs release lower basal ATP levels compared to healthy RBCs, as we expected. The effect of leptin has only been investigated in isolated RBCs so far, so it is of interest to test leptin in whole blood, which contains other proteins, molecules, and cells. Leptin was capable of enhancing RBC-derived ATP in 7% whole blood as well, but the ATP release was lower compared to 7% RBCs. This may be due to the presence of molecules and enzymes, such as apyrase, in the blood stream that can break down ATP.

The potential mechanism of ATP release by RBCs was also investigated. In anucleated RBCs, ATP is generated via glycolysis, and the presence of glucose in the cells is necessary for glycolysis to occur. Glucose is transported to RBCs via glucose transporter-1 (GLUT-1) that ducts to the cell surface from the protein pool inside the cell upon stimulation, such as a high glucose extracellular matrix. Therefore, monitoring GLUT-1 translocation may be beneficial in explaining the increase in ATP release observed in treated RBCs. Tiffany Janes has previously shown that samples treated with zinc and C-peptide enhance GLUT-1 translocation in the presence of albumin, and no change was observed when albumin was not present. This data was coupled with ATP release from RBCs, and the results confirmed Janes's findings. The preliminary study on GLUT-1 translocation showed that leptin further enhances GLUT-1 translocation in the presence of albumin. This experiment was studied in more detail by investigation of T1D and healthy RBCs in the presence and absence of albumin. T1D RBCs showed lower GLUT-1 translocation compared to healthy RBCs, which may explain the lower RBC-derived ATP release from T1D samples. Samples containing zinc and C-peptide increased GLUT-1 translocation in both healthy and T1D RBCs using PSS. However, no

effect was observed from zinc and C-peptide treated RBCs in the absence of albumin. Leptin, zinc, C-peptide treated RBCs increased GLUT-1 translocation in healthy and T1D samples in the presence and absence of albumin. It is worth noting that the levels of GLUT-1 translocation in leptin treated T1D RBCs was restored to the basal levels observed in healthy control RBCs. Also, the increase observed by leptin treated RBCs in albumin-free PSS was statistically the same as the increase observed by zinc and C-peptide treated samples in PSS. This confirms the hypothesis of leptin serving as a carrier for zinc and C-peptide. Next, GLUT-1 translocation was examined in 7% whole blood and the increase observed in samples treated with leptin, zinc and C-peptide was statistically the same as the increase observed in 7% RBCs. This may suggest that the presence of other proteins and molecules does not interfere with the function of leptin.

To date, the effect of leptin on RBCs has been investigated and the results suggest that the presence of leptin in the blood stream may be beneficial in improving blood flow. It is important to investigate the role of leptin mechanistically in order to be able to understand the observed effects. Therefore, the effect of leptin on the binding between RBCs and zinc and C-peptide was studied. It has been previously shown by Liu *et al*. that the presence of C-peptide is necessary to observe zinc uptake by RBCs, but the presence of zinc is not necessary to observe C-peptide uptake by RBCs. However, the presence of albumin is necessary to observe zinc and C-peptide binding to RBCs.[31] Also, unpublished work has shown that zinc binds to RBCs non-specifically in the absence of albumin when samples contain both C-peptide and zinc.

Samples containing leptin, zinc, and C-peptide increased zinc uptake by RBCs, compared to the samples containing only zinc and C-peptide in PSS. However, non-

specific binding was observed in zinc and C-peptide or zinc, C-peptide, and leptin treated RBCs in the absence of albumin. Zinc uptake by RBCs was also investigated at varying concentrations of leptin, and a binding curve suggests a 2:1 ratio of leptin to zinc.

C-peptide uptake by RBCs was elevated in the presence of leptin, compared to the samples containing zinc and C-peptide only. As mentioned earlier, no C-peptide uptake by RBCs was observed in the absence of albumin in zinc and C-peptide treated samples. However, samples containing zinc, C-peptide, and leptin increased C-peptide uptake in the absence of albumin, and the increased observed was statistically the same as the increase observed in zinc and C-peptide treated samples, which further confirms the hypothesis of leptin acting as a carrier molecule. Varying the concentration of leptin increased C-peptide uptake by RBCs until the cells became saturated, and the uptake remained almost constant. Therefore, leptin may be involved in the delivery of C-peptide to RBCs, and more C-peptide is delivered to the cells as the concentrations of leptin increases.

It is important to understand how leptin is interacting with RBCs if it is involved in carrying zinc and C-peptide to them. Therefore, leptin uptake by RBCs was studied at different concentrations of leptin using ELISA. At first, we thought that a binding curve was observed for the interaction between leptin and RBCs and that the cells become saturated at concentrations higher than 15 nM leptin. However, after examining the experiment more closely and running the standards at higher concentrations we realized that the saturation was likely due to the saturation of the ELISA wells rather than the RBCs. Therefore, another experiment was performed and leptin uptake by RBCs was quantified after 3 hours and 6 hours of incubation while adding fresh buffer to the cells

after 3 hours of incubation. These results suggested that the binding between leptin and RBCs is non-specific because leptin equilibrated between the cells and buffer after 3 hours of incubation as well as 6 hours of incubation. Even though leptin may not bind specifically to the cells, there might be a receptor for leptin-ligand complex binding to RBCs.

5.2 Future Directions

In order to learn more about diabetes and its complications, a stable and reusable device is necessary to mimic an *in vivo* resistance vessel. Our group has used 3D printing to accomplish this for the past several years.[45] 3D printing has been used for nearly 30 years and is very popular because it's more rugged and transferable to automation.[46] As the fabrication steps are depicted in Figure 5.1, the process starts with designing the model using computer aided design (CAD) software. Then, this file will be saved in a .STL format that is compatible with the 3D printer. The printer manufactures the model layer by layer. The nozzle of the printer lays down a layer of monomer and cures that layer using UV light. Support material holds everything in place as it's being cured and when the device is completed it can be removed using high pressure water or aggressive sanding. The printer continues this process until the object is completely printed. 3D printed devices have been previously used in Spence group to mimic vessel circulation and investigate cell-to-cell communication.

The suggested device this study should contain 6 channels to allow for multiple analyses. A model of this device has been printed and is shown in Figure 5.2. Four of these channels are straight channels with transwell inserts above each channel. The

inserts have porous membranes in the bottom allowing small molecules to diffuse through. These channels will be used for appropriate control samples. The last two channels will be used to study the effect of leptin, zinc, and C-peptide in the circulation. There are two inserts above each of these channels on the first part of the device to culture rat insulinoma cell lines (INS-1 cells, pancreatic beta cell mimics) and human adipocytes on the first and second channels respectively. ATP released from RBCs due to the effect of INS-1 cell secretion or adipocytes secretion will be measured in the second insert on each channel. Then, these two channels will be mixed forming one channel, and the ATP released due to the effect of INS-1 cells and adipocytes simultaneously will be measured. Samples will be loaded using a syringe pump, and the dimensions of this device are such that it fits in the plate reader allowing ATP measurements using the chemiluminescence assay directly.

The transwell insert membranes are coated with a fibronectin solution prior to cell culture. Rat INS-1 cells will be cultured in cell culture flasks in RPMI-1640 medium. Upon reaching confluence, cells will be detached using trypsin and centrifuged at 1000*g* for 4 minutes. The pellets will be resuspended in the media and diluted to a final cell density of 0.1 million/μL. Then, an aliquot of the cells will be cultured in the insert and incubated at 37°C for 2 hours. Then, the media will be replaced by 200 μL fresh media and incubated at 37°C for 24 hours. After 24 hours incubation, medium will be removed followed by three rinses with Krebs buffer. Finally, inserts will be incubated for another 1 hour before being placed on the device to induce secretion.

As mentioned earlier, this device will be used to mimic *in vivo* vessel circulation. Based on the result obtained in the static experiment to quantify ATP, the level of ATP

released by RBCs when the samples in the channels are mixed together should be higher than the amount of ATP released by the sample in the channels before mixing.

One of the challenges in this experiment is to clear the support material from the Y-shaped channel. One possible way to solve this problem is to print the device in two separate pieces. The top part contains the channels and inserts and the bottom part contains inlets and outlets. Devices printed in two pieces might have leaking problem, which can be potentially solved by using black materials (rubber-like) on the surface of the device. The family of rubber-like materials (Tango family) offers a variety of elastomer characteristics that makes them an appropriate choice for knobs, grips, pulls, handles, gaskets, seals, and hoses. Another challenge is the length of the channels that contain inserts with INS-1 cells and Adipocytes. The length of the channel might not give enough time to the samples to mix thoroughly with the secreted proteins and hormones from the inserts. In addition, when the two channels are mixed, they might not mix completely before they reach the insert for ATP measurement.

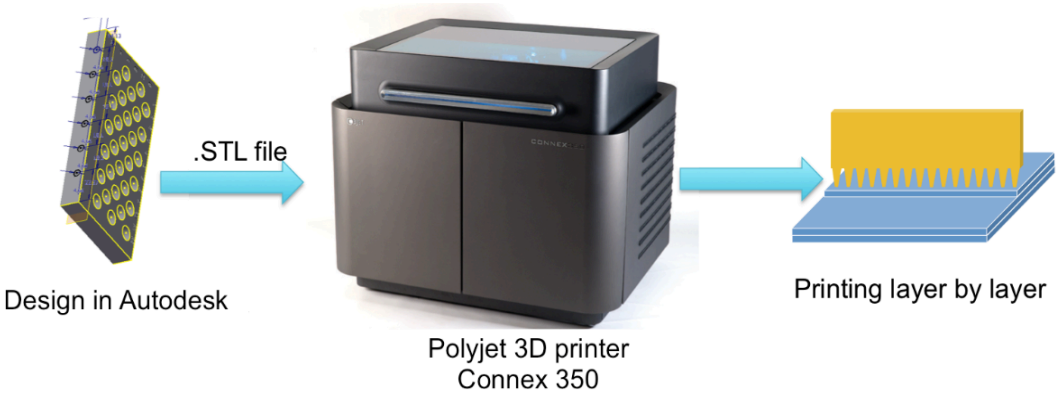

Figure 5.1. Fabrication Steps of 3D Printed Device. The model is designed using CAD software and saved in .STL format. The printer manufactures the model layer by layer until it is completed.

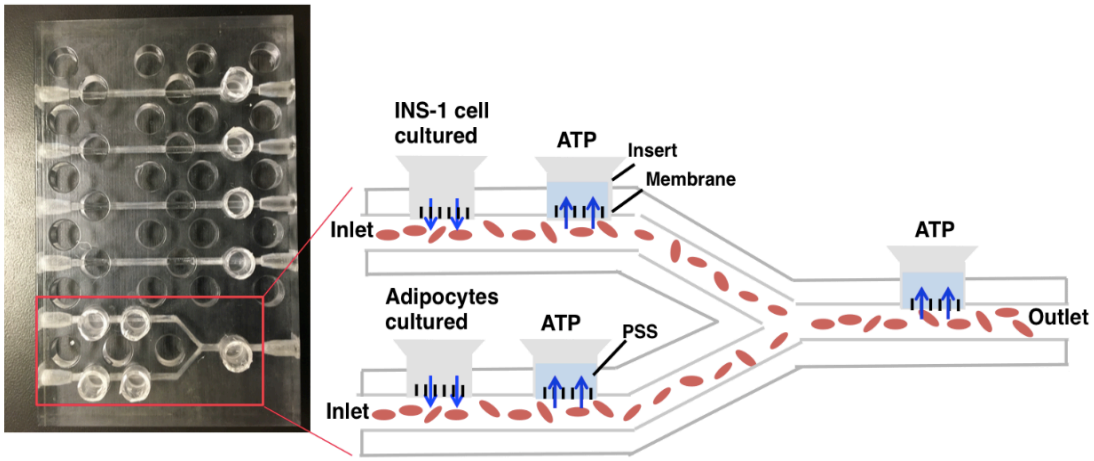

Figure 5.2. Schematic View of the Channels of 3D Printed Device. INS-1 cells and adipocytes will be cultured in the first insert of each channel. The amount of ATP released due to the secretion effect of each cell into the channels will be measured separately on the second insert. Then, the channels will be mixed and ATP release from RBCs due to the secretion effect of both cells will be measured on the third insert.

Leptin binding to RBCs needs to be investigated further, and a suggested experiment would be to quantify leptin uptake every three hours for 3-4 intervals with fresh buffer added after each interval. This will help to observe non-specific binding between leptin and RBCs when leptin starts to equilibrate between the fresh buffer and RBCs at each interval. The same experiment can be performed on samples containing C-peptide and leptin, and this will be beneficial in confirming the hypothesis of leptin-ligand specific binding to the cells.

Next, the interaction between leptin and C-peptide should be investigated since we hypothesized that leptin may serve as a carrier molecule for C-peptide. It is important to understand how leptin is binding to C-peptide and what kind of interaction occurs. This can be investigated using isothermal titration calorimeter (ITC) via measurement of the heat of the reaction. Additionally, Cody Pinger has developed a 3D printed dialysis

device in which molecules can be separated based on the pore size of the dialysis membrane. Leptin and C-peptide can be added to one side of the device and free C-peptide should be able to pass through the membrane based on the pore size. However, free leptin or leptin bound to C-peptide cannot pass through the membrane since they are larger than membrane's pore size. The amount of C-peptide that passes through the membrane can be subtracted from the initial amount of C-peptide added to quantify the amount of C-peptide bound. However, these methods will not give information about the binding site of the two molecules.

So far we hypothesized that leptin can potentially act as the carrier molecule for zinc and C-peptide and may be beneficial in diabetes complications by its effects on RBCs. However, further investigations is required to understand the interaction of leptin with C-peptide as well as RBCs.

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