LEPTIN DELIVERS β-CELL-DERIVED C-PEPTIDE TO ERYTHROCYTES

By

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ABSTRACT

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Diabetes mellitus, a global health concern, is a disorder of chronic hyperglycemia associated with deficiency or resistance to insulin. Although manageable with treatments aimed at increasing the body's utilization of insulin, often, complications associated with poor microvascular blood flow persist. Erythrocytes play an important role in regulation of microvascular blood flow by their ability to release ATP, a known stimulus of NO. Although insulin produced by the pancreatic β-cells acts on GLUT4, erythrocytes and most other bloodstream cells possess GLUT1, suggesting that a different molecular secretion may stimulate GLUT1 trafficking to the cell membrane. Fewer microvascular complications are observed for diabetic patients who possess residual pancreatic β-cell activity, suggesting that other pancreatic secretions are important.

C-peptide, the 31 amino acid peptide that connects the alpha and beta chains of insulin during its synthesis, is released into the bloodstream in equimolar quantities to insulin after its cleavage from proinsulin. The Spence group, and others, have shown that C-peptide increases erythrocyte-derived ATP release and downstream NO production. However, unlike all other groups, the Spence group has only observed cellular activity of C-peptide in the presence a carrier protein and transition metal. In vivo, the candidate metal is likely Zn^{2+}, due to its high concentrations in the pancreatic β-cells. In 2015, it was verified by ITC that HSA can carry C-peptide, with a K_a of $1.75 \pm 0.64 \times 10^{-5}$ M^{-1} and binding stoichiometry of two C-peptide to a single HSA.

Due to the long half-life of HSA (~12-21 days) it is subject to the slow process of non-enzymatic glycation, impacting its ability to act as a carrier molecule, which is particularly problematic in conditions of persistent hyperglycemia. For this reason, Spence group hypothesized that other molecules be able to carry C-peptide and Zn^{2+} to the erythrocyte. Leptin, a recently discovered adipocyte-derived hormone with a well-known role in metabolism, and a half-life of ~25 minutes, has been implicated in a variety of physiological processes, including glucose regulation, nitric oxide release and blood flow. It was confirmed that leptin, like albumin, can carry C-peptide and Zn^{2+}, increase erythrocyte-derived ATP release and stimulate GLUT1 trafficking.

This dissertation investigates of leptin's binding interactions with erythrocytes and C-peptide and how they are impacted by Zn^{2+}, albumin, and hyperglycemia. The results reported herein demonstrate that leptin has a saturable and specific binding site on the erythrocyte with K_d = (1.79 ± 0.46) x 10^{-7} M and a binding affinity in the presence of 20 nM C-peptide with K_d = (2.05 ± 0.20) x 10^{-7} M. Specific binding between C-peptide and leptin has been confirmed by SPR, with a K_d of 2.40 x 10^{-6} M. The data presented herein provide important information toward understanding the mechanism by which C-peptide and leptin are involved in erythrocyte energetics and will aid in creation of more targeted therapeutics to address the microvascular complications of diabetes.

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KEY TO ABBREVIATIONS

AC	Adenylyl cyclase
AF-PSS	Albumin-free physiological salt solution
Akt	Protein kinase B
ATP	Adenosine triphosphate
A1C	Glycated hemoglobin
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CFTR	Cystic fibrosis trans membrane conductance regulator
cGMP	Cyclic guanosine monophosphate
DDW	Double deionized water
EDHF	Endothelium-derived hyperpolarizing factor
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ERY	Erythrocyte
ESI-MS	Electrospray ionization mass spectrometry
FCS	Fluorescence correlation spectroscopy
FITC	Fluorescein isothiocyanate
GC	Guanylyl cyclase
gBSA	Glycated bovine serum albumin

gHSA	Glycated human serum albumin
GLUT1	Glucose transporter 1
GLUT4	Glucose transporter 4
HABA	4’hydroxyazobenzene-2-carboxylic acid
HLA	Human leukocyte antigen
HPLC	High performance liquid chromatography
HSA	Human serum albumin
HYNIC	Hydrazinonicotinamide
ITC	Isothermal Titration Calorimetry
JAK	Janus kinase
LC-MS	Liquid chromatography mass spectrometry
LED	Light-emitting diode
L-NAME	N^{w}-nitro-L-arginine methylester
MEK	Methyl ethyl ketone
MWCO	Molecular weight cut-off
NO	Nitric oxide
NPY	Neuropeptide Y
PBS	Phosphate buffered saline
PKA	Protein kinase A
PSS	Physiological salt solution
SADH	Streptavidin in dextran hydrogel
SP	Surface plasmon
SPR	Surface plasmon resonance

SRP	Signal recognition particles
STAT	Signal transducer and activator of transcription
TLC	Thin-layer chromatography
TRIS	Tris(hydroxymethyl)aminomethane

Chapter 1: Diabetes History, Complications and C-peptide

1.1 Diabetes Statistics

Diabetes mellitus (commonly known as diabetes) is a cluster of metabolic diseases characterized by hyperglycemia (elevated concentrations of bloodstream glucose) ensuing from defects in either insulin action or secretion, or both. Chronic hyperglycemia results in long-term damage, including organ dysfunction and failure, affecting in particular the kidneys, eyes, vasculature and nerves.[1] According to the 2016 World Health Organization (WHO) Global report on diabetes, worldwide, approximately 422 million adults were living with diabetes as of 2014, compared with 108 million in 1980. The global incidence of diabetes has almost doubled since 1980, from 4.7% to 8.5% of the adult population because of an increase in risk factors including being obese or overweight. Diabetes caused 1.5 million deaths in 2012, with another 2.2 million deaths associated with higher-than-ideal blood glucose, which is a risk factor for cardiovascular and other diseases. Of these deaths, 43% occurred before the age of 70, with the percentage of deaths due to high blood glucose or diabetes occurring before age 70 being higher for low- and middle-income countries than for high income countries. Over the past decade, the incidence of diabetes has risen faster for low and middle income countries relative to high income countries.[2]

In the United States as of 2018, 34.2 million people, or 10.5% of the U.S. population, had diabetes. Of these, 34.1 million, or 13%, were adults (age 18 or over), and another 7.3 million adults met laboratory criteria for diabetes but were not aware of

or did not report having diabetes. The percent of adults with diabetes increased with age, reaching 26.8% for those 65 or older. From 1999-2016, age adjusted prevalence of adults significantly increased, with estimates of 9.5% from 1999-2002 and 12% from 2013-2016.[3]

1.2 Classification of Diabetes

Many individuals do not fit into a certain classification for diabetes and deciding a type often depends on circumstances at the time of diagnosis. For a person with gestational diabetes for example hyperglycemia may continue even after delivery and result in a diagnosis of type 2 diabetes. On the other hand, a person who develops diabetes due to a high dose of exogenously administered steroids may become normoglycemic after steroids are discontinued, only to develop diabetes years later. Another example is a person treated with thiazides (a diuretic) who develops diabetes many years later. Because thiazides themselves rarely lead to hyperglycemia, it is likely that individuals who develop diabetes after thiazide use have a predisposal to type 2 diabetes that is provoked by the drug.[1]

The amount of hyperglycemia may change over time depending on the underlying disease process and degree of progression (Figure 1.1). The disease process can impair fasting glucose and/or glucose tolerance while not fulfilling the diagnostic criteria for diabetes. In some patients, sufficient glycemic control may be achieved with weight reduction, exercise and/or a glucose lowering agent, and the patient will not require administration of insulin. Other individuals will have residual insulin secretion yet still

require exogenous insulin to achieve normoglycemia but can survive without it. Still some others will have widespread β-cell destruction with no residual insulin secretion and require administration of exogenous insulin to survive. The severity of metabolic abnormality in any one case may progress, regress, or stay the same. The degree of hyperglycemia is reflective of the severity of disease and the prescribed treatment more than the origin of the disease itself.[1]

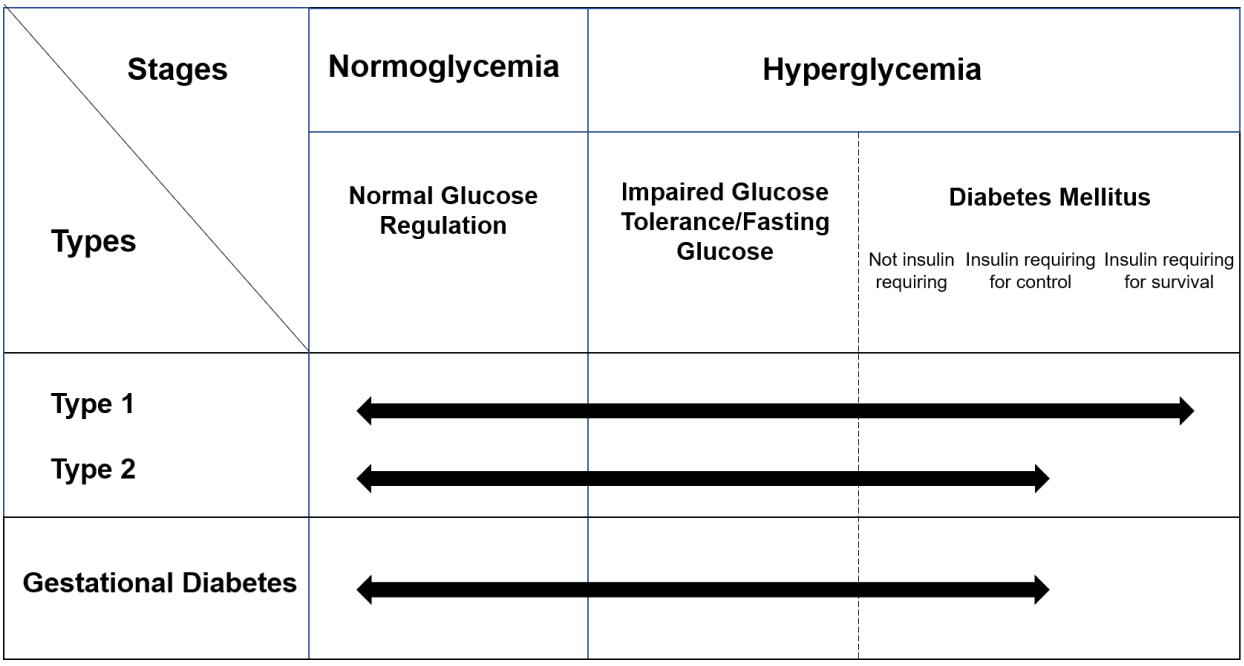

Figure 1.1. Disorders of glycemia – stages and etiologic types. Adapted from American Diabetes Association, Diagnosis and classification of diabetes mellitus, 2014.

1.2.1 Type 1 Diabetes

Type 1 diabetes is an immune-mediated form of diabetes and includes only 5-10% of those with diabetes. It has formerly been known by the terms insulin-dependent diabetes, or juvenile-onset diabetes, and arises from a cellular-mediated autoimmune

destruction of the pancreatic β-cells. Although this form of diabetes commonly occurs in childhood or adolescence, it can occur at any age, including the last decades of life. It is marked by the presence of autoantibodies[a,4] to the pancreatic islet cells, insulin, glutamic acid decarboxylase and tyrosine phosphatases.[1] One or more of these autoantibodies are usually detected in 85-90% of those with fasting hyperglycemia. Type 1 diabetes has strong links to variations in the human leukocyte antigen (HLA), particularly to HLA DQ beta and HLA DR beta, which can be either causative or protective.[1]

The rate of β-cell destruction in type 1 diabetes can be vastly different; rapid in some (usually infants and children) and slow in others (particularly adults). Some, particularly children and adolescents, may present with the first symptom of acute ketoacidosis,[b, 4] while others will have moderate fasting hyperglycemia that can quickly change to severe hyperglycemia with or without ketoacidosis in the course of infection or other stress.[1, 5] Still others (usually adults) will have enough residual β-cell function to avoid ketoacidosis for many years, although such individuals usually eventually require administration of exogenous insulin to survive.[1]

Risk factors for autoimmune destruction of the pancreatic β-cells includes genetic predisposition and environmental factors, many of which are still under investigation. Obesity is not normally correlated with type 1 diabetes but may be present, and usually,

[a] An autoantibody is an antibody that is made by an organism in response to a component of its own tissues.

[b] Ketoacidosis is a serious complication of diabetes in which there are high levels of blood acids called ketones. Without enough insulin, the body begins to break down fat as fuel, which leads to this build-up.

individuals with type 1 diabetes have increased susceptibility to other autoimmune disorders.[1]

1.2.2 Type 2 Diabetes

Type 2 diabetes has previously been known as adult-onset or non-insulin dependent diabetes and includes individuals who have insulin resistance and normally relative rather than complete insulin deficiency. In the beginning, and often throughout life, individuals with type 2 diabetes will not require administration of exogenous insulin to survive. There are likely many different factors that may lead to a diagnosis of type 2 diabetes, and some are not known. However, autoimmune destruction of the β-cells does not occur.[1]

Obesity is usually associated with type 2 diabetes, and obesity itself may cause some degree of insulin resistance. Individuals with type 2 diabetes who are not obese usually have a raised percentage of body fat distributed mainly in the abdominal region. Unlike type 1 diabetes, ketoacidosis rarely occurs spontaneously with type 2 diabetes (although it may be seen during stress of another illness or infection).[1] Type 2 diabetes can go undiagnosed for many years as the hyperglycemia develops gradually and at early stages is typically not severe enough to cause the characteristic symptoms of diabetes. (*i.e*. increased thirst, hunger, urination, fatigue, blurred vision)[1, 6] For these reasons, such individuals are at an increased risk of developing complications.[1]

While individuals with this form of disease may have normal or even elevated insulin levels, with normal β-cell function, the higher blood sugar levels observed in these

individuals would be expected to result in higher insulin levels as a response. Therefore, insulin secretion is considered defective because it is insufficient to make up for insulin resistance. While insulin resistance may improve with weight reduction through diet and exercise or by treatment of hyperglycemia with pharmaceuticals, it is rarely returned to normal. Risk of developing type 2 diabetes increases with age, obesity, and sedentary lifestyle. It is seen more commonly in women with a prior diagnosis of gestational diabetes and individuals with hypertension or dyslipidemia (abnormal blood lipid levels) and varies in frequency between different racial and ethnic groups. Interestingly, although the pathophysiology is complex and largely undefined, type 2 diabetes is more associated with a genetic predisposition than type 1 diabetes.[1]

1.3 Diagnostic Criteria for Diabetes

The diagnosis of diabetes for years was based on glucose measures, either oral glucose tolerance test or fasting plasma glucose[1] until in 1997 the first Expert Committee on the Diagnosis and Classification of Diabetes Mellitus updated the diagnostic criteria using the observed relationship between retinopathy and fasting plasma glucose levels as a main factor to identify threshold glucose level.[7] The analyses of epidemiologic studies evaluating the link between retinopathy and glycemia by means of fasting plasma glucose, 2 hour plasma glucose and A1C[c, 8] showed that below a certain level of glycemia

[c] A1C – Also known as hemoglobin A1c, glycohemoglobin, or HbA1c is a measure of glycation of hemoglobin, or how much glucose is attached to the body's hemoglobin protein.

there was little evidence of retinopathy, while above that level prevalence of retinopathy increased in a linear fashion. The analyses lead to a new diagnostic cut off point of greater than or equal to 126 mg/dl (7.0 mmol/L) for fasting plasma glucose and corroborated the long-standing diagnostic 2 hour plasma glucose level of greater than or equal to 200 mg/dl (11.1 mmol/L).[7]

A1C is a broadly used marker of chronic glycemia because it reflects an average of blood glucose levels over the span of 2-3 months. A1C plays an important role in the control of diabetes because it correlates with the presence of vascular complications and is an indicator of glycemic management. Although previously A1C has not been used as a diagnostic tool due to a lack of standardization for assays, now A1C assays are highly standardized and results can be consistently applied for a single patient at different time points as well as between patient populations. [1] In 2009, an International Expert Committee, in a decision confirmed by the American Diabetes Association, after examination of established and emerging epidemiological evidence, began to recommend the use of A1C test to diagnose diabetes with a cutoff of greater than or equal to 6.5%.[1, 9]

1.4 Vascular Complications of Diabetes

The life expectancy of adults with diabetes is decreased by an average of 5-10 years, and annual mortality is about 5.4%, or approximately double that for non-diabetic adults. While this increase is primarily due to cardiovascular disease, deaths for non-cardiovascular causes are also increased. Diabetes increases the risk for development

of vascular complications that are largely irreversible. While duration of diabetes is a key factor in progression of pathogenesis, coexisting health issues such as hypertension or high cholesterol and lifestyle factors such as cigarette smoking may contribute.[10] Microvascular complications of diabetes affect the small blood vessels in the body and include retinopathy, nephropathy and neuropathy, while macrovascular complications affect the large vessels and include coronary and peripheral artery disease as well as stroke.[11]

1.4.1 Microvascular Complications of Diabetes

There is a strong correlation between the occurrence and advancement of microvascular complications and glycemic control. In the Diabetes Control and Complications trial, which was a study of type 1 diabetes, in patients given intensive treatment with insulin the number of clinically relevant microvascular complications was reduced by 34-76% in patients administered rigorous treatment with insulin, leading to a 10% mean reduction in glycated[d, 12, 13] hemoglobin levels, to 7.2% from 8%, but the patients had more frequent hypoglycemic events.[14] For patients with type 2 diabetes, in a United Kingdom prospective diabetes study, tight glucose control diminished glycated hemoglobin concentrations by an average of 0.9% compared to standard treatment with

[d] Glycation is a covalent, non-enzymatic, post-translational, substate concentration-dependent and irreversible process linking the aldehyde group of glucose and other reducing sugars to proteins.

a mean glycated hemoglobin level of 7.0% vs. 7.9%, leading to a 25% reduction overall for microvascular complications.[10]

One of these complications, known as diabetic retinopathy, is a progressive disorder associated with various clinical abnormalities and is the most common cause of blindness for individuals 30-69 years. Retinal damage can arise from microvascular leakage and occlusion (blockage). Almost 1/5 of patients with type 2 diabetes have retinopathy to some degree at the time of diagnosis, although in type 1 diabetes retinopathy virtually never happens in the first five years post diagnosis or prior to puberty. However, after 15 years, virtually all patients with type 1 diabetes and nearly 2 out of 3 patients with type 2 diabetes will have some background retinopathy.[10] Retinopathy (Figure 1.2) is a term describing damage to any portion of the retina of the eyes, which may lead to vision impairment.[15] In type 1 diabetes, vision threatening retinopathy is often due to neovascularization, or formation of new blood vessels within the microvasculature in response to poor blood flow. Unfortunately, these vessels often leak, leading to a cloudy vitreous and resultant reduced vision. In type 2 diabetes, retinopathy often presents as maculopathy[10], or a pathological condition of the macula, a small spot in the retina that is responsible for highly sensitive and accurate central vision.[16]

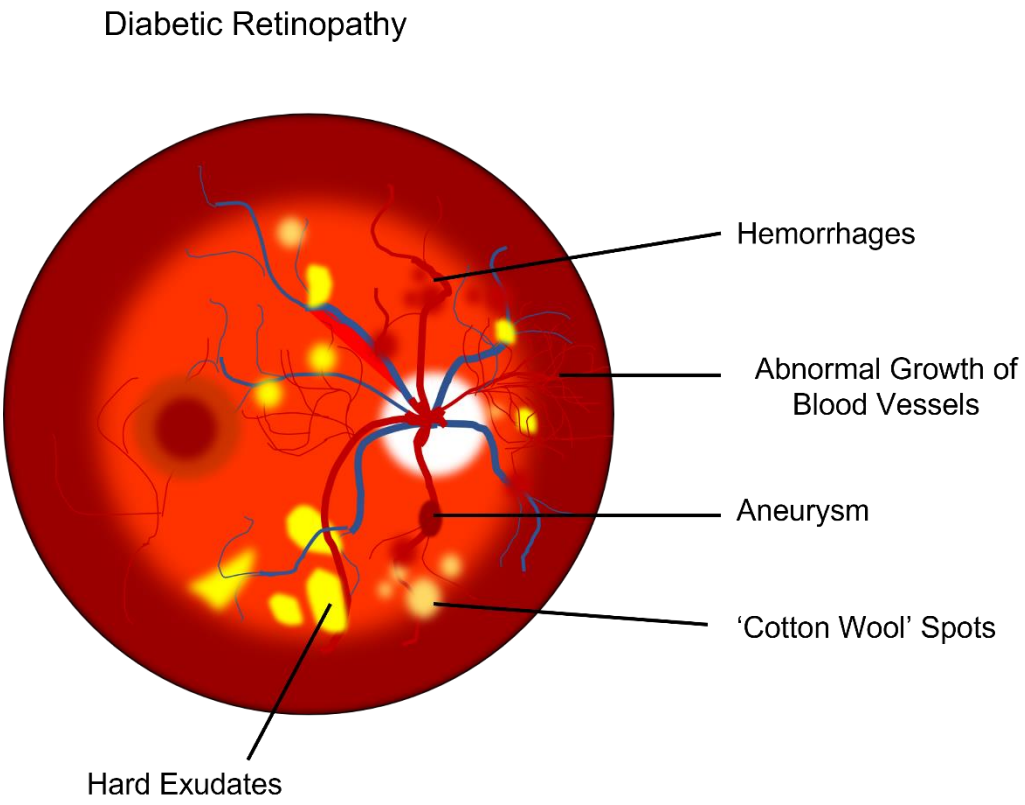

Figure 1.2: Eye with diabetic retinopathy. Poor blood flow associated with diabetes results in damage to the blood vessels of the retina. New, weakened vessels grow, clouding the vitreous and resulting in poor eyesight or blindness.

Diabetic nephropathy, also known as renal or kidney disease, is a term describing damage of a kidney, and is characterized by protein secreted in the urine (proteinuria) greater than 300 mg over 24 hours, increased blood pressure and diminishing renal function. The most severe form of diabetic nephropathy leads to end stage renal disease that requires dialysis or transplantation, but early stages, or incipient nephropathy, are characterized by microalbuminuria, where the urine contains small quantities of protein that are not detectable by conventional dipstick testing. Microalbuminuria is defined by an albumin excretion rate of 20-300 mg over 24 hours or 20-200 μg/minute in a timed collection and is foretelling of development of acute nephropathy, for type 1 diabetes.

While rate of decline in glomerular function can vary greatly between individuals, treatment of hypertension can greatly slow the decline in renal function and increase survival. Development of proteinuria is a marker of pervasive vascular damage and marks a heightened risk for later end stage renal disease and macrovascular complications, especially for cardiovascular disease. Both microproteinuria and proteinuria are linked to lessened survival for type 1 and type 2 diabetes.[10]

Neuropathy describes dysfunction of one or more peripheral nerves, and typically presents as numbness or weakness.[4] Diabetic neuropathy can present in many ways, and in the most common form presents as a diffuse progressive polyneuropathy that primarily affects the feet. This condition is predominantly sensory and can be asymptomatic, affecting 40-50% of all patients with diabetes.[10] Neuropathy can be detected by a monofilament test to assess sensory neuron and motor responses.[17] Patients with sensory neuropathy and other risk factors of diabetes must be careful with their foot care to minimize the risk of ulceration. While neuropathic foot ulcers are distinct from vascular ulcers, it is common for these to occur together.[10]

1.4.2 Macrovascular Complications of Diabetes

Atherosclerosis, a disease where the walls of the arteries develop abnormal lesions,[18] accounts for most of the excess mortality in patients with diabetes. However, the relationship between glucose control and macrovascular events is less dominant than for microvascular complications, with risk factors such as smoking, high blood pressure,

proteinuria and high cholesterol being more important for development of large vessel disease in patients with diabetes.[10]

Hyperlipidemia, or the abnormal elevation of lipids or lipoproteins in the blood,[19] is not more common in patients with type 1 diabetes than the general population.[10] However, in patients with type 2 diabetes, although the total and low density lipoprotein concentrations are similar to those of non-diabetics, there is a more atherogenic lipid profile, in that there are lower than normal concentrations of high density lipoprotein and higher than normal concentrations of small density lipoproteins.[10]

At least half of patients with diabetes suffer from hypertension; tight blood pressure control can reduce the risk of stroke, heart failure, diabetes-related deaths and microvascular complications.[10] Although a single direct cause for chronic diabetic complications is not evident, several reports suggest that an underlying dysfunction at the interface between the blood stream and peripheral tissues leads to vessel pathology.[20, 21]

1.5 History of Diabetes Research

Clinical features of diabetes were first described 3000 years ago by the ancient Egyptians. In the Ebers papyrus, which dates to 1550 BC there are descriptions of various diseases, among which there is a polyuric syndrome that is likely diabetes.[22] The Verdic medical treaties of ancient India also elaborate congenital and late-onset diabetic conditions.[23] The term diabetes was given by Araetus of Cappadocia (81-138 AD) who described diabetes as a polyuric wasting disease. Diabetes is a Greek word meaning ‘to run through or siphon’, due to the excessive thirst and urination associated with the

disease.[24] The word mellitus, meaning ‘honey sweet’, was added by Thomas Willis of Britain in 1675, to describe the sweetness of urine and blood of patients.[25]

In modern times, experimental influences of modern medicine have increased our understanding of diabetes, with two significant achievements leading the way. The first was the use of chemistry as a diagnostic tool beginning in the second half of the 18th century, and the second was the rise of endocrinology as a proper discipline because of the works of Claude Bernard (1813-1878) and Brown-Sequard (1817-1894). Bernard introduced the concept of organs of internal secretions (glands) and Brown-Sequard showed that infusing blood from healthy animals could delay death from adrenalectomy (removal of adrenal glands).[26] In 1776, Mathew Dobson, a physician of Liverpool, confirmed that there is sugar in the urine and blood of diabetic patients. In 1815, a French chemist named Michael Chevreal showed that this sugar was glucose.[27] Claude Bernard, who is considered the founder of experimental medicine for applying physical and chemical methods to artificially induce disease, proposed the idea of excess glucose production in patient’s livers with the aid of an enzyme and described the formation of liver glycogen and its fate in the liver and other places in the body.[26]

Proof that diabetes is due to a defect of the pancreas was the outcome of critical works of Joseph von Mering (1849-1908) and Oskar Minkowski (1841-1904), who showed that pancreatectomized dogs developed rapidly fatal diabetes that was not related to loss of flow of pancreatic juices to the intestine, implying that the pancreas plays some role in metabolism of carbohydrates.[28] Even though the role of pancreatic islets in the disease process of diabetes was known by the end of the 19th century, it took another 30 years for insulin to be discovered, isolated and be used clinically.[29]

Frederick Banting predicted that it may be possible to separate internal pancreatic secretions by ligating the pancreatic ducts, which would lead to atrophy of the acinar cells (the exocrine cells of the pancreas responsible for production and transport of enzymes)[30] to decrease contamination of the tissue extracts with digestive enzymes.[31] The suggestion was presented to J. J. R. Macleod, a well-known physiologist at the University of Toronto, who made available to Banting a summer laboratory, dogs for experiments, and arranged for Charles Best to assist Banting. By the fall of 1921, Banting and Best were able to separate a substance from pancreatic extracts which significantly increased the lifespan of dogs made diabetic by excision of the pancreas, and by winter of 1922, Banting and Best were able to save the life of a young by treatment with the separated substance. Aid was acquired by Macleod to allow for large scale commercial preparation of insulin, with the patent received by the University of Toronto. By 1923, large enough quantities of insulin were available for widespread treatment of diabetes.[31]

1.6 Insulin

Insulin is a peptide hormone secreted by the pancreatic β-cells in the islets of Langerhans that facilitates maintenance of normal blood glucose levels by aiding cellular glucose uptake, regulating lipid, carbohydrate and protein metabolism and assisting cell growth and division by mitogenic effects.[32] Although the monomeric structure of insulin was first discovered using X-ray crystallography and reported in 1926,[33] it took another 40 years for the structure of hexameric insulin containing zinc to be solved solved.[34-37] 2D NMR studies have provided information on the monomeric, dimeric and hexameric insulin

conformations, providing insight on the native insulin structure and amino acids that specifically bind to the insulin receptor.[38] The conformational state of insulin is influenced by its concentration and the surrounding pH,[39] with interactions among hydrophobic amino acids favoring aggregation of insulin monomers as concentration rises, leading to formation of dimers, and in the presence of around 10 mM Zn^{2+} and pH 6, aggregation into densely clustered 'granules' of insoluble crystalline hexamers, with a concentration of approximately 40 mM.[39, 40] Hexameric insulin is composed of six molecules of insulin peptide arranged as three dimers.[38] In the insulin hexamer, two zinc atoms are coordinated with the imidazole groups of three histidine residues and three water molecules.[40] Interactions are weaker between the dimers within the hexamers than for the interactions within the dimer, with greater van der Waals forces between the dimers within the hexamers than for the monomers within the dimers, making the hexamer configuration less stable and likely to disassemble when concentration fluctuates *(i.e.*, during secretion into the bloodstream). When insulin hexamers are secreted from the β-cell and diffuse down their concentration gradient into the blood, electrostatic forces, decreased insulin concentration and high pH[41] favor dissociation of insulin into its active, monomeric form.[38]

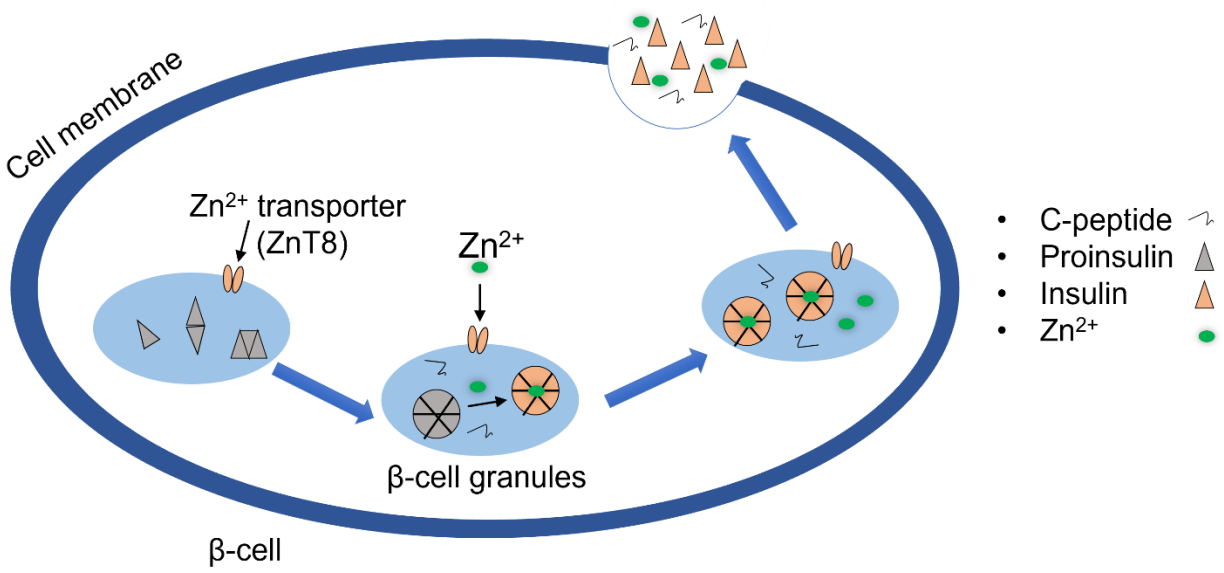

Figure 1.3: Maturation process of insulin. As concentration rises, insulin monomers aggregate. Within the insulin hexamer, two Zn^{2+} ions are coordinated with the imidazole groups of three histidine residues and three molecules of water. Upon secretion from the β-cell, with fluctuations in concentration and pH, insulin hexamers dissociate. Adapted from Medawala et al., *Rev. Diabet. Stud.* **2009**, *6* (3), 148-58.

Even though secreted insulin is comprised of 51 amino acids, the gene for insulin encodes a 110-amino acid precursor called preproinsulin that like other secreted proteins includes a hydrophobic N-terminal signal peptide that interacts with cytosolic ribonucleoprotein signal recognition particles (SRP).[42] SRPs help to transport preproinsulin across the rough endoplasmic reticulum membrane into the lumen by the peptide-conducing channel,[43, 44] where a signal peptidase cleaves the signal peptide of insulin to reveal proinsulin.[45] Proinsulin folds and forms three disulfide bounds,[46] in a process requiring a diverse set of endoplasmic reticulum chaperone proteins. [47] Before maturation of the three-dimensional conformation, the folded proinsulin is transported from the endoplasmic reticulum to the Golgi apparatus where it moves into immature secretory vesicles and is cleaved to produce insulin and C-peptide, the latter being a 31-

amino acid peptide.[48] Before secretion into the bloodstream, C-peptide and insulin are stored in the secretory granules alongside less abundant β-cell secretory products.[48, 49]

Administration of exogenous insulin, which is the standard treatment for type 1 diabetes, does not completely alleviate microvascular complications, and insulin itself has no direct effect on blood stream cells.[50,51] Insulin stimulates glucose transport through glucose transporter 4 (GLUT4) but most blood stream cells, including erythrocytes, neutrophils, macrophages and T-cells do not have GLUT4, and accomplish glucose transport through glucose transporter 1 (GLUT1).[51, 52] Also, since the half-life of insulin in the bloodstream is in the order of 4-6 minutes,[53] its role in control over bloodstream properties is likely minimal, suggesting a role for other pancreatic β-cell secretions.

1.7 C-peptide

C-peptide was first described by Steiner and colleagues in 1967 as a precursor in the biosynthesis of insulin.[54] Although the physical properties of insulin and its amino acid sequence had been known and described for a number of species,[55, 56] little was known about how this molecule was made. However, it was known that the two polypeptide chains of insulin do not readily combine in high yield under normal physiological conditions.[57] It was initially speculated that analogous to another pancreatic secretion, the digestive enzyme chymotrypsin, the A and B chains of insulin may be synthesized sequentially and then cleaved after the native structure, including proper formation of disulfide bridges, had been established.[58] Steiner studied the biosynthesis of insulin in two β-cell tumors removed from individuals suffering from recurrent severe

hypoglycemia.[54, 59] Gel filtration data for protein purified from tumor slices showed presence of an earlier eluting component that might contain insulin bound to other polypeptide material in the form of a complex.[54, 59] These studies with islet cell tumors,[54, 59] along with studies of rat[54, 60] and codfish islets[61] and fetal calf pancreas[62] demonstrated that insulin is formed from a larger single chain polypeptide precursor, proinsulin.

In between the A and B chains of porcine[63] and bovine[64] proinsulin, there is a connecting sequence of 30 (bovine) or 33 (porcine) amino acids. Although the sequences differ between the two species, identical pairs of basic amino acids occur at each end for both species, and this basic segment can be freed with limited tryptic digestion.[63, 65] In later experiments in rat islets, a large peptide containing most of the connecting peptide and similar to the tryptic peptide was found.[60] The peptide has also been identified in extracts of human and bovine pancreas, with the bovine peptide having an identical sequence to the bovine proinsulin connecting segment, without the terminal basic residues.[66] This peptide that is now known as C-peptide is held in the β-cell with insulin in equimolar amounts following cleavage from proinsulin (Figure 1.4),[66] and is secreted with insulin from the β-cell into the circulation. The presence of C-peptide and insulin in equimolar quantities in pancreatic extracts and labeling experiments with islets hinted that C-peptide may be important for granule formation, stability or release.[67]

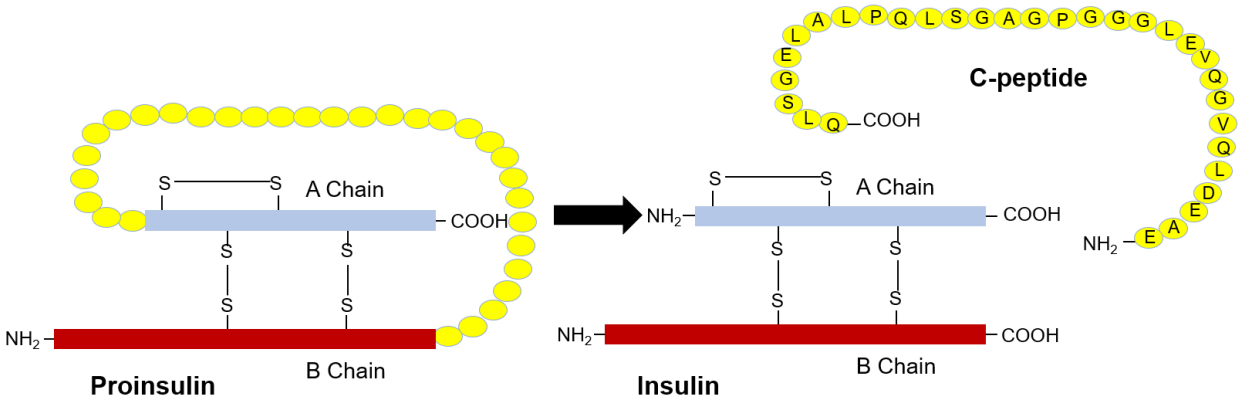

Figure 1.4: Depiction of insulin and C-peptide liberation from the proinsulin molecule. In proinsulin, the A and B chains of insulin are connected by C-peptide and disulfide bonds. C-peptide is excised by β-cell peptidases prior to release into the bloodstream.

1.7.1 Early Studies of C-peptide and Clinical Use

C-peptide fulfills an important biological role by supporting formation of the correct secondary and tertiary structure of insulin by correct positioning of disulfide bonds. The question has also been raised whether C-peptide is secreted from the β-cell with insulin as a convenient method of disposal, or if it plays a regulatory biological role.[68] Several early studies examined possible biological effects of C-peptide,[69] and while insulin-like effects on blood glucose levels or glucose level after glucose loading have not been detected,[70, 71] studies of rat C-peptide showed that C-peptide decreases glucose-stimulated insulin release both in vivo and in vitro.[72-76] It has also been reported that C-peptide inhibits arginine-stimulated glucagon release from isolated perfused rat pancreas[75] and inhibits fat-stimulated gastric inhibitory polypeptide secretion by intestinal cells.[72] Despite the data suggesting that that exogenously administered C-peptide decreases the need for insulin by facilitating glucose disposal, for a number of years, it

was thought that C-peptide did not possess biological activity apart from its role in insulin biosynthesis.[69]

Due to the fact that C-peptide is secreted into the circulation in equal quantities to insulin, but excreted from the body in a more constant rate over a longer time,[77] C-peptide is clinically useful as a method of determining insulin secretory status in diabetes treated with insulin and patients with hypoglycemia.[78] The rate of degradation of C-peptide in the body is slower compared to that of insulin (the half-life is ~30-35 minutes for C-peptide[79] whereas it is ~4-6 minutes for insulin[53]) and for this reason gives a more stable testing window for the fluctuating β-cell response.[78] The plasma concentration of C-peptide in the fasting state in healthy individuals is 0.3-0.6 nmol/l,[80] and although half of all secreted insulin from the pancreas is metabolized by the liver in first-pass metabolism, C-peptide has little hepatic clearance. C-peptide is cleared from peripheral circulation at a constant rate, while insulin is inconsistently cleared, which makes direct measurement of insulin less steady. Also, in diabetes, because C-peptide is not administered with insulin, measuring C-peptide allows determination of endogenous vs. exogenous insulin.[78]

Studies have demonstrated that there is a strong correlation between incidence of microvascular complications and glycemic control in diabetes.[81, 82] Several investigations in the 1980s showing of patients with type 1 diabetes have detectable C-peptide (measured in plasma or urine) have demonstrated that enduring insulin production is a key factor.[83, 84] In 1987, Östman and coworkers verified through analysis of C-peptide levels in plasma and urine that patients with type 1 diabetes who have residual β-cell function experience less microvascular complications and improved glycemic control compared to those who do not.[85] This important finding initiated a revival of the

investigation of C-peptide as a bioactive molecule, and between 1990 and 2000, more than 100 peer-reviewed articles were published examining the therapeutic potential and bioactive capacity of C-peptide.[86]

1.7.2 Biological Effects of C-peptide

Research has provided insights into the mechanism of signal transduction of C-peptide and cellular effects, with impacts on nerve and renal function, and glucose utilization. While most species only produce one form of proinsulin, mice and rats produce two isoforms, 1 and 2, that differ from three (mouse and two (rat) amino acid residues on the C-peptide portion of proinsulin.[69] Although rat C-peptides 1 and 2 differ by two amino acid residues,[87] they can stimulate sodium-potassium adenosine triphosphatase (Na^+-K^+-ATPase) activity to an equal degree,[88, 89] with a scrambled form of C-peptide having no discernable effect. Other studies have shown that C-peptide can stimulate Na^+-K^+-ATPase activity in rat sciatic nerve,[90, 91] granulation tissue,[90] pancreatic islets[92] and erythrocytes.[93] C-peptide also improves deformability of erythrocytes from patients with type I diabetes.[94]

It has been proposed that C-peptide may act in cooperation with other hormones. The dose response curve for C-peptide and Na^+-K^+-ATPase activity in renal tubular cells is shifted two orders of magnitude to the left in the presence of subthreshold concentrations of neuropeptide Y (NPY), which indicates increased C-peptide effectiveness in the presence of NPY, while no synergism has been observed for C-peptide and insulin for Na^+-K^+-ATPase activity.[88] A low insulin concentration, however,

enhances the Na^+ -K^+ -ATPase activity of human C-peptide on rat muscle arterioles.[95] Additional studies will increase understanding of these phenomena.

For patients with type 1 diabetes, administration of C-peptide overlaps with a circulatory response, with an increase in blood flow in skeletal muscles at rest and during exercise, redistribution of skin microvascular blood flow and enhanced capillary blood cell velocity.[96] C-peptide increases blood flow, capillary filtration coefficients and permeability surface area product in isolated and perfused rat hindquarter, which signifies recruitment of capillaries.[97] Reports suggest that C-peptide achieves vasodilation through an increase in intracellular Ca^{2+} concentration that stimulates endothelial nitric oxide synthase (eNOS) activity.[98-100] C-peptide has been shown to increase nitric oxide (NO) release from bovine aortic endothelial cells in a concentration-dependent way, and this release was abolished by addition of an NO synthase inhibitor.[99] Forearm blood flow improvements caused by C-peptide in type 1 diabetic patients can be prevented with an NO synthase blocker.[98] It has also been reported that C-peptide can stimulate a concentration-dependent dilation of rat skeletal muscle arterioles that occurs by an NO mechanism.[95]

Thus C-peptide, through activation of Ca^{2+}-dependent signal transduction pathways, stimulates Na^+ -K^+ -ATPase[88-90, 93] and eNOS activities.[95, 98, 99] Both of these enzymes are known have decreased activity in type 1 diabetes, particularly in renal and nerve tissue.[69] There is evidence that C-peptide replacement in type 1 diabetes leads to improved renal function, which is demonstrated by alleviation of glomerular hyperfiltration,[101-103] decreased urinary albumin secretion[101, 104] and elimination of nerve dysfunction.[104, 105]

Diabetes is associated with morphological and functional changes in the microcirculation, and it is firmly established that erythrocyte properties of diabetic patients are abnormal.[106-109] Abnormalities of diabetic erythrocytes include decreased deformability,[110, 111] decreased membrane viscosity,[112] as well as increased erythrocyte aggregation.[113] Although the molecular basis for these erythrocyte abnormalities is not known, it is thought to be related to non-enzymatic glycation of proteins and proceeding antioxidative imbalance.[114, 115] Erythrocyte deformability is important for sufficient perfusion in the microvasculature. The modified lipid composition of diabetic erythrocyte membrane and disruptions in ion homeostasis are linked to decreased Na^+ -K^+ -ATPase activity, with the latter leading to intracellular accumulation of sodium and calcium ions.[106, 116, 117]

The finding by Forst et al. that replacement of C-peptide in type 1 diabetic patients led to improvements of microvascular blood flow to levels similar to those of healthy subjects,[118] and the discovery of C-peptide activation of endothelial nitric oxide synthase by intracellular calcium ion influx[99] led to the hypothesis that C-peptide may affect rheologic[e, 4] properties of erythrocytes.[94] Using PC-based laser diffraction, Kunt et al. investigated the deformability of erythrocytes in type 1 diabetic subjects in relation to normal subjects to determine that erythrocyte deformability is significantly lower in type 1 diabetic patients relative to normal patients. When the erythrocytes were incubated with several concentrations of C-peptide, it was found that treatment with C-peptide could restore the deformability of diabetic erythrocytes to a level statistically like control

[e] Rheologic – relating to the science of deformation and flow of matter.

erythrocytes, but C-peptide had no effect on control erythrocytes.[94] Since different concentrations of C-peptide had a comparable effect on erythrocyte deformability, it was thought that basal physiological levels of C-peptide are enough to restore deformability of diabetic erythrocytes.[99] Follow up studies by Forst et al. supported the notion that C-peptide influences microvascular blood flow in humans, providing the first in vivo evidence of an effect of human C-peptide on endothelial NO metabolism and erythrocyte Na^+-K^+-ATPase activity.[119]

1.7.3 C-peptide Binding and the Search for a Receptor

No identification of a conserved active site or established binding assay has long been a barrier to recognition of C-peptide as a bioactive hormone.[69] Initial attempts at examining the binding interactions between C-peptide and cell membranes occurred in 1986, when binding of tyrosylated ^{125}I-labeled rat C-peptide (1) was evaluated using cultured rat islet tumor cells.[120] Although evidence of specific binding was reported, evaluation of the results was complicated by enduring secretion of C-peptide from the cells during the binding studies and existence of two different rat C-peptides.[87] Other studies looking at C-peptide interaction with skeletal cell membranes by radioligand technique had difficulty demonstrating cellular binding,[121] possibly because of relatively few binding sites per unit cell surface area.[120] In research done by Ido et al. in the late 1990s, C-peptide was found to have helpful actions on vascular and neural dysfunction in experimentally induced diabetes in rats.[90] These effects with native C-peptide and also

D-enantiomer[f] and sequence reverse C-peptide, leading to the idea that nonreceptor membrane interactions may explain some of C-peptide's effects.[90, 122]

Intracellular effects of C-peptide have been observed during examination of fresh segments of proximal rat nephron, a well-defined experimental model and established target of peptide hormones.[123, 124] Rat C-peptide (1) has been found to stimulate Na^+ -K^+ -ATPase activity in a concentration dependent way in single, proximal convoluted tubules separated from rat kidneys. Pretreatment of tubules with pertussis toxin, known to influence the α-subunit of G_i proteins, completely blocked the effect. This, and other observations, led to the notion that the C-peptide signal transduction pathway may involve a G-protein interacting with a ligand[g]-activated receptor.[88]

These studies were continued by investigating Na^+ -K^+ -ATPase activity for a set of C-peptide fragments. Two sets of C-peptide fragments were found to elicit cellular Na^+ -K^+ -ATPase activity, one at the -COOH terminal region of the C-peptide molecule, and the other in the midsegment as shown in Figure 1.5. For the -COOH terminal region, maximal activity was observed for a pentapeptide segment (EGSLQ).[89] This segment gave full replacement of C-peptide activity which is characteristic of a ligand-receptor interaction. Indeed, residues at positions 1 (glutamic acid) and 5 (glutamine) of the pentapeptide segment are conserved in most mammalian species. The studies on the pentapeptide segment were in agreement with findings from other studies involving rat

[f] Enantiomer – One of two stereoisomers that are non-superimposable mirror images. L enantiomers have the hydroxyl group attached on the left side of the asymmetric carbon furthest from the carbonyl, whereas D enantiomers have the hydroxyl group on the right side.

[g] Ligand (biochemistry) - a molecule that binds another (typically larger) molecule.

pancreatic islets.[125] For the midsegment region (ELGGGFGAG), only 80% of activity of the intact molecule was attained, with the data for this segment suggesting a second site of non-specific interactions with receptor-atypical behavior.[89]

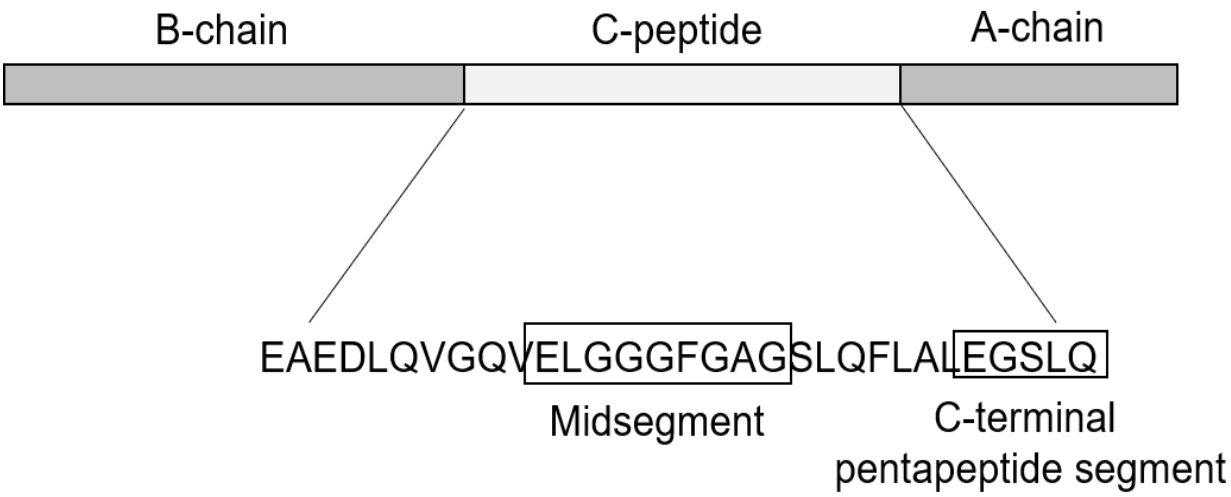

Figure 1.5: Linear representation of human proinsulin molecule showing amino acid sequence of C-peptide, highlighting the positions of the mid and pentapeptide segment that are associated with C-peptide cellular activity. The pentapeptide region (EGSLQ) mimics C-peptide in assays of Na^+ -K^+-ATPase activity and binding. Figure adapted from Wahren et al., *Amer. J. Physiol. Endocrinol. Metab.* **2000**, *278* (5), E759-68.

Fluorescence correlation spectroscopy (FCS) monitors Brownian movements[h, 4] of a fluorophore-labeled ligand after excitation with a fluorophore-focused laser beam.[126, 127] Measurements are carried out in a small volume element that can be shifted from the incubation medium with labeled ligand to the membrane surface of cells growing in culture.[69] Determination of diffusion time of the labeled ligand when it is free in the medium or bound to the cell membrane is allowed by the autocorrelation function of fluctuations

[h] Brownian motion – describes the erratic, random motion of microscopic particles in fluid, which results from unremitting bombardment from molecules in the surrounding medium.

in fluorescence intensity.[126, 127] FCS has higher detection sensitivity and enhanced signal-to-noise ratio with sub-micron resolution in comparison with radioligand binding analysis.[126, 127] In 1999, Wahren and coworkers applied FCS analysis to the characterization of C-peptide membrane interactions.[128]

FCS of human C-peptide labeled with tetramethyl-rhodamine at the N-terminus revealed specific binding to cultured human renal endothelial cells, skin fibroblasts and saphenous vein endothelial cells. This binding was determined to be stereospecific because a peptide with the same amino acid combination but random sequence or the D-enantiomer of C-peptide could not displace the bound C-peptide. Also, addition of the COOH-terminal pentapeptide segment could competitively displace bound C-peptide. Although proinsulin contains the pentapeptide segment, it could not competitively displace bind C-peptide, indicating that the free -COOH terminus is necessary for binding. Insulin, insulin-like growth factor and neuropeptide Y could not displace the binding.[128] C-peptide could not displace labeled insulin bound to cell membranes.[121, 128] The finding that proinsulin could not displace C-peptide binding was in contrast to reports which suggested that C-peptide may bind with low affinity to a proinsulin receptor.[129, 130] The FSC data also indicated species specificity, because at physiological concentrations rat C-peptide could not bind human cells.[69]

In agreement with Na^+-K^+-ATPase data,[88, 89] preincubation of cells with pertussis toxin eliminated measurable FSC binding of C-peptide at physiological concentrations.[128]

This was consistent with an allosteric[i] signal transduction mechanism, since before treatment with there was an apparent high affinity interaction of C-peptide with its receptor, but after treatment, only a possible low affinity interaction, which may be explained by a change in receptor configuration.[128, 131]

It was anticipated that in healthy humans, C-peptide receptor saturation is reached at physiological C-peptide concentrations (0.6 nM), as C-peptide effects have not been demonstrated in normal animals or healthy subjects[71, 76, 90, 102, 132-134] only in animal models of diabetes or in patients with type 1 diabetes.[94, 98, 100-105, 118, 134-136] This is in agreement with FSC data for renal tubular cells where half-saturation of C-peptide binding was determined to occur at a concentration of 0.3 nM, and full saturation at 0.9 nM.[128]

Although existing data provide insights into C-peptide membrane interactions, including stereospecific binding of C-peptide to a cell surface receptor that occurs at low nanomolar concentrations and is thought to be G-protein-coupled and mediated by the -COOH pentapeptide region, as well as nonspecific, nonchiral membrane interactions localized to the midsegment region, a more detailed understanding will require further evaluation and characterization of the receptor structure.[69]

[i] Allosteric – relating to the changing of activity of a protein by the binding of an effector molecule at a designated site.

1.8 Erythrocyte-derived ATP Release

Work relating to C-peptide involvement with erythrocytes and endothelial nitric oxide synthase activation[137] was important because of the work by Sprague et al. which showed that erythrocytes release adenosine triphosphate (ATP, Figure 1.6), an established stimulus of the potent vessel dilator NO from the endothelium. Erythrocytes release ATP in response to various stimuli, including hypoxia, mechanical deformation and other agonists.[138-140] Sprague showed that erythrocyte-derived ATP is a contributing factor of vascular resistance in the isolated rabbit lung.[141]

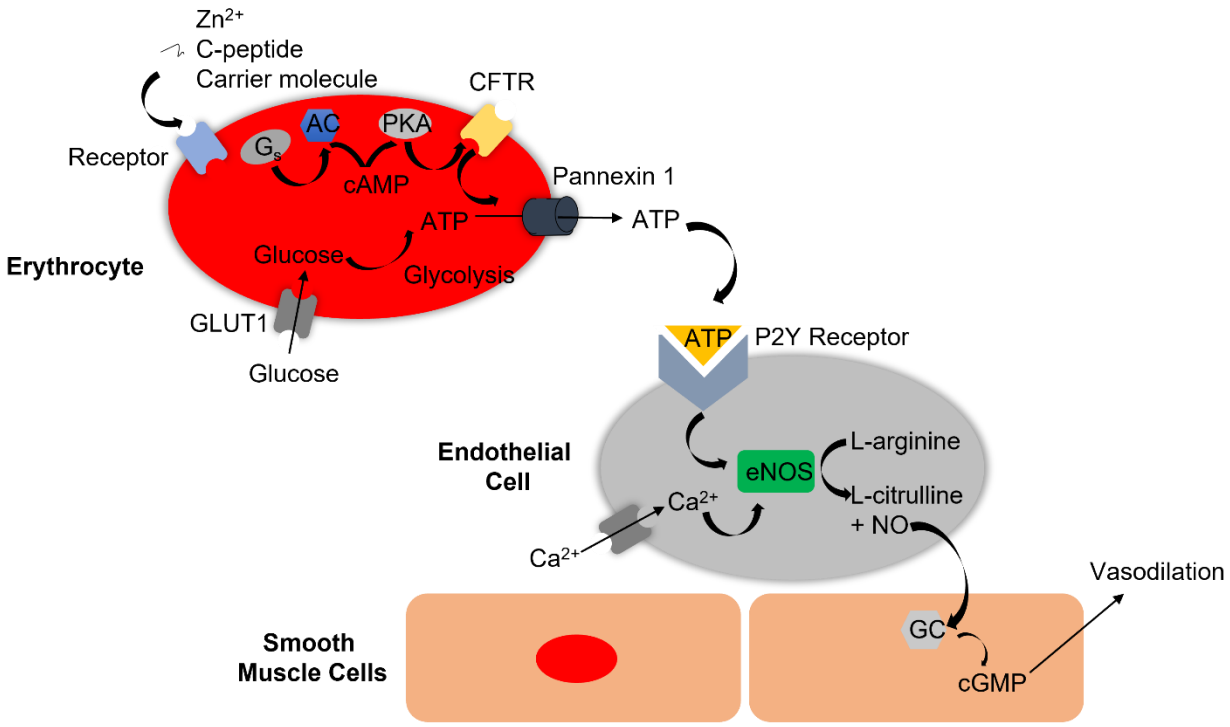

Figure 1.6: Proposed pathway for erythrocyte-derived ATP release and downstream NO release from endothelial cells. A stimulus (i.e., low O_2, shear stress) activates a cyclic guanosine monophosphate pathway leading to ATP release from the erythrocyte that binds to the P2Y receptor of an endothelial cell inducing nitric oxide release and leading to vasodilation. CFTR, cystic fibrosis trans membrane conductance regulator; PKA, protein kinase A; AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate, GC, guanylyl cyclase. Adapted from Sprague et al., *Diabetes* **2006**, *55* (12), 3588-93.

Combined reports showing that C-peptide can enhance blood flow in the microvasculature and glucose transport in specific cell types[100, 102, 121], and reports showing that erythrocytes can release ATP in response to various stimuli[138-140] led the Spence group to suggest that C-peptide may facilitate production of endothelium or platelet-derived NO by increasing ATP release from the erythrocyte.[142] In 2008, the Spence group showed that erythrocytes can release increased concentrations of ATP when incubated with C-peptide, but only in the presence of transition metals, Fe^{2+} or Cr^{3+}.The erythrocytes could release increased concentrations of ATP over a period of around 24 hours when incubated with C-peptide in the presence of Fe^{2+}, and continued ATP release over a period of 3-5 days with C-peptide in the presence Cr^{3+}. Erythrocytes incubated with C-peptide and these metals had augmented transport of ^{14}C-labeled glucose through the major glucose transporter found in the erythrocyte, GLUT1.[142] An increase in GLUT1 activity would increase glycolysis, which is the main path by which erythrocytes make ATP, and therefore activate the reported cyclic AMP-dependent pathway through which ATP is released from the erythrocyte.[143]

1.9 C-peptide and Zn^{2+} Delivery to Erythrocytes

Although the need for metal activation for C-peptide effects was an important finding in the investigation of bioactivity of the peptide because it offered a possible explanation for the lack of successful clinical trials, questions remained surrounding the role of metals in the mechanism. The choice of metals used to elicit cellular activity in the initial investigations was not arbitrary; the observation that within 24 to 36 hours after

preparation in water, C-peptide exhibited markedly reduced activity, particularly in its ability to increase concentrations of erythrocyte-derived ATP, lead to analysis of the 'inactivated' peptide by electrospray ionization mass spectrometry (ESI-MS), which showed that the peptide had not undergone any substantial change or degradation. A high-resolution scan of a triply charged region of the spectrum showed that the peptide had formed adducts with different ions including K^+ and Na^+, and Fe^{2+}. Addition of Fe^{2+} to fresh C-peptide solution in a molar ratio of 2:1 caused formation of this adduct in plenty. To lengthen the activity of C-peptide, a metal with a slower exchange rate with ligands in solution, Cr^{3+}, was added to a recently prepared C-peptide solution in a molar ration of 2:1 and again analyzed by ESI-MS, which showed the formation of C-peptide adduct with Cr^{3+}. In support of the idea that a transition metal was required for activation, C-peptide that had been purified by high performance liquid chromatography to remove trace metal impurities remaining from the manufacturing process showed no activity in the presence of erythrocytes. For these reasons, in initial studies, erythrocytes were incubated in C-peptide solutions containing Fe^{2+} or Cr^{3+}.[142]

In vivo in the pancreatic β-cell granules, C-peptide cohabits with insulin in its hexameric form[144] of six insulin molecules and two zinc atoms. Zinc transporter proteins in the β-cells keep zinc concentrations at millimolar levels,[142] and because the pH of the granules is highly acidic, granular C-peptide is protonated and thus would not easily bind to Zn^{2+}.[145, 146] The Spence group thought that when exposed to the bloodstream, C-peptide would become de-protonated and competitively bind and dislodge Zn^{2+} from hexamerized insulin, leading to the suggestion that in vivo, the metal necessary for C-peptide activity was Zn^{2+}.[142] Indeed, mass spectrometric analysis revealed that in the

absence of any insulin hexamer, C-peptide can effectively bind Zn^{2+}.[147] This finding was in agreement with previous reports that C-peptide can disaggregate hexameric insulin and that interaction between hexameric insulin and C-peptide is significantly impacted by pH, zinc ions and zinc chelators like ethylenediaminetetraacetic acid (EDTA).[148] A follow up study confirmed that Zn^{2+}, in the presence of C-peptide, can stimulate ATP release from erythrocytes.[147]

Although many methods were tried, the Spence group was not able to establish substantial binding between C-peptide and Zn^{2+} alone.[149] Since the addition of either C-peptide or Zn^{2+} alone did not increase erythrocyte-derived ATP, it was at first hypothesized that Zn^{2+} may assist delivery of C-peptide to the erythrocyte.[147] However, this was incorrect, as C-peptide enzyme-linked immunosorbent assay (ELISA) and $^{65}Zn^{2+}$ radioisotopic data demonstrated that C-peptide binds to erythrocytes in the presence or absence of Zn^{2+}, but Zn^{2+} transfer to the erythrocyte needs C-peptide.[149]

Because of the absence of binding between C-peptide and Zn^{2+}, it was postulated that another molecule in the system was contributing to the delivery of C-peptide and Zn^{2+} to the erythrocyte. The physiological salt solution (PSS) used as the buffer in all previous studies by the Spence group involving C-peptide contained albumin. Albumin is the most abundant protein in human plasma with a normal concentration between 35 and 50 g/l and known to have a diverse set of functions in vivo including oncotic pressure regulation, binding and transport, as well as antioxidant properties.[150] In 2015, isothermal titration calorimetry (ITC) analysis of binding provided important evidence that albumin is a determinant in C-peptide and Zn^{2+} binding and potentially delivery to the erythrocyte, which initiated a reevaluation of ELISA-based measurements of C-peptide binding to

erythrocytes and radioisotopic determination of Zn^{2+} delivery to erythrocytes.[149] As predicted, C-peptide binding to the erythrocyte was not found in the absence of albumin. Also, Zn^{2+} delivery to the erythrocyte was also absent in albumin-free PSS, whether or not C-peptide was present.[149]

Because in the literature it is well-known that the glutamic acid residue at position 27 (E27) of C-peptide is necessary for cellular activity[96, 151] and many linkages between albumin and other peptides or proteins occur through carboxylate linkages, further exploration assessed the ability of a E27 mutant to alanine (E27A) to bind albumin. However, it was determined by ITC that the E27A mutant did not bind albumin, and by radioisotopic assay, it was shown that the mutant, when in the three-component system with albumin and Zn^{2+}, could not deliver Zn^{2+} to the erythrocyte. Because the mutant could not be detected by C-peptide ELISA, binding of the mutant to the erythrocyte itself was not determined.[149]

Although the data showed that albumin is needed for Zn^{2+} and C-peptide delivery to the erythrocyte, static measurements did not offer any evidence toward the mechanism by which C-peptide can improve blood flow in animal and human type 1 diabetic patients. Because of this, a 3D-printed fluidic device (Figure 1.7) was designed and created to allow active, flow-based studies of erythrocyte interaction with C-peptide, Zn^{2+} and albumin. In line with the binding data, studies with the fluidic device demonstrated an increase in ATP release from erythrocytes incubated with C-peptide, $Zn^{2+}$and albumin.[149]

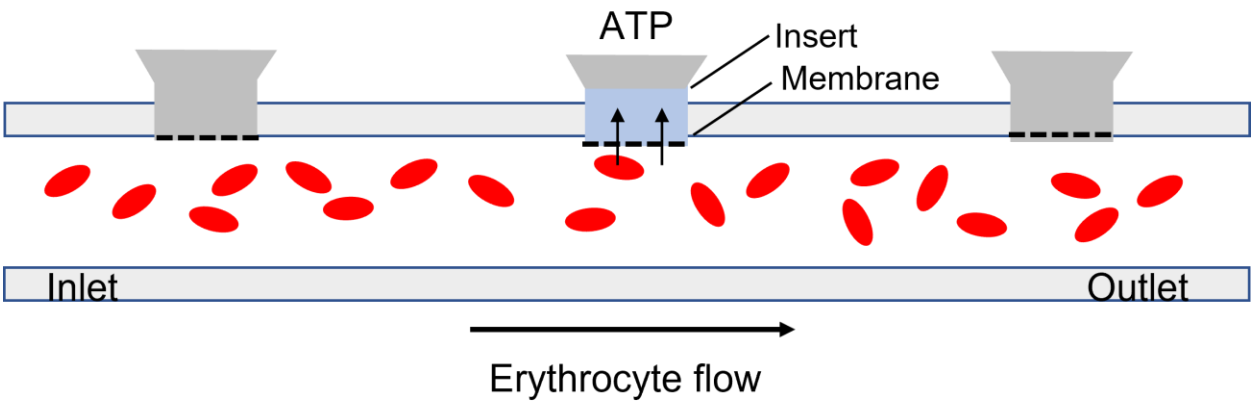

Figure 1.7: Depiction of 3D-printed fluidic device used to measure erythrocyte-derived release of ATP. As shown, erythrocyte samples are pumped through channels of the device. ATP released from the erythrocytes diffuses from channels of the device through the porous membrane of a transwell insert (which is loaded with 50 µL of PSS buffer immediately prior to erythrocyte delivery to device channels). The moles of ATP diffusing into the transwell insert are proportional to the amount of ATP in the channel, allowing the amount of ATP released from the erythrocytes to be quantitatively determined. Adapted from Liu et al., *Integr. Biol.* **2015**, *7*, 534-43.

To simulate in vivo conditions, the system was updated to include INS-1 cells, a widely used rat islet β-cell model more closely more closely in diabetes research that can secret both insulin and C-peptide and contains ZnT-8 protein (the transporter found in β-cell granules responsible for Zn^{2+} transport) cultured on 6 mm transwell membrane inserts and positioned inside the fluidic device. When the INS-1 cells were stimulated with glucose, ATP release and downstream NO production by endothelial cells was only noted when the endothelial cells and erythrocytes were present, and the erythrocytes were flowing in a stream of albumin-containing buffer. Importantly, the C-peptide secretion concentrations into the insert over a time of two hours and the concentration of C-peptide that could diffuse into the device channels and influence the erythrocyte flowing stream were between 0.5-2 nM, which is comparable to what has been reported in vivo. The downstream physiological importance of C-peptide, Zn^{2+} and albumin effects was

demonstrated by measurement of the vasodilator NO. The use of PPADs, a known inhibitor of the ATP binding site on endothelial cells, decreased endothelium-derived NO production, supporting the idea that the increase in measured NO was because of ATP release which in turn was incited by albumin delivery of Zn^{2+} and C-peptide to the flowing erythrocytes.[149]

Due to this demonstrated importance of Zn^{2+} for C-peptide cellular and tissue effects,[149] the Spence group proposed an alternative explanation for reports which have shown that addition of EDTA to C-peptide formulations eliminated cellular effects. It had previously been suggested that EDTA was binding to Ca^{2+} stores which were necessary for cellular function.[152] However, based on the relative binding affinities of EDTA to Ca^{2+} and Zn^{2+}, and because EDTA would be unable to enter the cells due to its charge (EDTA has a charge of -4, from four oxygen atoms with lone pairs) the Spence group hypothesized that a more plausible explanation was that EDTA was competitively binding to Zn^{2+} prior to delivery to cells in vivo. Although a C-peptide receptor has not yet been discovered, the finding by the Spence group in 2015 that C-peptide requires a carrier protein to bind to cells was important for advancing these efforts. For example, to accelerate the search, it may be possible to grow crystals containing albumin, Zn^{2+} and C-peptide in order to discover structural features of the assembly necessary for receptor-based binding.[149]

1.10 Binding Analysis of Albumin

Although albumin can bind and transport a wide range of small size metabolites,[153, 154] due to its long half-life time compared to other proteins (~12-21 days) and its high concentration, albumin is exceptionally sensitive to the effects of non-enzymatic glycation.[150] Glycation modifications can cause conformational changes in protein structure,[150] and many ligands have a modified affinity to albumin when the protein is glycated.[155-159] Increased levels of glycated albumin are often seen in diabetes, and have important effects on cellular function.[150] To gain a better understanding of the binding relationship between human serum albumin (HSA), C-peptide and Zn^{2+}, in 2018, the Spence group developed an ultrafiltration method (Figure 1.8) to quantitatively determine binding constants. Utilizing 3D-printing technology, a custom ultrafiltration device was designed which could hold any membrane size and be fitted to the end of a commercially available 1 mL plastic syringe. The device was used to determine binding between various albumins and C-peptide, and binding affinity between Zn^{2+} and normal or glycated human serum albumin (gHSA).[160]

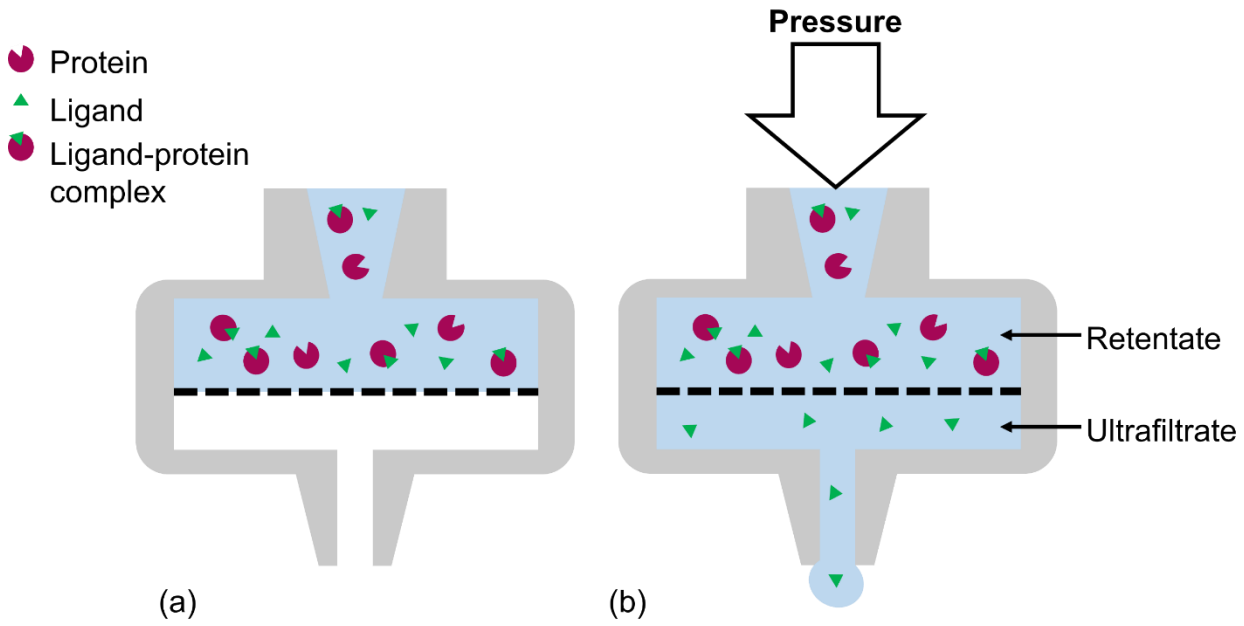

Figure 1.8: Illustration of the principle of ultrafiltration binding studies. In (a), a sample mixture of protein and ligand is loaded into a device fitted with a size-exclusion membrane. The pores of the membrane are large enough to allow passage of the ligand but small enough to prevent passage of the protein and any ligand-protein complexes. In (b), pressure is used to push a small amount of solution containing the unbound ligand through the membrane pores. The concentration of unbound ligand in the ultrafiltrate is equivalent to the concentration of unbound ligand in the retentate. Adapted from Castiaux et al., *Anal. Bioanal*. **2018**, *410*, 7565- 73.

Results for normal human serum albumin/Zn^{2+} binding found using the ultrafiltration method (K_d = 5.77 ± 0.19 × 10^{-7} M)[160] were statistically equal to reported results that used different methods.[149] Interestingly, the amount of Zn^{2+} bound to normal human serum albumin was significantly different from the glycated human serum albumin (97 ± 2% protein bound vs. 91 ± 3%, respectively).[160] The binding affinity of C-peptide to normal human serum albumin (K_d = 2.4 ± 0.3 × 10^{-6} M)[160] was in agreement with values described in the literature using traditional methods.[149] The binding of C-peptide to normal human serum albumin, unlike Zn^{2+} binding, was statistically equivalent to its binding to glycated human serum albumin (77.7 ± 6.2 and 78.8 ± 7.4%, respectively).[160]

The data imply that C-peptide replacement therapy in people with type 1 diabetes may be very dependent upon the binding characteristics of Zn^{2+} to human serum albumin.[160] It also means that in vivo, in conditions of chronic hyperglycemia, other circulating proteins with shorter half-lives may be more important for maintenance of factors normally controlled by albumin. Leptin, an adipocyte-derived signally factor involved in regulation of energy balance,[161] with a half-life in human circulation of approximately 25 minutes,[162] is one such candidate.

Chapter 2: Leptin's Role in Regulation of Blood Flow

2.1 Discovery of Leptin

For many decades, evidence has been increasing for the existence of a physiological system for homeostatic control of body weight.[163] In 1953, Kennedy proposed a relationship between ingested calories and energy expenditure in connection with energy storage in adipose tissue.[164] Kennedy foresaw a homeostatic mechanism that can monitor changes in energy stores and prompt compensatory changes in food intake and energy expenditure to hold adipose mass at a set level. This mechanism is supported by the well-established fact that adipose tissue mass remains mostly stable over long periods in many mammals. This adipostatic model of body weight regulation is consistent with observations of changes in adiposity with fasting, surgical resection or forced overfeeding.[165-167]

The term 'parabiosis' (Figure 2.1) refers to the artificial connection of the circulatory systems of animals through surgical resection, so that they share a physiological system.[168] It is a common technique used in many fields of research, including obesity, aging, diabetes, and endocrinology.[169] In 1959, Hervey demonstrated that parabiosis between rats made obese by lesions in the ventromedial hypothalamus and control rats lead to death by starvation for the controls. These results led Hervey to the idea that increased levels of a circulating satiety factor from the obese rats could inhibit food intake in the control rats. The obese rats could not respond to elevated levels of the satiety factor due to the hypothalamic lesion.[168]

Figure 2.1: Image of two normal mice whose circulatory systems have been artificially connected by surgical resection (parabiosis). Reproduced from Hervey, *J. Physiol.* **1959**, *145* (2), 336-52.

The concept of a satiety factor in the circulation was supported by the finding of recessive mutations, diabetes (*db*) and obese (*ob*) (Figure 2.2) that cause hyperphagia (increased consumption of food), diminished energy expenditure and early onset obesity in the mouse.[170] Hauseberger[171] and Coleman[172, 173] demonstrated that parabiosis of *ob*/*ob* and wild type mice avoided weight gain in the *ob/ob* mice, however, parabiosis to *db/db* mice resulted in extreme hypophagia (reduction in feeding) in the wild-type lean mice. Together, these results demonstrated that the *ob* locus was needed to produce a circulating satiety factor while the *db* locus coded for a molecule necessary for response

to the factor.[174] The predictions of Coleman were confirmed decades later by cloning of the *ob*[175] and *db*[176] genes, the product of the *ob* gene termed leptin, from Greek '*leptos'* meaning 'thin', because it resulted in a clear reduction in body weight, body fat and food intake when injected into leptin-deficient or normal mice.[177]

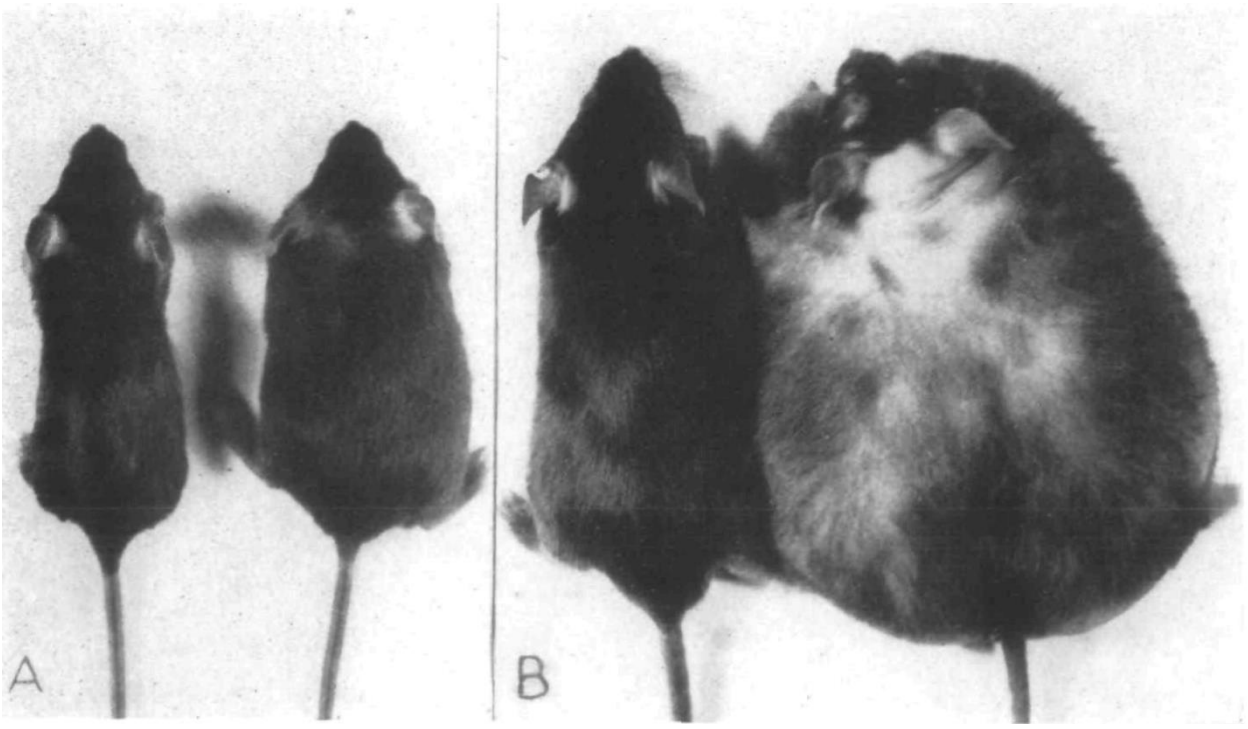

Figure 2.2: Image A shows a normal (left) and *ob*/*ob* mouse (right) at 21 days of age. Image B compares the same mice at 10 months of age. The mice are indistinguishable for the first four to six weeks of life, at which point the *ob*/*ob* mouse begins to gain weight rapidly. Reproduced from Ingalls et al., *J. Hered.* **1950**, *41*, 317.

An increase in leptin concentration was suspected to prevent obesity by decreasing appetite and increasing thermogenesis through actions in the brain.[178, 179] However, as more has been discovered about leptin, its original view as an anti-obesity hormone has evolved into a more complicated one. Now it is known that leptin is involved

in many physiologic processes, from immune and neuroendocrine function, metabolism, to reproduction, growth and development.[163]

2.2 Leptin and Glucose Homeostasis

Together with obesity, *db* and *ob* mice have a phenotype like type 2 diabetes, with features like hyperinsulinemia, insulin resistance and hyperglycemia. Because of this, *db* and *ob* mice are broadly used as animal models of diabetes.[178, 180-184] Although it is possible that the modified glucose metabolism that accompanies leptin receptor or leptin deficiency is secondary to hyperphagia and obesity, many lines of evidence suggest that leptin can independently modulate glucose metabolism from food intake and body weight..[185] Along with transient hypoglycemia,[184, 186] hyperinsulinemia occurs in *ob/ob* mice[183, 184, 186, 187] before the initiation of obesity, insulin resistance and hyperphagia. In rodents having interrupted leptin receptor function, including corpulent (obese) rats, *db/db* mice and Zucker fatty (*fa*/*fa*) rats, early hyperglycemia is common.[182, 188-191] Humans and rodents with a large or entire loss of adipose tissue (lipodystrophy), analogous to leptin-deficient *ob/ob* mice, experience insulin resistance, hyperinsulinemia, and hyperglycemia and are hypoleptinemic.[192, 193]

2.2.1 Mechanisms of Leptin Action on Glucose Homeostasis

Leptin receptors are pervasively expressed throughout in central nervous system and peripheral tissues.[194] Various studies have been performed to determine the target

tissues that control leptin influence on glucose homeostasis.[185] The hypothalamus and many extra-hypothalamic sites within the brain have been recognized as important targets of leptin,[195-197] and direct leptin signaling in peripheral tissues can influence glucose metabolism.[185]

2.2.2 Leptin Effects on Type 1 Diabetes

In type 1 diabetes mouse models, mono-therapy with leptin (no use of exogenously administered insulin/other compounds) improves the pathologic results of insulin deficiency connected with diabetes.[198, 199] A single year of treatment with leptin vastly improved lipid and glucose profiles for individuals affected by both acquired generalized lipodystrophy and type 1 diabetes. In those individuals, although therapy with insulin was not completely halted, leptin treatment enhanced insulin sensitivity enough that insulin doses were markedly reduced (30-50% compared to pre-leptin administration doses).[200] These pre-clinical and clinical discoveries ignited interest in leptin as a type 1 diabetes therapeutic.

A clinical trial (NCT01268644) aimed at determining the efficacy of leptin therapy in patients with type 1 diabetes was launched in 2010. Metreleptin, a recombinant analog of human leptin, was administered subcutaneously (0.08 mg/kg/day females and 0.04 mg/kg/day males) to patients with type 1 diabetes (mean age 33 years) twice a day for 20 weeks. No significant changes in glycated hemoglobin levels were found, but a small reduction in body weight (2.6 kg at 12 weeks and 4.7 kg at 20 weeks) and daily insulin dose (by 12.6% at 12 weeks and 15% at 20 weeks) were observed. It was noted that

some patients are likely more responsive to leptin therapy than others, but contributing factors were not identified in the data from the pilot study.[201]

2.2.3 Leptin Effects on Type 2 Diabetes

Findings from many pre-clinical investigations in mouse models of type 2 diabetes show that leptin improves lipid and glucose profiles and lessens insulin resistance.[202-205] Nevertheless, results from two recent clinical trials imply that leptin therapy is only minimally proficient for improving insulin resistance in obese individuals with type 2 diabetes.[206, 207] Because it is possible that the anti-type 2 diabetes action of leptin would be evident in patients with type 2 diabetes who are not obese and whose circulating leptin levels are low or normal, such as Asian type 2 diabetic populations with low general adiposity, more clinical trials are necessary.[208]

2.3 Leptin Receptor Isoforms

There are at least six plasma membrane receptors that bind leptin, ranging from Ob-Ra to Ob-Rf. Ob-Rb, the main isoform with the longest intracellular domain, is located mainly in the hypothalamus of the brain and activates Janus kinases (JAKs) that phosphorylate signal transducers and activators of transcription (STATs). When STATs are phosphorylated, they translocate to the nucleus where they regulate target gene transcription.

The short isoforms of the leptin receptor, Ob-Ra, Ob-Rc, Ob-Rd and Ob-Rf have short intracellular domains and are unable to activate the JAK-STAT pathway, but can induce signal transduction by other mechanisms, like mitogen-activated protein kinases or phosphatidylinositol 3-kinase.[209] Although short forms of the leptin receptor are located ubiquitously in human tissues, their function is less obvious. Ob-Ra is thought to perform transport functions, particularly in the choroid plexus.[210] Ob-Re, the soluble leptin receptor, represents the main leptin-binding activity in human blood[211-213]. The physiological function of Ob-Re is thought to be related to regulation of steady-state leptin levels by binding with leptin in the circulation to prevent its degradation and clearance,[214, 215] which could mean that high soluble leptin receptor levels that have been in the blood of lean humans serve as a reservoir of bioactive leptin.[216]

2.3.1 Effect of Leptin on the Sympathetic Nervous System

The autonomic nervous system is made up of two main divisions: the sympathetic and parasympathetic nervous system, which often function in opposing ways. The motor outflow of both systems is formed by two consecutively connected sets of neurons. The first set, which originates in the spinal cord or brain stem are called preganglionic neurons, and the second set, which lies outside the central nervous system in collections of nerve cells termed autonomic ganglia, are called ganglion cells or postganglionic neurons. The parasympathetic nervous system mainly controls internal organs such as glands. Although it provides control for many tissues, the parasympathetic nervous system is not crucial for maintenance of life.[217] The sympathetic nervous system regulates a vast array

of internal functions and during times of extreme emotional or physical stress, stimulates the cardiovascular and adrenal catecholamine[j] systems to adjust homeostasis.[218,219] It is the sympathetic nervous system that is in control of the body's rapid and involuntary 'fight or flight' response.[219]

The first identified effect of leptin related to its hemodynamic or blood flow properties was stimulation of the sympathetic nervous system. Leptin reduces food intake and increases energy expenditure by stimulating sympathetic outflow to brown adipose tissue to increase thermogenesis. Leptin also influences sympathetic trafficking in other tissues including the kidney, adrenals and hindlimbs.[220] Studies suggest that leptin reflexively stimulates the sympathetic nervous system by activating afferent (conducting inward or toward) nerve endings in adipose tissue.[209] Leptin injected into the epididymis (a duct behind the testes) not only stimulates efferent (conducting outward or away from) sympathetic nerves to the adipose tissue of the epididymis, but also to other tissues including brown adipose tissue, the adrenal medulla (a small organ on top of the kidney that makes chemicals including epinephrine and norepinephrine, which are involved in sending nerve signals), pancreas and liver.[221] Additionally, injection of leptin into perirenal (surrounding the kidney) adipose tissue enhances renal sympathetic nervous activity in a dose-dependent way. These studies suggest that leptin released in adipose tissue may

[j] Catecholamines are hormones made by the adrenal glands and released by the body in response to physical or emotional stress. The main types of catecholamines are epinephrine (adrenaline), dopamine and norepinephrine.

be able to act locally on afferent fibers to stimulate the sympathetic nervous system by a paracrine (having only an effect within the vicinity of the secreting gland) mechanism.[222]

However, the majority of studies provide evidence backing the idea that the central nervous system plays a major role in the sympathoexcitatory effects of leptin.[209] In support of this, intracerebroventricular (direct injection into the cerebrospinal fluid of the cerebral ventricles in order to bypass the blood-brain-barrier) injection of leptin increases sympathetic nervous system activity at doses which do not increase systemic leptin level, and damage to the hypothalamic arcuate nucleus eliminates the sympathoexcitatory effect of leptin.[220, 223]

Evidence suggests that the effects of leptin on brown adipose tissue and renal sympathetic nerves are regulated differentially.[224, 225] Stimulation of arterial baroreceptors (receptors located in the carotid sinus and aortic arch, which sense blood pressure and relay information to the brain[226]) by increasing blood pressure lessens the effect of leptin on renal sympathetic fibers but does not influence its effect on fibers innervating brown adipose tissue.[224, 225] In contrast, hypothermia enhances the effect of leptin on brown adipose tissue but not on renal sympathetic nerves. The data suggest that the effect of leptin on renal sympathetic fibers is related to leptin's role in hemodynamics, but leptin's effect on the sympathetic nerve activity of brown adipose tissue is related to its role in regulation of thermogenesis.[224, 225]

In humans, some[227-229] but not all[230, 231] studies suggest that leptin regulates sympathetic nervous system activity. The differences may be explained by differences in study populations and method (i.e., indirect vs. direct) used to measure sympathetic nerve activity. Many studies have demonstrated that although central and peripheral

administration of leptin can stimulate the sympathetic nervous system, blood pressure increases only when leptin is centrally administered,[223, 232-234] suggesting that leptin stimulates counterbalancing depressor activities outside of the central nervous system.[209]

2.3.2 Leptin and Nitric Oxide

In 1999, Frühbeck demonstrated that a bolus, or a single, large dose, of intravenously administered leptin increases plasma concentration of NO metabolites (nitrites and nitrates). Although leptin administration by itself had no direct effect on blood pressure, for rats that received the NO synthase inhibitor, N^{w}-nitro-L-arginine methylester (L-NAME) together with leptin, blood pressure was higher for rats administered L-NAME alone. Leptin lowered blood pressure in rats in which the sympathetic nervous system was inhibited pharmacologically.[235]

Bełtowski et al. demonstrated that intraperitoneal[236] or intravenous[237] administration of leptin raises the plasma concentration and increases urinary elimination of NO metabolites and of the NO second messenger, cyclic guanosine monophosphate, in a time as well as dose-dependent manner. These results were in agreement with in vitro studies (using rat vessels) in which leptin-induced NO-dependent vasorelaxation was observed.[238-240] It has been shown that leptin enhances NO production in cultured bovine pulmonary artery endothelial cells, human aortic endothelial cells and isolated rat aortic rings.[240, 241]

For many years, NO synthase was categorized only as a Ca^{2+}-dependent enzyme, and for this reason, many stimuli able to modulate endothelial NO formation have not

been characterized on the basis of their Ca^{2+}-dependence.[242] Vecchione et al. have reported that leptin can stimulate NO release in the absence of extracellular Ca^{2+}. Acetylcholine is a common stimulus used to test endothelial NO release. In direct measurements of NO release from vascular tissue using a fluorescent probe, it was found that the absence of Ca^{2+} could prevent acetylcholine-induced fluorescence but did not influence the effect of leptin on NO production.[240] Serine threonine kinase Akt, which influences various cellular processes including glucose metabolism and cell survival, is another important regulator of endothelial NO production[243, 244] and activates eNOS through phosphorylation on serine.[1177] It has been reported that leptin enhances Akt phosphorylation in endothelial cells and isolated vessels, which can induce eNOS phosphorylation on serine[1177], which is a site specific to Akt phosphorylation of the enzyme.[240]

Not all studies support the role of NO in the blood flow controlling effects of leptin. Haynes and colleagues found that leptin has no direct effect on mesenteric, hindlimb or renal vascular flow in conscious rats, even following administration of the α_1-adrenoreceptor antagonist prazosin or NO synthase inhibitor, L-NAME. The data suggested that the absence of effect from leptin is not the result of a balance between sympathetic nervous system activity and peripheral NO mechanisms.[245] Infusion of leptin for three hours had no observed effect on the decrease in renovascular flow brought on by stimulation of splanchnic sympathetic trunk (sympathetic innervation of the abdomen), implying that, even if leptin can stimulate NO production, the overall influence of leptin on blood flow is minimal.[246] Administration of high levels of leptin did not modify vascular flow

measured for two to three hours simultaneously in vascular beds of several conscious animals.[234, 247]

It is unlikely that the sympathetic nervous system and NO-dependent effects of leptin are so perfectly matched as to have no impact on blood flow properties. There are several possible explanations for the observed vasodilatory effects of leptin. One possibility is that leptin stimulates NO production mainly in large conduit arteries, which contribute minimally to overall vascular resistance.[209] For in vivo studies, it is doubtful that the source of leptin-stimulated NO production can be definitively established. Leptin can stimulate NO synthesis in many vascular tissues including adipocytes,[248] erythrocytes[249] and cardiomyocytes.[250] NO stimulating effects of leptin have been observed only at pharmacological concentrations.[209] Studies of administration of leptin at pharmacological concentrations caused NO-dependent relaxation of isolated rat or canine coronary arterioles,[251] but there was no effect on coronary blood flow in vivo for anesthetized dogs if leptin was given in doses corresponding to the 'obese' range. Interestingly, it was observed by Sprague et al. in 1996 that rabbit and human, but not dog erythrocytes, release ATP in response to mechanical deformation.[138] If high levels of leptin can induce an ATP release (and thereby stimulate NO) in other cell types, this may offer a possible explanation for the lack of NO stimulatory effects observed in dogs when leptin was administered above pharmacological concentrations.

2.3.3 NO Independent Vasodilatory Effects of Leptin

While the majority of studies investigating the role of leptin in the vasculature support a role for NO, some studies have shown that leptin may elicit vasorelaxation independent of NO.[209] For instance, leptin decreases blood pressure in rats in which both the sympathetic nervous system activity and NO metabolism had been pharmacologically inhibited.[238] In vivo, leptin prevents blood pressure increase induced by L-NAME, which supports the hypothesis that leptin may stimulate other depressor mechanisms when NO synthase is inhibited. In human studies, leptin infusion into the coronary[252] or brachial artery[253] lead to vasorelaxation that is not prevented by NO synthase inhibitors. However, since L-NAME is a competitive inhibitor with L-arginine for eNOS, even in the presence of L-NAME, high levels of eNOS stimulation may still lead to production of NO.

Also in support of a NO-independent vasodilatory effect of leptin, apart from NO, the vascular endothelium releases two other factors with a known role in vasodilation: prostacyclin and endothelium-derived hyperpolarizing factor (EDHF).[209] While EDHF activity could be accounted for by various mechanisms, cytochrome P450-dependent arachidonate metabolites, along with isomers of epoxyeicosatrienoic acid (EET) appear to be the major contributors.[254] A study by Lembo et al.[238] suggested that EDHF contributes to the vasodilatory effect of leptin in in isolated rat mesenteric arteries, although a study by Kimura et al.[239] refuted this. Pretreatment of rats receiving L-NAME with either a mixture of charybdotoxin and apamin (which inhibit intermediate and small conductance potassium channels, respectively, and are used frequently to block EDHF activity) or sulfaphenazole (an inhibitor of EET synthesis) stimulates the pressor effect

(increasing blood pressure by stimulating blood vessel constriction) of leptin, demonstrating that leptin may prompt EDHF/EET-dependent vasorelaxation in vivo when NO availability is decreased.[209]

Still other studies have offered evidence that leptin can modulate vascular tone independent of its effect on the endothelium.[209] Frühbeck and colleagues found that while leptin alone had no effect on rat aortic rings from which endothelial tissue had been removed, leptin significantly reduced the vasoconstrictor effect of angiotensin II in this setting by inhibiting Ca^{2+} release from intracellular stores.[255]

2.3.4 Renal Effects of Leptin

Serradeili-Le Gal et al. identified specific binding sites for leptin in the rat renal medulla and demonstrated a substantial rise in diuresis, or production of urine, after intraperitoneal injection of leptin in mice.[256] Many follow-up studies demonstrated that leptin administered locally[257], intraperitoneally,[258] intravenously[237, 259] into the renal artery enhances natriuresis (excretion of sodium into the urine) without changing glomerular filtration rate, renal blood flow or K^+ secretion, hinting that the natriuretic effect of leptin is the result of tubular Na^+ reabsorption inhibition. Tubular Na^+ reabsorption is primarily regulated by Na^+,K^+-ATPase contained in the basolateral membranes of tubular cells.[260] Locally[261] or Systemically[258, 262] administered leptin leads to a dose- and time-dependent fall in Na^+,K^+-ATPase activity in the renal medulla. In agreement with the effect specific to the renal medulla is the exclusive localization of leptin receptors there, most likely in the medullary collecting duct.[256, 263] Unlike Na^+,K^+-ATPase, leptin has no effect on two

associated enzymes: ouabain-sensitive H^+ ,K^+-ATPase that is found on the apical membranes and has roles in K^+ reabsorption and urine acidification and ouabain-resistant Na^+-ATPase, found only in the proximal tubule and responsible for reabsorption of approximately 10% of filtered Na^+.[262, 264]

2.4 Leptin and Its Interaction with Pancreatic Hormones

It is interesting to note that leptin did not modify glycated hemoglobin levels (a marker of elevated blood glucose) in clinical trials of individuals with type 1 diabetes,[201] a group who would be expended to have low or no exogenously produced insulin, and therefore little to no C-peptide. Moreover, evidence indicates that although type 2 diabetes is typically associated with obesity, metabolic syndrome and accompanied by elevated leptin levels and leptin resistance, studies have indicated that leptin therapy may be beneficial for promoting glucose homeostasis in combination with diet and exercise to promote weight loss.[265-270] C-peptide has been shown to have a direct effect on blood flow in animal models of diabetes as well as in small scale human trials, suggesting it has a possible direct effect in the bloodstream. However, the discovery by the Spence lab in 2015 that C-peptide and Zn^{2+} delivery to the erythrocyte and subsequent downstream effects requires the presence of the albumin,[149] presents a problem for utilizing C-peptide as a diabetes therapeutic, where there are increased levels of glycated albumin which can less effectively act as a carrier molecule.[150, 160, 271] The known role of leptin in glucose homeostasis,[185, 272] and studies reporting that leptin has a relaxing effect on blood vessels that is mediated by NO release from the endothelium[239] inspired the Spence

group to investigate of a potential role for leptin in C-peptide and Zn^{2+} associated erythrocyte-derived ATP release.

2.4.1 C-peptide and leptin Effect on Erythrocyte Cellular Activity

Preliminary studies demonstrated that static ATP release from erythrocytes was not increased when the cells were incubated with leptin alone, but when the cells were incubated with 3.8 nM leptin in the presence of 20 nM of Zn^{2+} and C-peptide, there was a 30% increase in chemiluminescence intensity corresponding to a 30% increase in ATP release. Incubation of leptin with either Zn^{2+} or C-peptide alone did not significantly increase ATP release. While the exact features of the interaction were not investigated, the preliminary evidence suggested a possible mechanism of leptin-mediated glucose clearance involving the erythrocyte and modulated by C-peptide and Zn^{2+}.[273]

These studies were continued and confirmed that leptin amplifies the cellular energetic effects of C-peptide when in combination with C-peptide, Zn^{2+} and albumin.[274] ATP release from erythrocytes in the presence of leptin, C-peptide and Zn^{2+}, and combinations were measured, while also monitoring GLUT1 translocation to the erythrocyte membrane (Figure 2.3).[275] In the presence of C-peptide and Zn^{2+}, a 30% increase in erythrocyte-derived ATP was measured, and the signal was increased by another 20% when leptin was added. Importantly, leptin's effect on ATP release was enhanced in high glucose conditions (not shown), like those that would occur in the bloodstream of a patient with diabetes. It was found that in the presence of C-peptide and Zn^{2+}, leptin increases GLUT1 translocation by 20% as compared to control samples.[274]

Combined, these data suggest that levels of leptin in the bloodstream may correlate with glucose concentrations and the ability of erythrocytes to use glucose.

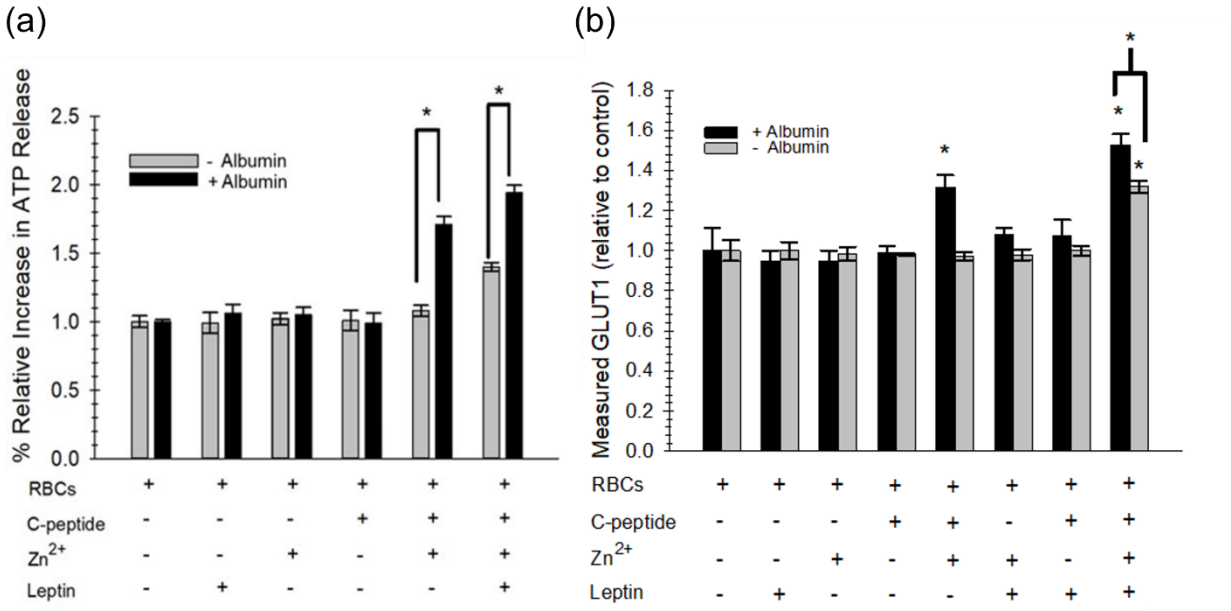

Figure 2.3: Leptin effect on C-peptide and Zn^{2+}-related cellular activity of erythrocytes. Depicted in (a), ATP release from erythrocytes is enhanced in the presence of C-peptide and Zn^{2+}, but only in buffer containing albumin. In (b), measured GLUT1 levels in erythrocyte membranes after incubation with C-peptide, Zn^{2+}, leptin and their combinations with or without albumin are shown. In all data sets, black bars indicate albumin containing buffer, while grey bars indicate albumin free. For all samples, n = 5, while significance from the control is indicated by an * ($p<0.001$). Reproduced from Keshavarz et al., *Mol. Pharm.* **2021**, *18* (6), 2438-47.

2.4.2 Mechanistic Studies of Leptin's Effect on Erythrocytes

In initial studies, leptin's effect on C-peptide and Zn^{2+} binding to erythrocytes and the interaction between leptin and erythrocytes was investigated. Zn^{2+} binding, or uptake, was examined by incubating erythrocytes with combinations of $^{65}Zn^{2+}$, C-peptide and leptin in albumin containing or albumin free PSS. Experiments were performed in

PSS with or without albumin to separate the effects of leptin from the known involvement of albumin in C-peptide's mechanism of action. Interaction of C-peptide and leptin with erythrocytes were examined by ELISA.[274]

2.4.2.1 Zn^{2+} Binding to Erythrocytes is Increased by Leptin

Binding of 20 nM $^{65}Zn^{2+}$ in the presence of 20 nM C-peptide and varying concentrations of leptin, with or without albumin was investigated. It was found that 2.6 $\pm$ 0.3 nM ($p<0.4$) of $^{65}Zn^{2+}$ is bound, or taken up by erythrocytes incubated with 20 nM $^{65}Zn^{2+}$ and 20 nM C-peptide in albumin-containing PSS,[274] which was agreement with prior work.[149] This was increased to approximately 3.6 $\pm$ 0.6 nM ($p<0.4$) in the presence of 3.8 nM leptin,[274] while $^{65}Zn^{2+}$ bound non-specifically, with or without leptin, in the absence of albumin. When the concentration of leptin was increased in increments ranging from 0.8 to 12 nM, $^{65}Zn^{2+}$ binding was observed to increase proportionally. $^{65}Zn^{2+}$ binding to erythrocytes was demonstrated to increase when leptin was added into the system with $^{65}Zn^{2+}$, C-peptide and albumin. When leptin levels were incrementally increased in the presence of erythrocytes incubated with $^{65}Zn^{2+}$ in albumin containing PSS, binding appeared to increase in a linear way, and did not reach a plateau as would be observed in a true saturation binding curve.[274] For this reason, more studies are required to determine where leptin binding saturates under the experimental conditions.

2.4.2.2 C-peptide and leptin Binding to Erythrocytes

C-peptide and leptin binding to erythrocytes were also studied using ELISA. For C-peptide binding experiments, erythrocytes were incubated with Zn^{2+}, C-peptide, leptin, and combinations in albumin-free or albumin-containing PSS. In addition, C-peptide binding to erythrocytes was investigated at varying concentrations of leptin (from 0.4 to 10 nM, corresponding to ranges for normal and obese leptin concentrations[276]) while Zn^{2+} and C-peptide levels were held constant (20 nM). For leptin binding experiments, varying amounts of leptin were added to samples to achieve final concentrations of 0-25 nM in a solution of 7% erythrocytes in albumin containing PSS. No Zn^{2+} or C-peptide were added to the samples. All samples were incubated at 37 °C for three hours, centrifuged at 500*g* for five minutes, and then supernatant was collected and used for the ELISA analysis of C-peptide or leptin content, respectively. Analyte[k,] concentration bound to erythrocytes was determined by subtracting the amount measured in the supernatant from the initial amount added.[274]

For erythrocytes incubated with Zn^{2+} and C-peptide and albumin in the presence or absence of leptin, it was determined that C-peptide binding was increased in comparison to erythrocytes incubated with Zn^{2+}, C-peptide and albumin alone. Although erythrocytes incubated with only Zn^{2+} and C-peptide showed no C-peptide binding, when leptin was added, even in the absence of albumin the erythrocytes could bind C-peptide.[274]

[k] Analyte – (chemistry) A substance whose chemical constituents are being identified and measured.

Although construction of binding curves was attempted for C-peptide and leptin binding to erythrocytes, the results were difficult to interpret due to errors of experimental design. It was thought that the apparent saturation points of C-peptide and leptin binding were due to samples being inappropriately diluted for the ELISA plate rather than a true saturation binding curve, since further unpublished experiments revealed that increasing the dilution of samples before ELISA analysis resulted in a straight line, indicating saturation of binding had not been reached.

In this dissertation, molecular interactions of leptin with C-peptide, Zn^{2+}, albumin and erythrocytes are further characterized. In this effort, various experimental techniques have been utilized, including ELISA, radiolabeling with ^{99m}Tc, ultrafiltration binding analysis and surface plasmon resonance. The interaction of leptin with erythrocytes was further probed in the presence and absence of Zn^{2+}, C-peptide and albumin utilizing ELISA analysis and radiolabeling with ^{99m}Tc-labeled leptin. 3D-printed ultrafiltration devices were created and used to investigate binding of C-peptide and leptin and confirm their interaction as well as determine a binding affinity constant using surface plasmon resonance (SPR). Lastly, investigations were initiated for C-peptide binding to erythrocytes in normal and high sugar conditions and in the presence of glycated albumin. These studies supplement the existing knowledge of the role of C-peptide and leptin in regulation of blood flow and will hopefully aid in the design of novel therapeutics for addressing the microvascular complications of diabetes.

Chapter 3 – Investigation of Leptin Binding to Erythrocytes

3.1 Drug-receptor Interaction (Theory)

Receptor theory is the use of receptor models to define drug behavior.[277] Paul Ehrlich and John Newport Langley put forth the concept of a receptor that would assist drug action at the beginning of the 20th century, and Alfred Joseph Clark was the first to measure drug-induced biological responses. Essentially all quantitative theoretical modeling of receptor role, to the current date, has focused on G protein-coupled receptors and ligand-gated ion channels.[278]

The central ideas of receptor theory, which are generally attributed to Clark, are the following:

- Drugs interact with receptors in a reversible way to produce a change in the receptor.
- The relation may be modeled mathematically and adheres to the Law of Mass Action[l,279]
- The binding of receptor and drug decides the quantitative connection involving dose and effect.

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[l] Law of Mass Action – statement that the rate of any chemical reaction is related to the product of the masses of the reacting substance, with each mass raised to a power equivalent to the coefficient in the chemical equation.

- Shared affinity of receptors and drugs governs selectivity of drug effects.
- Mutually exclusive molecules in competition for the same receptors determines agonist, partial agonist and antagonist drug activity.[280, 281]

The occupancy theory of drug-receptor interaction defines the numerical connection between drug concentrations and the responses that occur because of the interaction of those drugs with receptors, and also defines the behavior of agonists and antagonists.[282]

3.2 Ligand Binding Assays

Binding studies of pharmacological receptors aim to determine dependable approximations of the affinity of ligands to a receptor of notice together with associated errors and assess the mechanism of interaction of ligands with the receptor solely and in combination. Many stages are in the development of a binding assay: initial choices, establishment of assay conditions, validation, application to new ligands and quantitative analysis of the ensuing data to determine binding parameters for the ligands.[283]

3.2.1 Types of Binding Assays

Using radioligand binding as an illustration, three main types of receptor binding assays are possible: kinetic, competition/modulation and saturation. In a kinetic experiment, the binding of one or more concentrations of radioligand is quantified at an

incrementing succession of time points and afterwards analyzed to approximate the association (k_{on}) and dissociation rates (k_{off}). In modulation or competition experiments, the binding of one or more unchanging concentrations of a radioligand is quantified at equilibrium in the company of an incrementing succession of non-labelled compound, and the data is evaluated to decide the binding constant of the compound for the unliganded receptor as well as the cooperativity between the radioligand and compound for binding to the receptor. In saturation experiments, the binding of an accumulative series of concentrations of a radioligand *L*, is assessed at equilibrium and studied to decide its binding constant (affinity constant, K_a, or dissociation constant, K_d) and concentration of specific binding sites for radioligand (R_T); experimentally established R_T is typically described as $B_{max.}$[283]

3.2.2 Non-Specific Ligand Binding

In addition to binding to receptors of notice, ligands also bind to other sites. This binding to the receptor of interest is called specific binding, while binding to other sites is referred to as non-specific binding.[284, 285] Although the molecular details are not clearly defined, non-specific binding depends on charge or hydrophobicity of the molecule and not its exact structure. Non-specific binding can represent binding to cellular receptors, transporters or proteins that are not of interest to the researcher. Non-specific binding can also be interaction of the ligand with the sample container (or, depending on experimental set up, the membranes or filters used to separate bound from free ligand).[284]

Typically, non-specific binding is proportional to the amount of radioligand added. That is, adding twice as much radioligand will result in twice as much non-specific binding. Therefore, non-specific binding must be accounted for in receptor binding studies. Non-specific binding can be detected by measuring radioligand binding in the presence of a saturating amount of an unlabeled ligand/drug which binds to the receptors. In principle, virtually all the receptors of interest will be occupied by the unlabeled drug, so that any radioligand binding that is measured constitutes non-specific binding. In this way, specific binding can be determined by subtracting non-specific radioligand binding from the total ligand binding at that concentration.[284]

Determination of the specific unlabeled drug to be used to determine non-specific binding is also important. If no other drug/ligand is known to bind to the receptors (or the receptor of interest has not been identified), a logical answer is to use the same compound as the radioligand, but in its unlabeled form. In the majority of cases however, researchers try to avoid using the same compound and instead determine non-specific binding with a drug that is chemically different from the radioligand, but can bind to the same receptors.[286] Enough concentration of unlabeled drug should be added to block effectively all the specific radioligand binding, but not so much that general physical changes occur to the membrane, which could alter binding. For a well-characterized receptor, it is generally acceptable to use a concentration of unlabeled compound that is equal to approximately 100x its K_d for the receptors.[284]

3.2.3 Evaluating Ligand Binding Data – Non-linear Regression

Before the advent of non-linear regression, researchers had to transfer curved binding data into straight lines to analyze by linear regression. A common way to do this is by using a Scatchard Plot. A Scatchard plot can be a useful way to display data to show a change in B_{max} or $K_{d.}$ However, since linear transformation of the data distorts experimental error, the slope and intercept of a linear regression line should not be used to determine the values for B_{max} and K_d.[286]

There is more than one way to determine the B_{max} and K_d using nonlinear regression. The first method, and the method used in this research, is to subtract non-specific from total binding and analyze only the specific binding.[284] Or, one can simply analyze the total binding, and determine the amount of nonspecific binding from the shape of the total binding curve.[284, 285] But perhaps the best method, depending on the capabilities of available ligand binding analysis software, is to globally analyze total and non-specific binding at one time.[284]

3.3 Evaluating Leptin Binding to Erythrocytes by Enzyme Linked Immunosorbent Assay

Immunoassays are a frequently used analytical technique to quantitatively determine a wide range of analytes in medical, clinical, environmental, and biotechnological fields. The antibody molecule that recognizes the matching antigen (analyte), offers a high specificity of analysis. Immunoassays are useful in terms of sensitivity, selectivity, speed and cost effectiveness.[287, 288] The ELISA, depicted in Figure

3.1[289] in particular, is exceptional to other types of immunoassays in terms of kinetics, sensitivity and selectively. These benefits originate from selective antibody-antigen reactions, employment of surplus capture antibody and enzyme-antibody conjugate and chemical-augmentation with enzyme-conjugates that enable detection of exceedingly low (0.01 pg mL^{-1} to 100 ng mL^{-1})[290] analyte concentrations.[291-293] However, the ELISA is a multistep, labor-intensive procedure that entails long incubation periods and many incubation steps and washes.[294]

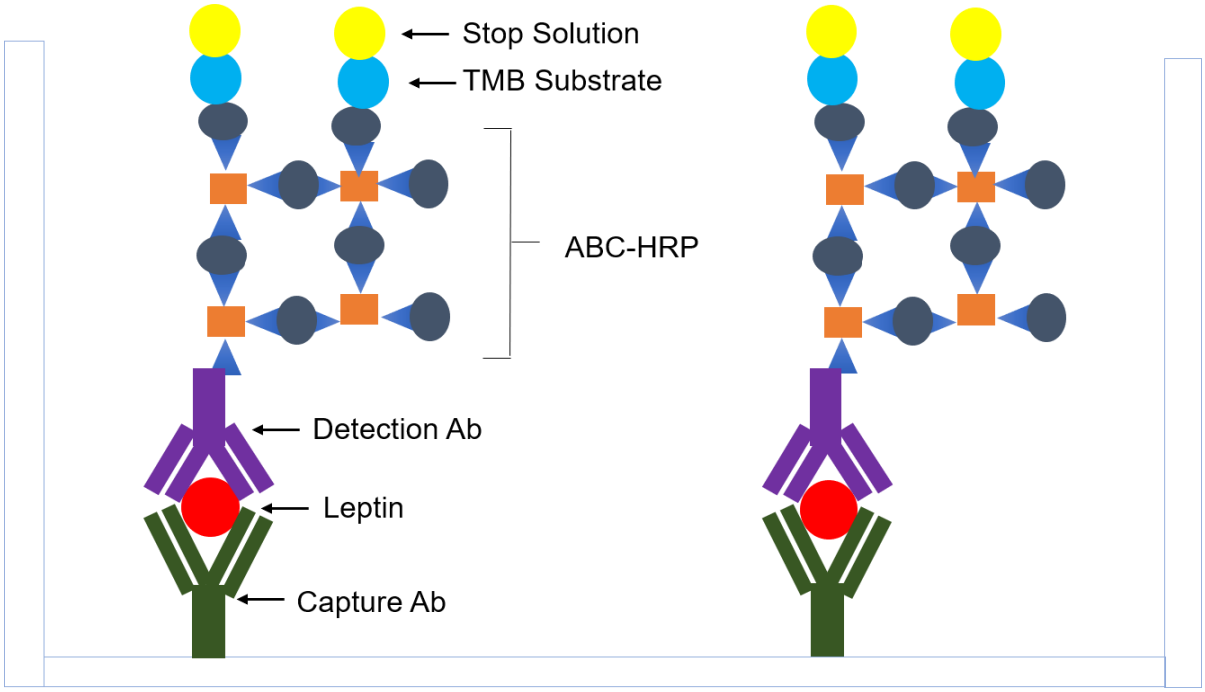


Figure 3.1: General schematic for a human leptin ELISA kit. The wells of a 96 well strip-plate are pre-coated with a leptin-specific antibody (capture antibody). The detection antibody is a leptin-specific biotinylated antibody. To measure human leptin, standards and samples are added to wells, then biotinylated detection antibody is added. The wells are washed with PBS, and then avidin-biotin-peroxidase complex (ABC-HRP) is added. Unbound ABC-HRP is washed away with PBS, and 3,3',5,5'-tetramethylbenzidine (TMB) color developing reagent is added. As a HRP substrate, the TMB is catalyzed to produce a blue color product, which changes to yellow after adding acidic stop solution. Boster Bio. Human Leptin ELISA Kit PicoKine. https://www.bosterbio.com/human-leptin-picokine-trade-elisa-kit-ek0437-boster.html (accessed July 20, 2021).

Leptin ELISA was initially used to evaluate leptin binding to erythrocytes and how it is influenced by C-peptide. That is, erythrocytes were incubated with leptin with or without C-peptide at 37 °C for a defined increment of time, centrifuged, followed by determination of leptin content remaining in the supernatant via leptin ELISA.

In the principle of the ELISA assay, the absorbance of the product (which in this case is yellow, as in Figure 3.1 and absorbing at 450 nM) is linearly proportional to the amount of analyte (in this case, human leptin) in the sample. When the absorbance of the yellow product in each well is read with a spectrophotometer, the sample readings can be benchmarked against a standard curve to determine the concentration of human leptin in the sample.[289]

However, the instrument response has a limited dynamic range, determined by many factors (i.e. signal-to-noise ratio, compound chemistry, instrument sensitivity, optical path length, specificity of optical filters, etc.).[295] This linear range is typically known or must otherwise be determined in advance of running the experiment. Thus, one noteworthy challenge of using an ELISA kit to quantify analyte concentration is appropriately diluting samples so that the analyte concentration falls within the target range. If samples are too concentrated, one may simply be measuring saturation of the ELISA kit wells rather than saturation of analyte binding to the target. If samples are too dilute, the absorbance signal from the analyte may be indistinguishable from background.

Although the experimental procedure for leptin was optimized so that samples were appropriately diluted for data collection, difficulties remained in quantitation of analyte concentration. Specifically, the concentration of leptin measured in the supernatant was often higher than the amount of leptin initially added to samples.

Although the cause of this error was not definitively determined, it was hypothesized that it may have been the result of the leptin ELISA being an indirect method of measurement. That is, the amount of analyte bound to cells was determined by measuring analyte in the supernatant from the cells, and then subtracting this value from the known amount of leptin originally added. However, because the ELISA depends on color change for detection, it was not possible to measure leptin binding to erythrocytes directly by this method. For this reason, an alternative, direct method was used to evaluate leptin interaction with erythrocytes and C-peptide, using leptin labeled with technetium-99m (^{99m}Tc).

3.4 Radiolabeling with ^{99m}Tc for Direct Binding Analysis of 99mTc-C-peptide and leptin to Erythrocytes

The vast use of ^{99m}Tc for medical imaging applications because of its favorable cost, physical properties and availability ignited interest in the development of ^{99m}Tc protein conjugates.[296] High specific activity ^{99m}Tc labeling can be accomplished using bifunctional coupling agents, and the most commonly used is 6-hydrazinonicotinic acid (HYNIC).[297] Schwartz et al. were the first to report preparation of hydrazine-modified proteins and their use for synthesis of ^{99m}Tc-protein conjugates.[296] Subsequently, the same research group reported on the use of the ^{99m}Tc-tricine precursor complex for radiolabeling HYNIC protein conjugates. The development of the ^{99m}Tc-tricine precursor further improved the efficiency of this labeling technique. Using this refined procedure, proteins are labeled on primary amino groups with the N-hydroxysuccinimide ester of

HYNIC (succinimidyl 6-hydrazinonicotinate hydrochloride).[298] The resulting HYNIC derivative can then be labeled with ^{99m}Tc to produce ^{99m}Tc-labeled proteins in high yield.[296, 298] Tin is used to reduce the $^{99m}TcO_4^-$, while tricine serves as the coligand reagent to create a stable chelation complex with HYNIC.[298] Although the exact structure of Tc-99m-HYNIC-co-ligand complexes remain uncertain, liquid chromatography mass spectrometry (LC-MS) and stability studies have revealed insights (Figure 3.2).[299]

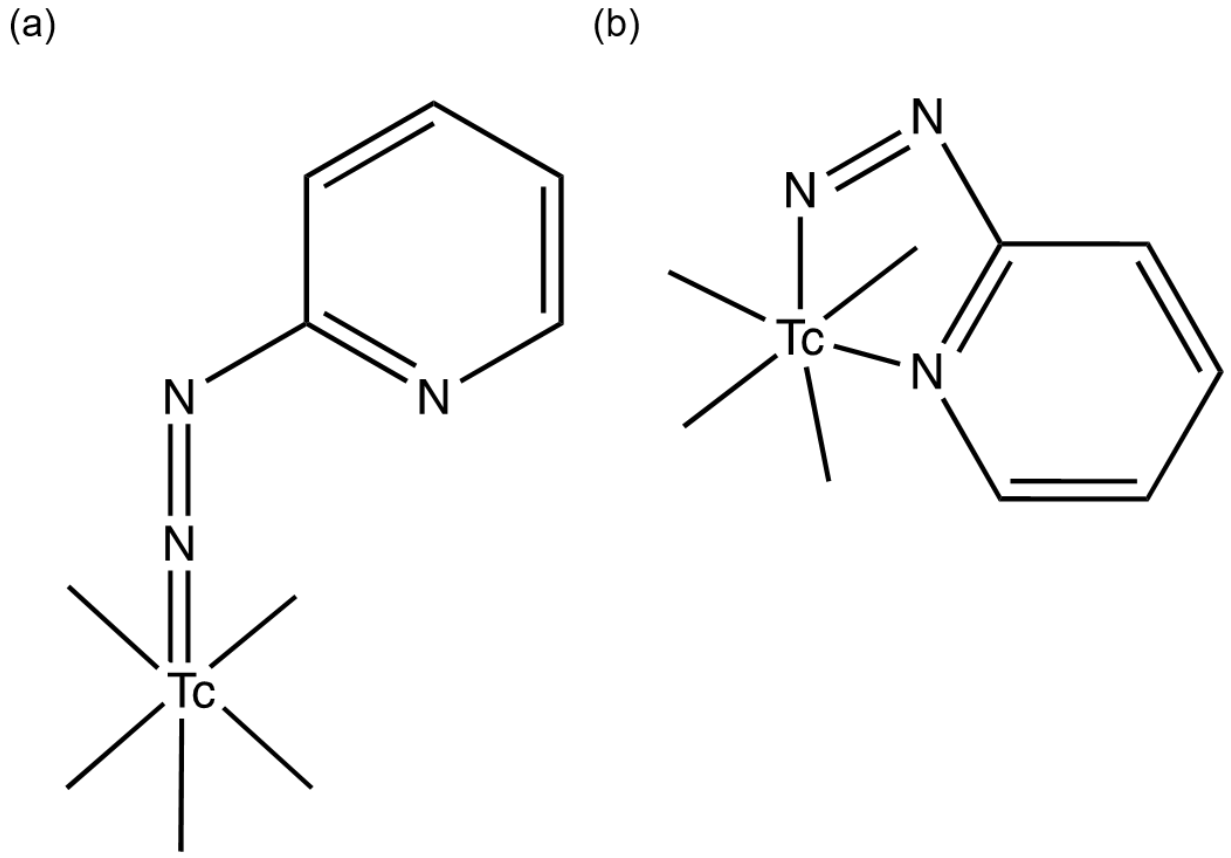


Figure 3.2: Possible structures of ^{99m}Tc-HYNIC-peptide complexes showing (a) monodentate (attached to the central atom of a coordination complex by one bond) and (b) bidendate (attached by two bonds) binding of HYNIC to technetium. Adapted from King et al., *Dalton. Trans.* **2007**, *43*, 4998-5007.

3.5 Experimental Methods

3.5.1 Preparation of Lyophilized HYNIC reagent

1.2 mg of HYNIC (given by Dr. Gary Bridger, AnorMED, Inc., Langley, British Columbia, Canada) was diluted in 1 mL of dimethylformamide (DMF; Acros Organics, Geel, Belgium). For leptin-HYNIC kits, 9.9 µL of formulated solution was used (for a 3:5 ratio of HYNIC:leptin). The solution was relocated via pipet into a 5 mL lyophilization vial (Wheaton, Millville, NJ), and the septum (Fisher Scientific, Waltham, MA) was lightly positioned on top. The vials were moved to a freeze-dryer (VirTis AdVantage, Gardiner, NY) that was precooled to -80 °C. The vacuum (Leybold, Cologne, Germany) was selected for 10 seconds and then freeze was set for 45 minutes. Following, the condenser was turned on, and after reaching -90 °C, the freezer was turned off and the vacuum was turned on. The heat was put to -70 °C, the vials were left overnight, and the next morning, the heat was set to 10 °C for 1 hour. Next, an air pump (Craftsman; Stanley Black and Decker, New Britain, CT) was connected to the freeze dryer, and the air valve was opened. To assist closure of the septa on the vials, the stopper of the freeze-dryer was engaged three times. Finally, caps were screwed onto the vials, and they were kept at -20 °C until needed.

3.5.2 Preparation of Lyophilized Tin Tricine Reagent

Using a procedure adapted from the Tait research laboratory at the University of Washington,[300] 3.6 g of tricine (Sigma Aldrich, St. Louis, MO) were put in a beaker with 98 mL of 18.2 MΩ, double deionized water (DDW). The pH was adjusted to 7.1 with dropwise supplementation of 5 M NaOH while stirring. The solution was moved to a vacuum flask before degassing the solution using a sonicator (Branson, Danbury, CT) and vacuum. Next, 80 mg of $SnCl_2$ (Acros Organics) were added to 1.7 mL of 100% ethanol (Decon Labs, Inc., King of Prussia, PA). When the tricine was totally degassed, 100 µL of $SnCl_2$ solution were added to the tricine and mixed. 1 mL portions of the solution were pipetted into 5 mL lyophilization vials (Wheaton). The freeze-drying method outlined in section 3.4.1 was used to lyophilize the tin tricine reagent, allowing preparation of a maximum of 30 vials per batch.

3.5.3 Preparation of C-peptide

Crude C-peptide (85% pure; Peptide 2.0, Chantilly, VA) was purified by reverse-phase high performance liquid chromatography (HPLC; Shimadzu, Kyoto, Japan) utilizing an Atlantis T3 OBD Prep Column (10 mm x 150 mm; Waters, Milford, MA) for separation. HPLC parameters are shown in Table 3.1. The purified C-peptide was lyophilized overnight (Labconco Corporation, Kansas City, MO) and then characterized using mass spectrometry (Waters, MA). Lyophilized C-peptide was stored at -20 °C.

To use in experiments, a small amount of the lyophilized C-peptide (~0.2-0.3 mg) was resuspended in DDW, and its concentration deduced using a C-peptide ELISA kit (ALPCO Salem, NH). Subsequently, the C-peptide was diluted and kept as 8 μM aliquots in DDW at 4 °C, with final dilution to 800 nM in DDW immediately before use.

Table 3.1 C-peptide purification by HPLC gradient method. Solvent A: 0.1% HPLC-grade trifluoroacetic acid (TFA; EMD Millipore, Burlington, MA) in HPLC-grade water (Sigma Aldrich). Solvent B: 0.089% TFA/60% HPLC-grade acetonitrile (Sigma Aldrich) in HPLC-grade water.

Time (min)	Flow Rate (mL/min)	% Solvent A	% Solvent B
5	5	100	0
40	5	40	60
50	5	0	100
60	5	stop	stop

3.5.4 Preparation of Zn^{2+}

$ZnCl_2$ (Columbus Chemical Industries, Inc., Columbus, WI) was used for the Zn^{2+} source. Zn^{2+} was prepared as a 1.5 mM solution, then diluted and maintained as an 80 μM solution in DDW, with dilution to 800 nM in DDW immediately prior to use.

3.5.5 Preparation of ^{99m}Tc-leptin

Recombinant human leptin (R&D systems, Minneapolis, MN) was used as the leptin source in radiolabeling experiments. For this purpose, a 5 mg vial of lyophilized

leptin, which was stored at -20 °C upon receipt from manufacturer, was resuspended to around 5 mg/mL in 1-3 mL of phosphate-buffered saline (PBS), relocated to a 10 kDa molecular weight cut-off (MWCO) dialysis cassette (Thermo Scientific, Rockford, IL), and exchanged in PBS overnight at 4 °C (this was done to remove TRIS present from manufacture, that having a free amine group, could compete for binding to HYNIC in the protein labeling procedure). The PBS contained in mM, 1.8 KH_2PO_4 (Sigma Aldrich), 137 NaCl (Sigma Aldrich), 2.7 KCl (Thermo Fisher) and 10 Na_2HPO_4 (Sigma Aldrich) in DDW at pH 7.4. The concentration of leptin after dialysis, typically between 200-400 µM, was quantitated using a Human Leptin ELISA Kit (Picokine, Boster Biological Technology, Pleasanton, CA). The leptin was kept in aliquots of 200 µL at -20 °C.

^{99m}Tc radiolabeling was accomplished by conjugation with HYNIC. HYNIC was coupled to leptin by incubating for one hour in the dark, one of the 200 µL exchanged leptin aliquots in PBS with 37.5 µmol of lyophilized HYNIC, from a kit that was prepared beforehand as described in section 3.4.1, in 600 µL of Na_2PO_4 buffer (0.15 M, pH 7.8). After, the mixture was dialyzed overnight in a 10 kDa MWCO cassette (Thermo Scientific) at 4 °C in PBS to separate unconjugated, free HYNIC.

After dialysis, around 50 mCi of pertechnetate ($^{99m}TcO_4^-$; Cardinal Health, Swartz Creek, MI) in 0.3-0.5 mL of saline were chelated to the leptin-HYNIC conjugate. By a previously described method, tricine was utilized as the transchelator.[298] To start, all of the pertechnetate was added to a lyophilized tin tricine reagent ‘kit’ containing 0.2 mmol of tricine and 0.567 µmol of $SnCl_2{\cdot}2H_2O$ (previously prepared as described in section 3.4.2) and incubated for 15 min to reduce ^{99m}Tc. Next, 100-250 µL of the tricine-^{99m}Tc chelation complex were added to the leptin-HYNIC conjugate, which had exchanged in

PBS overnight. The mixture was allowed to incubate for 30 minutes before purification with a 10 mL, 6 kDa MWCO polyacrylamide desalting column (Thermo Scientific).

Radiochemical purity of the fractions containing radiolabeled leptin was checked by thin-layer chromatography (TLC)[301] using Tec-Control Chromatography Strips (Biodex, Shirley, NY). ^{99m}Tc-leptin (2-5 µL) was dotted onto the origin line of two separate chromatography strips and eluted with PBS or methyl ethyl ketone (MEK; Acros Organics). Subsequently, the strips were cut in half, placed into exclusive microcentrifuge tubes (VWR, Radnor, PA) and counted in a 2480 WIZARD^2 automatic gamma counter (Perkin Elmer, Inc., Waltham, MA). The idea of the TLC assay was that radiolabeled leptin would not move, or move only marginally, on the strips while using PBS for elution, in this way separating the labeled leptin from free ^{99m}Tc (either as ^{99m}Tc-tricine complex or pertechnetate). Using MEK for elution, only pertechnetate should move along the strips. The percentage of unconjugated ^{99m}Tc could be determined for the fraction(s) by dividing the counts at the top of the strip by the total counts. After purity of the fraction(s) was confirmed by TLC, the Lowry assay for protein concentration was carried out against known concentrations of unlabeled leptin standards (from stored 200 µL aliquots in PBS). This step allowed forthcoming association of protein concentration with gamma emission from the labeled leptin.

3.5.6 Isolation and Purification of Erythrocytes

All methods for obtaining whole blood were approved by the Human Investigation Committee Branch of the Institutional Review Board of Michigan State University. Whole

blood was acquired by venipuncture from healthy, consenting human donors and drawn into vacutainer heparinized (Fisher Scientific) or citrated tubes (Franklin Lakes, NJ). Following, the blood was centrifuged at 500*g* for 10 minutes. After removal of the buffy coat and plasma, the left-over erythrocytes were rinsed by resuspending in physiological saline solution (PSS) or an albumin free version of this solution (AF-PSS). The PSS contained, in mM, 2.0 $CaCl_2$ (Thermo Fisher), 140.5 NaCl, 4.7 KCl, 12 $MgSO_4$ (Thermo Fisher), 21.0 tris[hydroxymethyl]aminomethane (Invitrogen, Waltham, MA), 5.5 dextrose and 0.5% bovine serum albumin (BSA, Sigma Aldrich) in deionized and distilled water (DDW, 18.2MΩ) at pH 7.4 (BSA not included for AF-PSS). After at least three washings of the erythrocytes with the correct form of PSS, the final hematocrit of the erythrocytes (normally around 70%) was evaluated using a StatSpin MP microhematocrit centrifuge (Beckman Coulter, Brea, CA) and StatSpin CritSpin digital hematocrit reader (Beckman Coulter).

3.5.7 Standard and Sample Preparation for Radiolabeling Experiments

Saturation binding experiments were performed to determine the amount of specific binding of ^{99m}Tc-leptin to erythrocytes. ^{99m}Tc-leptin standards were prepared in AF-PSS using the purified ^{99m}Tc-leptin stock described above (as ^{99m}Tc-leptin concentration remaining on erythrocytes after several wash steps was dramatically reduced, following initial experiments standards were adjusted from 0-1000 nM to a more appropriate range of 0-200 nM). All samples (for total and non-specific binding analysis) were prepared by first adding either adding 25 µL of 800 nM C-peptide (which resulted in

a concentration of 20 nM in 1 mL final sample volume, or 20 pmol per sample) or 25 µL of DDW (to later determine ^{99m}Tc-leptin binding without C-peptide) to 1.7 mL microcentrifuge tubes. Next, for total binding samples, AF-PSS was added to 1.7 mL tubes followed by addition of the relevant volume of ^{99m}Tc-leptin in AF-PSS (between 0-500 µL) to reach the intended final concentration of leptin in the sample solution (0-1000 nM). Samples for analysis of non-specific ^{99m}Tc-leptin binding were prepared in a similar manner to those for total binding, only that a surplus of unlabeled leptin or BSA was added to the samples prior to incubation. For samples using unlabeled leptin as the ligand to determine non-specific binding, unlabeled recombinant human leptin (R&D systems), which had been dialyzed in PBS and concentration verified using leptin ELISA, (Boster Bio) was added to AF-PSS before (so that each 1 mL sample contained 10 µM, or 10 nmol of unlabeled leptin) prior to addition of erythrocytes washed in AF-PSS. For samples using BSA as the ligand to determine non-specific binding, PSS (containing 75 µM BSA) was used as the sample buffer instead of AF-PSS. Erythrocytes were also washed in PSS. The result was that each sample contained around 60 µM in 1 mL volume, or 60 nmol of BSA. Finally, for all samples, erythrocytes that had been washed in the designated sample buffer were added in a volume to attain a 7% solution in 1 mL total volume.

Standards and samples were incubated at 37 °C for two hours with intermittent inverting. After, samples were washed and centrifuged at 750*g* for one minute, with following removal of the supernatant from the erythrocytes and replacement with AF-PSS. After five of these wash steps, gamma radiation emission from the radiolabel was

evaluated using a gamma counter. The amount of label bound to each erythrocyte was analyzed using a standard curve generated with ^{99m}Tc-leptin standards.

For samples used to examine total leptin binding *only*, at lower, physiologic concentrations, the experimental setup was as described above except that no samples were prepared with excess ligand to block non-specific binding. Also, although the data is not shown, for samples containing lower concentrations of ^{99m}Tc-leptin (0-5 nM), gamma radiation emission was evaluated and recorded after each wash step to ensure that the signal recorded at the final (fifth) wash step was not below the detection limit of the instrument.

3.6 Results

The first experiments performed to examine ^{99m}Tc-leptin binding to erythrocytes were used to construct a saturation binding curve in the absence of C-peptide. To construct the binding curve shown in Figure 3.3 (a), various concentrations of ^{99m}Tc-leptin were incubated with erythrocytes, both alone (shaded circles), and in the presence of an excess, or saturating amount of unlabeled leptin (open circles). By analyzing ^{99m}Tc-leptin binding in the presence of excess unlabeled leptin, the amount of non-specific binding was experimentally measured, and specific binding was determined as the difference between total and non-specific binding (Figure 3.3 (b)).

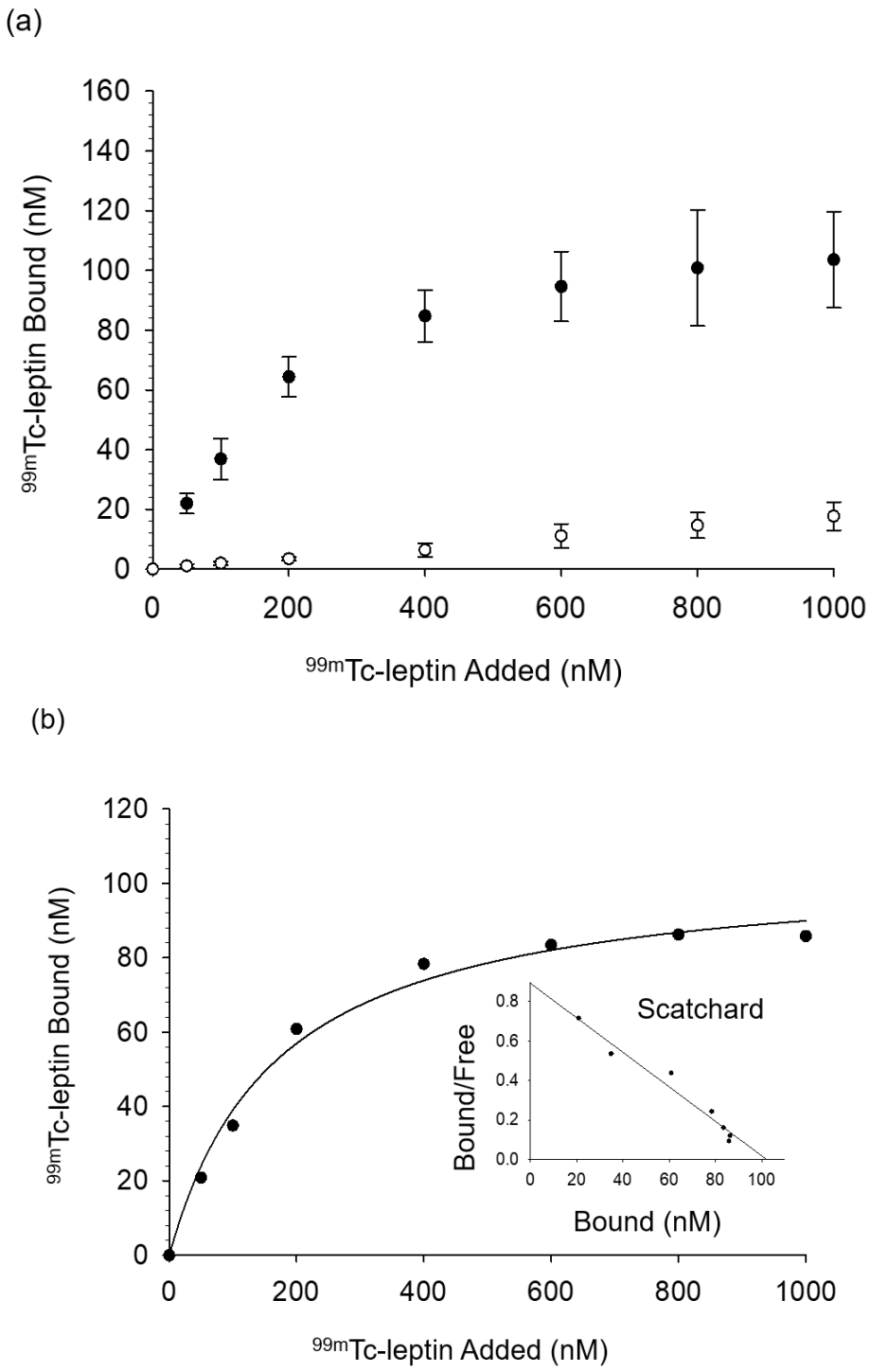


Figure 3.3: ^{99m}Tc-leptin binding to erythrocytes in the absence of C-peptide. In (a), experimentally determined total ^{99m}Tc-leptin binding (closed circles) and non-specific ^{99m}Tc-leptin binding (open circles) are displayed [^{99m}Tc-leptin] = 0-1000 nM, 7% erythrocytes. *n* = 3, error = sd. In (b) specifically bound ^{99m}Tc-leptin was determined and analyzed using non-linear regression software (SigmaPlot 13.0), assuming a one-site binding model. (K_d = (1.69 ±0.24) x 10^{-7} M, B_{max} = (1.05 ±0.04) x 10^{-7} M (goodness-of-fit (R^2) = 0.9896).

After saturation binding experiments were performed in the absence of C-peptide, the next step was to conduct the same experiments with the addition of 20 nM C-peptide to samples (Figure 3.4).

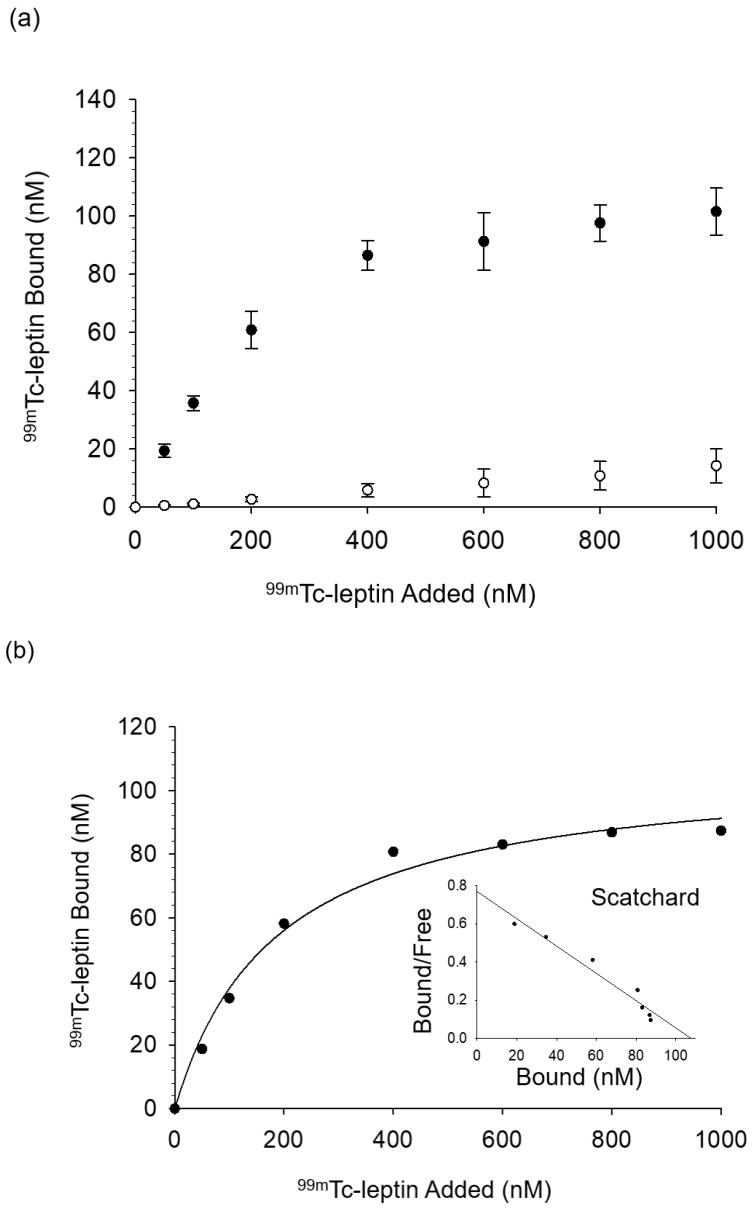


Figure 3.4: ^{99m}Tc-leptin binding to erythrocytes in the presence of C-peptide. In (a), experimentally determined total ^{99m}Tc-leptin binding (closed circles) and non-specific ^{99m}Tc-leptin binding (open circles) are displayed. [^{99m}Tc-leptin] = 0-1000 nM, [C-peptide] = 20 nM, 7% erythrocytes. n = 3, error = sd. In (b) specifically bound ^{99m}Tc-leptin was determined and analyzed using non-linear regression software (SigmaPlot 13.0), assuming a one-site binding model. (K_d = (1.86 ± 0.29) x 10^{-7} M, B_{max} = (1.08 ±0.05) x 10^{-7} M (goodness-of-fit (R^2) = 0.9884).

In Figure 3.5, comparison of specific binding of ^{99m}Tc-leptin in the presence or absence of C-peptide, as determined using leptin as the unlabeled ligand, is shown.

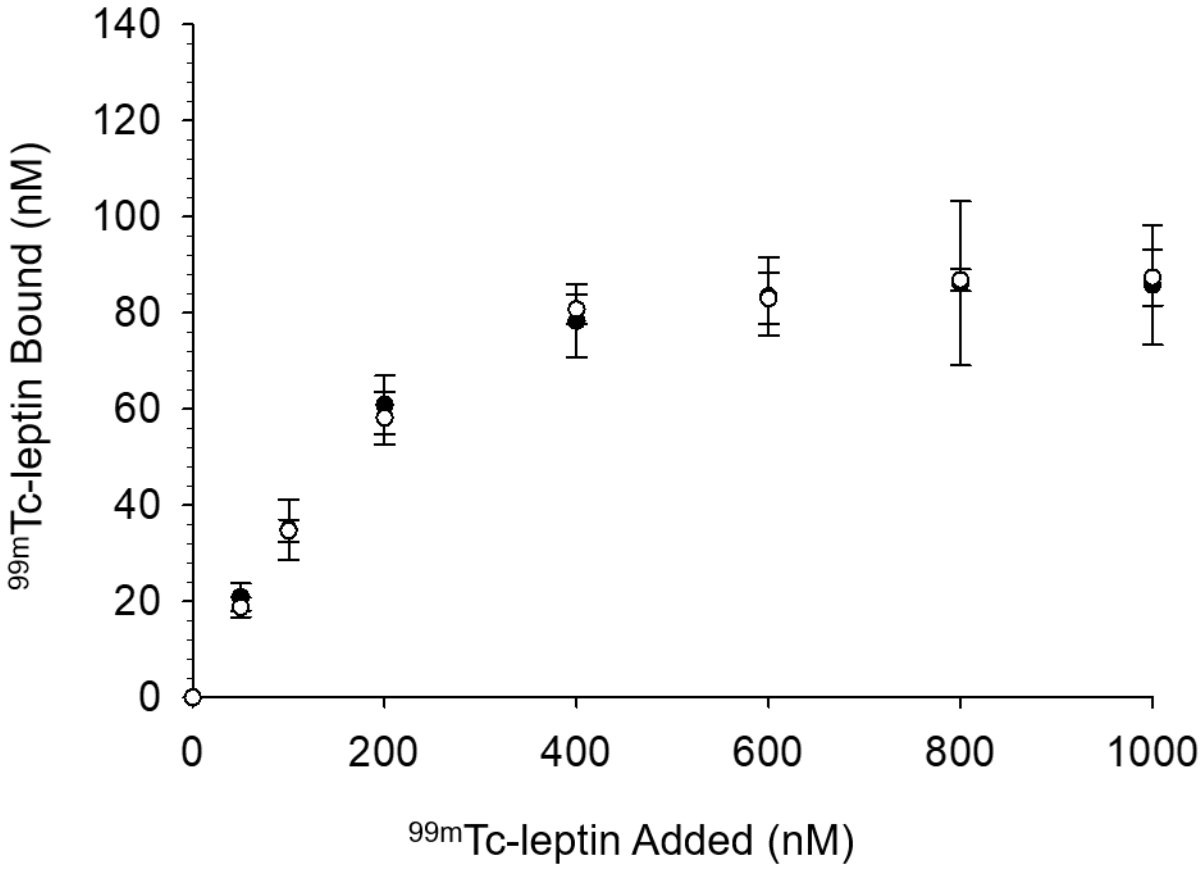


Figure 3.5: Specific binding of ^{99m}Tc-leptin to erythrocytes in the absence (closed circles) or presence (open circles) of 20 nM C-peptide, using unlabeled leptin to determine non-specific binding. [^{99m}Tc-leptin] = 0-1000 nM, [C-peptide] =0 or 20 nM, 7% erythrocytes. *n* = 3, error = sd.

After specific binding of leptin in a saturating concentration of unlabeled leptin in the presence or absence of C-peptide was evaluated, the effect of different concentrations of C-peptide in the sample buffer on total ^{99m}Tc-leptin binding to erythrocytes was examined, as displayed in Figure 3.6. Concurrent radiolabeling work by another Spence group member indicated that saturation of C-peptide binding to the

erythrocyte may occur near 100 nM, as opposed to 20 nM, as had previously been determined in the Spence group. Therefore, the effect of additions of much higher levels of C-peptide was investigated by adding concentrations of unlabeled C-peptide between 0-1000 nM.

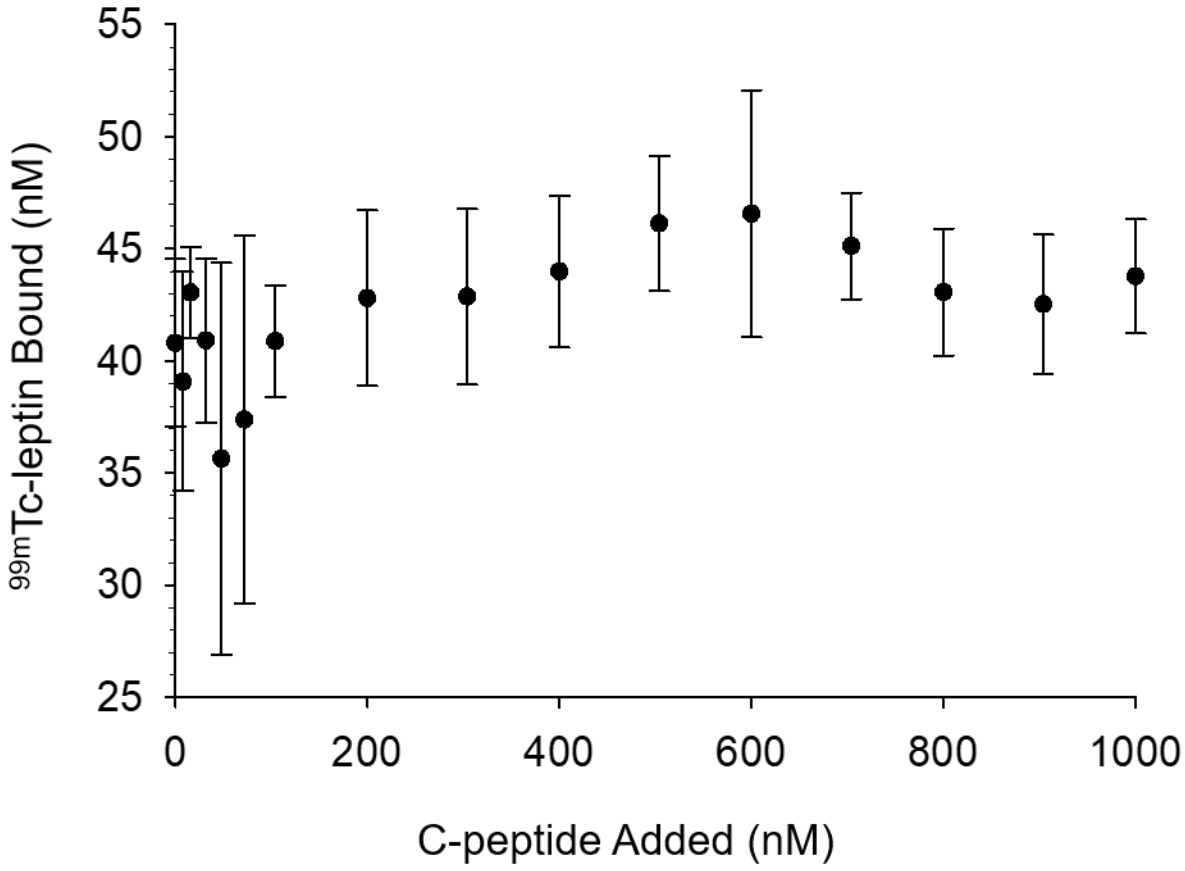


Figure 3.6: Total ^{99m}Tc-leptin binding to erythrocytes in the presence of increasing amounts of C-peptide. [^{99m}Tc-leptin] = 100 nM, [C-peptide] = 0-1000 nM, 7% erythrocytes, *n* = 3, error = sd.

Next, in Figure 3.7, the effect of C-peptide on ^{99m}Tc-leptin binding at physiologically relevant concentrations of C-peptide and leptin is shown.

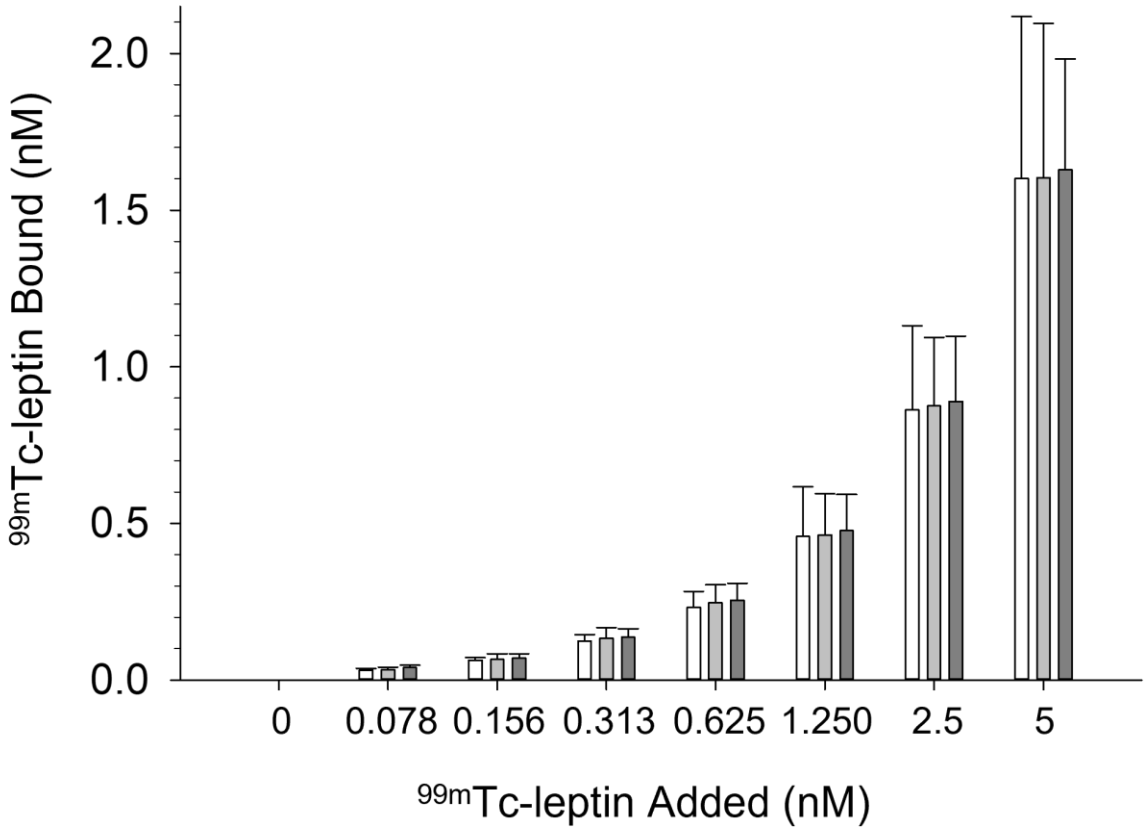


Figure 3.7: Analysis of the effect of C-peptide on total ^{99m}Tc-leptin binding to erythrocytes at physiologically relevant levels. Physiological concentrations of leptin (0-5 nM) were added to erythrocyte samples containing either 0 nM (white), 0.6 nM (light grey) or 20 nM (dark grey) C-peptide. After several wash steps, ^{99m}Tc-leptin binding to erythrocytes was radiometrically determined. 7% erythrocytes, *n* = 3, error = sd.

Although Zn^{2+} is not involved in binding of the C-peptide/carrier protein complex to the erythrocyte, because $Zn^{2+}$is necessary for C-peptide-related erythrocyte derived ATP and downstream NO release, the effect of Zn^{2+} on ^{99m}Tc-leptin binding, in the presence of C-peptide was examined, as shown in Figure 3.8. A literature search revealed an average physiological serum leptin level of approximately 0.5 nM, with upper values approaching 5 nM, so labeled leptin concentrations were prepared in these regions.[276] Because previous experiments in the Spence lab have revealed a saturating

concentration of C-peptide to erythrocytes of approximately 20 nM, but average serum C-peptide levels are near ~0.6 nM,[302] both of these concentrations were evaluated. For Zn^{2+} concentrations, an amount was added that was equimolar to the C-peptide saturating concentration, as well as a value near the average physiological serum level, of approximately 10 µM.[303]

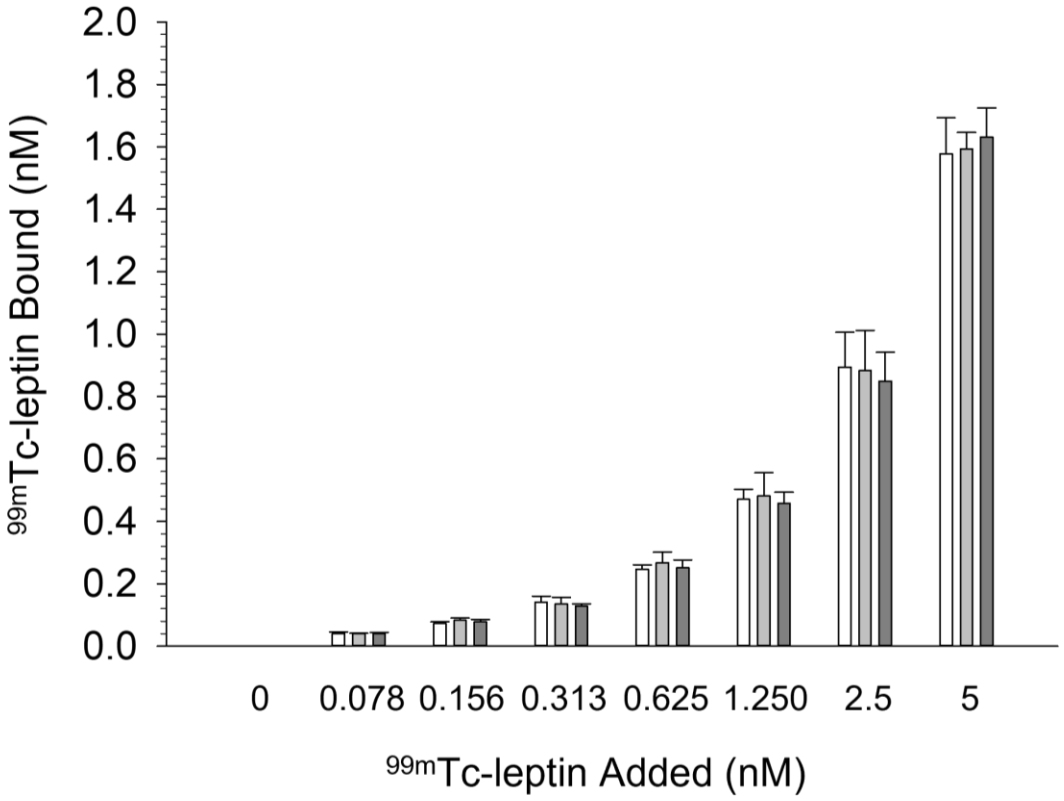


Figure 3.8: Analysis of the effect of Zn^{2+} in the presence of C-peptide on ^{99m}Tc-leptin binding to erythrocytes at physiologically relevant levels. Physiological concentrations of leptin (ranging from 0-5 nM) were added to erythrocyte samples containing 20 nM C-peptide and either 0 nM (white), 20 nM (light grey) or 10 µM (dark grey) Zn^{2+}. After several wash steps, ^{99m}Tc-leptin binding to erythrocytes was radiometrically determined. 7% erythrocytes, *n* = 3, error = sd.

In the following experiments, shown in Figure 3.9, to increase the number of replicates, ^{99m}Tc-leptin binding to erythrocytes for single, physiologically relevant concentrations of ^{99m}Tc-C-peptide and leptin were evaluated, focusing on the average

serum leptin level of approximately 0.5 nM with a saturating concentration of C-peptide of 20 nM. Without C-peptide, after several wash steps, 0.13 ± 0.04 nM ^{99m}Tc-leptin bound to the erythrocytes, with C-peptide, a minor increase was observed of 0.14 ± 0.06 nM.

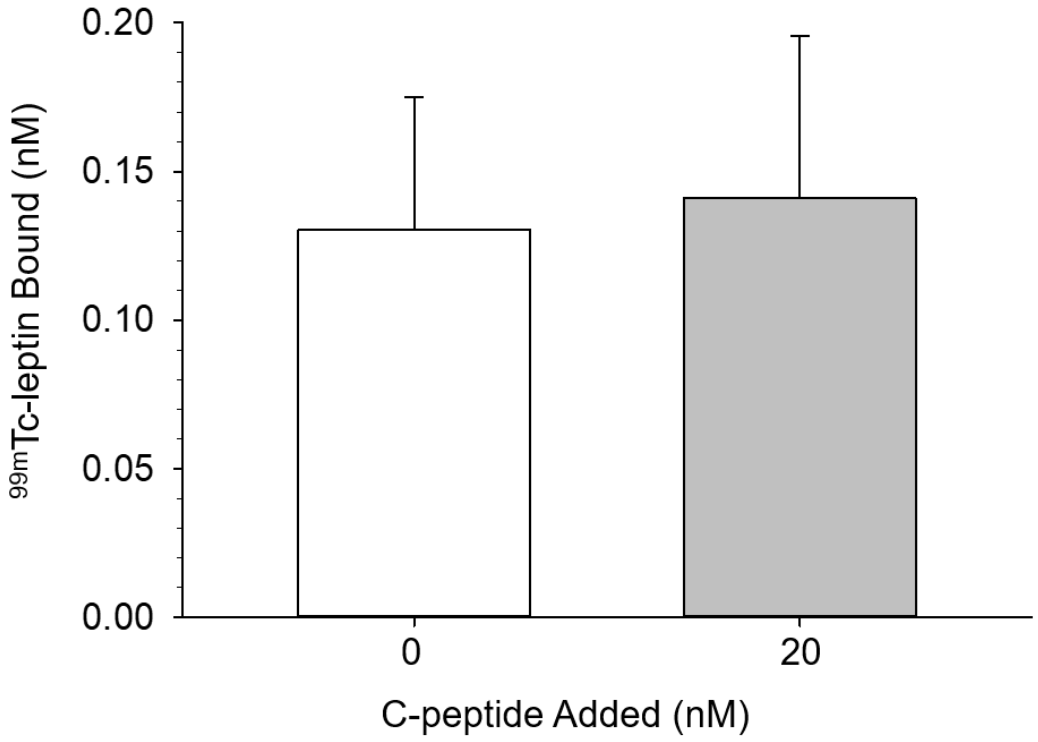
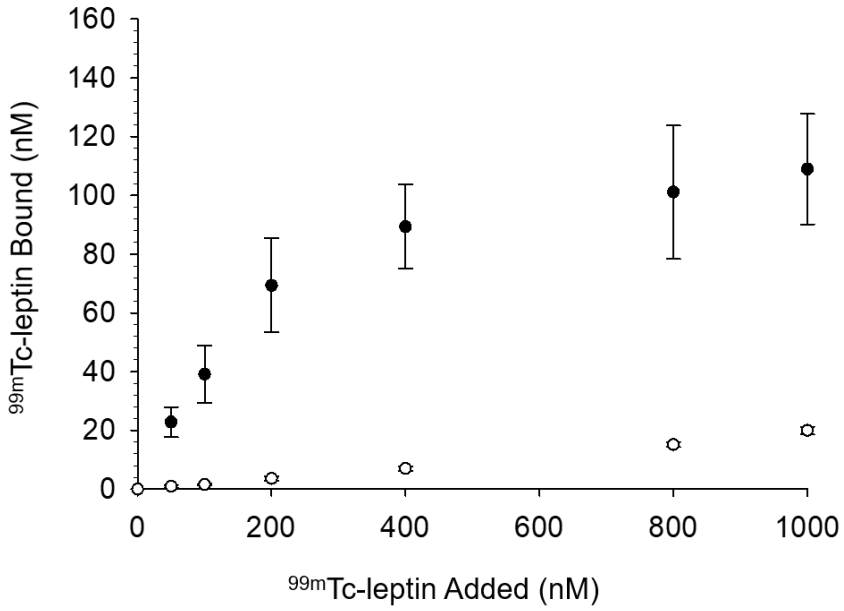


Figure 3.9: Comparison of total ^{99m}Tc-leptin binding to erythrocytes without (plain bar) or with C-peptide (light grey bar). A single concentration of ^{99m}Tc-leptin (0.5 nM, representing the average serum leptin level under normal physiological conditions) was chosen to the effect of a saturating concentration of C-peptide (20 nM) on leptin binding to erythrocytes.7% erythrocytes, *n* = 3 (3 biological replicates, 11 technical replicates), error = sd.

Next experiments evaluated binding constants for specific ^{99m}Tc-leptin binding to erythrocytes in the absence or presence of C-peptide, using BSA as the agent to determine non-specific binding (as opposed to unlabeled leptin). First, as shown in Figure 3.10, specific binding of leptin to erythrocytes was evaluated in the absence of C-peptide.

(a)



(b)

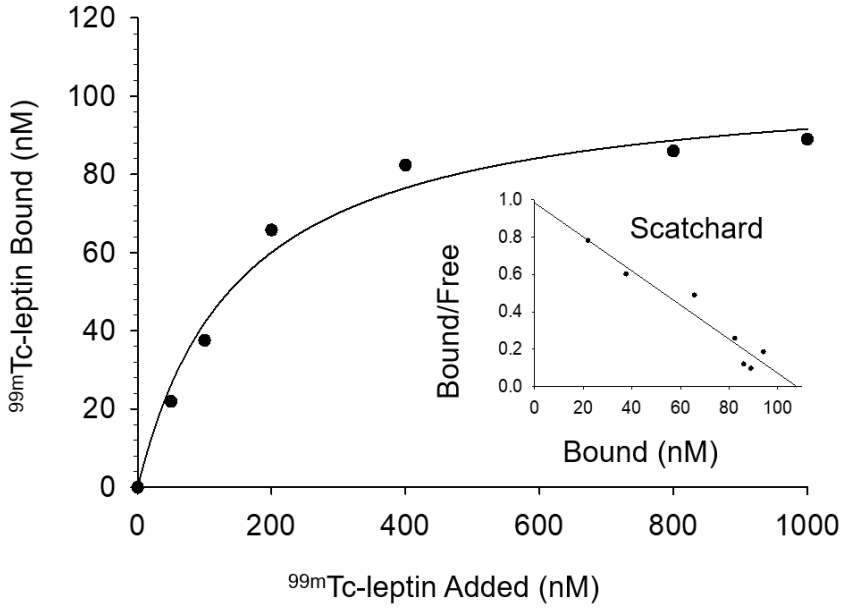
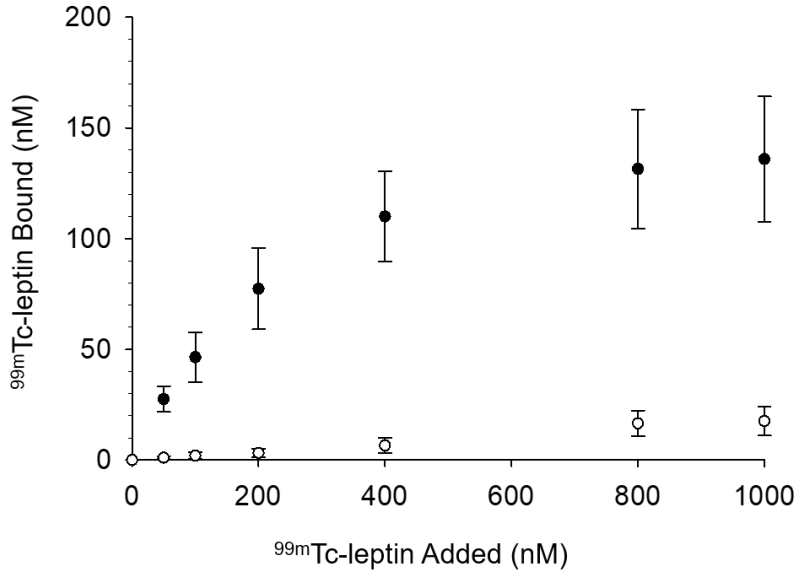


Figure 3.10: ^{99m}Tc-leptin binding to erythrocytes in the absence of C-peptide. In (a), experimentally determined total ^{99m}Tc-leptin binding (closed circles) and non-specific ^{99m}Tc-leptin binding (open circles) are displayed. [^{99m}Tc-leptin] = 0-1000 nM, 7% erythrocytes. n = 3, error = sd. In (b) specifically bound ^{99m}Tc-leptin was determined and analyzed using non-linear regression software (SigmaPlot 13.0), assuming a one-site binding model. (K_d = (1.51 ±0.28) x 10^{-7} M, B_{max} = (1.05 ±0.06) x 10^{-7} M (goodness-of-fit (R^2) = 0.9840).

Next, as shown in Figure 3.11, specific binding of ^{99m}Tc-leptin, again using BSA to determine non-specific binding, was evaluated in the presence of C-peptide.

(a)



(b)

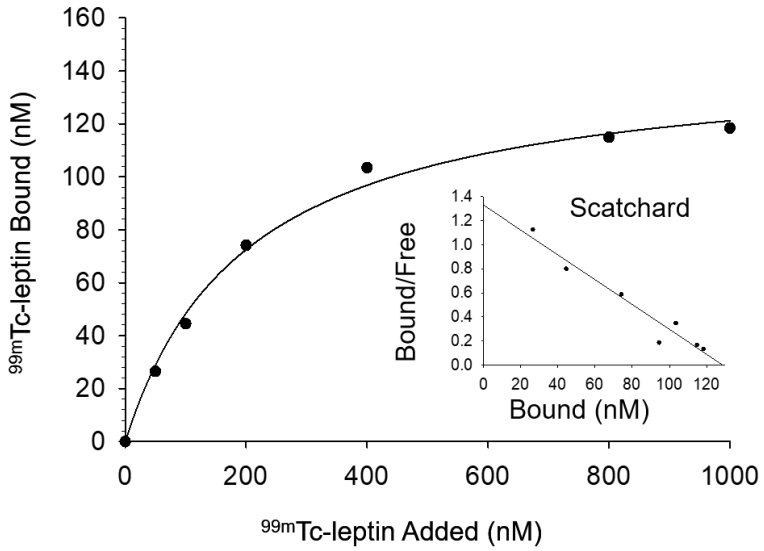


Figure 3.11: ^{99m}Tc-leptin binding to erythrocytes in the presence of C-peptide. In (a), experimentally determined total ^{99m}Tc-leptin binding (closed circles) and non-specific ^{99m}Tc-leptin binding (open circles) are displayed. [99mTc-leptin] = 0-1000 nM, [C-peptide] = 20 nM, 7% erythrocytes. *n* = 3, error = sd. In (b) specifically bound ^{99m}Tc-leptin was determined and analyzed using non-linear regression software (SigmaPlot 13.0), assuming a one-site binding model. (K_d = (2.01 ±0.23) x 10^{-7} M, B_{max} = (1.46 ±0.06) x 10^{-7} M (goodness-of-fit (R^2) = 0.9940).

Finally, a comparison between specific ^{99m}Tc-leptin binding, as determined by using BSA as the ligand to determine non-specific binding, in the presence or absence of C-peptide, is shown in Figure 3.12.

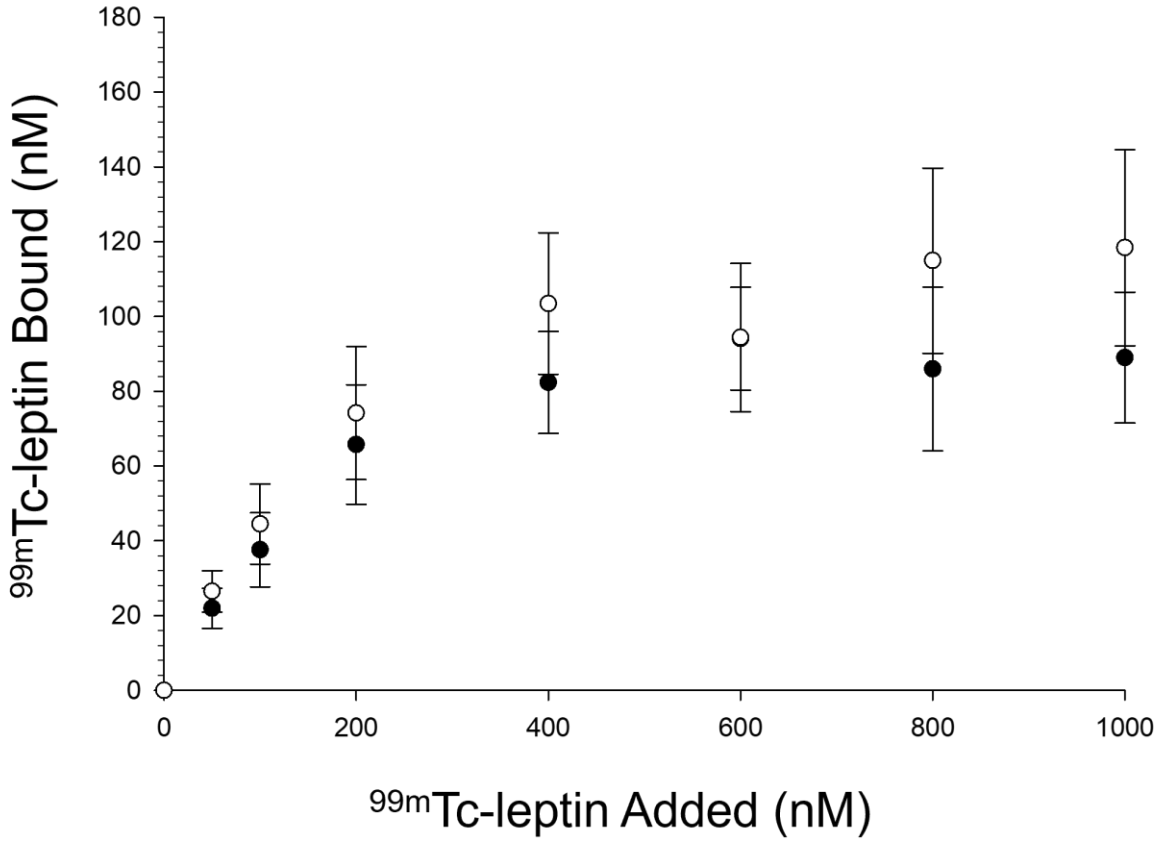


Figure 3.12: Specific binding of ^{99m}Tc-leptin to erythrocytes in the absence (closed circles) or presence (open circles) of 20 nM C-peptide, using unlabeled albumin to determine non-specific binding. [^{99m}Tc-leptin] = 0-1000 nM, [C-peptide] =0 or 20 nM, 7% erythrocytes. *n* = 3, error = sd.

3.7 Discussion

Leptin has long been known to be implicated in energy balance in vivo,[170, 173, 175] particularly in terms of the control of appetite and obesity.[177, 178] Also, leptin has been implicated as having a role in blood flow properties.[235, 238, 253] Related reports imply that this adipocyte-derived cytokine hormone interacts with components of the bloodstream. In agreement, previous work utilizing a fluorescence isothiocyanate (FITC)-labeled form of leptin reported binding to erythrocytes in a specific and saturable way.[304] Here, it is reported that leptin delivers a pancreatic hormone to the erythrocyte, a delivery that may be hindered or negatively affected by the FITC label (Figure 3.13)[305-308] itself, so a ^{99m}Tc label was used. Of note, the binding data that was obtained using the ^{99m}Tc-labeled leptin was comparable to results in the literature using the FITC label,[304] verifying that leptin has a specific and saturable binding site on the erythrocyte membrane. Importantly, the results provide evidence that the FITC label did not interfere with leptin binding to the erythrocyte in a previous report.[304]

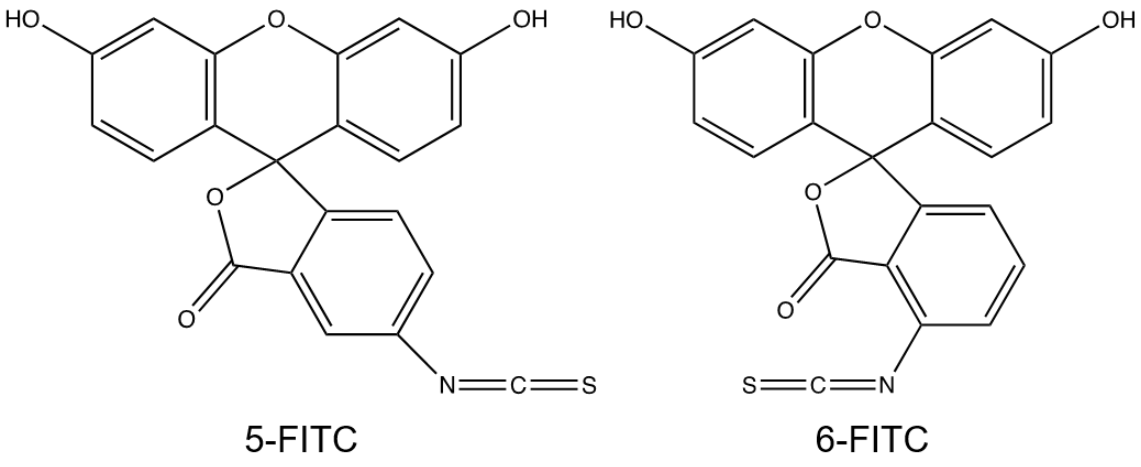


Figure 3.13: Isomers of fluorescein isothiocyanate (FITC), a derivative of fluorescein used in many applications such as flow cytometry. FITC is reactive toward nucleophiles, amine, and sulfhydryl groups on proteins.

Here, several aspects of ^{99m}Tc-leptin binding to erythrocytes were examined to discern any influence from C-peptide and investigate the possibility of a receptor binding complex. Saturation binding experiments were performed, in which a B_{max} and K_d for leptin binding to erythrocytes were determined in the presence and absence of C-peptide, using two different ligands to determine non-specific binding: leptin and BSA. Leptin was chosen because the receptor of interest on the erythrocyte is not definitively known, and BSA was chosen because it is a common agent used for blocking, and it is know from prior work[149] that it has an important relationship to C-peptide.

While there was not a statistically significant difference in K_d, it is noteworthy that in both instances, whether blocking with leptin or BSA, there was an increase in the amount of leptin binding to erythrocytes in the presence of C-peptide, as indicated by a higher B_{max} value. This may be indicative of a separate binding complex for a leptin/C-peptide complex vs. leptin alone. Moreover, it is notable that there is a much more striking difference in leptin binding in the presence or absence of C-peptide when non-specific binding was determined using BSA. The reason for this is not known and warrants investigation in future studies.

Because the increase in binding in the presence of C-peptide that was observed when using leptin as the ligand to determine non-specific binding was very minor, it was necessary to determine if a statistically significant difference in leptin binding was simply being overlooked due to the high levels of leptin needed to achieve saturation. To evaluate this idea, samples were prepared with concentrations of ^{99m}Tc-leptin, C-peptide and Zn^{2+} at closer to their respective physiological values (because these experiments examined total binding, the choice of blocking ligand was not relevant).

While a statistically significant difference in binding at physiologically relevant levels was not observed, it is interesting to note that for Figure 3.7, for each concentration analyzed for each biological replicate, there was a subtle increase in binding in the presence of C-peptide. In agreement with prior work from the Spence group, this trend was not observed in figure 3.8, which analyzed the effect of Zn^{2+}. For these reasons, the experiment was repeated, dialing in on a single concentration of C-peptide and leptin (Figure 3.9) in order simplify the experimental procedure (thus minimizing the potential for error) and shorten time to results. The original intent in collecting data for Figure 3.9, was to increase the number of biological replicates examined. However, this was not feasible due to time constraints and availability of donors. Therefore, it was decided to instead increase the number of technical replicates (typical experiments where several concentrations were prepared only used one technical replicate each, however, by focusing on a single concentration, this number was increased to 11). Although a statistically significant difference in binding was still not observed, perhaps a change in experimental design in future experiments to increase the number of biological replicates per study would aid in proper analysis.

Clark's occupancy theory (1934) describes the relationship between dose and response as a linear fashion, assuming that the maximal response to the drug is equivalent to the maximal tissue response.[281] While this theory is most suitable to describe the behavior of full agonists, there have been many divergent evolutionary steps in the development of occupancy theory, which have changed the concept to account for differences between the model and the observed results of experiments. In 1956, Stephenson proposed that other possibilities than the one assumed by Clark for the

relationship between the proportion of receptors occupied and the response should be considered.[309] Stephenson broadened the application of receptor theory to include the following hypotheses:

- The largest effect can be produced by an agonist while occupying only a small share of the receptors.
- Response Is not necessarily linearly proportional to the occupied number of receptors.
- Different drugs may have different abilities to start a response and for this reason occupy different portions of the receptors while creating equal responses (i.e., drug efficacy).[309]

Stephenson's hypotheses may be relevant to the nature of Zn^{2+}/C-peptide/leptin binding to an erythrocyte receptor and the associated cellular response. Namely, a large change in binding or binding affinity may not be necessary to produce the observed biological effects (i.e., erythrocyte-derived ATP release and GLUT1 translocation). Thus, it may be that a minor change in binding affinity, when amplified through a downstream pathway, can lead to a substantial, observable biological effect. Moreover, if a leptin/C-peptide complex can bind a different receptor than leptin alone, binding affinity would not be directly comparable. Also, receptor binding can be associated with promotion, inhibition, or simply no change in biological activity.

While presenting data on analysis ^{99m}Tc-leptin binding to erythrocytes in the presence of C-peptide, a question that was often posed was whether the interaction of

leptin with C-peptide had been examined. This strongly guided next step experiments, where binding interactions between C-peptide and leptin were analyzed using two different experimental techniques, namely, ultrafiltration and surface plasmon resonance, which will be discussed in the next chapter.

Chapter 4: Binding Analysis of C-peptide and Leptin

4.1 Introduction

Many analytical techniques have been developed for measuring the binding affinity of a ligand to a protein, such as ITC, SPR,[310, 311] mass spectrometry,[312] equilibrium dialysis,[311] ultrafiltration,[313] ultracentrifugation[314] and ultrafast affinity extraction chromatography.[315, 316] Each has benefits and drawbacks, and the user must decide which is most practical and appropriate for their needs.

Early Spence group published data examining the interaction between C-peptide, Zn^{2+} and albumin utilized ITC.[149] However, the ongoing use of this technique for experiments was not feasible due to lack of access to an ITC instrument on campus that was readily accessible and in commission. Purchasing an ITC instrument was not a realistic option due to their expense. Furthermore, the necessity of experiments to be performed under near physiological conditions (temperature, concentration range, competitive binding, etc.) would be difficult to replicate for ITC analysis.[317] For these reasons, the Spence lab moved away from using ITC for these types of experiments and instead utilized equilibrium dialysis, which is a traditional binding analysis technique that could be performed within the Spence lab and did not require outside equipment. Furthermore, use of 3D-printing equipment in the Spence lab allowed for customization of devices to perform measurements.[318]

Equilibrium dialysis is an uncomplicated technique that can be used to analyze binding affinity between a protein and ligand. It enables for reproducible, accurate, and

cost-effective analysis of binding constants, and can be carried out when a ligand and protein of relevance (analyte) have a great enough difference in molecular weight (a minimum of 5-fold difference).[319-321]

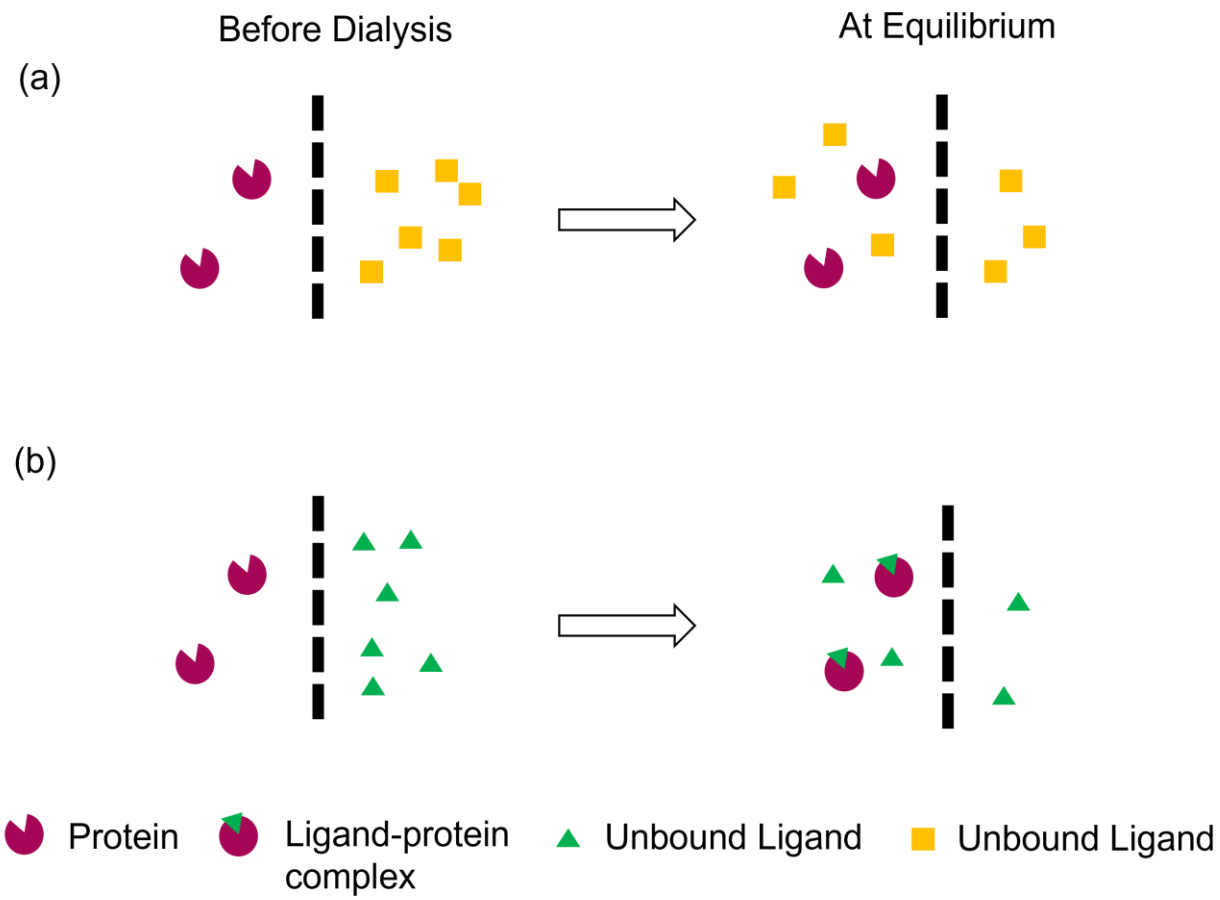


Figure 4.1: Illustration of the principle of equilibrium dialysis. Part (a) depicts a protein and ligand that do not bind. The ligand is free to cross the membrane, and at equilibrium, the concentration of ligand in the assay chamber is exactly half of what was placed in the initial sample chamber. Part (b) depicts and protein and ligand that bind to form a complex. In this situation, bound ligand is unable to diffuse across the membrane and will remain in the sample chamber. Free ligand concentration will still be equal on either side of the membrane upon reaching equilibrium. The final concentration of ligand concentration in the assay chamber will be equal to the total amount of ligand added minus the total amount of ligand bound divided by two. Adapted from https://www.harvardapparatus.com/overview-equilibrium-dialysis.

As shown in Figure 4.1, in an equilibrium dialysis experiment, a ligand and protein are placed in a solution within in a sample compartment on one side of a permeable membrane containing just buffer solution on the other side. The membrane has pores big

enough to allow the ligand to diffuse through, but small enough to hold the protein and any created ligand-protein complex in the sample compartment. After many hours of incubation on a shaker, the amount of free ligand is equal on either side of the membrane.[322] At this point, the concentration of ligand-protein complex may be determined using these equations:

$$\text{Equation 4.1:} \quad [\text{Bound Ligand}] = \frac{\text{Total Ligand (Moles)} - \text{Free Ligand (Moles)}}{\text{Volume in Sample Compartment}}$$

$$\text{Equation 4.2:} \quad \%\ \text{Protein Bound} = \frac{\text{Bound Ligand (Moles)}}{\text{Total Ligand (Moles)}} * 100$$

$$\text{Equation 4.3:} \quad K_a = \frac{[\text{Bound Ligand}]}{[\text{Free Ligand}] * ([\text{Total Receptor}] - [\text{Bound Ligand}])}$$

If sought, this information can be used to determine a single-point affinity constant, create a Hill plot, Scatchard plot or saturation binding curve to assess the characteristics of the binding interaction.[322] Despite its simplicity, a drawback of equilibrium dialysis is the necessity of long incubation time for free ligand to reach equilibrium concentration on both sides of the membrane, may take anywhere from 6-24 hours.[323] If the system is not allowed to establish equilibrium, as a consequence, concentration of the free ligand will not reach equilibrium concentrations in the sample and buffer compartment, with the result of deceivingly low estimates of free ligand and K_d (falsely implying a strong binding affinity).[324]

Due to these limitations, the Spence group ultimately developed an ultrafiltration system to perform binding affinity measurements. Ultrafiltration takes advantage of pressure to push solution and low molecular weight compounds across a size-exclusion

membrane while holding high molecular weight compounds. The technique necessitates a small volume of sample to be separated from the main solution for analysis, because if the volume is kept low (less than 10% of total sample volume), the equilibrium binding of the system is not affected because there is only a small change to the protein concentration. If this condition holds true, the concentration of ligand in the ultrafiltrate is equal to the concentration of free ligand in the greater part of the sample.[313, 325, 326] Although ultrafiltration devices are commercially available, in order to increase cost effectiveness and allow greater control over factors such as freedom from contaminants and choice of membrane size, the Spence group design a 3D printed ultrafiltration device that could be fabricated in-house. As was discussed in chapter 1, the initially developed ultrafiltration method, which relied on a commercially available syringe to pump sample into an attached 3D-printed ultrafiltration device, was used to quantitatively determine binding constants for albumin, C-peptide and Zn^{2+}.[160] This ultrafiltration method, which relied on a syringe attached to a custom 3D-printed syringe filter, was later refined into a centrifuge-compatible device which could fit into a standard 1.7 mL tube (see Figure 4.2). Refinement of the device increased throughput allowing greater flexibility in experimental design because up to 24 devices could be centrifuged simultaneously. The updated device was used to compare in vivo binding affinity between albumin and Zn^{2+} for control and plasma samples from people with diabetes collected in-house.[327]

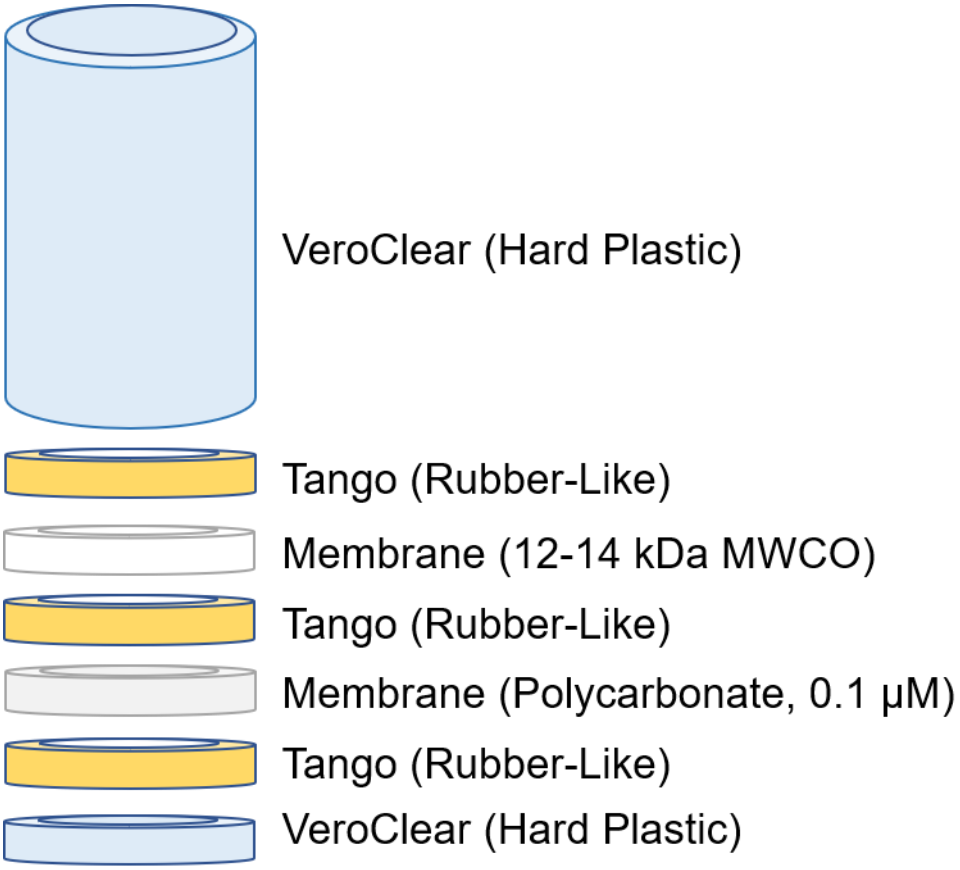


Figure 4.2: Depiction of 3D-printed ultrafiltration device used to perform binding affinity measurements. Adapted from Jacobs et al., *Metallomics* **2020**, *12* (7), 1036-43.

With this device, first, the equilibrium dissociation constant was evaluated for Zn^{2+} and a commercially acquired normal HSA with a glycation level of 11.5% ($K_d = 2.1 \pm 0.5 \times 10^{-7}$ M). Then, a glycated portion of the normal HSA was enriched (glycated HSA, 65.5%) and separated with boronate-affinity chromatography. The separated albumin was determined to have a 2.3-fold decrease in Zn^{2+} binding affinity ($K_d = 4.8 \pm 0.8 \times 10^{-7}$ M) as compared to the normal HSA sample. The glycation level of HSA in control plasma (13.0% ± 0.8, n = 3 donors) and plasma from individuals with diabetes (26.9% ± 6.6, n = 5 donors) was evaluated by mass spectrometry. HSA from plasma obtained in-house

from an individual with type 1 diabetes was determined to have a glycation amount of 24.1% and K_d = 3.3 ± 0.5 × 10^{-7} M for Zn^{2+}, and a 1.5-fold decrease in binding affinity compared to normal HSA.[327] Together, the data suggest that increased levels of glycated HSA, resulting from covalent attachment of sugar (can be glucose, fructose, or other derivatives) to the protein by a slow, non-enzymatic process, reduce binding to Zn^{2+} and may have implications in diabetic complications and the use of C-peptide replacement as a therapy for type 1 diabetes.[327]

4.1.1 Adapting Ultrafiltration Dialysis to Investigate C-peptide and leptin Interaction

Due to the prior success of ultrafiltration dialysis in the Spence lab in performing fast and accurate binding measurements, an ultrafiltration method was used in initial attempts to analyze binding interactions between C-peptide and leptin. However, there were several challenges in attempting to evaluate C-peptide and leptin interaction by this technique.

In initial experiments, there were issues with non-specific binding to the device preventing measurement of C-peptide. Due to a high amount of non-specific binding, measurement of C-peptide through commercially purchased ultrafiltration devices was not successful, and for this reason, 3D-printed devices were created. An additional finding was that even with devices created in-house, measurement of C-peptide in ultrafiltrate was not possible without the addition of a sample component to help reduce non-specific binding. For this reason, BSA was added to the samples. However, this further complicated the experiment, since it was known from prior Spence group work that

albumin can bind C-peptide.[149] Then, once BSA was added to the system, there was a question of how much should be added to optimize measurement of C-peptide while minimizing interference from C-peptide interaction with albumin.

There was also a question of what concentrations of C-peptide and leptin were optimal for experiments. Since 20 nM is the reported saturating concentration of C-peptide to erythrocytes,[149] this concentration was initially chosen for C-peptide. The optimal level of leptin to add to evaluate its interaction with C-peptide was not known. Since previous experiments which analyzed C-peptide and leptin interaction with erythrocytes by ELISA method had focused on physiological concentrations,[274] leptin levels were initially added between of 0, 0.5 and 5 nM, corresponding to control, normal/average, and 'obese' serum leptin concentrations.[276] However, a statistically significant difference in free C-peptide was not measured in the ultrafiltrate under these conditions.

A literature search revealed a guide to designing simple and informative binding assays, indicating that a fixed concentration of one of the reactants that is lower than the K_d (specifying that approximately 10x lower than the K_d would suffice) with enough of the other reactant so that virtually all is free under the experimental conditions should be reasonable.[324] Basing concentrations of C-peptide and leptin on their K_d was not possible as the K_d was not known and was the value being sought. However, it was known by 2015 analysis using ITC that HSA and C-peptide interact with a K_a of $1.75 \pm 0.64 \times 10^{-5}$ M^{-1} (and therefore a K_d of $1.75 \pm 0.64 \times 10^{-5}$ M), with a binding stoichiometry of 0.53 ± 0.03, indicating two molecules of C-peptide bound per HSA molecule.[149] Based on this information, leptin levels were increased to 50 and 500 nM in subsequent experiments,

to ensure that all available C-peptide could interact with leptin. However, again, a statistically significant difference in free C-peptide was not observed.

It was considered that membrane pore size in the ultrafiltration devices might be a factor. Initial experiments used devices with 12-14 kDa membranes as this device had already been characterized in prior work.[317] In an equilibrium dialysis/ultrafiltration system, some of the higher molecular weight ligand (molecule to be retained) will always pass through the membrane, and the amount being dependent on the membrane pore size. Typically, in the definition of a molecular weight cut off value, this amount is less than 10%.[328] Using a larger pore size, while allowing more passage of ligand, can be beneficial for ultrafiltration because it decreases the centrifugation time and speed needed to separate ultrafiltrate from the sample. Using a smaller pore size, while optimal for preventing passage of ligand through the membrane pores and therefore interfering with the measurement of unbound analyte, can increase effects of the larger ligand (leptin) trapping the analyte (C-peptide) of interest, and requires that the device be centrifuged at increased speeds and times.[329] The potential drawbacks of using a device with a smaller pore size were experienced in this research when attempting to switch from performing experiments with well characterized 12-14 kDa membranes[317] to 3.5 kDa membranes. First, it was found that the 3.5 kDa ultrafiltration devices often failed at the centrifugation speeds and times necessary for enough ultrafiltrate to pass through the device to perform the ELISA analysis. In addition, as mentioned above, using a smaller membrane size required further optimization of experimental conditions. Specifically, the devices had to be loaded with at least 50 nM C-peptide to attain enough C-peptide in the ultrafiltrate for its detection by C-peptide ELISA. A 7 kDa MWCO membrane size was

commercially available and would perhaps be a better size choice to use, since 7 kDa is an intermediate value in between the molecular weights of C-peptide and leptin. However, shipping delays from several manufacturers during the COVID-19 pandemic prevented obtainment of the 7 kDa membrane size for the duration of these experiments.

Another consideration for the ultrafiltration experiments was a whether a period of sample incubation was necessary, and if so, how long of a time and what temperatures would be optimal? Typically, equilibrium binding is reached more rapidly at higher temperatures. It was not known if C-peptide and leptin specific binding, if it existed, would be relatively instantaneous or require a certain amount of time to occur (as a reminder, the half-life of leptin is approximately 25 minutes, while the half-life of C-peptide in vivo is around 30 minutes). In attempting to answer this question, it was found that it was difficult to test longer incubation times and higher incubation temperatures using the ultrafiltration method, unless the sample was cooled down to 4 °C after the incubation period. Increasing the temperature, even to room temperature, increased failure of devices (devices would frequently break during the centrifugation process). In an adaptation from the method of Hage and coworkers,[315, 330] and taking into consideration the half-lives of the two proteins in vivo, an incubation time and temperature of 37 °C for 30 minutes was used in order to ensure the sample had enough time to reach equilibrium prior to the centrifugation step. However, it was found that incorporating this step required that samples be cooled to 4 °C prior to loading into the devices.

Then, after a successful ultrafiltration experiment, C-peptide concentration in the ultrafiltrate had to be determined. The method employed for this was the C-peptide ELISA, due its previous success in the Spence group.[147, 149, 317] But, using the C-peptide

ELISA assay to perform the measurement raised additional questions. Specifically, it seemed leptin itself may be interfering with measurement of C-peptide by ELISA.

Perhaps most importantly, the final consideration that led to the search for another method of analysis of C-peptide and leptin binding, was the requirement for size difference to separate a ligand and analyte by an equilibrium dialysis/ultrafiltration method. Recall that separating a ligand and analyte by an equilibrium dialysis method requires a difference in molecule weight of at least 5x difference.[319-321] There is approximately a 5x difference in molecule weight between leptin (16 kDa) and C-peptide (~3 kDa), placing them just at the cut off for difference in molecule weight, which could perhaps explain the challenges of this experiment.

For the above-mentioned reasons, despite the simplicity of the ultrafiltration method and the availability of materials in-house, C-peptide and leptin interaction was ultimately investigated using another method. Due to its frequent application in the study of molecular interaction/drug binding and the discovery of available instrumentation (and expertise) in a neighboring drug developing and repurposing core, surface plasmon resonance (SPR) was a logical choice.

4.1.2 Surface Plasmon Resonance - Background

The first optical chemical sensors were designed for measurement of CO_2 and O_2 concentration and focused on measurement of changes in absorption.[331] Subsequently, a wide assortment of optical methods have been adjusted for use in biosensors and chemical sensors incorporating spectroscopy (luminescence, phosphorescence,

fluorescence, Raman), spectroscopy of guided modes in optical waveguide structures (grating coupler, resonant mirror), interferometry (model interferometry in optical wavelength structures, white light interferometry), ellipsometry, and SPR.[332] In these methods, a chosen quantity is analyzed by quantifying refractive index, absorbance and fluorescence properties of a chemo-optical transducing medium or analyte molecules.[333]

Surface plasmons (SPs) (Figure 4.3) are waves that disseminate along the surface of a conductor (normally a metal).[334] Basically, they are light waves confined to the surface because of their interaction with free electrons of the conductor (firmly speaking, surface plasmons should be denoted as surface plasmon polaritons to indicate this hybrid character).[335] In this interaction, the free electrons counter by cooperatively oscillating in resonance with the light wave. This resonant interaction between the surface charge oscillation and the electromagnetic field of the light comprises the surface plasmon and determines its distinctive properties.[334]

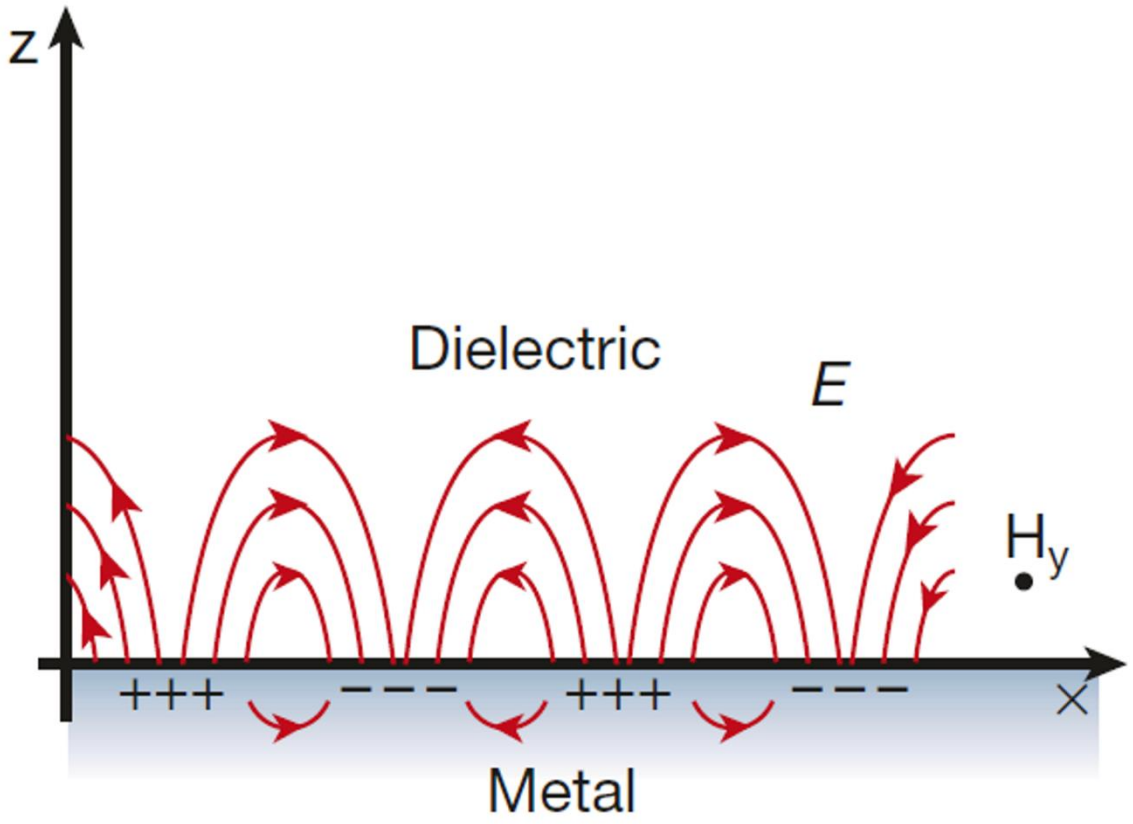


Figure 4.3: Schematic illustration of an intensity dispersion of the magnetic field and field components of a surface plasmon polariton at a metal-dielectric interface. The surface plasmons have a combined surface charge and electromagnetic character. SPs are transverse magnetic (H is in the y direction), with the generation of surface charge requiring an electric field normal (perpendicular) to the surface. The combined nature also leads to the field component perpendicular to the surface being increased near the surface while decaying exponentially with distance away. Reproduced from Barnes et al., *Nature* **2003**, *424*, 824-30.

Irregular diffraction on gratings because of excitation of surface plasma waves was initially detailed by Wood at the start of the twentieth century.[336] In the late sixties, Otto[337] and Kretschmann[338] confirmed optical excitation of surface plasmons by reduced total reflection. An SPR optical sensor commonly is comprised of an electronic system allowing data processing and sustaining the optoelectronic components of the sensor, a transducing medium that connects the (bio)chemical and optical domains and an optical

system.[332] The transducing medium[m] relates variations in the analyte of interest to changes in the refractive[n] index which can be analyzed by optically searching the SPR.[332, 339] The optical section of the SPR sensor contains a source of optical radiation and an optical structure in which SPW are excited and cross-examined. While probing the SPR, an electronic signal is produced and overseen by the electronic system. While resolution, sensitivity and sensor stability are decided by properties of both the transducing medium and optical system, response time and selectivity of the sensor are primarily decided by properties of the transducing medium.[332]

Since the propagation of SPWs is always greater than that of the optical wave in the dielectric (Figure 4.4), the SPW cannot be enhanced directly by an incident optical wave at a two-dimensional metal-dielectric boundary. Because of this, the momentum of the incident optical wave needs to be heightened to equal that of the SPW. This is usually accomplished using attenuated total reflection in prism couplers or optical waveguides, or diffraction at the surface of diffraction gratings. To excite surface plasmon polaritons in a resonant way, electron bombardment or incident light can be used (normally visible or infrared).[340] When light is used to excite surface plasmon waves, there are two common configurations: Kretschmann and Otto. In the Otto configuration, light irradiates the wall

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[m] In SPR biosensors, the transducing medium is typically created as a layer or matrix of biomolecules that can bind the analyte molecules.

[n] Refractive index is a measure of the bending of a ray of light while transitioning from one medium into another. It is equal to the velocity of light *c* of a given wavelength in empty space divided by its velocity *v* in a component, or n = *c*/*v*.

of a glass block, is wholly internally reflected and a thin metal film is placed close enough to the prism wall so that an evanescent wave can intermingle with the plasma waves on the surface to excite the plasmons. In the Kretschmann configuration (most common), the metal film is evaporated onto the glass block, and light analogously illuminates the glass block so that an evanescent wave infiltrates the metal film, exciting plasmons at the outer portion of the film.[341]

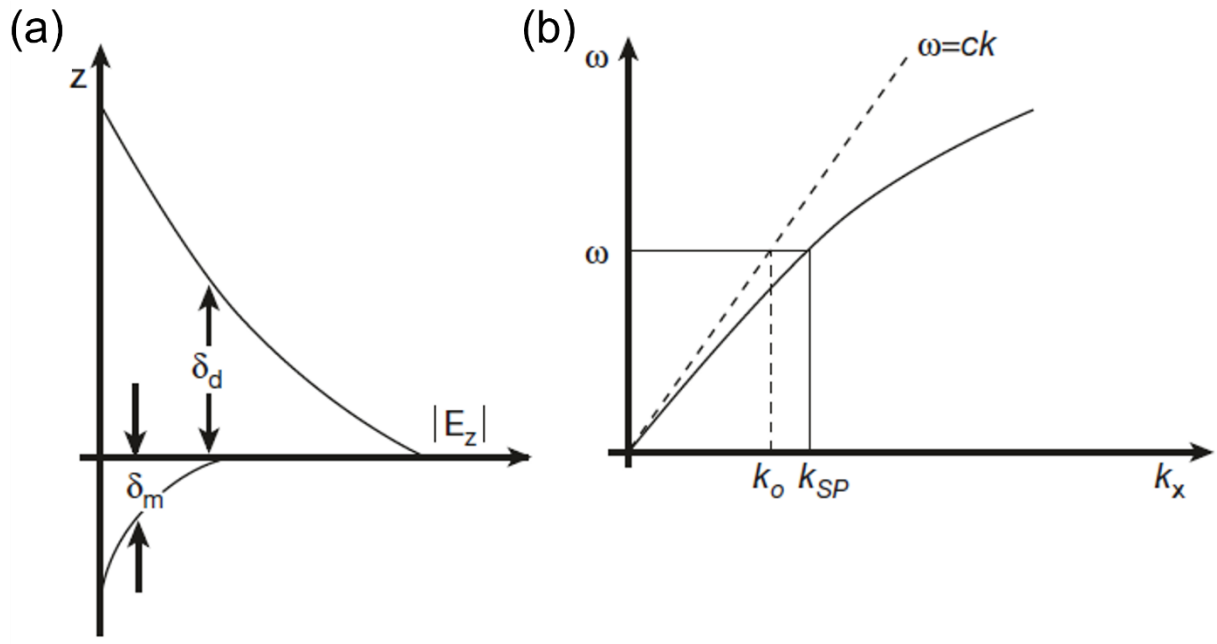

Figure 4.4: Scheme for surface plasmon polaritons (SSPs) a) shows the infiltration distance of SSPs into the metal and dielectric. The field in the perpendicular direction is termed evanescent, due to the attached, non-radiative nature of SPs that blocks power from disseminating away from the surface. In the dielectric field above the metal (usually air or glass), the decay length of the field, δ_d, is on the order of approximately half the wavelength of light implicated, while the decay length into the metal, δ_m, is determined by the skin depth. b) shows the dispersion curve for a SP mode, showing the momentum mismatch issue that must be surmounted to couple SP modes and light, with the SP mode always beyond the light line, having larger momentum ($\hbar K_{SP}$) than a free space photon ($\hbar K_0$) of the same frequency ω. Reproduced from Barnes et al., *Nature* **2003**, *424*, 824-30.

The current work used a FortéBio Pioneer SPR system with a sensor (designed in the Kretschmann configuration), disposable SPR sensor chip, and optical reflection guide. The guide, permanently fixed in the system, is made up of a glass prism with optical surfaces allowing for light from an emitting diode (LED) to undergo SPR and be returned onto a photodiode array on the same plane. In the detector, the single channel optical system is replicated in triplicate in order to form three sensing channels.[342]

4.1.3 Surface Plasmon Resonance Biosensor Chip Design

In a standard SPR biosensor assay, as shown in Figure 4.5 (a) below, a ligand (leptin) is covalently immobilized on the biosensor surface and the analyte (C-peptide) travels over the surface through a microfluidic flow cell.[275, 343] SPR detectors track the change in refractive index of the solvent layer close to the surface enticed by association and dissociation of the analyte-ligand complex formed and generate real-time response data as a sensorgram as shown in Figure 4.5 (b).[343] In the association phase of the interaction, the analyte binds to the immobilized ligand, which generates a rise in response. Over time, as equilibrium between the bound and free analyte is attained, the response intensity levels off. During the dissociation phase, the instrument switches back to running buffer, which makes it feasible to attain information about the stability of the complex.[344]

An important feature of the SPR biosensor is the interface between the sensor surface and immobilized ligand.[343] The current work utilized a SADH sensor chip, a glass chip coated with a semi-transparent gold film, with a surface chemistry of streptavidin

immobilized in a three-dimensional carboxymethyl dextran hydrogel for attachment to the affinity ligand,[342] allowing for selective, irreversible capture of biotin-containing ligands (i.e., biotinylated leptin) while minimizing non-specific binding. The chip, with moderate to high capacity, it ideal for analysis of intermediate to large molecule kinetics.[345] In addition, due to high stability of the dextran layer on the biosensor chip, several hundred binding cycles may be run over an immobilized ligand if desired.[343, 345]

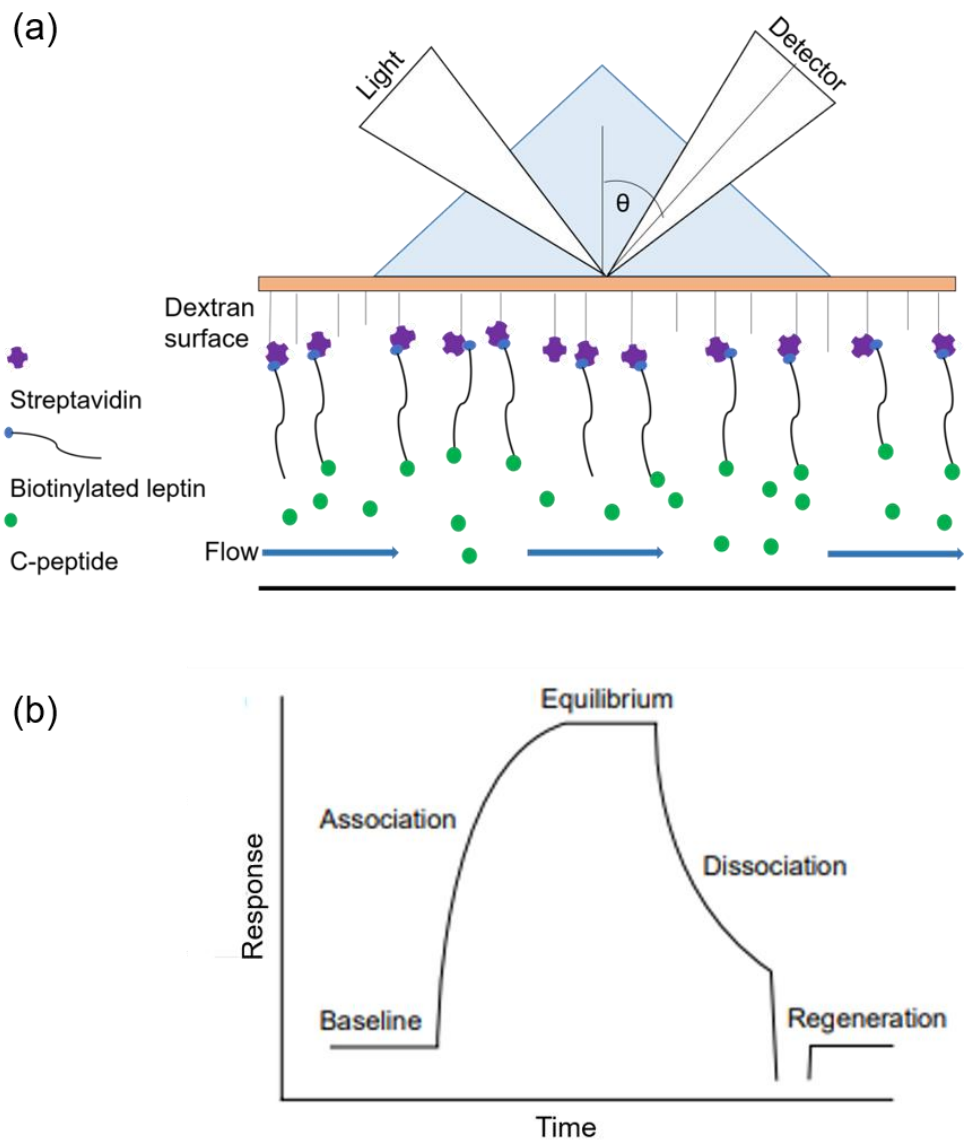

Figure 4.5: SPR biosensor assay to evaluate leptin interaction with C-peptide. In (a) the ligand (leptin) is adhered on the SADH biosensor chip surface. The analyte (C-peptide) travels through a microfluidic flow cell and SPR is utilized to track the modification of refractive index as analyte collects on the sensor surface. (b) Portrayal of the usual features of an SPR sensorgram. Before introducing the analyte, the baseline response must be constant. A rise in response during the association phase indicates complex formation in real-time. When an equal number of analyte molecules associate with and dissociate from the surface simultaneously, equilibrium is attained. If wanted, the surface may be washed to attain the decay rate of the complex during the dissociation phase. After regeneration, the binding response should revert to baseline. Adapted from Rich & Myszka, *Curr. Opin. Biotechnol.* **2000**, *11*, 54-61.

Compared with other technologies available for monitoring intermolecular interactions, SPR biosensors offer several distinct advantages (Table 4.1) , including no labeling requirement, real-time measurement, ability to perform the measurement without disturbing equilibrium, monitoring stability of the immobilized ligand, use in qualitative formats (i.e. drug screening), and in determination of binding affinity and kinetic rate constants, ability to monitor simultaneous interactions at multiple surfaces, automated instrumentation, low use of material for analyses (generally less than ~1 μg of protein ligand to make a single surface) and experiments can be performed using buffers containing organic solvents.[344] SPR biosensors are well adapted to pharmaceutical discovery, particularly for characterization of target macromolecules and biopharmaceuticals. SPR biosensors can directly monitor a molecule's binding activity, which associates with its function.[343]

While SPR offers several advantages, like any method of analysis of ligand binding, there are also potential shortcomings (Table 4.1).[346] One potential limitation of SPR is that the ligand may not maintain its native configuration when immobilized to the sensor chip surface. Another possibility is that its orientation on the chip may sterically hinder analyte binding. One way to circumvent this issue is to use an 'upside down' orientation, by functionalizing the analyte of interest to the biosensor chip. However, immobilization strategies that use antibodies, biotin, or other molecular 'tags' prevent this issue by uniformly orienting the ligand, thereby avoiding heterogeneity of binding due to different orientations of ligand immobilized to the sensor surface. Another potential limitation that occurs when a ligand is physically fixed onto a surface is that it is physically separated from the analyte in the bulk solution. For this reason, it is important to verify

that transfer of the analyte to ligand at the sensor surface is not restricted, or else the analyte concentration near the surface will be different from the bulk concentration (i.e., mass transport limitation). Non-specific binding events at the sensor surface also must be carefully regulated by experimental design.[346]

Table 4.1: Advantages and disadvantages for using SPR to monitor molecular interactions. Adapted from Helmerhorst et al., *Clin. Biochem. Rev.* **2021**, *33* (4) 161-73.

Advantages	**Disadvantages**
Label-free	Expense of chips and instrumentation
Real-time and continuous measurement	Non-specific binding to surfaces
Sensor chips can be regenerated	Immobilization effects
Small sample amount and volumes	Steric hindrance with binding events
Highly sensitive	Mass transport limitations
Generic methods for diverse molecules	Misinterpretation of data common
Quick testing	Requires precise control experiments
Calibration-free concentration analysis	
Measure ‘active’ concentrations	
Specific to the binding event	

4.2 Methods

4.2.1 Preparation of C-peptide

For ultrafiltration experiments, C-peptide was made as described in 3.4.3. For SPR experiments, C-peptide was made as described in 3.4.3, except that the top stock concentration (which in this instance was 38.5 μM) was used for SPR along with dilutions of 8 μM-8 nM in DDW.

4.2.2 Preparation of Leptin for Ultrafiltration Experiments

Leptin for ultrafiltration experiments was prepared from a lyophilized leptin stock (R&D systems, Minneapolis, MN) per manufacturer instructions by resuspended to 1 mg/1 mL in TRIS-NaCl (Sigma Aldrich, St. Louis, MO), pH 8.0, assayed by leptin ELISA (Boster Biological Technology, Pleasanton, CA) to confirm concentration, and stored at 4 °C prior to use.

4.2.3 Preparation of Leptin For SPR

Due to the need to remove TRIS present in the lyophilized leptin stock which would interfere with biotin labeling, leptin used for SPR experiments was prepared and stored the same way as leptin used for radiolabeling (described in section 3.4.5).

4.2.4 Binding Analysis with Commercial Ultrafiltration Devices

An initial experimental was designed to load ultrafiltration devices with a prepared solution of C-peptide and leptin in buffer, centrifuge to separate a small amount of ultrafiltrate, and analyze C-peptide content of the ultrafiltrate by C-peptide ELISA assay. Initial attempts to analyze the C-peptide interaction with leptin utilized Amicon Ultra-0.5 Centrifugal Filter Devices with 10 kilodalton (kD) molecular weight cut off (MWCO) membranes (MilliporeSigma, Burlington, MA).[347] To adapt the commercially available centrifugal filters for performing ultrafiltration measurement, an optimal centrifuge speed and time had to be determined (recall that the volume of ultrafiltrate must be kept low to maintain equilibrium). To perform this optimization step, an ultrafiltration device was filled with 500 µL of DDW and tested a variety of centrifugation times and speeds (Table 4.2). Based on these data, an appropriate centrifugation speed and time was determined.

Table 4.2 Optimization of centrifugation time and speed for commercial ultrafiltration devices. A series of centrifugation speeds and times were tested and ultrafiltrate was measured to determine optimal conditions for initial experiments. *n* = 3, volumes are approximate.

Centrifugation Speed (*g*)	Time (min)	Ultrafiltrate (µL)
8,000	4	250
5,000	1	30-40
4,000	2	50-75
4,000	1	20-35
3,000	2	35-55
2,000	3	35-50
2,000	2	20-35
2,000	1	15-20
1,000	5	25-35

After constructing the table, for the initial experiments a centrifugation time of five minutes at 1,000*g* was used. An initial set of samples was prepared containing 20 nM C-peptide in PSS, added 200 µL of sample above the filters, and centrifuged. But, when the first experiments with C-peptide and leptin were attempted, it was found that much less filtrate passed through the unused membranes than what was measured in initial testing. It is thought that glycerol coating on the membrane may wash off after multiple centrifugation steps and thus change the volume of ultrafiltrate passing through the device. Proper optimization of the device would require several commercially purchased devices, additional time and use of shared equipment. For these reasons, through a

series of trial and error, a new centrifugation time and speed was decided upon, of 4,000*g* for 2 min, to consistently obtain a filtrate volume of approximately 25-35 µL for an unrinsed membrane.

After determining an optimal centrifuge speed and time, initial samples were prepared with 20 nM C-peptide in PSS buffer, as a basic test to determine the amount of C-peptide bound to leptin. Samples were prepared, aliquoted the appropriate amount into the devices, centrifuged and then collected the ultrafiltrate for analysis of C-peptide concentration by ELISA. However, despite numerous attempts, C-peptide could not be detected in the ultrafiltrate when using the commercially purchased devices. Initially, as it was thought that C-peptide may be sticking to the glycerol coating on the membrane, the membranes were prerinsed with DDW and even 0.1 M NaOH, in procedure indicated by the Amicon Centrifugal Filter User Manual[347] to eliminate this issue. However, this had no effect on experimental outcome. Changing the buffer from PSS to a higher salt buffer like PBS or tris(hydroxymethyl)aminomethane sodium chloride (TRIS-NaCl), which is another method indicated to reduce non-specific binding by reducing charge interactions between the analyte and membrane/device surface,[348] was also not effective. It was considered that the method of Lee et al., of treating the membranes with Tween 20 or Tween 80,[349] may be adapted. This is a method that may be able to reduce non-specific binding due to hydrophobic interactions between the analyte and sensor surface.[348] However, in the meantime, while materials were being acquired, a successful experiment was performed with 3D-printed 12-14 kDa membrane MWCO ultrafiltration devices, so future experiments used devices that were created in-house.

4.2.5 Creation of 12 and 3.5 kDa MWCO Ultrafiltration Devices

Spectra/Por® flat sheet dialysis membranes (12-14 and 3.5 kDa MWCO, regenerated cellulose, Spectrum Laboratories, Inc. Rancho Dominguez, CA) were used as acquired. The membrane sheets were positioned between two sheets of wax paper and cut with a metal hole punch to produce circles with a diameter of 6 mm.

The 3D-printed ultrafiltration device had earlier been designed using CAD software (Autodesk Inventor Professional, San Rafael, CA) and sent to a J750 Multi-Material PolyJet 3D-printer (Stratasys, Eden Prairie, MN) as a series of .STL files.[327] The device was printed in support-free mode by a previously described method,[160] which stops the printer from laying a waxy substrate that is used to preserve structural integrity of particular devices. The printer was set to print without laying support material by opening the Stratasys Parameters Manager page on the computer connected to the printer. These parameters were changed from their fixed denominations to zero millimeters: Carpet_height, Carpet_protectorZ and ImproveSupport_thickOfPedestal. Forgoing placement of support material enabled direct placement of the membrane into the device, generating a seamless seal without the need for adhesives or glue. Membranes were placed into the device by a previously described Print-Pause-Print procedure.[318]

To make the centrifuge-enabled ultrafiltration device, three .stl files were used. The bottom layer, a hard plastic support (veroclear), the next two layers, a tacky Tango+ material that is intended to hold a polycarbonate membrane, the third and fourth layers designed to hold the cellulose dialysis membrane, and the fifth layer, a cylinder to make the membrane ‘cup’ to retain the liquid sample. The ensuing device is a small cup with a

permeable bottom that fits into a standard 1.7 mL microcentrifuge tube, as shown in Figure 4.2. As previously detailed, the printer was set to 'support-free' mode and the first layer was printed to finish. Then, the printer settings were changed to tell the printer to lower the build tray to a distance equal to the height of the object formerly printed. The second layer was sent to the printer as a new device, which tells the printer to lay material (in this case, a 0.1 mM layer of Tango+) on top of the prior object. Once the second layer was finished, a polycarbonate membrane with 0.4 µm pores was meticulously placed on top of the layer, and once more the build tray was lowered, and the next layer (0.1 mM Tango+) was printed. Following, the dialysis membrane of the desired pore size was put in, and the method of lowering the print tray and printing a layer of Tango+ was repeated. The final part of the device, which holds a liquid sample, was then printed to produce the final construct. Preferred centrifuge time and speeds were determined in previous work.[317]

4.2.6 Optimization of Experiment Conditions with 3D-printed Ultrafiltration Devices

For all ultrafiltration experiments, C-peptide standards were prepared, at the same time as samples, in PSS from an 800 nM stock in DDW, in concentrations of 0, 4.8, 10.2, 14.8, 20 and 24.8 nM, along with 2 x 20 nM controls. Standards were incubated at the same temperature and duration of time as the corresponding sample set.

12-14 kD ultrafiltration devices had been previously optimized[317] for a volume of 200 µL and a centrifugation time and speed of 90 minutes at 15000*g*, which allowed for collection of approximately 10-13 µL of ultrafiltrate per sample. All reported results for 12-

14 kDa MWCO membranes contained 75 μM BSA (PSS buffer) to prevent non-specific binding of C-peptide to the device. Samples were incubated for a period of 30 minutes at 37 °C, followed by a brief period of refrigeration to cool the sample before transferring to the device. In the samples, 25 μL of 800 nM C-peptide in DDW was added to achieve a final concentration of 20 nM, and leptin was diluted from a concentrated stock in PBS, into PSS buffer, to achieve final concentrations in samples of 0, 0.5, 5, 50 and 500 nM.

For 3.5 kDa devices, an experiment was first performed, based on the available optimization data for the 12-14 kDa MWCO devices,[317] to evaluate the suitability of two hours at 20000*g* for centrifugation speed and time (Table 4.3). First, the mass of several 1.7 mL microcentrifuge tubes was recorded. Then, the tubes were fit with custom 3D-printed 3.5 kDa ultrafiltration devices. Following, 200 μL of DDW was added to each device. After centrifugation, devices were removed, and the microcentrifuge tubes were massed again to determine the average amount of ultrafiltrate passing through the membrane pores. Once centrifugation speed and time were experimentally verified to produce an average ultrafiltrate volume of less than 10% of the total sample volume (approximately 7%), ultrafiltration experiments were performed with C-peptide and leptin. Experiments with 3.5 kDa devices, that were able to successfully measure C-peptide, used a C-peptide concentration of 50 nM and leptin concentrations of 0, 50 and 500 nM. Samples contained 75 μM BSA and were incubated at 37 °C for 30 minutes prior to centrifugation and subsequent ELISA analysis.

Table 4.3: Optimization of centrifuge speed and time for 3.5 kDa devices.

1.7 mL Tube	Initial mass (g)	Final mass (g)	Ultrafiltrate (µL)
1	1.0449	1.0573	12.4
2	1.0481	1.0613	13.2
3	1.0422	1.0579	15.7

4.2.7 C-peptide ELISA for Ultrafiltration Experiments

C-peptide ELISA (ALPCO, Boston, MA) analysis followed all successful ultrafiltration experiments. All successful ELISA assays used a standard dilution of 1:50, and a sample dilution of 1:3 in DDW prior to loading the samples onto a 96 well plate for assay. Assay was performed per manufacturer instructions.

4.2.8 Biotinylation of Leptin for SPR Experiments

Before use in SPR experiments, leptin was biotinylated for functionalization to the avidin biosensor chip. Like the procedure utilized for leptin labeling with HYNIC described in 3.4.2, leptin that was labeled with biotin for SPR experiments (recombinant human leptin, R&D systems) was made by resuspending 5 mg of lyophilized leptin in 1 mL of PBS overnight and evaluating its concentration using a human leptin ELISA kit (Boster Biological Technology). Biotinylation was carried out with a Thermo Scientific™ EZ-link NHS-PEG_4-biotinylation kit. First, a 200 µL aliquot of the dialyzed and assayed leptin was diluted to 1 mL in BupH™ PBS (Thermo Scientific). The BupH™ PBS, a modified

Dulbecco's PBS, contained, in mM, 8.0 sodium phosphate, 2.0 sodium phosphate, 0.14 sodium chloride, and 10.0 potassium chloride. By kit instructions, the number of millimoles of NHS-PEG_4-Biogtin to add to the reaction to obtain a 20-fold molar excess were determined. A 20 mM solution of NHS-PEG_4-Biotin was made following kit instructions, and 62.6 µL of the biotin solution were added to the leptin solution. While the leptin was let to incubate with the biotin for 60 minutes at room temperature, a Thermo Scientific Zeba Spin Desalting Column was readied per kit instructions. After 60 minutes of incubation, the leptin/biotin solution was put on the column, the solution was permitted to soak into the column resin, and then the column was centrifuged at 1000*g* for two minutes. The flow through, or eluate, was gathered as the purified leptin sample. To evaluate the amount of biotin incorporation in the purified leptin sample, a 4'hydroxyazobenzene-2-carboxylic acid (HABA) assay was carried out and to analyze the final concentration of leptin in the purified sample, a bicinchoninic acid (BCA) assay was performed.[275]

4.2.8.1 Procedure for HABA assay

To evaluate the level of biotin integration, a solution with biotinylated leptin was added to a mixture of HABA and avidin. Because of the higher affinity for avidin, biotin displaces HABA from its interaction with avidin, and the absorbance at 500 nm decreases correspondingly.[350] In the assay method, an unknown quantity of biotin present in a solution is approximated in a single cuvette (or microplate well) by quantifying the absorbance of the HABA-avidin solution prior to and after addition of the biotin-containing

sample.[351] The change in absorbance is correlated with the amount of biotin in the sample.[351]

To perform the assay, the 180 µL of HABA/avidin was pipetted into a microplate well,[351] and absorbance was measured at 500 nm and recorded as A_{500}HABA/avidin. To the well, 20 µL of biotinylated leptin (collected from column flow-through) was added and mixed using the pipet. Absorbance of this solution as measured at 500 nm until the value remained constant for 15 seconds (at least 3 reads), and the value was recorded as A_{500}HABA/avidin sample. Moles of biotin incorporated per mole of leptin were then calculated as outlined in the Thermo Scientific™ EZ-link NHS-PEG_4-biotinylation kit procedure.[351]

4.2.8.2 Procedure for BCA assay

A BCA assay, which combines the property of Cu^{2+} reduction to Cu^{1+} by protein in alkaline medium (biuret reaction) with the very sensitive colorimetric detection of the cuprous cation (Cu^{1+}) by BCA,[352, 353] was used to estimate biotinylated leptin concentration in the column flow through. A modified procedure of the Pierce™ BCA protein assay kit[354] was carried out by first preparing BSA standards in PBS by diluting 100 µL of the 1 g/1 mL kit BCA standard with 700 µL PBS to make a 125 µg/mL solution. The 125 µg/1 mL standard was used to create a 62.5 µg/mL standard, and that standard was used to make 31.25 µg/1 mL. These standards and a blank were loaded into the bottom of a clear bottom plate in a volume of 25 µL each, along with 3 x 25 µL aliquots of unknown biotinylated leptin sample, which was diluted to bring the estimated

concentration close to approximately 100 µg/1 mL. Working reagent was prepared by mixing 2.45 mL of kit reagent A, an aqueous solution of 1% BCA-Na_2, 2% Na_2CO_3 * H_2O, 0.16% tartrate, 0.4% NaOH and 0.95% $NaHCO_3$, with 50 µL of kit reagent B, which was 4% $CuSO_4$ * $5H_2O$ in deionized water.[353]

4.2.9 SPR analysis

SPR experiments were carried out with a FortéBio Pioneer SPR System at the MSU Drug Discovery and Repurposing Core with the help of Dr. Joseph Nichols. A biotinylated leptin sample, with an estimated leptin concentration of 55 µg/mL, was integrated onto a FortéBio SADH Biosensor chip. C-peptide was run over the chip (in PBS running buffer) in amounts of 8 nM, 100 nM, 300 nM, 1 µM, 8 µM, and 38.5 µM in DDW. Binding affinity data (resonance units vs. time) was gathered and evaluated using Qdat software in the Pioneer SPR system.[275]

4.3 Results

Figure 4.6 shows the results for analysis of C-peptide and leptin binding by the ultrafiltration method using 12-14 kDa devices to measure free C-peptide. In these initial experiments, C-peptide concentration in the ultrafiltrate and in the retentate were determined by ELISA for comparison. In the ultrafiltrate, for 0 nM leptin, 0.64 ± 0.11 nM C-peptide was measured, by comparison, when C-peptide in the retentate was measured, for samples with no leptin, C-peptide concentration was 14.5 ± 1.51 nM, and with 0.5 nM

leptin, decreased to 14.3 ± 0.79 nM, and for 5 nM, C-peptide measured in the retentate was only 11.2 ± 4.47 nM, meaning that for the samples with no leptin, approximately 4.84 nM of the initially added C-peptide was accounted for, for samples with 0.5 nM leptin, this number increased to 5.24 nM, and for samples with 5 nM leptin, to 8.35 nM.

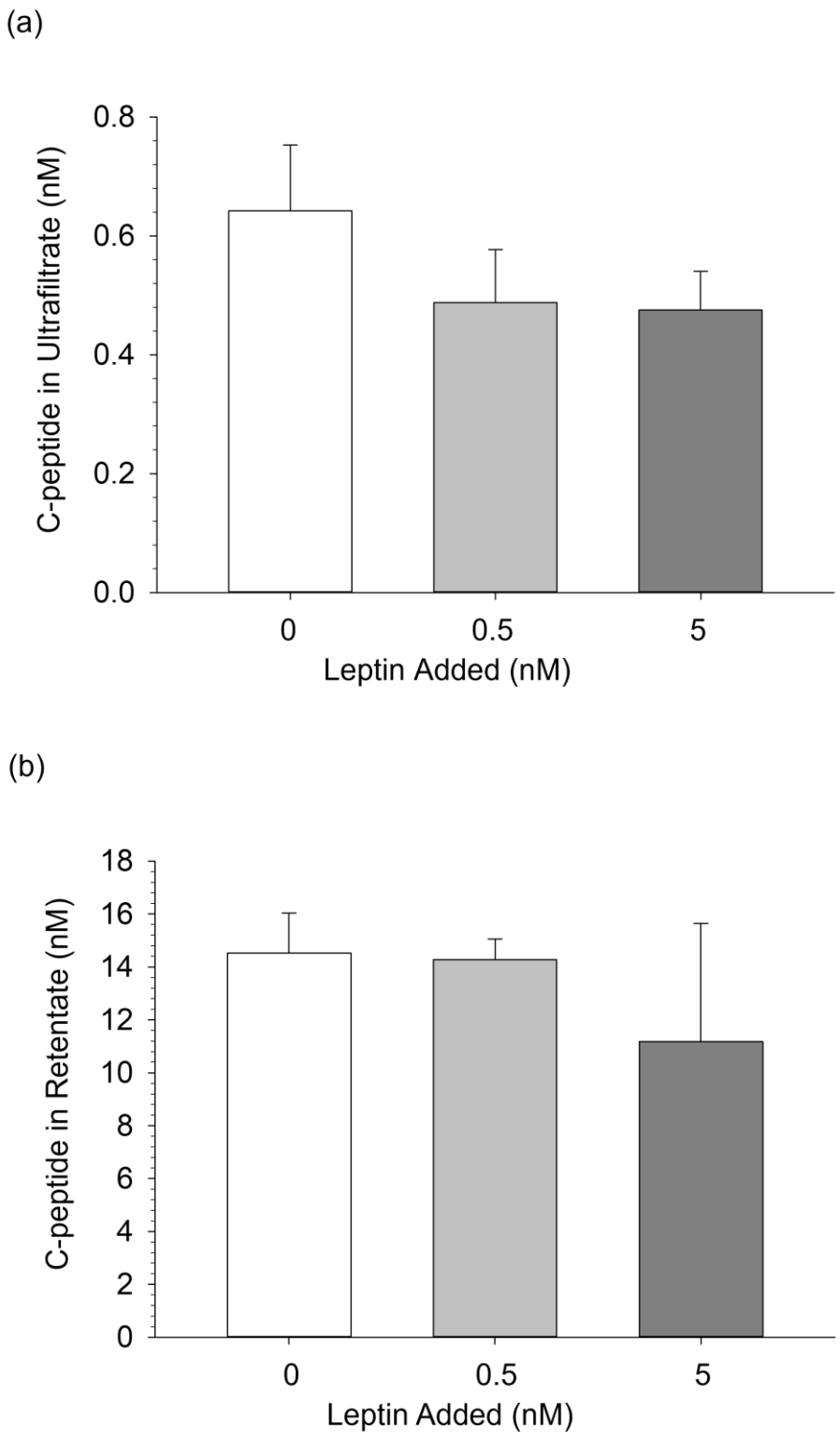

Figure 4.6: Ultrafiltration binding analysis of 20 nM C-peptide and 0, 0.5 or 5 nM leptin using 12-14 kDa MWCO 3D-printed devices. In (a), C-peptide measured in ultrafiltrate is displayed while (b) shows the C-peptide measured in retentate. *n* = 3-4, error = sd.

In the next set of experiments, shown in Figure 4.7, a method was devised to analyze the effect of leptin on the measurement of C-peptide by the ELISA method. For these four sets of C-peptide standards were prepared: one standard set was pre-diluted to the required concentration range for the ELISA assay. The other three sets were prepared in concentrations of 0-24.8 nM. To each of these three sets, 10 nM leptin in PSS was added in the appropriate volume to reach a final concentration of 0, 0.5 or 5 nM, respectively, in 1 mL samples. After their preparation in PSS buffer, samples were immediately diluted 1:50 in DDW to reach the required C-peptide concentration for the assay. The assay was performed, and the C-peptide content of the three standard sets was determined relative to the pre-diluted C-peptide standard curve.

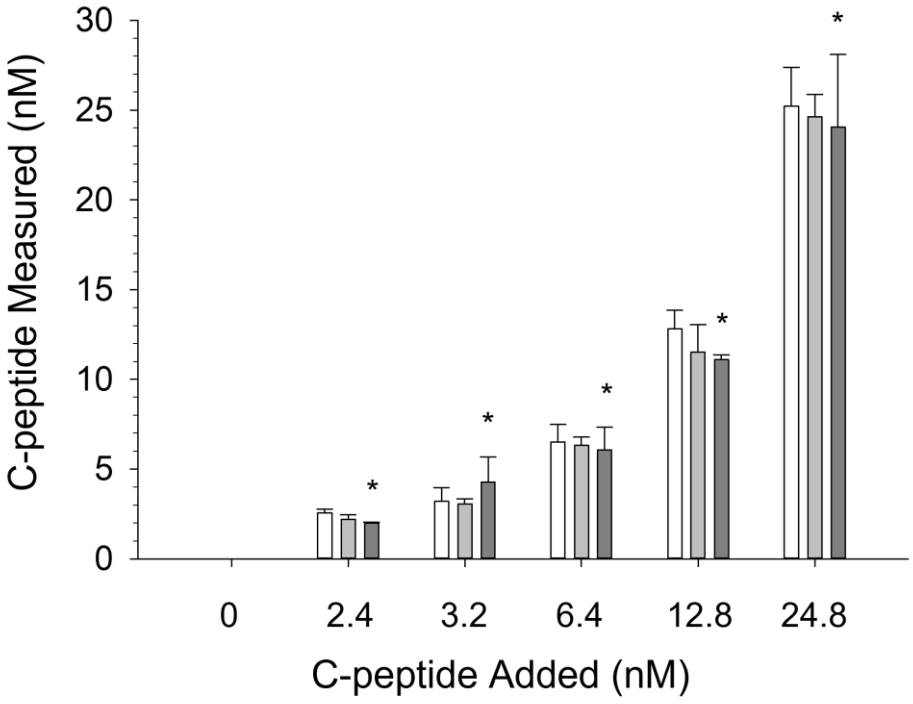

Figure 4.7: C-peptide and leptin standard curves. White = 0 nM leptin, light grey = 0.5 nM leptin, dark grey = 5 nM leptin. *n* = 3, error = sd. Significance from control indicated by * ($p<0.05$) (overall).

In Figure 4.8, to maximize the ability of leptin to interact with and complex to C-peptide, ultrafiltration experiments were performed with 20 nM C-peptide, and either 0, 50 or 500 nM leptin.[324] C-peptide interaction with leptin in the presence of higher concentrations of leptin, namely 50 and 500 nM, were evaluated. For these experiments, when no leptin was added, a C-peptide concentration of 1.37 ± 0.05 nM was measured in the ultrafiltrate. When 50 nM leptin was added, this value decreased to 1.10 ± 0.24 nM. When 500 nM leptin was added, C-peptide measured in the ultrafiltrate was 1.19 ± 0.13 nM.

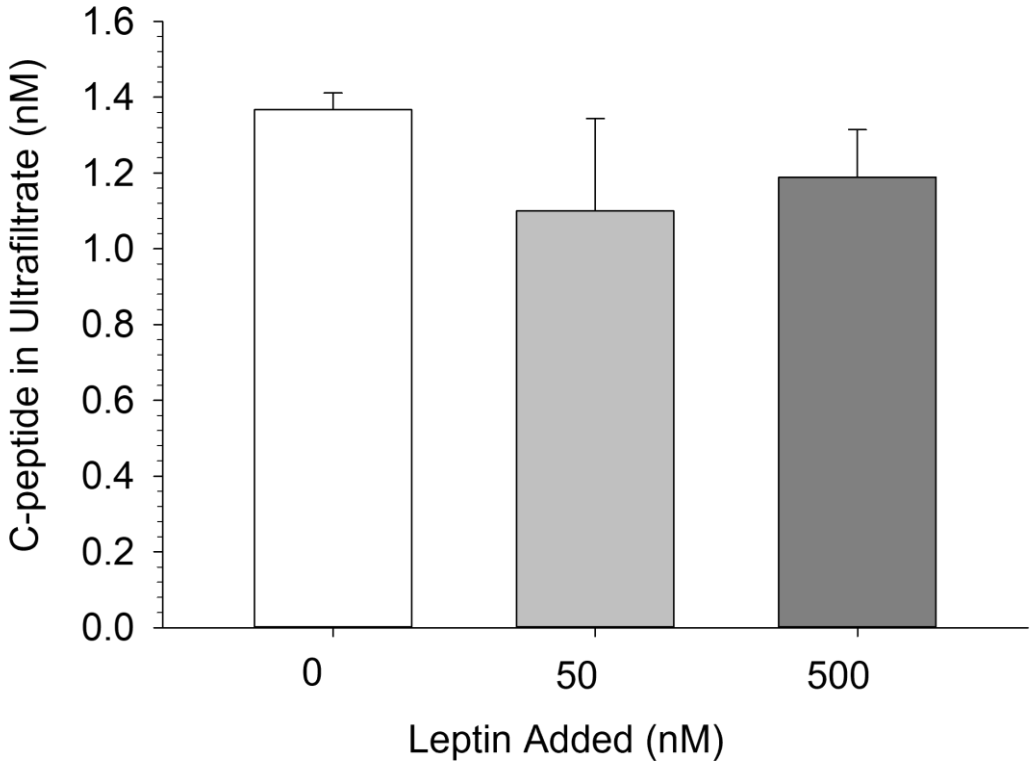

Figure 4.8: Ultrafiltration binding analysis of C-peptide and 50 or 500 nM leptin using 12-14 kDa MWCO 3D-printed devices. *n* = 3. error = sd.

Because it was suspected that leptin crossing the pores of the 12-14 kDa membrane may be interfering with measurement of C-peptide and the true equilibrium binding value, next the ultrafiltration experiment, shown in Figure 4.9, was attempted using 3D-printed devices with 3.5 kDa MWCO cut off membranes. Experiments were performed with 20 nM C-peptide and 0, 50 or 500 nM leptin. For samples with no leptin, 0.82 ± 0.07 nM C-peptide was measured in the ultrafiltrate, when 0.5 nM leptin was added, 0.83 nM C-peptide was measured. For 500 nM leptin, C-peptide measured in the ultrafiltrate decreased to 0.56 ± 0.18 nM.

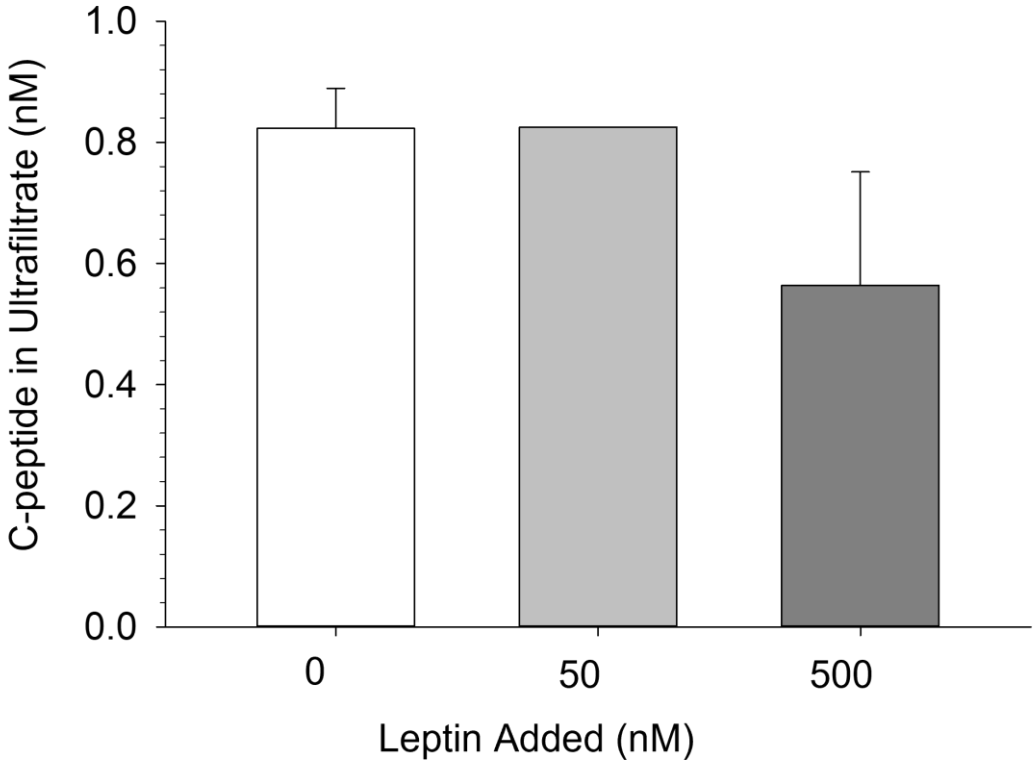

Figure 4.9: Ultrafiltration binding analysis of C-peptide and 50 or 500 nM leptin using 3.5 kDa MWCO 3D-printed devices. *n* = 2-4. error = sd.

Next, C-peptide and leptin interaction was analyzed by the method of SPR In this procedure leptin was immobilized onto a biosensor chip which was placed into the instrument. Then, several concentrations of C-peptide in DDW (ranging from 8 nM to 38.5 μM) were prepared and loaded into the instrument. The system was programmed to allow the C-peptide to flow over the leptin biosensor chip in PBS running buffer over the course of several hours. Changes in the refractive index from sensor chip surface as buffer flowed over, and bound leptin interacted with C-peptide, were recorded by the instrument as response units vs. time as shown in Figure 4.10. The assembled data produced binding curves, which were then analyzed using Qdat software in the Pioneer system, to confirm specific binding of C-peptide and leptin with a K_d of approximately 2.4×10^{-6} M.[275]

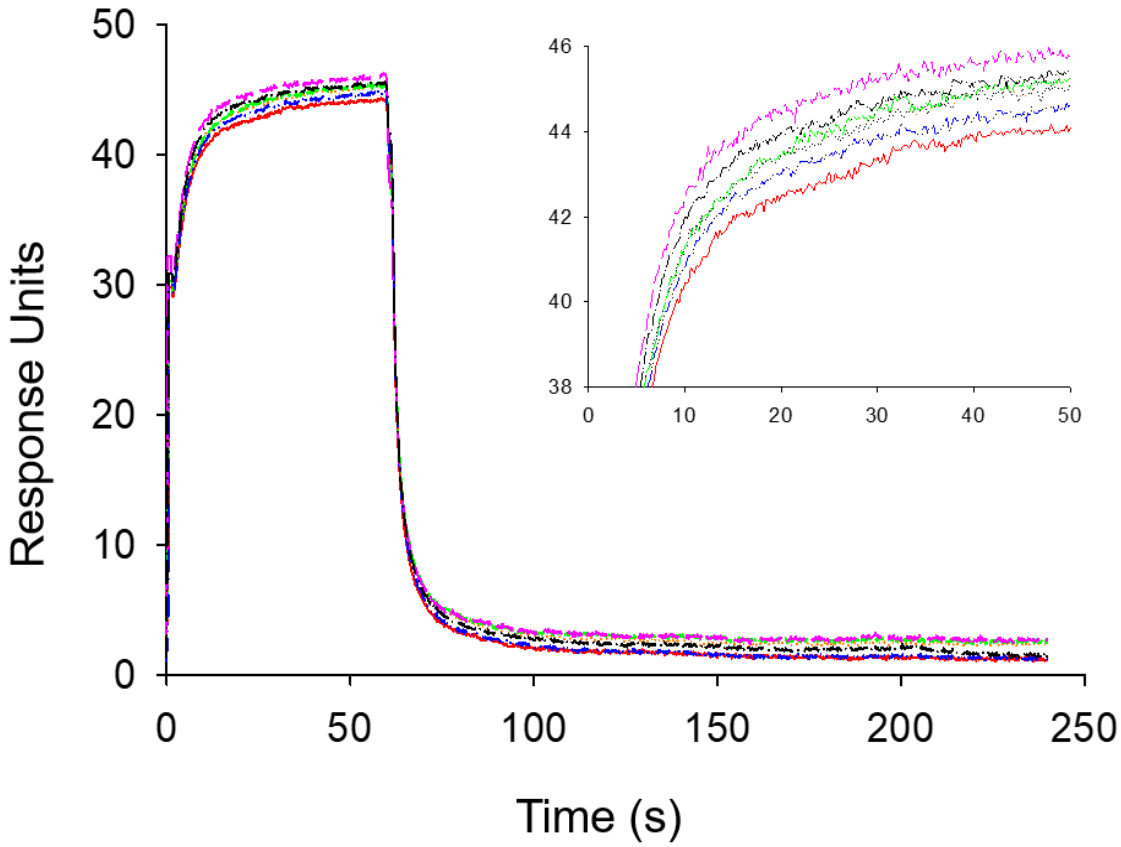

Figure 4.10: SPR was carried out to confirm leptin binding to C-peptide. The inset displays resolved binding curves for C-peptide concentrations of 8 nM, 100 nM, 300 nM, 1 µM, 8 µM and 38.5 µM in DDW. Binding affinity data (resonance units vs. time) was collected and evaluated using the Qdat software in the pioneer SPR system. The resultant K_d = 2.4 x 10^{-6} M. Adapted from Keshavarz et al., *Mol. Pharm.* **2021,** *18* (6), 2438-47.

4.4 Discussion

Although the precise reason(s) for the difficulty in attaining values for binding constants for C-peptide and leptin by the ultrafiltration method are not known, it is thought to be primarily due to the similarity in size between C-peptide and leptin. Another observation that may have been a factor in the ultrafiltration method's inability to analyze binding between C-peptide and leptin is that leptin may interfere with the detection of C-

peptide by ELISA assay. As shown in Figure 4.7, when samples containing C-peptide and leptin were assayed for C-peptide concentration without undergoing ultrafiltration, a comparison of average values vs. control samples (with 20 nM C-peptide and no leptin) revealed that there is a statistically significant difference in C-peptide measured when at least 5 nM of leptin is present in the system. Although higher levels of leptin were not evaluated, it is suspected that at greater leptin levels, there is likely a greater change in measurement of C-peptide by ELISA. This could produce significant interference, particularly with ultrafiltration experiments that use larger membrane sizes (i.e., 12-14 kDa) with higher levels of leptin (50-500 nM).

As indicated by the manufacturer, the C-peptide ELISA kit itself is formulated with different types of antibodies that have high affinity and specificity against distinctly different epitopes[o, 4] of C-peptide, which allows reaction between various C-peptide antibodies and native C-peptide to occur in the microplate wells without competition or steric hindrance.[355] However, any substance that can alter the measurable concentration of analyte or alter antibody binding can potentially cause interference in an immunoassay. While the occurrence of interference is typically low in assays that contain blocking agents that neutralize or inhibit the interference, it is often higher in new, untested immunoassays. In the latter case, the analysis may require use of a different assay, or supplemental measurements, before and after treatment with more blocking reagent.[356] In terms of the use of C-peptide ELISA for analyte measurement in ultrafiltrate, potential for interference is greatly minimized since samples were prepared in PSS buffer instead

[o] Epitope (biochemistry) – the part of an antigen molecule to which an antibody attaches itself.

of human blood serum. In addition, since the C-peptide ELISA method has been in use since the mid-1970s, it should be considered a well characterized immunoassay. However, the recent discovery of leptin (1994) means that its interaction with a variety of biomolecules has not been tested, so the potential for interference from leptin is unknown. Although it is known that many peptides circulate bound to serum proteins that modify ligand bioactivity and bioavailability, to date, investigations of leptin binding activity in human serum have mainly focused on the interaction of leptin with its soluble receptor.[211-213]

SPR experiments provided novel data, in confirming leptin's ability to specifically bind C-peptide, with a K_d of 2.4 x 10^{-6} M.[275] While not a perfect comparison, in 2015, using ITC, the Spence group confirmed specific binding between HSA and C-peptide with a K_a of 1.75 $\pm$ 0.64 x 10^5 M^{-1} ($1/K_a = K_d$ = 1.75 $\pm$ 0.64 x 10^{-5} M).[149] Thus the K_d of C-peptide and leptin by this comparison would represent a higher binding affinity interaction. Caro and coworkers, by radioimmunoassay, have identified a mean serum leptin concentration of approximately 0.47 $\pm$ 0.58 nM in healthy non-obese humans, while it is 1.96 $\pm$ 1.51 nM in healthy obese humans.[276] In perspective, the average concentration of HSA, the most abundant circulating plasma protein, in healthy humans, is approximately 751.88 μM, while in diabetic humans, it is 601.50 μM.[357] Thus, in terms of physiological relevance, a higher binding affinity for C-peptide and leptin than for HSA and C-peptide would support the Spence group observation that addition of leptin to erythrocyte samples containing albumin, Zn^{2+} and C-peptide enhances C-peptide binding and GLUT1 translocation compared to erythrocyte samples containing albumin, Zn^{2+} and C-peptide alone.[275]

The SPR data, together with the ^{99m}Tc-leptin labeling data shown in chapter 3, add an important contribution to leptin as an arbitrator of energy balance in vivo, especially because leptin has the potential to alleviate phenomena that occur in the bloodstream of an individual with diabetes.[275] For example, in states of chronic hyperglycemia, where more albumin is glycated and cannot as efficiently carry molecules,[150, 160, 271] leptin, with its fairly short half-life of ~25 minutes[162] in relation to albumin with a half-life of ~12-21 days would not be as affected by non-enzymatic glycation, which is a drawn-out process occurring over the progression of several hours to days.[271] For this reason, leptin delivery of C-peptide to the erythrocyte may be part of a mechanism that the body employs in an effort to control dysregulated glucose homeostasis, and in this way, improve microvascular blood flow.[275]

The binding of a leptin/C-peptide complex which involves Zn^{2+} is physiologically relevant in terms of the established role of the erythrocyte in control of vascular tone and overall blood flow.[275] Interestingly, in agreement with work from Spence group, there are reports in the literature of beneficial effects of leptin addition to people with type 2 diabetes (who still have circulating leptin),[208] while in contrast, there is scarce evidence of improvement in blood flow to people with type 1 diabetes, a group that would be expected to have little to no circulating C-peptide due to destruction of pancreatic β-cells.[275] While previous research from the Spence group research has examined C-peptide interaction with erythrocytes in the presence of leptin at physiological concentrations under normal glycemic conditions,[274] C-peptide binding to the erythrocyte has not been examined in the presence of leptin under hyperglycemic conditions. These data are important to substantiate the hypothesis that leptin may be able to serve as a carrier molecule for

C-peptide when albumin cannot. Therefore, in the next chapter, progress on investigating C-peptide interaction with erythrocytes in the presence of leptin under hyperglycemic conditions as well as future directions for research will be discussed.

Chapter 5: Current Studies, Conclusions and Future Directions

5.1 Investigation of C-peptide Binding to Erythrocytes in the Presence of Leptin in Hyperglycemic Conditions

Despite the absence of an identified receptor, it is established that C-peptide specifically binds to human renal tubular cells, fibroblasts and venous endothelial cells.[128] In 2015, the Spence group reported analogous binding characteristics of C-peptide to human erythrocytes,[149] however, albumin was a required component in the solvent (buffer) system for any measurable C-peptide binding to cells. The report provided evidence that albumin is needed for erythrocyte uptake of C-peptide and Zn^{2+}. The uptake of C-peptide and Zn^{2+} led to enhancement in erythrocyte membrane deformability, which could explain the observed ATP release when erythrocytes are subjected to flow-induced sheer stress. The ability of C-peptide and Zn^{2+} to have effects in combination with molecules secreted from other tissues was also investigated at this time. Specifically, it was demonstrated that leptin further enhances ATP release from erythrocytes by an additional 30% when administered with C-peptide and Zn^{2+}.[273]

The Spence group continued these investigations for a potential role of leptin, involving C-peptide and Zn^{2+}, in the amplification of glucose trafficking in the blood stream confirming that leptin increases erythrocyte-derived ATP release when delivered in combination with C-peptide and Zn^{2+}, and furthermore providing evidence that the combination of C-peptide/Zn^{2+}/leptin induces GLUT1 translocation on the erythrocyte cell membrane.[274] As discussed in section 2.4.2 of this dissertation, the Spence group also

began initial investigations into the binding interactions of Zn^{2+}, C-peptide and leptin with the erythrocyte cell membrane.[275]

For studies of $^{65}Zn^{2+}$ binding by erythrocytes, for samples incubated with $^{65}Zn^{2+}$ and C-peptide in PSS buffer, there was a $^{65}Zn^{2+}$ binding of 1.68 ± 0.3 nM, $p<0.04$, compared to a $^{65}Zn^{2+}$ binding of 3.6 ± 0.6 nM, $p<0.001$ for samples incubated with $^{65}Zn^{2+}$ and C-peptide in PSS buffer with 3.8 nM leptin.[274] The effect of leptin on C-peptide binding was also examined. For erythrocytes incubated with 20 nM C-peptide and 20 nM Zn^{2+} in PSS, C-peptide binding of 1.7 ± 0.1 nM was observed.[358] When leptin was added in the amount of 3.8 nM, C-peptide binding increased to 3.1 ± 0.1 nM.[274]

Here, these studies are continued by investigating C-peptide binding to the erythrocyte under conditions reminiscent to that which would occur in the bloodstream of a patient with diabetes. Namely, C-peptide binding to the erythrocyte is measured in the presence of leptin and Zn^{2+} in normoglycemic conditions, in comparison to hyperglycemic conditions reminiscent to those which occur in diabetes mellitus, and furthermore, in the presence of an increased ratio of glycated albumin.

5.2 Methods

5.2.1 Preparation of C-peptide

Crude C-peptide (Peptide 2.0 Inc., Chantilly, VA) for erythrocyte binding studies was prepared as described in section 3.5.3.

5.2.2 Preparation of Zn^{2+}

Zn^{2+} was prepared as described in section 3.5.4.

5.2.3 Preparation of Leptin

Lyophilized recombinant human leptin (R&D systems, Minneapolis, MN) was prepared and stored per manufacturer instructions, as described in section 4.2.2.

5.2.4 Preparation of Glycated Bovine Serum Albumin

Glycated bovine serum albumin (gBSA) was purchased as a lyophilized powder (Sigma Aldrich, St. Louis, MO) and stored at -20 °C prior to use. For use in experiments, a small amount of the glycated BSA (~5 mg) was massed in a 1.7 mL tube. Then, 1 mL of AF-PSS was added. A small aliquot of this stock was diluted 1:100 for determination of concentration by BCA assay, as described in section 4.2.8.2. Following, the stock was diluted with AF-PSS to a final concentration of 5,000 nM.

5.2.5 Blood Collection and Erythrocyte Isolation

Blood collection and isolation of erythrocytes was performed as described in section 3.5.6. Erythrocytes for all C-peptide binding experiments were washed in PSS (containing 75 µM BSA).

5.2.6 Standard and Sample Preparation for Binding Experiments in Normal and High Glucose PSS

C-peptide standards were prepared as described in section 4.2.6. Samples were prepared by first adding 25 µL of 800 nM Zn^{2+} in DDW followed by 25 µL of 800 nM C-peptide in DDW to 1.7 mL microcentrifuge tubes. These tubes were allowed to sit for at least three minutes to allow the Zn^{2+} and C-peptide to mix before addition of other sample components. Simultaneously, leptin was diluted in the appropriate buffer (normal (5.5 mM) or high glucose (15 mM) PSS) to a final concentration of 10 nM. Then, leptin was added to the appropriate sample tube(s) in enough volume to prepare samples of 0, 0.5 and 5 nM leptin. The appropriate form of PSS was added next, followed by erythrocytes so that the final sample volume was 1 mL with 7% erythrocytes. Samples were inverted to mix and then allowed to incubate at 37 °C, with inverting every 30 minutes for a total of two hours. Following, samples were centrifuged for 500*g* for five minutes, and the supernatant was stored at 4 °C prior to C-peptide ELISA analysis (ALPCO, Boston, MA).

5.2.7 Standard and Sample Preparation for Binding Experiments with gBSA

Standards were prepared as in section 4.2.6, except that instead of using regular PSS for the buffer, a PSS buffer was prepared that was 5,000 nM in BSA by massing ~25 mg of regular BSA (Sigma Aldrich, St. Louis, MO) in a 5 mL centrifuge tube and resuspending with 5 mL of AF-PSS. BCA assay was performed as described in section

4.2.8.2 to determine the exact concentration, and then the stock was diluted to 5,000 nM using AF-PSS.

Samples were prepared similarly to what is described in section 5.2.6 except that instead of using PSS, samples were prepared using the 5,000 nM BSA/gBSA stocks, so that samples were either all BSA or 50:50 BSA/gBSA. It was not possible to wash the erythrocytes in AF-PSS, as it was found that this caused cell lysis and interfered with measurement of C-peptide. Because using regular PSS for cell isolation and wash steps, which contained 75 μM BSA increased final BSA concentration in the samples by approximately 5,000 nM (or 5 nmol per 1 mL sample), it was not possible to prepare samples containing exclusively gBSA. For this reason, only samples with 10,000 nM BSA or 5,000 nM BSA/5000 nM gBSA were analyzed. Table 5.1 below shows an example of sample preparation for binding experiments which examined the effect of gBSA.

Table 5.1 Sample preparation for analysis of C-peptide binding to erythrocytes in the presence of leptin and gBSA. All listed values are volume in microliters (µL). 800 nM Zn^{2+}, 800 nM C-peptide (Cp), 10 nM leptin (Lp), 5,000 nM BSA in AF-PSS (BSA), 5,000 nM gBSA in AF-PSS (gBSA), and erythrocytes (RBCs) were prepared as described in methods section.

*Final volume of erythrocytes and buffer were determined after wash steps and unique for each experiment.

Sample	Zn^{2+}	Cp	Lp (BSA)	Lp (gBSA)	BSA*	gBSA*	RBCs*
1	25	25	0	0	850	0	100
2	25	25	0	0	425	425	100
3	25	25	50	0	850	0	100
4	25	25	25	25	425	425	100
5	25	25	500	0	850	0	100
6	25	25	250	250	425	425	100

5.2.8 C-peptide ELISA

Standards and samples were diluted 1:50 in DDW for C-peptide ELISA. C-peptide ELISA was performed per manufacturer instructions.

5.3 Results

In initial experiments, it was first attempted to establish a baseline value for C-peptide binding to erythrocytes in the presence of leptin in albumin containing buffer (PSS). The results of these studies are shown in Figure 5.1. Incubation of 20 nM

Zn^{2+}/C-peptide with no leptin in normoglycemic conditions (in PSS buffer with 5.5 mM glucose) resulted in a C-peptide binding of 1.44 ± 1.99. When 0.5 nM leptin was added, a C-peptide binding of 1.80 ± 1.11 nM was observed. With the addition of 5 nM leptin, a C-peptide binding of 2.96 ± 1.29 was observed.

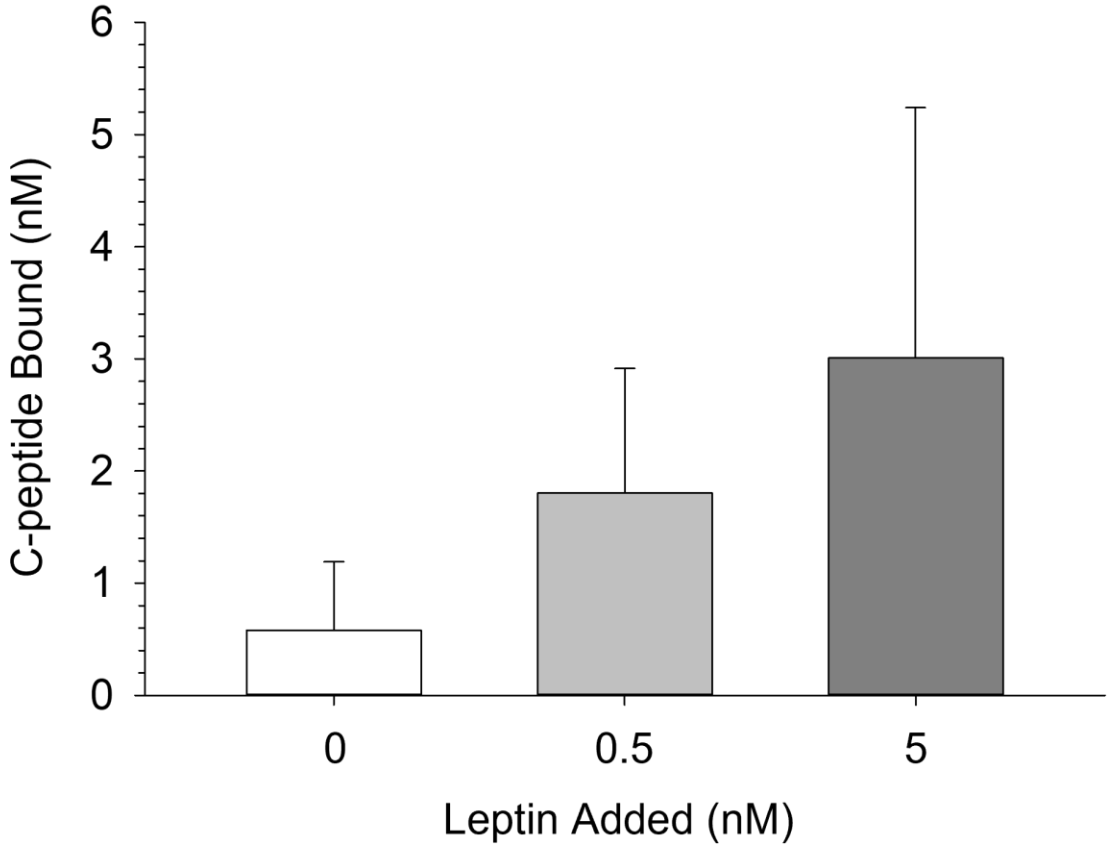

Figure 5.1: C-peptide binding to erythrocytes in the presence of leptin in normoglycemic conditions. Zn^{2+}/C-peptide = 20 nM, 7% erythrocytes, glucose = 5.5 mM, *n* = 4-5, error = sd.

For Figure 5.2, which examined the effect of hyperglycemic conditions (in PSS buffer with 15 mM glucose), when no leptin was added, a C-peptide binding of 1.20 ± 0.27 was determined. Adding 0.5 nM leptin resulted in a binding of 2.09 ± 0.47. Addition of 5 nM leptin resulted in 3.30 ± 0.90 nM of C-peptide binding. While there was no statistical difference between the results obtained for Figure 5.1, for Figure 5.2, in which erythrocytes were incubated in high glucose conditions with 20 nM C-peptide, a statistical difference ($p<0.05$) was observed between the samples with no leptin and the samples that had 0.5 or 5 nM leptin, respectively.

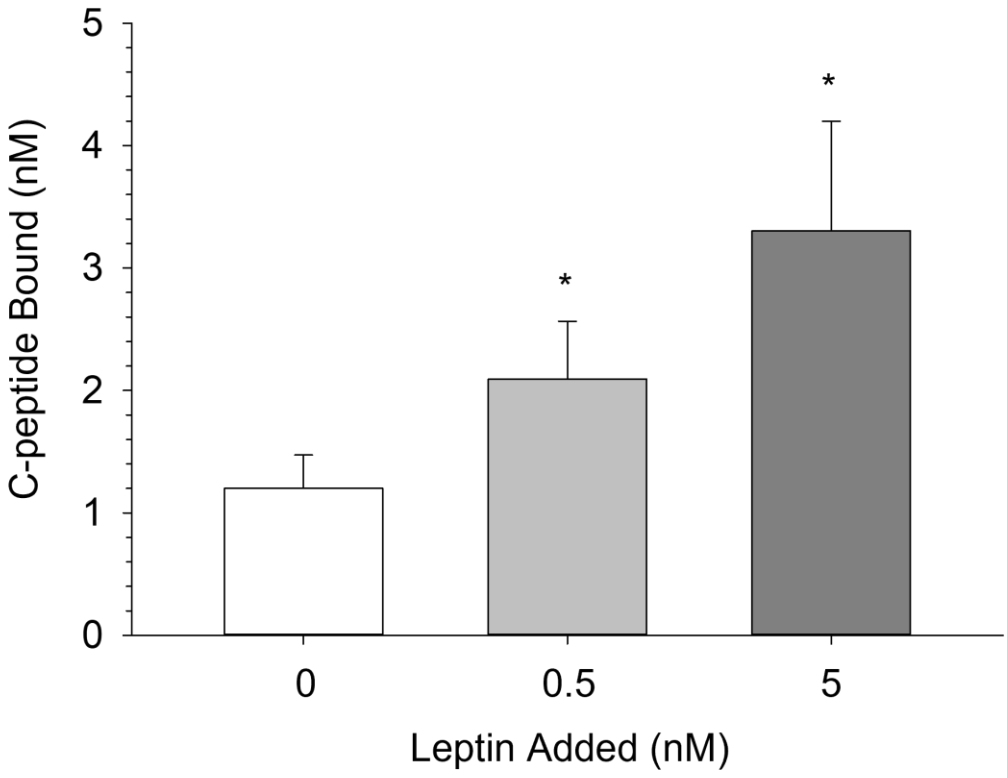

Figure 5.2: C-peptide binding to erythrocytes in the presence of leptin in hyperglycemic conditions. Zn^{2+}/C-peptide = 20 nM, 7% erythrocytes, glucose = 15 mM, *n* = 4, error = sd. Significance from control indicated by * ($p<0.05$).

Figure 5.3 shows the results of initial studies examining the effect of glycated albumin on C-peptide binding to erythrocytes in the presence of leptin.

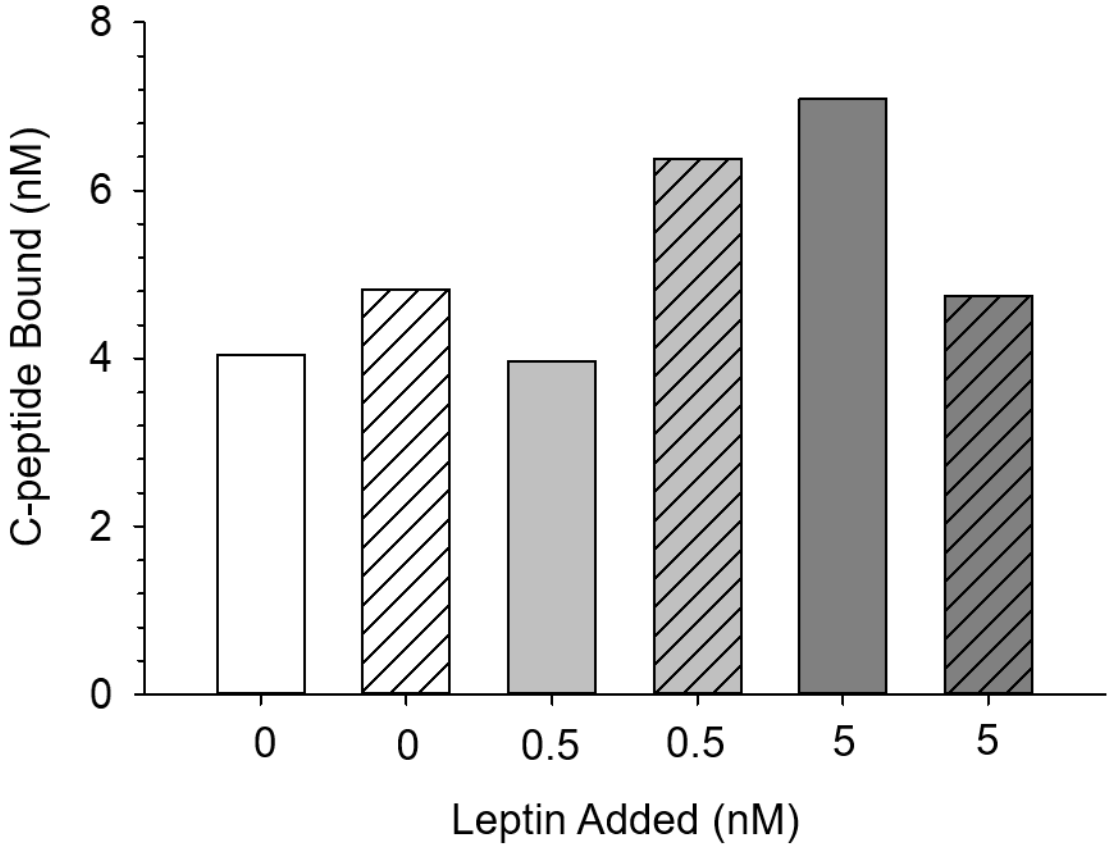

Figure 5.3: C-peptide binding to erythrocytes in the presence of leptin under normoglycemic conditions with glycated albumin. Zn^{2+}/C-peptide = 20 nM, 7% erythrocytes, glucose = 5.5 mM, n = 1. Solid bars = 10,000 nM BSA, striped bars = 5,000 nM BSA/5,000 nM gBSA, colors correspond to leptin concentration (white= 0 nM, light grey= 0.5 nM, and dark grey= 5 nM).

5.4 Discussion

Due to high variability of results for C-peptide binding experiments, more studies/biological replicates will be necessary for proper analysis. While no conclusions can be definitively made based on the current data, it is hypothesized that incubating erythrocytes with C-peptide in hyperglycemic conditions, with normal control erythrocytes and normal albumin, may, perhaps counterintuitively, result in an increase in cellular binding (or uptake) of C-peptide, especially in the presence of leptin. Since these studies have focused on normal, healthy cells which have not been chronically exposed to hyperglycemic conditions (and therefore have not accumulated oxidative stress/cell rigidity associated with diabetes), the consequence could be that cellular utilization/metabolism of glucose would initially increase, to lower blood glucose levels. If C-peptide/albumin/leptin are key players in glucose metabolism of the erythrocyte, it follows that C-peptide binding and C-peptide-associated cellular activity (ATP release, GLUT1 translocation and perhaps downstream NO release) should increase under these circumstances. Further studies are needed to investigate this hypothesis, and comparison between normal and diabetic erythrocytes will be essential.

To investigate the ability of leptin to carry C-peptide in the presence of gBSA (Figure 5.3), which can less effectively act as a carrier molecule compared to BSA,[150, 160, 271, 327] experiments were performed with buffer containing a known amount of gBSA. Initial experiments failed due to the cells being washed in AF-PSS, which increased cell lysis and prevented accurate measurement of C-peptide. Therefore, a successful experiment was only performed once. It is hard to make any speculations based on the results of a

single experiment. While it appears there may be a trend of increased C-peptide binding to erythrocytes in the presence of leptin, the effect of gBSA cannot be established. It is hypothesized that reducing the amount of BSA in the samples would result in increased non-specific binding of C-peptide to the microcentrifuge tubes. Therefore, the apparent increase in binding of C-peptide in the presence of AF-PSS containing 5,000 nM BSA (4 nM, in comparison to typical results[149] for binding of C-peptide to erythrocytes in regular PSS buffer (containing 75 μM BSA) of approximately 2 nM, or 2 pmol) may simply be a consequence of experimental design. As a reminder, the C-peptide ELISA measurement of C-peptide binding to erythrocytes is an indirect method measurement based on the measured amount of C-peptide remaining in the supernatant after incubation subtracted from the initial amount added. Therefore, if control samples indicated that exactly 20 nM C-peptide was added to the samples and approximately 16 nM C-peptide was measured in the supernatant of the sample with 10,000 nM BSA and no leptin (white unshaded bar, far left), it could mean that 4 nM C-peptide bound to the erythrocytes. However, what is more likely is that a decrease in BSA in samples would lead to an increase in non-specific binding, so that 2 nM (4 nM minus 2 nM), or more, of the unaccounted 4 nM of C-peptide, may be bound to the microcentrifuge tube or non-specifically to the cell surface, instead of specifically bound to cell receptor(s) of interest.

It may be useful in future research to modify the method of analysis of C-peptide binding to erythrocytes. This was attempted in the current research, by labeling C-peptide with ^{99m}Tc. While C-peptide can be successfully labeled by attachment of HYNIC to its N-terminus, purification of the labeled compound and determination of its concentration was problematic. More specifically, due to the small size of the peptide, separation of the

labeled compound from free ^{99m}Tc could not be achieved consistently. Separation was attempted using size-exclusion column chromatography as well as high performance liquid chromatography.[359] Because of the half-life of ^{99m}Tc (~six hours), a fast and efficient separation method is important for successful use of this label. Another issue that was encountered was determination of concentration of the labeled compound. Because C-peptide does not contain aromatic amino acids (tryptophan, phenylalanine, and tyrosine), Lowry assay could not be used to determine its concentration. BCA assay is dependent on the presence of cysteine, tyrosine, or tryptophan residues, which C-peptide does not contain. Finally, Bradford assay could not be used because C-peptide lacks arginine and lysine amino acids. While C-peptide ELISA could be used, there were additional safety requirements as the whole ELISA would then be considered radioactive. Moreover, using ELISA assay to determine protein concentration adds additional time to the experiment. If a fast and efficient purification method of C-peptide from free ^{99m}Tc can be realized, perhaps this method can be useful in future studies. Alternatively, C-peptide could be labeled with a radioisotope with a longer half-life, such as ^{14}C, which has a half-life of 5,730 $\pm$ 40 years.[360] However, trace metal contaminants in the commercially purchased peptide would still be a concern, so additional purification would be required (and verification of purity), which would raise concerns for equipment contamination.

In terms of advancing the current studies, experiments with normal/high glucose and gBSA should be continued. Experiments could also investigate the combined effect of hyperglycemic and gBSA on leptin interaction with the erythrocyte in the presence of C-peptide. Most importantly, investigations of C-peptide binding to diabetic erythrocytes in the presence of leptin should be pursued.

5.5 Overall Conclusions and Future Directions

This dissertation has assessed leptin interaction with erythrocytes using different techniques, including leptin ELISA, as well as via measurements with a newly created ^{99m}Tc-leptin conjugate. These interactions were successfully analyzed directly using ^{99m}Tc-leptin in a variety of conditions, including high and low concentrations of C-peptide and leptin, as well as in the absence or presence of Zn^{2+}, C-peptide, and albumin. Analysis of C-peptide and leptin interaction was attempted using ultrafiltration dialysis and successfully evaluated using SPR. Finally, this dissertation launched investigation of how C-peptide and leptin interact with erythrocytes in hyperglycemic conditions. However, despite the progress that has been made, there are many questions that remain unanswered and areas of interest that remain unexplored that will be useful for advancing our knowledge of the role of C-peptide and leptin in microvascular blood flow dynamics in future research. Some of the key areas will be discussed next.

5.5.1 Identification of C-peptide and Leptin Receptor(s) on the Erythrocyte

The current studies have investigated leptin from the standpoint of its association with C-peptide[361] and a potential collaborative role with Zn^{2+} and C-peptide in promoting microvascular blood flow. From this standpoint, there are several related studies that could be performed to confirm this activity and identify a candidate receptor. For instance, studies of erythrocyte deformability and endothelial NO release associated with a Zn^{2+}/C-peptide/leptin combination will be useful for providing additional evidence in

support of a role for Zn^{2+}/C-peptide/leptin in regulation of microvascular blood flow, which would provide additional evidence supporting the existence of a target receptor on the erythrocyte.

Indeed, erythrocytes possess several receptors of unknown function.[362] Although specific binding of leptin to erythrocytes has been established in separate reports,[275, 304] a leptin receptor (or receptors) on the erythrocyte has not been definitively identified. Koliakos and colleagues confirmed specific binding of FITC-leptin to the erythrocyte.[304] In these studies, a role for leptin in activation of the Na^+ -H^+ exchanger (NHE 1), which is involved in functions including regulation of intracellular pH and cell volume,[363] was investigated. Although leptin was shown to increase NHE 1 activity, and specific binding of FITC-leptin was demonstrated independently using Scatchard analysis,[361] a cellular receptor for leptin was not determined. It was hypothesized that the receptor in question must be a short form of the leptin receptor (OB-Rs), since the long form of the leptin receptor is located primarily in the hypothalamus.[364, 365]

In terms of how a leptin related C-peptide receptor on the erythrocyte might be identified, one possibility is to start with what is currently known regarding the signal transduction mechanism of C-peptide. Specifically, C-peptide has been demonstrated to initiate intracellular signaling cascades that are typically associated with activation of a G-protein-coupled receptor (GPCR), including activation of protein kinase C and mobilization of calcium.[366] The effects of C-peptide have been shown to be sensitive to pertussis toxin[366, 367] which catalyzes adenosine diphosphate-ribosylation of the α_i subunits of the heterotrimeric G-protein, thereby preventing G-proteins from interacting with GPCRs on the cell membrane.[368] A place to start may be to perform investigation of

the known C-peptide and leptin associated erythrocyte activities, including as ATP release and GLUT1 translocation, and perhaps erythrocyte deformability and endothelial NO release if they are confirmed, in the presence of pertussis toxin. If the Zn^{2+}/C-peptide/leptin effects are reduced or eliminated by pertussis toxic, it would provide evidence that the mechanism has similar associations. If this is the case, a receptor knockdown strategy may be useful to narrow down the list of possible candidate GPCRs.[80]

Although capture of receptor complexes in complete integrity and in sufficient quantity and purity for characterization remains a key challenge in receptor research,[369] it may be possible in future research to utilize a receptor pull down strategy to isolate a leptin/C-peptide receptor. Recently developed methods to identify proteomic[p,4] components of receptor complexes in cells may be useful in characterization of a C-peptide/leptin receptor on erythrocytes. Many methods have been developed to elucidate the proteomic portion of receptor complexes on in cells,[370, 371] and a frequently used approach involves use of 1- or 2-dimensional gel electrophoresis for separating an isolated receptor component, following with mass spectrometry analysis to identify proteins and determine the specifics of their primary sequence.[372] Verification of protein interactions may be accomplished using Western blotting and colocalization of native receptor and known binding protein within cells.[373] Advantages offered by mass spectrometric analysis including high-throughput capabilities, easy automation and rapid

[p] Proteomics – The study of proteomes (the entirety of proteins that may be expressed by an organism, tissue, or cell) and their functions.

protein identification make the technique well-adapted for proteomic evaluations of complex protein mixtures including those of native receptor complex in cells.[372]

5.5.2 Investigation of Leptin and Zn^{2+} Interaction

Studies which have shown that obese individuals have low zinc and high leptin levels hint that there is a relationship between zinc and nutrition, and as a consequence, between zinc and leptin.[374] Nascimento Marreiro et al. investigated the effect of zinc supplementation on serum insulin levels and insulin resistance in obese women. Over the course of the 4-week study, it was found that circulating insulin levels decreased significantly in the zinc supplemented group compared to a control group. Leptin levels did not change, although it was noted that more studies would be needed, including studies on individuals with documented insulin resistance and supplementation with different dosages of zinc for different durations of time.[375] Other studies have suggested that zinc may regulate serum leptin production in humans.[376, 377]

Although this dissertation emphasized analysis of the interaction between C-peptide and leptin due to the known relationship of C-peptide with Zn^{2+} and albumin that was investigated in 2015,[149] it will be important in future investigations to analyze the binding relationship between leptin and Zn^{2+}. While previous studies have analyzed Zn^{2+} interaction with the erythrocyte in the presence of leptin,[275] binding analysis between the ligands themselves will be important in future studies. Although equilibrium dialysis was not successful for the analysis of C-peptide and leptin interaction due to their size similarity, it should be readily adaptable for a simple and cost-efficient analysis of leptin

and Zn^{2+} interaction. Creation and optimization of a 3D-printed ultrafiltration device fitted with 7 kDa molecular weight cutoff membrane could be utilized to study this binding relationship, and equilibrium binding constants could be determined using $^{65}Zn^{2+}$ as the analyte and recombinant human leptin as the ligand.

5.5.3 Other Considerations for Development of a Zn^{2+}/C-peptide/Leptin Diabetes Therapeutic

It is important to note, as previously discussed in this dissertation, that while there are reports of beneficial effects of leptin addition to individuals with type 2 diabetes,[208] there is very little evidence of improvement in blood flow upon addition of leptin to individuals with type 1 diabetes. A lack of endogenous C-peptide in the circulation of people with type 1 diabetes may explain this difference and offer a potential solution for this group. However, association with Zn^{2+}, as well as a carrier molecule appear to be a requirement for C-peptide biological activity.[149, 275] This raises concerns to how beneficial administration of C-peptide alone would be for improving microvascular blood flow in type 1 diabetes, a group that would be expected to have less functional albumin, due to increased non-enzymatic glycation in the presence of chronically elevated blood glucose. Indeed, this may explain the lack of successful clinical trials for administration of C-peptide as a type 1 diabetes therapeutic. Leptin, which is not subject to non-enzymatic glycation due to its short half-life, could be useful in formulation of a Zn^{2+} and C-peptide therapeutic in this circumstance.

There are also factors to consider for development of a peptide hormone-based therapeutic that would be beneficial for improving microvascular blood flow in type 2 diabetes. Metabolic syndrome, which increase the risk for type 2 diabetes as well as cardiovascular disease, is a major public health issue worldwide.[378] There are a constellation of abnormalities associated with metabolic syndrome, including glucose intolerance, insulin resistance, central obesity, dyslipidemia and hypertension.[378] Although results of preclinical studies have shown that leptin can improve insulin resistance, glucose and lipid imbalances in mouse models of type 2 diabetes,[202-205] results of at least two recent clinical trials have indicated that leptin is either not effective or only minimally effective in improving diabetes and insulin resistance in obese people affected by type 2 diabetes.[206, 207] The potential of leptin administration to restore metabolic imbalance appears to be greatest in those who are leptin deficient.[208] However, in metabolic syndrome, leptin levels are paradoxically elevated, and individuals are resistant to the effects of leptin. Furthermore, it has also been reported erythrocytes from a type 2 diabetic rat model display an apparent resistance to Zn^{2+} -activated C-peptide.[147]

Although the cause of resistance to C-peptide, insulin and leptin effects are not definitively known, different theories have been proposed[32, 147, 379] and it is likely to be multifactorial. While pharmaceuticals have been developed to help alleviate this resistance and associated metabolic abnormalities, pharmaceuticals have many potential side effects and risks and must be carefully considered for benefits and drawbacks. However, it is important to note, that in the case of metabolic syndrome, perhaps the most important therapeutic intervention may simply be diet and lifestyle interventions.[378, 380]

5.5.4 The Role Adipocyte-Derived Cytokine Hormones in Blood Flow

It has recently been discovered that adipose tissue, in addition to leptin, secretes a variety of other cytokines, collectively termed adipokines, that are of metabolic importance. Adiponectin, for instance, appears to antagonize the effects of leptin, with studies showing that adiponectin has anti-inflammatory properties and may negatively regulate atherogenesis.[361] Resistin, a member of a recently discovered family of cysteine-rich secretory proteins termed 'resistin-like molecules' (RELM) or 'found in the inflammatory zone' (FIZZ), was first identified in a screen of potential targets for the thiazolidinedione (TZD) class of insulin sensitizers in 3T3-L1 adipocytes.[381] Resistin appears to be necessary for regulation of adipogenesis, glucose homeostasis and possibly inflammation.[382] Adipokines network in an intricate manner to regulate endothelial function and further studies will increase our understanding of this process. Development of therapeutics involving adipokines, such as leptin, is an attractive idea with implications for treatment of atherosclerosis and diabetes.[361]

5.5.5 Interaction of C-peptide and Leptin with Other Proteins of Interest

Recent (unpublished) Spence lab research has investigated potential interaction of casein, transferrin, and collagen with C-peptide. Casein is an inclusive name for a family of milk proteins consisting of four peptides which differ in amino acid, phosphorus and carbohydrate content but are similar in amphiphilic character. In most conditions, casein molecules congregate into spherical micelles. Due to its unique properties and

abundant availability in bovine milk (~2.75% of content) casein has applications in science, pharmaceutical and food industries. Due to its ability tendency form micelles, casein has potential to serve as a carrier for delivery biologically active agents in pharmaceutical applications.[383] Much like bovine serum albumin, casein is a commonly used blocking agent in biological assays. Research (unpublished) research in the Spence group has demonstrated by C-peptide ELISA analysis (following incubation of 20 nM C-peptide and 7% (normal, healthy control) erythrocytes in AF-PSS with phycological levels of casein for two hours at 37 °C) that C-peptide has no detectable binding to erythrocytes in the presence casein (but no albumin or leptin), for this reason, it may be useful to substitute casein as a blocking agent (in place of BSA) in future leptin/C-peptide/Zn^{2+} studies to investigate now these components interact and how biological activities are affected in the absence of albumin.

Transferrin is a metal-binding β-globulin found in vertebrate blood plasma and other fluids outside the cell. It is believed to modulate iron changes between sites of absorption, storage and use, with the most important center storage and utilization being the bone marrow.[384] Free iron can enhance the production of free radicals which leads to oxidative damage like lipid peroxidation. Transferrin plays an important role in antioxidant defense by converting iron into a redox inactive form. Abnormalities in transferrin function due to changes in concentration and increased glycation seen in diabetic states may be related to chronic complications.[385] By C-peptide ELISA analysis in recent Spence group studies, similar to the studies with casein, no detectable interaction of C-peptide with erythrocytes was observed in the presence of transferrin.

Another protein of interest is collagen. Collagen is the most abundant protein found in animals.[386] Much like casein, collagen has also been widely used for medical applications. Due to its safety and biocompatibility because of its biological characteristics like weak antigenicity and biodegradability, collagen is useful as a drug delivery system in many settings.[387] Nonenzymatic glycation of collagen which leads to crosslinking of the protein has been implicated in diabetic complications.[388-390] Recent Spence group research on analysis of (20 nM) C-peptide binding to (7%) healthy control erythrocytes in the presence of physiological levels of collagen, by C-peptide ELISA analysis, has revealed a minimal C-peptide uptake/binding to the erythrocytes of 0.21 $\pm$ 0.14 pmoles (in comparison to a C-peptide uptake/binding of 0.74 $\pm$ 0.11 picomoles when the erythrocytes were incubated with leptin under similar conditions).

5.5.6 Glycated Hemoglobin and Diabetic Complications

Glycated hemoglobin, in a similar manner to glycated albumin, is formed by the non-enzymatic linkage of glucose or other reducing sugars to the amino-terminal valine of the β-chain of hemoglobin.[12] Data from the Diabetes Control and Complications Trial (2008) assessed the effect of A1C variability on the risk of nephropathy and retinopathy in individuals with type 1 diabetes, to find that long term fluctuations in blood glucose control contribute to development of these microvascular complications.[14] Therefore, it may be desirable in future research to conduct studies evaluating the relationship between glycated hemoglobin level and leptin binding. For instance, a study may be performed in which known amounts of glycated hemoglobin are introduced into

erythrocyte samples and incubated with an incrementing series of ^{99m}Tc-leptin with or without 20 nM C-peptide to determine leptin binding as a function of HbA1c. Similar studies could be performed to evaluate the effect on C-peptide binding using C-peptide ELISA or a future optimized C-peptide labeling method. Similarly, it will be desirable to examine C-peptide biological activity on erythrocytes, in combination with Zn^{2+}, as a function of A1C level. Understanding the relationship between C-peptide to other circulating molecules their interaction with erythrocytes in the pathophysiological environment of diabetes will be important in future studies aimed at addressing microvascular complications.

APPENDIX

“*Any intelligent fool can make things bigger and more complex. It takes a lot of genius and a lot of courage to move in the opposite direction.*”

– Albert Einstein

“*No human is limited*.”

– Eliud Kipchoge

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