

Does chronic leptin treatment decrease glucagon responsiveness in STZ-induced type 1 diabetic rats?

by

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Abstract

Central leptin administration has been shown to normalize blood glucose concentrations in streptozotocin (STZ)-induced diabetic rats. We hypothesize that the mechanism of this normalization of blood glucose concentrations in STZ rats occurs via a leptin-mediated decrease in glucagon responsiveness resulting in a decrease of gluconeogenesis.

Continuous infusion of somatostatin was used to inhibit endogenous glucagon, allowing for the examination of glucose production in response to a pyruvate challenge in the presence of high levels of glucagon. Responses to glucagon after an intraperitoneal injection of pyruvate in each treatment group were compared to control blood glucose measurements from the pyruvate challenge (in absence of glucagon). Both diabetic and non-diabetic vehicle-treated rats had an increased response to the pyruvate challenge in the presence of high levels of glucagon. In contrast, no significant difference was found between the pyruvate challenges in the presence of high levels of glucagon and the control trial pyruvate challenge in diabetic leptin-treated rats.

Leptin-treatment of diabetic rats did not alter plasma glucagon concentrations after the infusion of glucagon, but rather blocked the production of glucose in response to pyruvate. This suggests that central leptin treatment decreases the hepatic responsiveness to pyruvate in the presence of high levels of glucagon. Therefore, it is

suggested that chronic central leptin treatment in diabetic rats normalizes blood glucose concentrations due to a leptin-mediated decrease in glucagon responsiveness.

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Chapter 1

Introduction

Diabetes mellitus is an epidemic affecting the United States and many parts of the world. The American Diabetes Association reported in 2011 that 25.8 million children and adults, or 8.3% of the United States population, had been diagnosed with diabetes. Another 7 million were undiagnosed, and around 79 million were considered pre-diabetic (1). According to the World Health Organization in 2013, global diabetic prevalence was estimated at 347 million (2) and this is forecasted to increase to 552 million adults or 9.9% of the population, by the year 2030 (3).

The metabolic disease diabetes mellitus (DM) is typically divided into several different types. The predominant forms are type 1 and 2 diabetes. These types of diabetes are characterized by hyperglycemia derived from defects in insulin secretion, insulin action, or both. Type 1 DM (T1DM) is characterized by insulin deficiency as a result of the autoimmune destruction of the beta (β) cells of the pancreas. Type 2 DM (T2DM) is characterized by abnormalities that result in resistance to insulin action (4). Onset of T2DM is frequently accompanied by obesity, which exacerbates insulin resistance.

Insulin resistance is a condition in which insulin is produced by the body, but it becomes less effective within the body. Higher levels of resistance require larger quantities of insulin to bring blood glucose concentrations back to homeostasis. The

basis of the abnormalities in carbohydrate, fat, and protein metabolism in diabetes is deficient action of insulin on target tissues (4). This results from inadequate insulin secretion and/or diminished tissue responses to insulin at one or more points in the complex pathways of hormone action. Impairment of insulin secretion and defects in insulin action frequently coexist in the same patient.

Untreated diabetes is commonly characterized by fasting hyperglycemia. A large contributor to diabetic hyperglycemia is the overproduction of hepatic glucose (5), caused by increased rates of gluconeogenesis, that is the production of glucose from non-carbohydrate precursors, and glycogenolysis, the production of glucose from the breakdown of glycogen. Gluconeogenesis and glycogenolysis are hormonally mediated. Glucagon stimulates these processes by aiding in the phosphorylation of regulating enzymes. In gluconeogenesis, the two key gluconeogenic enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) are upregulated. Low blood glucose stimulates the release of glucagon and subsequently the hormone aids in the breakdown of glycogen resulting from increased G6Pase expression and activation. Glucagon increases intracellular cyclic AMP levels, which increases phosphoenolpyruvate carboxykinase (PEPCK) transcription acting through a cAMP response element (CRE) (6). All the while, the catabolic process of glycolysis is inhibited by these concerted anabolic processes (7).

Insulin promotes glycolysis, inhibiting hepatic glucose output and counteracting the effect of glucagon. In the absence of insulin or with insulin resistance in diabetes, elevated levels of glucagon and rates of hepatic glucose production are present (8).

Leptin, a hormone primarily produced from white adipose tissue (9), has been found to play a role in energy homeostasis and the normalization of blood glucose through an inhibitory effect on hepatic glucose production (10). This adipocyte-derived hormone interacts with its receptor (ObR) both centrally and peripherally. The long form of the leptin receptor, ObRb, is thought to signal predominantly within the hypothalamus of the brain (11). It has been suggested that a lack of leptin signaling in the brain may lead to insulin resistance and hyperglycemia (12). Obesity has a profound impact on both leptin and insulin resistance. Despite high circulating levels of both insulin and leptin, obese subjects often suffer from hyperglycemia and its associated complications as a result of improper signaling or resistance. Numerous studies involving leptin have suggested that within the central nervous system (CNS) leptin may play a role in glucose homeostasis independent of insulin (13-17) providing an alternative mechanism to correct hyperglycemia. Intracerebroventricular (ICV) administration of leptin has been found to normalize blood glucose in streptozotocin (STZ)-induced diabetic rats (14, 18). Pair-fed trials found leptin's effect to be independent of a leptin-induced decrease in food intake and body weight (7-23).

Elevated rates of gluconeogenesis and glycogenolysis contribute to hyperglycemia in uncontrolled diabetes (uDM). Hepatic glucose production is enhanced by a lack of insulin and the unopposed effects of glucagon. One potential mechanism by which leptin could regulate glucose metabolism is through an inhibitory effect on glucagon, resulting in a decrease in hepatic glucose production. Currently there are no studies examining the effects of central leptin administration on glucagon responsiveness.

The ultimate objective of this study is to determine the mechanism by which central leptin administration normalizes blood glucose concentrations in type 1 diabetic rats. A more specific goal is to determine the ability of leptin-treated STZ-induced diabetic rats to increase blood glucose in response to exogenous glucagon. To decrease the potential complication factor of endogenous glucagon, continuous infusion of somatostatin will be used to inhibit endogenous glucagon. This will allow for the examination of glucose production in response to an infusion of exogenous glucagon in the type 1 diabetic rats chronically treated with either leptin or vehicle after endogenous glucagon has been removed.

Hypothesis

We hypothesize that leptin-treated diabetic rats will have an attenuated response to pyruvate under conditions of high glucagon. This would support our contention that normalization of blood glucose in leptin treated STZ-induced diabetic rats is due to a leptin-mediated decrease in glucagon responsiveness.

Chapter II

Literature Review

2.1 Diabetes Prevalence and Outcomes

The prevalence of diabetes mellitus (DM) is increasing at an alarming rate. Along with obesity, diabetes has reached epidemic proportions in the developed world (24). The diagnosis of diabetes in the United States is projected to increase from the current 1 in 10 people to a range of 1 in 5 to 1 in 3 by the year 2050 (25). In 2013, world prevalence of diabetes among adults was estimated at 347 million (2), and this is forecasted to increase to 552 million adults, or 9.9% of the population, by the year 2030 (3). The chronic nature of DM yields astronomical costs in treatments and healthcare. In 2007, the Centers for Disease Control and Prevention (CDC) reported total annual cost of diabetes in the United States, including undiagnosed, gestational, and pre-diabetes, at an estimated \$218 billion (26). The estimated global healthcare expenditures to treat and prevent diabetic complications are projected at \$471 billion (USD) and are expected to exceed \$595 billion by the year 2030 (27).

Classic symptoms resulting from hyperglycemia in untreated diabetes include polyuria, polydipsia, polyphagia, and weight loss. Untreated diabetes may also result in a myriad of acute and chronic complications. The acute condition of ketoacidosis, or the lowering of blood pH, is a condition in which the body utilizes fat as a fuel source instead of glucose either because there is not enough insulin or none at all. This condition often accompanies hyperglycemia and can be a life-threatening consequence

of uncontrolled diabetes. Other acute conditions include diabetic coma, lactic acidosis, non-ketotic hyperosmolar syndrome, respiratory infections, and periodontal (gum) disease. Chronic hyperglycemia of DM is associated with long-term damage, dysfunction, and failure of various organs, including the eyes, kidneys, nerves, heart, and blood vessels. Impairment of growth and susceptibility to certain infections may also accompany chronic hyperglycemia. Associated co-morbidities and complications include heart disease and stroke, hypertension, neuropathy, retinopathy, nephropathy, abnormalities of lipoprotein metabolism, and risk of limb and extremity amputations (28). Patients with DM are also at an increased risk for peripheral arterial and cerebrovascular disease. (29). In the year 2007, diabetes was ranked as the seventh leading cause of death. Diabetes was listed as a contributing factor to a staggering total of 231,404 deaths (30). As this trend of diabetes and insulin resistance increases, a search for novel therapeutic agents in the treatment of this epidemic continues.

2.1.1 Types and Diagnosis of Diabetes

The predominant forms of diabetes mellitus are T1DM and T2DM; however, other forms such as gestational diabetes, and a possible type 3 also occurs. The diagnosis of DM is based on criteria that include appearance of multiple symptoms and a random plasma glucose concentration ≥ 200 mg/dl (11.1 mmol/l). As previously discussed, DM is characterized by classic symptoms of polyuria, polydipsia, and polyphagia, as well as a fasting plasma glucose of (FPG) ≥ 126 mg/dl (7.0 mmol/l). A 2-hour postload glucose ≥ 200 mg/dl (11.1 mmol/l) during an oral glucose tolerance test (OGTT) is another possible diagnostic criterion. The test uses a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water (4).

T1DM, also referred to at one time as juvenile-onset diabetes, is characterized by insulin deficiency as a result of autoimmune destruction of the β cells of the pancreas. Hyperglycemia in fasting conditions can range from moderate to severe, especially in times of stress or illness/infection. Contrary to previous notions, T1DM is not limited to childhood. A study observing new cases of type 1 diabetes diagnosed during a 3 year period found that type 1 incidence peaked during ages 0-9 and then again at ages 50-80 (31). Some individuals may retain some β cell function sufficient to prevent ketoacidosis for many years. However, as β cell function declines eventual dependence on insulin is necessary for survival. T1DM accounts for ~ 5–10% of total cases of diabetes (4).

T2DM is characterized by abnormalities that result in resistance to insulin action. Previously referred to as non-insulin-dependent diabetes, T2DM, or adult-onset diabetes, encompasses individuals who have insulin resistance and usually eventually over time have relative insulin deficiency. This predominate form of diabetes includes ~90–95% of those with diabetes. The cause of T2DM is a combination of resistance to insulin action and an inadequate compensatory insulin secretory response. Many patients with this form of diabetes are classified as obese (≥ 30 kg/m²). Obesity is considered the highest risk factor for development of insulin resistance (32, 33). Patients who are not obese by traditional weight criteria may have an increased percentage of body fat distributed predominantly in the abdominal region (4).

Gestational diabetes is defined as any degree of glucose intolerance with onset or first recognition during pregnancy. This form of diabetes affects ~135,000 cases (~4%) of all pregnancies in the United States. Deterioration of glucose tolerance occurs

normally during pregnancy, particularly in the 3rd trimester. Classification of gestational diabetes includes a fasting plasma glucose level >126 mg/dl (7.0 mmol/l) or a casual plasma glucose >200 mg/dl (11.1 mmol/l) (4).

Type 3 diabetes has recently undergone research to further define the characteristics of the disease. According to de la Monte et al. (2008), type 3 diabetes is an accurate term for depicting Alzheimer's disease. This is due to the fact that Alzheimer's results from resistance to insulin in the brain. This selective involvement in the brain, as well as other molecular and biochemical features that overlap with both T1DM and T2DM, suggest a strong correlation between the diseases (34).

2.1.2 Insulin Release

Insulin is a 51 amino acid containing protein hormone that binds to an insulin receptor, a hetero-tetrameric glycoprotein membrane composed of two α and two β subunits linked together by disulfide bonds (35). In a normal, non-diabetic post prandial state, glucose enters the β cells of the pancreas through the glucose transporter, GLUT2. Within the cell, the enzyme glucokinase phosphorylates glucose to glucose-6-phosphate (G6P) generating multiple high-energy adenosine tri-phosphate (ATP) molecules through the processes of glycolysis and the respiratory cycle (24). The increasing levels of ATP within the beta cell increases the intracellular ATP: ADP ratio and therefore closes the ATP-sensitive potassium (K^+) channels. Potassium ions are prevented from exiting causing an accumulation of positive potassium ions within the cell. The cell surface membrane becomes depolarized due to the increased positive charge with respect to the outside. This depolarization causes voltage-gated calcium ion

(Ca²⁺) channels to open allowing calcium ions to move into the cells by facilitated diffusion. Increased intracellular calcium causes the activation of phospholipase C, which cleaves the membrane phospholipid phosphatidyl inositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (33). IP₃ binds to receptor proteins in the plasma membrane of the endoplasmic reticulum (ER) allowing the release of calcium ions from the ER via IP₃-gated channels, and further raises the intracellular concentration of calcium ions. These increased amounts of calcium stimulate the release of previously synthesized insulin that has been stored within secretory vesicles of pancreatic β cells in the islets of Langerhans (24, 36).

A common characteristic of diabetes is the decreased uptake and phosphorylation of glucose resulting in a repression of synthesis and activity of glucokinase (37). Repression of this enzyme results in a lack of response to a fall in the insulin: glucagon ratio. Recent evidence has found impaired insulin secretion in individuals before the development of impaired glucose tolerance. This supports the theory that impairment of insulin secretion, rather than insulin resistance alone is the primary cause of the development of T2DM (38).

2.2 Insulin Resistance, Obesity, Inflammation, and Substrate signaling

Myocytes (muscle cells) and adipocytes (fat cells) are among the most insulin sensitive tissues, and are the most strongly influenced by insulin (24). Upon release, insulin binds to the extracellular α subunits of the insulin receptor in its dimeric form, phosphorylating multiple tyrosine residues on the receptor. Activation of the insulin receptor induces an increased rate of the glucose transporter, GLUT4, vesicle

translocation to the plasma membrane allowing more glucose to move into myocytes and adipocytes (35, 39, 40), lowering glucose concentrations in the blood. The receptor then binds and phosphorylates insulin receptor substrate-1 (IRS-1). IRS proteins are a critical link in hepatic insulin signaling. The 2 major IRS isoforms, IRS-1 and IRS-2, are highly expressed in normal conditions in the liver. IRS-1 is involved primarily in glucose homeostasis and IRS-2 is associated with lipid metabolism (41) as well as promotion of adult β cell regeneration and central control of nutrient homeostasis (22). Following phosphorylation, IRS-1 binds the enzyme phosphatidylinositol-3-kinase (PI3K). Through a series of phosphorylation events of the insulin signaling cascade, PI3K's interaction with IRS helps to regulate overall glucose uptake (40). On the other hand, when blood glucose concentrations decrease, insulin is low. This causes a movement of GLUT4 into intracellular vesicles and a lower amount of glucose within the plasma membrane. Overall, insulin stimulates the metabolic processes of glycolysis and glycogen synthesis, while suppressing gluconeogenesis and glycogenolysis.

However, in the case of insulin resistance, the body produces insulin, but does not use it efficiently. This is characterized by impaired sensitivity to insulin-mediated glucose disposal (24). This results in a higher demand for insulin to promote entry of glucose into cells. Pancreatic β cells release excessive insulin to compensate for the insensitivity, thus producing a state of hyperinsulinemia.

Exposure to high insulin levels overtime decreases cellular GLUT4 content and reduces its translocation (42). The impaired insulin-stimulated GLUT4 translocation and glucose transport in T2DM skeletal muscle are associated with reduced insulin-stimulated IRS-1 tyrosine phosphorylation and PI3K activity. This is attributed to the

observation of physiological levels of insulin inducing a 2.0 fold increase in GLUT4 content in plasma membranes of skeletal muscle from control subjects (43). The suppression of GLUT4 concentrations and activation of GLUT4 occurs in an insulin resistant condition and this further exacerbates the resistance (35, 40). The pancreas eventually fails to compensate for the body's need of insulin, yielding increasing concentrations of glucose in the blood stream. Over time, the varying degrees of insulin resistance may progress into the most common disease state associated with insulin resistance, which is T2DM (24, 44).

There are multiple causes of insulin resistance. The insulin resistance of obesity and T2DM is characterized by defects at many levels. Some of the associated defects include impairment in insulin secretion (38), decreases in both insulin receptor concentration (35) and insulin receptor kinase activity, decreases in the concentration and phosphorylation of IRS-1 and IRS-2, decreases in PI3K activity, decreases in glucose transporter translocation, and decreases in the activity of intracellular enzymes (40). A hypothesis for T2DM is that insulin resistance in classic target tissues, such as liver, muscle and fat, coupled with resistance in beta-cell and other tissues, combine to produce the pathophysiology of T2DM (40).

Obesity, particularly central obesity (45, 46), is closely linked with insulin resistance (33). A key indication of whole-body resistance is thought to be correlated with the accumulation of fat in the abdominal region. It has been speculated that decreased insulin action in severely obese subjects is related to the impaired insulin-mediated glucose uptake accompanied by deficient insulin receptor signaling (47). Obesity-related signaling defects involve insulin's initial activation of the insulin receptor

tyrosine kinase. This activation results in phosphorylation of IRSs on numerous tyrosine residues. These phosphotyrosine residues act as docking sites for various SH2 (Src Homology 2) domain-containing proteins, including the regulatory subunit of PI3K. Binding the subunit of PI3K activates the lipid kinase that promotes glucose transport (46). Adipose and skeletal muscle are the most predominantly insulin-sensitive tissues and suffer the most from lack of insulin action. Although the liver does not require insulin for glucose entry into cells, the ability of the liver to regulate glucose levels via reducing its secretion of glucose in the presence of insulin is affected by insulin resistance (48).

Insulin resistance in muscle and fat cells reduces glucose uptake (and also local storage of glucose as glycogen and triglycerides, respectively), whereas insulin resistance in liver cells results in reduced glycogen synthesis and storage and a failure to suppress glucose production and release into the blood.

Insulin action in adipocytes involves changes in gene transcription. The transcription factor adipocyte determination and differentiation factor-1/sterol regulatory element-binding protein-1c (ADD-1/SREBP-1c) is believed to play a crucial role in the actions of insulin to regulate adipocyte gene expression by stimulating genes involved in lipogenesis and suppressing those involved in fatty acid oxidation (46). Circulating free fatty acids (FFAs) derived from adipocytes are elevated in many insulin resistant states. Accumulation of FFAs in insulin-sensitive non-adipose tissues, such as muscle and liver, can impair insulin-mediated glucose uptake in these tissues (45). This has been suggested to contribute to the overall insulin resistance of diabetes and obesity by inhibiting glucose uptake, glycogen synthesis and glucose oxidation, and by increasing hepatic glucose output (40).

A characteristic signaling defect of obesity is the increased expression and activity of several protein tyrosine phosphatases (PTPs). These PTPs terminate insulin signaling through dephosphorylation of tyrosine residues. Three PTPs of importance, PTP1B, leukocyte antigen–related phosphatase (LAR), and SH2, are increased in expression and/or activity in muscle and adipose tissue of obese humans and rodents (46). PTP1B and LAR dephosphorylate the insulin receptor and IRS-1 in vitro (46). Inhibitory phosphorylations such as serine phosphorylation may attenuate or block signaling by decreasing insulin-stimulated tyrosine phosphorylation. This provides negative feedback to insulin signaling, affecting various other pathways and cascades to produce insulin resistance (40).

IRS proteins are a critical link in hepatic insulin signaling and decreased expression of these proteins in the liver may be a key component of hepatic insulin resistance. The 2 major IRS isoforms, IRS-1 and IRS-2, are highly expressed in normal conditions in the liver, but are down regulated to varying degrees in livers of diabetic subjects. IRS-2 signaling promotes regeneration of adult β cells and central control of nutrient homeostasis, which can prevent obesity and diabetes in mice (22). Recent investigation has proposed that enhancement of β cell IRS-2 expression in insulin-resistant patients might significantly delay islet destruction and onset of diabetes (49).

Obesity is characterized by a state of inflammation. A link between chronic inflammation and insulin resistance has been increasingly established by numerous studies examining the correlative and causative factors of insulin resistance. Accumulation of lipid in adipocytes, enlarging the cells, increases the probability of producing pro-inflammatory cytokines such as tumour necrosis factor- α (TNF- α),

Interleukin-6 (IL-6), resistin, monocyte chemoattractant protein-1 (MCP-1), and plasminogen activator inhibitor-1 (PAI-1). Production of these inflammatory substances elicits communication between pathways resulting in the production of pro-inflammatory cytokines to induce insulin resistance (50).

Obesity-induced insulin resistance was examined in the white adipose tissue (WAT) of mouse models of genetic and high-fat diet-induced obesity/diabetes and macrophage-specific or -enriched genes such as TNF- α , macrophage inflammatory protein-1 α (MIP-1 α), MCP-1, and others, were observed to be significantly upregulated (51). The cytokine, TNF- α , exhibits a strong influence on adipocyte glucose and lipid metabolism and has been implicated in the insulin resistance associated with obesity and T2DM (52). In obesity, enlarged adipocytes overexpress and secrete this cytokine. TNF- α signaling impairs insulin signaling, in part through serine phosphorylation of IRS-1, and can reduce GLUT4 gene expression (46).

2.3 Regulating Reactions Involved in Glucose Metabolism

Glucose homeostasis is achieved through a balancing of several antagonistic biochemical pathways. Normal fasting blood glucose ranges typically between 70-100mg/dL and fluctuates throughout the day depending on food consumption (53). Metabolic pathways are either activated or inhibited in response to blood glucose concentrations. Secretions of insulin or glucagon regulate this net balance in response to blood glucose levels. The pathways of glycolysis and glycogen synthesis are stimulated by insulin and function to remove glucose from circulation, thus decreasing blood glucose concentrations. Conversely gluconeogenesis and glycogenolysis are

stimulated by glucagon and act to add glucose to the circulation, increasing blood glucose concentrations. Insulin resistance causes an attenuated response to glucose and therefore has a significant effect on these pathways which will be later explored in greater detail.

2.3.1 Postprandial glucose metabolism and the role of insulin

In a postprandial (fed) state, normal pancreatic β cells secrete insulin in response to the influx of glucose. The binding of insulin to its receptor on the plasma membrane of target cells such as muscle and adipose cells results in rapid uptake of glucose. Skeletal muscle glucose is stored through the process of glycogenesis, while in adipose tissue glucose is converted to triglycerides through de novo fatty acid synthesis. In the liver, insulin acts to stimulate glycolysis and the formation of glycogen and triglycerides, while inhibiting the processes of glycogenolysis and gluconeogenesis (40, 54).

Glycolysis

The metabolic pathway of glycolysis is a ten reaction sequence that converts glucose into pyruvate. The free energy released from this process is used to form the high-energy compounds adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide (NADH). The process generates four molecules of ATP per glucose, but two are consumed in the preparatory phase, yielding a net of 2 ATP molecules per glucose. The ten intermediate compounds involved provide entry points to glycolysis. The glycolytic pathway has three irreversible reactions that are regulated by the rate-controlling enzymes specific to glycolysis which are hexokinase, phosphofruktokinase, and pyruvate kinase (55). The rate of glycolysis in the liver is

regulated to meet major cellular needs such as the production of ATP, provision of components for biosynthetic reactions, and functions to decrease blood glucose concentration. A fall in blood glucose produces a termination of glycolysis to allow for the process of gluconeogenesis to occur (56, 57).

Glycolysis does not use oxygen, so it can occur in both anaerobic and aerobic conditions. Aerobic glycolysis produces ATP at a rate that can maintain lower-intensity energy for muscle cells over an extended period of time. Oxygen is utilized from air as the final electron acceptor in the electron transport chain. Through a process called pyruvate decarboxylation, pyruvate, formed in glycolysis, is converted to acetyl-CoA and carbon dioxide (CO_2) and NADH_2 within the mitochondria. The acetyl-CoA enters the citric acid cycle, where it is fully oxidized to carbon dioxide and water, producing more NADH and FADH_2 . The NADH is oxidized to NAD^+ by the electron transport chain, using oxygen as the final electron acceptor. This process creates a hydrogen ion gradient across the inner membrane of the mitochondria. Finally, ATP synthase uses this hydrogen ion gradient to produce about 2.5 ATP for every NADH oxidized in a process called oxidative phosphorylation and 1.5 ATP for every FADH_2 oxidized. If the need for ATP is greater than can be supplied there is a shift to anaerobic glycolysis (56).

Under anaerobic respiration, glycolysis is only effective for energy production during short, intense bouts of exercise. Although less efficient, anaerobic respiration is a much faster method of producing ATP under low oxygen conditions. Production of 2 ATP molecules per glucose molecule (~38 ATP molecules under aerobic conditions) occurs to aid in quick replenishment of energy. To allow glycolysis to continue NAD^+

must be regenerated. This is accomplished under anaerobic conditions by lactate dehydrogenase, which converts pyruvate and NADH into lactic acid and NAD⁺. The build-up of lactic acid causes the pH in the cytoplasm to quickly drop due to an accumulation of hydrogen ions in the muscle. Through a process called the Cori cycle, the liver eliminates excess lactate by transforming it back into pyruvate and ultimately back into glucose. The glucose can then be released back into circulation. This may eventually inhibit enzymes involved in glycolysis (55).

The rate of glycolysis is significantly lower in diabetes. A 60% lower rate of glycolysis was found in hepatocytes of streptozotocin (STZ)-induced diabetic rats (37). The inhibition of glycolysis and increased cycling of pyruvate to glucose (85% of glucose molecules recycled) was proposed to be a consequence of elevated rates of endogenous fatty acid oxidation in diabetic hepatocytes (37). Several mechanisms contribute to increased blood glucose concentrations within the body. Decreased rates of glycolysis, as well as decreased glycogen synthesis, are a part of this framework of elevated glucose levels in circulation.

Glycogen synthesis

Glycogen synthesis is a process of clearing excess glucose from the blood through the storage of glucose as glycogen in the fed-state to be used for mobilization during times of energy deficit (58). Glycogen, a polymer of repeating units of glucose residues linked by α -1:4 glycosidic linkages and α -1:6 linkages at branch points, is the major storage form of carbohydrate in the body. Glycogen is predominantly stored in skeletal muscle, comprising nearly two-thirds of total glycogen, but stores are also found

in liver and adipose tissues as well as others (59). Insulin release in response to a rise in blood glucose stimulates glycogen accumulation through a coordinated increase in glucose transport and glycogen synthesis.

An activated form of glucose is made by the enzyme UDP-glucose pyrophosphorylase. This enzyme exchanges the phosphate of glucose-1-phosphate for UDP. The non-reducing end of the small protein glycogenin, which is autoglycosylated at a serine residue until ~8 glucose units long, is utilized along with the UDP-glucose by the enzyme glycogen synthase. This enzyme, in its activated/dephosphorylated state, acts to assemble glucose molecules into a chain, attaching units by α -1:4 bonds. Branches are formed by the branching enzyme, amylo- α (1:4) \rightarrow α (1:6)transglycosylase, which transfers the end of the glycogen chain onto an earlier part via an α -1:6 glycosidic bond (60).

Glycogen synthesis is under the hormonal control of counter-regulatory hormones insulin and glucagon. These hormones exert opposing effects on the rate and level of glycogen synthase phosphorylation (60). Under normal conditions, insulin activates the enzyme glycogen synthase by binding to the "a" form of glycogen phosphorylase to inactivate the enzyme, whereas glucagon secretion would promote the opposite effect. Glycogen phosphorylase, an enzyme catalyzing the rate-limiting step in glycogenolysis, stimulates the release of glucose-1-phosphate from the terminal alpha-1,4-glycosidic bond. The activation of glycogen synthase produces an inhibition of kinases such as protein kinase A (PKA) or glycogen synthase kinase-3 (GSK-3) and a reduction in levels of cyclic AMP (cAMP) (40, 61). These kinases and cAMP are part of

a natural negative feedback inhibition to inactivate/phosphorylate glycogen synthase when blood glucose is low.

Lack of insulin, insulin resistance, or increased levels of glucagon in diabetes yields an improper synthesis of glycogen. A decreased response to insulin results in the phosphorylation and inactivation of glycogen synthase yielding a diminished clearance of glucose from the blood by glycogen synthesis. The rate and the extent of activation of glycogen synthase in response to incubation with 60 mM-glucose was found to be greatly decreased in diabetic hepatocytes, and the conversion of synthase “b” into “a” was found to be 4.5-fold lower (61). The inactivated synthase fails to inhibit glycogen phosphorylase. Inactivation of glycogen synthase and activation of glycogen phosphorylase are favored in the diabetic state.

2.3.2 Pathways of fasted glucose metabolism and the role of glucagon

Glucagon

Glucagon, a 29-amino acid, 3485-Da peptide (62), is secreted from pancreatic alpha (α) cells during periods of starvation or low blood glucose. This hormone has been heavily researched in regards to its crucial role in energy homeostasis and pathogenesis of diabetes. Glucagon stimulates mobilization of fat from adipose tissue to skeletal muscle for β -oxidation and with the aid of glucocorticoids activates glycogenolysis and gluconeogenesis in the liver (54). An increased rate of hepatic glycogenolysis and an increased rate of total glucose production contribute to hyperglycemia in diabetes (8, 57, 63).

Glycogenolysis

Glycogenolysis is the breakdown of glycogen to glucose-1-phosphate and glucose. This process maintains blood glucose homeostasis in a time of low blood glucose or fasting conditions. Glycogenolysis occurs in myocytes and hepatocytes in response to hormonal and neural signals. Adipose tissue also contains minimal glycogen stores. Glycogen metabolism within adipocytes has been suggested to serve as a method of energy sensing in the coordination of glucose and lipid metabolism within adipose tissue, especially during the transition between fasted and fed states (64). Alpha cell secretions of glucagon enter the hepatic portal vein where it acts on G protein-coupled glucagon receptors in the liver. The result is an exchange in GDP/GTP and stimulation of the enzyme adenylyl cyclase. The stimulation utilizes ATP, which is converted into cyclic AMP (cAMP). Heightened levels of cAMP bind and release an activated form of protein kinase A (PKA). PKA phosphorylates phosphorylase kinase which in turn phosphorylates glycogen phosphorylase b, transforming it into the active glycogen phosphorylase a. This halts glycogen synthesis by phosphorylating and inactivating glycogen synthase. Inversely, insulin acts to suppress glycogenolysis. As previously described under glycogen synthesis, insulin opposes the breakdown of glycogen by activating glycogen synthase and inhibiting PKA (40).

Glycogen branches are broken down by the sequential removal of glucose monomers via phosphorylation and cleavage by the enzyme glycogen phosphorylase. Glycogen phosphorylase cleaves the bond linking a terminal glucose residue to a glycogen branch by substitution of a phosphoryl group for the α -1:4 linkage, forming glucose-1-phosphate (P). Glucose residues are phosphorolysed from branches of

glycogen until four residues before a glucose that is branched with a α -1:6 linkage. The glycogen debranching enzyme, α 1,6 glucosidase, cleaves the glucose residue by hydrolytic cleavage and then transfers three of the remaining four glucose units to the end of another glycogen branch. In the liver, glucose can be released into circulation or phosphorylated by glucokinase in the muscle or hexokinase in the liver. Glucose is converted to glucose-6-P and metabolized by hexokinase/glucokinase (65).

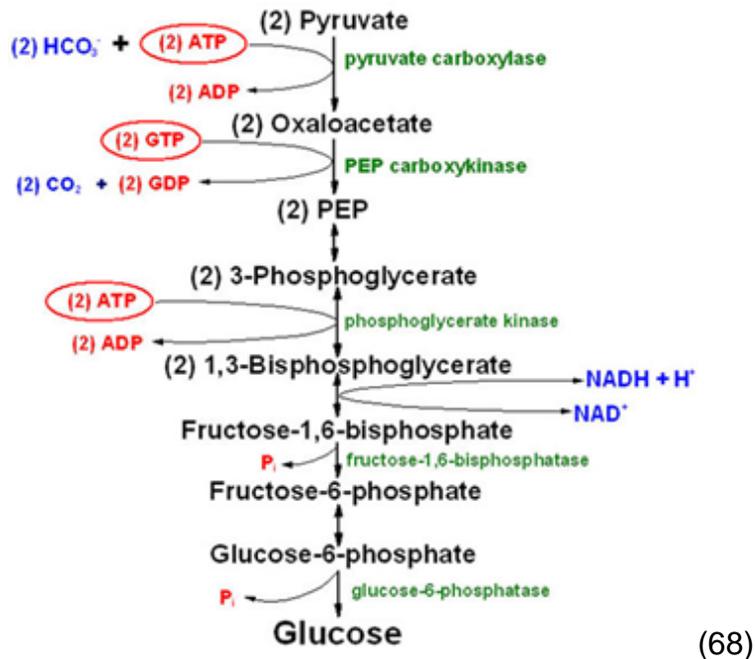
In order to provide energy for muscle contraction, glycogen is degraded in myocytes for an immediate source of glucose-6-phosphate. Glycogen degradation in hepatocytes releases glucose into the bloodstream for uptake by other cells. The phosphate group of glucose-6-phosphate is removed by the enzyme glucose-6-phosphatase, which is not present in myocytes, and the free glucose exits the cell via GLUT2 facilitated diffusion channels in the hepatocyte cell membrane (40).

Impaired suppression of lipolysis and hepatic gluconeogenesis, as well as decreased insulin-dependent glucose transport and metabolism in skeletal muscle, are some of the alterations of energy metabolism displayed in obesity-induced insulin resistance and T2DM. High circulating levels of nonesterified fatty acids (NEFAs) are strongly correlated with insulin resistance and the impairment of glucose metabolism due to adipose tissues' inability to appropriately store lipid, resulting in abnormal accumulation of fat in other tissues (64). Normal insulin signaling promotes triglyceride synthesis and storage in the central lipid droplet of adipocytes; however, this is altered in insulin resistance. Reduction of triglyceride mobilization is attributed to enhanced glycogen metabolism in adipose tissue (66).

Gluconeogenesis

Hepatic gluconeogenesis is a major pathway that maintains normal plasma glucose levels during prolonged starvation. It is one of two main mechanisms used to keep blood glucose concentrations from dropping to hypoglycemic levels, with the other being degradation of hepatic glycogen via glycogenolysis. The overall result of gluconeogenesis is the generation of glucose from non-carbohydrate carbon substrates such as pyruvate, lactate, glycerol, and glucogenic amino acids especially alanine and glutamine (67). Gluconeogenesis occurs mainly in the liver, but to some extent in the cortex of the kidney and small intestine. These organs utilize different gluconeogenic precursors. The liver uses primarily lactate and alanine, while the kidney uses lactate and glutamine.

Gluconeogenesis is an exergonic pathway consisting of a series of eleven enzyme-catalyzed reactions. The pathway may begin in the mitochondria or cytoplasm, depending on the substrate used. Many of the reactions are the reversible steps found in glycolysis. However, it is not the reverse of glycolysis due to the three irreversible reactions of glycolysis that must be bypassed in gluconeogenesis. The irreversible enzymes of glycolysis including hexokinase/glucokinase, phosphofructokinase, and pyruvate kinase are replaced with glucose-6-phosphatase (G6Pase), fructose-1,6-bisphosphatase, and phosphoenolpyruvate carboxykinase (PEPCK) in gluconeogenesis (56, 57). This system of reciprocal control allows the controlling factors of glycolysis and gluconeogenesis to inhibit each other and prevent the formation of a futile cycle. The overall reaction of gluconeogenesis is as follows:



Gluconeogenesis begins in the mitochondria with the formation of oxaloacetate by the carboxylation of pyruvate. This reaction also requires one molecule of ATP, and is catalyzed by pyruvate carboxylase. This enzyme is stimulated by high levels of acetyl-CoA, which is produced in β -oxidation in the liver, and inhibited, by insulin and the resulting high levels of pyruvate. Oxaloacetate is decarboxylated and then phosphorylated to form phosphoenolpyruvate (PEP) using the enzyme phosphoenolpyruvate carboxykinase (PEPCK). A molecule of GTP is hydrolyzed to GDP to drive the reaction and donate a phosphate group. The next steps in the reaction are the same as reversed glycolysis. However, fructose-1,6-bisphosphatase converts fructose-1,6-bisphosphate to fructose 6-phosphate, using one water molecule and releasing one phosphate. This rate-controlling step of gluconeogenesis is under allosteric control and is inhibited by AMP and fructose 2,6 bis-phosphate (69). The glucose-6-phosphate formed is used in other metabolic pathways or dephosphorylated to free glucose. Free glucose easily diffuses in and out of the cell, but glucose-6-

phosphate is trapped within the cell to control intracellular glucose levels. The final reaction of gluconeogenesis, the formation of glucose, occurs in the lumen of the endoplasmic reticulum (ER), where glucose-6-phosphate is hydrolyzed by glucose-6-phosphatase to produce glucose. Glucose is shuttled into the cytoplasm by glucose transporters located in the ER's membrane (54).

Most factors regulate the activity of the gluconeogenesis pathway by inhibiting the activity or expression of key enzymes. Gluconeogenesis control is hormonally mediated. Low blood glucose stimulates the release of glucagon from pancreatic α cells, stimulating the activity of the adjacent membrane bound adenylyl cyclase to catalyze the conversion of ATP to cAMP. The resulting increase in levels of cAMP stimulates protein kinase A (PKA) activity and promotes phosphorylation of cAMP responsive element binding protein (CREB). Phospho-CREB then interacts with the glucose homeostasis regulator, CREB-regulated transcription co-activator 2 (CRTC2/TORC2) (54). The CRTC2 increases transcription of the gluconeogenic enzymes PEPCK and G6Pase, and therefore stimulates hepatic glucose production (7) and inhibits glycolysis.

Epinephrine, an adrenomedullary hormone, works in combination with glucagon as a positive regulator of gluconeogenesis. Also referred to as adrenaline, epinephrine is primarily known for its role in stress and/or the fight-or-flight response. This hormone compensates for deficient glucagon secretion (70). The combination of glucagon and other glucose counter regulatory hormones, such as epinephrine, growth hormone, and cortisol, mobilize gluconeogenic precursors such as lactate, amino acids, and glycerol to the liver (62) aiding in the stimulation of transcription of PEPCK and G6Pase (7).

Insulin secretion acts as an opposing mechanism blocking both gluconeogenesis and glycogenolysis (40, 71). Expression of gluconeogenic genes as well as release of glucose is suppressed by insulin, therefore, decreasing hepatic glucose production (7). However, as plasma insulin levels decrease the dominant inhibition lessens. This unhampers the synthesis of the gluconeogenic enzyme PEPCK and allows hormones such as glucagon and β -adrenergic agonists to stimulate adenylyl cyclase and increase cAMP levels (57).

Elevated glucose production in diabetes is attributed to an increased rate of gluconeogenesis (8, 57, 63). The enzymes responsive to glucagon and catecholamines are already phosphorylated (57) therefore increasing the rate at which gluconeogenesis occurs. Increased substrate supply and changes in the concentration of various enzymes affect the overall elevated hepatic glucose production in DM (57).

2.4 Leptin as a Novel Therapeutic Approach to Diabetes

In the case of insulin resistance, hepatic glucose production is exacerbated to uncontrollable levels. Severe degrees of insulin resistance require copious amounts of insulin to restore euglycemia. This highlights the need for an insulin independent mechanism for controlling hepatic glucose production and overall elevated gluconeogenesis in diabetic subjects.

Leptin

Leptin, an adipocyte-derived 16-kDa protein hormone, is predominantly known for its roles in both energy and glucose homeostasis. Leptin's various roles in glucose and energy metabolism takes place within the central nervous system (CNS) as well as

peripheral tissues. The hypothalamus is the major connection between the CNS and leptin action to regulate feeding behavior and energy balance. The area known as the arcuate nucleus (ARC) has the highest amount of leptin receptors (72). Circulating concentrations of this adipocyte-derived hormone deliver information on peripheral energy stores to the CNS by interacting with a number of hypothalamic neuropeptide systems to regulate feeding behavior, energy expenditure, and blood glucose (10, 73).

2.4.1 Leptin Receptor Isoforms and Leptin Signaling

Leptin has multiple receptor isoforms. The short receptor isoforms, ObRa and ObRc, transport leptin across the blood brain barrier. However, the long leptin receptor, ObRb, containing four tyrosine residues (Tyr974, Tyr985, Tyr1077 and Tyr1138), is considered to be the predominant isoform for leptin signaling. The ObRb is expressed in several organs and throughout the CNS, particularly in the hypothalamus (11).

JAK/STAT pathway

ObRb contains intracellular motifs enabling activation of one of the main signaling cascades, the Janus tyrosine kinase 2 (JAK2)/ cytosolic signal transducer and activator of transcription protein 3 (STAT3) signal transduction pathway (74). The JAK2/STAT3 pathway comprises four non-receptor tyrosine kinases (JAKs) and seven transcription factors (STATs). A conformational change in the leptin receptor takes place following binding of leptin. This initiates tyrosine phosphorylation and activation of ObRb by JAK2, resulting in the phosphorylation of the three tyrosine residues (Tyr985, Tyr1077 and Tyr1138) that recruit and activate downstream signaling proteins (74). The phosphorylated leptin receptor then associates or recruits STAT3. This association with

STAT3 is upregulated by pharmacological doses of leptin as confirmed by studies observing the elevated levels of STAT3 (7, 23). Recruitment of STAT3 causes the leptin receptor to dimerize and translocate to the nucleus where it binds DNA to initiate gene transcription (12). STAT3 signaling is thought to participate in energy homeostasis through the melanocortin pathway (18, 74). Activation of STAT3 by leptin induces the transcription of the anorexigenic neuropeptide, proopiomelanocortin (POMC), in the ARC of the hypothalamus as well as suppresses agouti-related peptide (AgRP) (11, 74). Leptin also induces suppressor of cytokine signaling 3 (SOCS3), a negative-feedback mechanism of leptin signaling. It is suggested that SOCS3 plays a role in regulating leptin sensitivity and is a possible underlying mechanism in the development of leptin resistance in obesity (75).

Maintenance of energy balance is preserved by feedback inhibition of several mechanisms. There are two subtypes of leptin-sensitive neurons within the ARC, orexigenic and anorexigenic. Orexigenic neurons synthesize neuropeptide-Y (NPY) and agouti-related protein (AgRP). Binding of leptin to the NPY/AgRP-containing neurons causes a decrease in the release of NPY and AgRP. The second type of neuron is anorexigenic, which produces proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) (76).

POMC-expressing neurons in the hypothalamic melanocortin system of the ARC are depolarized by leptin leading to an increase in the release of the cleavage product α -melanocyte stimulating hormone (α -MSH). The α -MSH acts on downstream target neurons to reduce food intake, increase energy expenditure, and regulate glucose metabolism (73). This is confirmed by the findings that blood glucose is normalized in

POMC x Cre recombinase mice compared to wild type (WT) mice levels independent of weight change (18). In contrast, activation of arcuate nucleus (ARC) neurons expressing AGRP and neuropeptide Y (NPY) induce feeding, reduce energy expenditure, and reduces physical activity. AGRP/NPY neurons directly inhibit POMC neurons through synaptic release of inhibitory γ -aminobutyric acid (GABA) (73). An increase in GABAergic output was found in STZ-treated rats contributing to the overproduction of glucagon and epinephrine (78). SOCS proteins also inhibit signaling by binding to phosphorylated JAK proteins or interacting directly with tyrosine-phosphorylated receptors. Overall, leptin inhibits food intake by both stimulating anorexigenic neurons and inhibiting the orexigenic neurons.

PI3K

The JAK2/STAT3 cascade interacts with the phosphatidylinositol 3-kinase (PI3K) / phosphodiesterase 3B (PDE3B)/cAMP pathway to play a critical role in leptin signaling within the hypothalamus (74). The PI3K pathway, insulin's main intracellular signaling cascade, may also be stimulated by leptin. This pathway contributes to leptin's appetite-suppressing effects and resulting weight loss. Leptin stimulation of the PI3K pathway occurs primarily in the hypothalamus of the CNS, as well peripheral tissues such as adipose and liver. PI3K stimulates POMC neurons by activating ATP-sensitive potassium channels and voltage-gated calcium channels. Inducement of the PI3K pathway involves binding to IRS-1 and IRS-2, activation of PI3K and protein kinase B (AKT), and activation of phosphodiesterase 3B (PDE3B) leading to an eventual decrease in cAMP levels (79). As part of a negative feedback inhibition the insulin-PI3K

pathway hyperpolarizes POMC neurons, making them less sensitive to leptin. This is a proposed mechanism for corollary leptin and insulin resistance in obesity (11).

MAPK

The mitogen-activated protein kinase (MAPK) cascade is stimulated by short, ObRa, and long ObRb leptin isoforms. Extracellular signal-related kinases 1 and 2 (ERK 1/2) are the major MAPKs involved in leptin's central effects and play a key role in regulation of food intake, energy expenditure, and body weight. ERK 1 and 2 are stimulated by leptin via downstream signaling of SH2-containing protein tyrosine phosphatase 2 (SHP2) and a tyrosine of the ObRb after JAK2 activation. Following leptin administration, a response of ERK 1 and 2 is seen in POMC neurons. On the other hand, inhibition of the ERKs in POMC neurons results in an opposing effect characterized by an increase in food intake (11).

AMPK

Leptin signaling stimulates 5' adenosine monophosphate-activated protein kinase (AMPK) pathway in peripheral tissues (11, 80). This pathway contributes to leptin's involvement in glucose metabolism and fatty acid oxidation. In skeletal muscle fatty acid oxidation, and resulting reduced lipid content of cells, is stimulated by blocking the effect of the enzyme acetyl-CoA carboxylase (ACC), lowering malonyl CoA, and disinhibiting CPT1 (carnitine palmitoyl transferase 1) (74, 80). However, in the hypothalamus, leptin has an inhibitory effect on AMPK signaling. Stimulation of hypothalamic ACC and a significant reduction in food intake and weight gain are the result of this inhibited AMPK signaling (11).

2.4.2 Leptin and Energy Homeostasis

Leptin circulates at levels in proportion to body fat stores and enters the CNS, via the hypothalamus, in proportion to the plasma level of leptin (10). Therefore plasma leptin levels closely correlate with both body mass index (BMI) and total amount of body fat (74). The presence of extreme obesity in animals and humans with mutations of the leptin gene or the leptin receptor, ObR, demonstrates that normal leptin production and action are critical for maintaining energy balance (52).

Rodent ob/ob and db/db models are both characterized by obesity, diabetes, or insulin resistance, and have a specific gene defect in the leptin system. Through experiments involving parabioses of ob/ob, db/db, and WT mice the discovery was made that ob/ob mice are leptin deficient, whereas db/db mice have a mutated inactive form of the leptin receptor. Therefore, administration of exogenous leptin reduces body weight and reverses metabolic and endocrine disruptions in ob/ob mice (11). These rodent models have been used extensively to examine leptin's effect on obesity and leptin signaling.

Replacement of leptin in obese ob/ob mice corrected the metabolic defects and diminished obesity (12) and administration to young, lean rodents resulted in reduced food intake and weight loss (73). Hyperleptinemia induced by adipose proportionate rises in leptin levels fails to halt the progression of obesity (12). The high plasma leptin concentrations in most obese subjects have been interpreted to suggest that obesity is most often associated with resistance to the actions of leptin (52). Despite high plasma leptin concentrations, obese patients and rodents were found to have significantly lower

concentrations of the hormone within the hypothalamus as compared to non-obese subjects (15), suggesting that leptin signaling may work through a central mechanism. However, exogenous leptin administration to STZ-induced diabetic rats and mice has shown reductions in food intake (14, 18, 21, 80), resulting in weight maintenance (16, 20, 82) or a decreased body weight (21, 83).

Leptin levels are pulsatile and follow circadian rhythm with the highest levels being from midnight to early morning and lowest from early to mid-afternoon. Plasma levels are also affected by fed and fasted states exhibiting an acute decrease during fasting or energy restriction and an increase with re-feeding and overfeeding (52). Despite similar pulsatile characteristics of leptin secretion, obese individuals have higher pulse amplitudes as compared to lean individuals.

2.4.3 Leptin, DM, and Insulin Resistance

As discussed prior, DM is characterized by an exacerbated level of insulin resistance or lack of insulin and an elevated level of hepatic glucose production (5). Due to its roles in glucose metabolism, leptin has recently been under heavy investigation in regards to its role in the metabolic disease of DM.

Insulin is the major regulator of leptin expression and secretion by adipose tissue (9, 52). Kieffer and Habener (2000) proposed the idea of an adipoinsular axis, a bi-directional feedback loop existing between insulin and leptin (84). Insulin favors anabolism and increased body mass whereas leptin acts centrally to suppress food intake and increase energy expenditure. It is speculated that leptin may influence insulin release due to the expression of ObRb by pancreatic β cells (80). The idea behind this

feedback loop is that leptin acts to suppress insulin production from pancreatic β cells, whereas insulin increases leptin expression. Leptin can directly regulate glucose metabolism centrally and on the endocrine pancreas through this mechanism of insulin suppression (79, 85).

The inhibition of insulin release by leptin is mediated by the hyperpolarization of pancreatic β cell membranes, resulting in decreased electrical activity and a decline in Ca^{2+} signaling. However, the combination of glucose and glucagon-like peptide 1 (GLP-1) reverses the leptin-induced decrease of Ca^{2+} in pancreatic islets and overcame leptin suppression of insulin release (85). Nevertheless, the levels of GLP-1 needed to inhibit leptin suppression exceed physiologic ranges. The inhibitory effect of leptin on insulin secretion is suggested by Zhao et al. (1998) to represent a homeostatic control by insulin-sensitive adipose tissue (86). This effect is primarily regulated at the level of cAMP through activation of PDE3B, which is involved in the PI3K leptin signaling pathway.

Leptin and Insulin Sensitivity

Previous studies support leptin's role in increasing insulin sensitivity. Deficiency of leptin and decreased signaling within the brain has been associated with the progressive insulin resistance and hyperglycemia seen in uncontrolled DM (10, 12, 16, 22).

An increase of peripheral insulin sensitivity was observed upon administration of leptin to STZ-induced diabetic rats (22) as indicated by steady state plasma glucose (SSPG). This increased peripheral insulin sensitivity was attributed to the presence of

leptin in the brain. This finding was supported by German et al. (2009) through induction of adenoviral leptin receptors in the arcuate (ARC) nucleus in the hypothalamus of Koletsky fa^k/fa^k rats (87). It was proposed that the restoration of leptin receptor signaling contributed to this improvement in sensitivity by increasing the insulin-mediated suppression of hepatic glucose production (87).

2.4.4 Insulin-independent mechanisms of leptin

Differing from previous notions depicting the effect of leptin to increase hepatic insulin sensitivity (22, 87), German et al. (2011) observed a central ability of the brain to normalize diabetic hyperglycemia independent of insulin. German and colleagues centrally administered leptin to male Wistar rats with uncontrolled DM (uDM), resulting in dramatically reduced rates of glucose appearance to non-diabetic control values, as well as decreased levels of hepatic glucose production. The suppression of hepatic glucose production, despite trace plasma levels of insulin, contributed to normalization of blood glucose. This was made evident by a full amelioration of the hyperglycemia in STZ-induced diabetic rats by pharmacological doses of leptin (13) or adenoviral gene therapy (17) to induce a state of hyperleptinemia, despite extremely low plasma insulin levels. Numerous other studies also support leptin's insulin-independent euglycemic effect (14-16).

Administration of leptin within the hypothalamus has been discovered to normalize blood glucose concentrations in leptin-deficient *ob/ob* mice (13, 16) STZ or alloxan-induced diabetic rodents (14, 18). Blood glucose normalization through injections of exogenous leptin peripherally is attributed to augmented glucose disposal

imposed by central receptors after its entry across the blood brain barrier to the hypothalamus (15). Therefore, despite insulin suppression, leptin exhibits a significant effect on normalization of blood glucose.

Systemic administration of leptin at a dose that maintains normal physiological plasma leptin levels prevented the development of severe, progressive insulin resistance in rats with uDM, an effect that could not be explained by leptin-induced changes of food intake or body weight (19). Leptin is known for its role in reducing food intake and decreasing body weight (73), however, pair-feeding rodents did not normalize blood glucose concentrations suggesting that the effect of leptin was independent of reduced food intakes and decreased body weight (7, 14, 18-23).

2.4.5 Leptin and Hepatic Glucose Production

Hepatic glucose production is a major determinant of plasma glucose concentration contributing to hyperglycemia in diabetic subjects (5). A combination of impaired insulin-induced inhibition of hepatic glucose production and stimulation of glucose uptake results in the elevated blood glucose levels seen in diabetes. Although the exact mechanism of leptin-mediated euglycemia remains unknown it is believed that inhibition of hepatic glucose production in the liver is involved (10).

As previously discussed with regard to pancreatic β cells, leptin also exerts a similar effect on pancreatic α cells by hyperpolarizing the cell membranes resulting in decreased electrical activity and decreased Ca^{2+} signaling, decreasing glucagon secretion (88). Fluxes in cellular cAMP levels are predicted to arise because of feedback inhibition of adenylyl cyclase by Ca^{2+} (89). Hyper-secretion of glucagon from α

cells induces an elevation of cAMP levels stimulating hepatic glucose production, as well as glycogenolysis. Leptin acts to reduce both Ca^{2+} levels as well as glucagon-stimulated cAMP accumulation, through a PI3K-dependent activation of PDE3B (90). Therefore, despite exhibited hyperglucagonemia, leptin has been suggested to act as an inhibitor of plasma glucagon concentrations (17, 23, 82) and glucagon responsiveness in order to control blood glucose levels. Physiological leptin replacement, despite normalized plasma glucagon levels and reduction of gluconeogenic genes, only slightly improved hyperglycemia (19). This suggests that factors beyond leptin deficiency or depressed glucagon concentrations are needed to explain the hyperglycemia of uncontrolled DM (10). However, there is reason to believe that plasma glucagon is not necessarily inhibited, but rather there is a lack in responsiveness to the hormone. Numerous studies have shown normalization in glucagon levels as opposed to an actual significant decrease. This provides many opportunities for exploration into leptin's effects on glucagon responsiveness.

2.4.6 Glucagon Responsiveness and Somatostatin

The regulatory peptide, somatostatin, is produced by neuroendocrine neurons, inflammatory cells, and immune cells. Somatostatin acts as an endogenous inhibitory regulator of the secretory and proliferative responses of target cells that are widely distributed in the brain and periphery (91). There are five known membrane receptors (SSTR1-5) for somatostatin, and the SSTR2 and SSTR5 receptors regulate pancreatic endocrine function. Inhibition of glucagon release in pancreatic islets is primarily mediated via SSTR2, whereas insulin secretion is regulated primarily via SSTR5 (92). Somatostatin is regularly used in research for these inhibitory properties.

Glucagon, as explained prior, contributes to raising blood glucose concentrations in times of fasting. In diabetes, elevated glucagon concentrations are suggested to be one of the main contributing factors to increased hepatic glucose production and the resulting hyperglycemia. It is proposed that a decreased response to glucagon is the result of chronic central leptin treatment. Infusion of somatostatin to inhibit endogenous glucagon concentrations allows for precise measuring of glucagon responsiveness. This novel approach of examining glucagon responsiveness will aid in determining the overall mechanism by which central leptin administration normalizes blood glucose concentrations in type 1 diabetic rats.

Overall, leptin aids in blood glucose and energy homeostasis by exhibiting its effects centrally, as well as peripherally. Inhibition of the elevated rates of gluconeogenesis and hepatic glucose production is a primary mechanism by which the adipocyte hormone acts to regulate glucose metabolism. Gluconeogenesis suppression is manifested by these effects on glucagon, a reduction of hepatic cAMP response element-binding protein (CREB) (17, 23, 93), and decreased gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase (PEPCK) (14, 17, 19, 23, 93) and glucose-6-phosphatase (G6pase) (14, 17, 19, 20).

2.5 Research Hypothesis

Previously, chronic ICV leptin treatment has been found to normalize blood glucose concentrations in STZ-induced diabetic rats (14, 18, 81). Leptin-treated rats experience drastic decreases in blood glucose during a fast as compared to vehicle-treated animals. Therefore, we hypothesize that chronic ICV leptin administration

reduces the responsiveness to circulating plasma glucagon in STZ-induced diabetic rats, contributing to the suppression of gluconeogenesis, resulting in normalization of blood glucose concentrations.

Chapter III

Does chronic leptin treatment decrease glucagon responsiveness in STZ-induced type 1 diabetic rats?

Abstract: Central administration of leptin normalizes blood glucose (BG) concentrations in streptozotocin (STZ)-induced diabetic rats. We hypothesize that this is due to a leptin-mediated decrease in glucagon responsiveness.

Male Wistar rats (n=12) were implanted with an intracerebroventricular (ICV) cannula. Eight rats were induced with diabetes by an intraperitoneal (IP) injection of STZ (50 mg/kg), while four rats were given control injections. Half of the diabetic rats received daily injections of leptin (DL) (5 µg), while the other diabetic group (DV) and non-diabetic (NDV) group received daily injections of vehicle. BG concentrations were determined daily.

After BG concentrations of DL rats were normalized to NDV values, rats were fasted (DL rats for 6 hours and others for 21 hours). Additional fasting time was given so that the rats would be timed controlled to when they would be infused with glucagon. An IP injection of pyruvate (2 g/kg) was given and BG concentrations were determined periodically over the next 2 hours. Days later, rats were fasted (as described above) and the jugular vein and carotid artery were cannulated. Rats were intravenously (IV)

infused with somatostatin followed by a one hour IV infusion of somatostatin-glucagon. An IP injection of pyruvate was administered and BG concentrations were determined following prior pyruvate challenge measures, as described above. Both DV and NDV rats had an increased blood glucose response to the pyruvate challenge in the presence of high levels of glucagon. In contrast, the response to pyruvate was not increased by glucagon in DL rats.

This suggests that central leptin treatment decreases the hepatic responsiveness to pyruvate in the presence of high levels of glucagon, and therefore supports the contention that BG normalization in leptin-treated diabetic rats is due to a leptin-mediated decrease in glucagon responsiveness.

3.1 Introduction

In 2011, the American Diabetes Association reported that 25.8 million children and adults, or 8.3% of the United States population, had been diagnosed with diabetes (1). According to the World Health Organization in 2013, global diabetic prevalence was estimated at 347 million (2) and is forecasted to increase to 552 million, affecting 9.9% of adults, by the year 2030 (3).

Type 2 diabetes, accounting for 90-95% of total cases, is the most common form of diabetes. This increasingly prevalent disease is associated with insulin resistance and obesity (33). Obesity is considered the highest risk factor for development of insulin resistance (32). The hormone insulin is produced and secreted by the beta cells of the pancreas. Insulin acts to regulate glucose metabolism and help control blood glucose concentration by promoting the uptake of glucose into muscle and fat cells from the blood and inhibiting glucagon production by the pancreas. Uptake of glucose for either energy production or storage (in the form of glycogen) (94) aids in the lowering of blood glucose concentrations.

Insulin resistance is a physiological condition in which the cells of insulin-sensitive tissues fail to respond to the normal actions of insulin. These tissues cannot appropriately lower blood glucose concentrations for a given amount of the hormone. This decreased response to insulin results in an increase in glucose production and release by the liver, as well as a decrease in glucose uptake in muscle and fat tissues, producing high blood glucose concentrations (24).

Normal physiologic levels of insulin are insufficient to regulate blood glucose levels in patients with insulin resistance. Therefore, traditional methods of treatment for patients with diabetes have centered on increasing insulin secretion and increasing insulin sensitivity (95, 96). However, there is a possibility that blood glucose can be regulated independently of insulin. Leptin, an adipocyte-derived hormone, has effects on energy homeostasis through its regulation of food intake and body weight. Within the hypothalamus, leptin combines with leptin receptors to help maintain energy balance by inhibiting energy intake and increasing energy expenditure. In addition, leptin prevents the decrease in energy expenditure that is associated with a decrease in food intake (97, 98). Obesity is characterized by high circulating leptin levels, suggesting a resistance to leptin. This would contribute to elevated weight gain and feeding resulting in obesity (12, 99). However, several studies suggest leptin exhibits effects beyond just energy homeostasis. Recent evidence supports leptin's interaction with glucose utilization. Our lab and others have demonstrated that chronic intracerebroventricular leptin treatment can normalize blood glucose concentrations in streptozotocin-induced diabetic rats independent of changes in food intake and levels of serum insulin (22, 100, 101). This suggests that leptin acts independently of insulin to normalize blood glucose concentrations in diabetic rats.

The exact mechanism of this normalization of blood glucose in diabetes remains to be discovered. Our lab (22) has shown that, compared to vehicle-treated rats, leptin-treated rats cannot maintain normal blood glucose concentrations during a fast. The hormone glucagon plays a major role in glucose regulation by increasing blood glucose concentrations during a fast or in the case of diabetes, an overall elevated blood

glucose level. This suggests the possibility that leptin may mediate a decrease in blood glucose by decreasing the body's responsiveness to glucagon.

This study examined the response to a pyruvate challenge in diabetic, leptin-treated rats in the presence of high levels of plasma glucagon. Chronic leptin treatment blocked the ability of pyruvate to increase blood glucose in diabetic rats with high levels of blood glucagon.

3.2 Materials and Methods

Animals: Twelve male Wistar rats (~250g; Harlan, Indianapolis, IN) were housed in individual shoe box cages and kept in a temperature- (23°C ±3) and humidity-controlled room with a 12:12 hour light: dark cycle. Animals had free access to tap water and chow. The procedures involving animals were approved by Auburn University's Institutional Animal Care and Use Committee (IACUC).

Experimental Design: Rats were anesthetized and implanted with an intracerebroventricular (ICV) cannula into the lateral ventricle of the brain. The rats were then divided into 3 groups with 4 rats per group: diabetic-leptin treated (DL), diabetic-vehicle treated (DV), and non-diabetic-vehicle treated (NDV). The diabetic rats were given an intraperitoneal injection of streptozotocin (STZ) (50 mg/kg), while the other rats were given control injections of vehicle.

Hyperglycemia (≥ 300 mg/dL) was confirmed in the 8 diabetic rats. Half of the diabetic rats received daily ICV injections of leptin (5 ug/day) and the other half received vehicle (5 ug/day). The non-diabetic controls received daily ICV vehicle injections. Daily

body weights were measured and blood glucose concentrations were determined from the tail vein via a handheld glucometer.

After normalization of the blood glucose concentration in the leptin-treated diabetic rats occurred, the rats were fasted. Vehicle-treated rats were fasted for twenty-one hours, while the leptin-treated diabetic rats were fasted for six hours. The leptin treated rats' shorter fast prevented hypoglycemia. In a previous study from our laboratory it was found that blood glucose concentration of leptin-treated rats decreased by 50% after six hours of fasting (22). A pyruvate challenge was conducted. Blood glucose was determined via the tail vein prior to fasting, and then at specific time points to mimic the sampling regimen of the later glucagon responsiveness experiment. Blood glucose was determined every hour for 3 hours (0, 60, 120, 180), and at an additional 30 minutes. Pyruvate (2.0g/kg) was then injected and blood glucose was determined every 15 minutes for the next 2 hours (0, 15, 30, 45, 60, 90, and 120 min).

After a few days of recovery and confirmation of normalized blood glucose concentration in the leptin-treated diabetic, the rats were again fasted for the determination of glucagon responsiveness. All groups of rats underwent surgical implantation of cannulas into the jugular vein and the carotid artery. Immediately following implantation, blood glucose concentrations were determined from the tail vein. To determine glucagon responsiveness both groups of diabetic rats, as well as the control group underwent a series of infusions via the jugular vein by a syringe pump. First, somatostatin was continuously infused for 180 minutes to inhibit endogenous glucagon. This was followed by 180 minutes of continuous infusion of a solution of somatostatin and exogenous glucagon at a previously determined rate (0.33 μ g/min).

Lastly, the rats were subjected to a pyruvate challenge (after 60 minutes of the somatostatin-glucagon infusion) by an IP injection of 2.0 g/kg pyruvate to examine serum glucose concentration from hepatic glucose production. Blood glucose concentrations were determined from samples prior to fasting, prior to the infusion of somatostatin, every hour for three hours of somatostatin infusion, every fifteen minutes for one hour of somatostatin-glucagon infusion, every 15 minutes (0, 15, 30, 60, 90, and 120 min) for the first hour followed by every 30 minutes of the second hour of the pyruvate challenge. Three blood samples were taken from the carotid artery prior to each infusion. The blood (~0.5ml) was collected into EDTA treated tubes with a protease inhibitor (10µl) added to determine plasma glucagon concentrations. The rats were then euthanized by exsanguination.

Surgical procedure (ICV cannulation): The rats were given an IP injection of ketamine-xylazine (100mg/kg ketamine and 1mg/kg xylazine) to anesthetize the animals before surgery. The rats were implanted with an intracerebroventricular (ICV) cannula into the lateral ventricle of the brain using the following coordinates: -0.8 mm posterior and 1.4 mm lateral to bregma, 3.5 mm deep from the surface of skull. Anesthetized rats were placed in a stereotaxic apparatus and a midline incision was made in the skin on top of the skull. A small hole was drilled into the skull at the appropriate coordinates. Four stainless steel screws were placed around the hole in the skull and a 22-gauge stainless steel guide cannula was placed at the appropriate depth. Cranoplastic cement was then used to secure the cannula to the screws and a dummy cannula was placed into the guide cannula. The rats were then individually placed in warm shoebox cages for recovery from anesthesia.

To confirm proper placement of the ICV cannula, an angiotensin II drinking test was used after several days of recovery from surgery. Each rat was given an ICV injection of angiotensin II (40ng/4 μ l) and placed in an individual hanging cage with a graduated tube containing water. Rats that drank 3 ml of water or more within 15 min after injection were considered to have a positive response and correct cannula placement and were therefore included in the experiment. After the drinking test, 12 of 12 rats were used in this study.

Induction of Diabetes: A solution of streptozotocin (STZ) was prepared just prior to injection in the rats by adding 0.05M citrate buffer (pH=4.5) to a known amount of STZ (Sigma, St. Louis, MO). Diabetes was induced by an IP injection of STZ (50 mg/kg), while control rats were given an injection of citrate buffer only. After twenty-four hours, blood samples were collected from the tail vein and blood glucose concentration determined by a handheld glucometer. If the blood glucose concentration of the STZ-treated rats were 300 mg/dL or greater, the rats were considered to be diabetic. Diabetic rats whose blood glucose concentrations did not reach 300 mg/dL received a second injection of STZ.

Surgical Procedure (Jugular Vein and Carotid Artery Cannulation): The rats were given an IP injection of ketamine-xylazine (100mg/kg ketamine and 1mg/kg xylazine) to anesthetize the animals before surgery followed by continuous isoflurane gas during the procedures. The rats were placed on their backs under a dissecting microscope. A 1 inch ventral cervical skin incision was made along the midline. Tissue was separated by means of blunt dissection to visualize both the carotid artery and the jugular vein. Both

jugular and carotid are secured with nylon suture followed by a small incision and implantation of a polyethylene cannula.

Plasma glucagon concentration determination: Plasma glucagon concentrations were determined by a commercial radioimmunoassay (EMD Millipore Corporation, Billerica, MA).

Data & Statistical analysis: Statistical analyses of the results were performed by using statistical computer program JMP 10.0. Blood glucose data were expressed on an absolute basis and as the response due to glucagon based on the individual rat's baseline value at time zero. Changes in blood glucose concentrations were determined from the difference between the blood glucose concentrations in response to glucagon versus the response to vehicle. Body weights, daily blood glucose concentrations, and the blood glucose response to glucagon were analyzed by ANOVA with repeated measures to determine statistical differences. If necessary, contrast statements were used to determine differences between specific groups. Fed and fasted blood glucose data were determined by ANOVA without repeated measure. Again, if necessary, contrast statements were used to determine differences between specific groups. The Simpson rule was applied for determination of area under the curve (AUC). Two points (75 and 105 minutes) were estimated based on the means of other points in intervals of 15 minutes each and to satisfy the odd number of points required. P values of 0.05 or less were considered to be statistically different.

3.3 Results

Body Weight: Body weight of NDV increased gradually during the experiment, yielding ~25g change in body weight from baseline, the body weight measured on the day before STZ or vehicle treatment began. NDV rats body weight over time was significantly elevated as compared to DV and DL groups (Figure 1, $p < 0.0001$). Over the first 4 days after leptin treatment, body weight of DL rats was greater than that of the DV rats. After 4 days, there was no difference in the change in body weight between DV and DL rats.

Blood glucose concentrations: Initial daily blood glucose concentrations (Figure 2) of the STZ-induced diabetic groups (DL & DV) were greatly increased as compared to NDV rats ($p < 0.0001$) (~ 400 mg/dL vs ~ 120 mg/dL). After five days of daily ICV leptin or vehicle injections, blood glucose concentrations decreased in DL rats, producing statistically lower ($p = 0.002$) concentrations than DV rats. Day five also marked the normalization (non-significant difference) of blood glucose concentrations in DL rats to levels of NDV rats, ~95mg/dL.

Similar to daily blood glucose values, blood sampled in the fed state (prior to fasting) showed that blood glucose concentrations of DL rats were normalized to that of NDV rats (~100 mg/dL) and significantly less than DV rats (~475 mg/dL, $p < 0.0001$) (Figure 3). After fasting, blood glucose concentrations decreased in both NDV rats ($p = 0.0388$) and DL rats ($p = 0.0335$, ~85mg/dL). Contrary to expected results, the blood glucose concentration of the DV rats also decreased significantly ($p < 0.0001$) to values similar to DL and NDV rats. Overall, fasting decreased blood glucose in all groups ($p < 0.0001$)

Pyruvate challenge: After fasting, the rats' blood glucose was tested periodically before injection of pyruvate and after to replicate the blood glucose sampling during the somatostatin glucagon administration experiment. Blood glucose concentrations in response to an injection of pyruvate (2.0g/kg) are shown in Figure 4. No significant difference was found between NDV and DL rats. Likewise, there was no significant difference between NDV and DV rats. Although it was not statistically significant, a difference ($p < 0.148$) in blood glucose concentrations from time points of 60 through 120 minutes was found between DL and DV groups.

Pyruvate challenge in the presence of glucagon: After 3 hours of ICV somatostatin injection, a somatostatin-glucagon solution was administered. After the first hour of ICV somatostatin-glucagon infusion, an IP injection of pyruvate (2.0g/kg) was given and the blood glucose concentrations were measured (Figure 8). The response to glucagon after IP injection of pyruvate in each treatment group was compared to control blood glucose measurements from the pyruvate challenge (in absence of glucagon) (Figure 4). As expected, the NDV rats' response to glucagon was significantly higher ($p = 0.0075$) than measurements during the control trial (Figure 5). Similarly, the DV rats had a significantly increased ($p < 0.0001$) response to glucagon as compared to control values (Figure 6). However, in the DL rats the difference between controlled trials and glucagon administration was non-significant (Figure 7).

Comparison of responses in all groups to pyruvate in the presence of glucagon is listed in Figure 8. In the latter half of the injection period (60-120 minutes), blood glucose measurements of the NDV group were significantly decreased as compared to the DV group ($p = 0.0021$). However, both the NDV ($p = 0.0491$) and DV group ($p = 0.0013$) were

significantly elevated as compared to the DL group. The DL group remained around baseline values (~100mg/dL) without any significant change throughout the entirety of the response time.

AUC for Pyruvate Challenge: The AUC simplifies the expression of the glucose response to the pyruvate challenge into one value. It demonstrated the ability of the liver to produce glucose in a pyruvate challenge in either the presence or absence (control) of glucagon (Figure 9). In this summary of pyruvate responses across all groups, the data indicates an AUC for both NDV and DV rats under the presence of glucagon compared to their vehicle-treated controls. However, in DL rats, glucagon did not increase pyruvate-induced glucose production in DL rats.

Interestingly, across all groups of rats, there was a significant correlation ($p < 0.0001$) between the AUC for the response to pyruvate after glucagon and blood glucose concentrations during a fast. This suggests an underlying association between the blood glucose concentration after a fast and the responsiveness to glucagon (Figure 10).

Plasma Glucagon Concentration: Plasma glucagon concentrations were initially elevated in control blood samples of NDV and DL rats, followed by a significant decrease after ICV somatostatin infusion. Plasma glucagon concentrations were elevated in all groups after somatostatin-glucagon infusion (after glucagon). Leptin treatment of diabetic rats had no alteration on plasma glucagon concentrations as compared to DV rats after the infusion of glucagon.

3.4 Discussion

As previously found by our lab (22, 107), as well as others (14, 18, 108), chronic central leptin administration normalized diabetic rats' blood glucose concentration to non-diabetic values. This was also evident in the current study (Figure 2). Our laboratory has previously shown that leptin treatment, in both diabetic and non-diabetic rats, has an enhanced effect to reduce blood glucose concentrations after a 6-hour fast as compared to vehicle-treated rats (22). In that study, blood glucose concentrations of fasted leptin-treated rats were about 50% of that of the same rats in the fed state. However, blood glucose concentrations of vehicle-treated rats were not significantly changed from that of the fed state. In the present study, after the DL rats fasted for 6 hours, blood glucose concentrations also decreased by about ~50% (Figure 3). (Differences in fasting times between leptin- and vehicle-treated rats were used to prevent hypoglycemia from potentially developing in the leptin-treated rats). However, contrary to previous findings, after a 21 hour fast the blood glucose concentrations of DV rats were also severely decreased as compared to fed values. This evident difference between fed and fasted states may be attributed to the small number ($n=2$) of DV rats, or it may suggest a significant impact of fasting itself. Overall, the fast decreased all groups' blood glucose ($p<0.0001$). DL, as well as NDV rats exhibited a decrease in blood glucose concentrations, although not as great as the DV group.

To explore in greater detail the nature of a fast, the mechanisms for glucose production must be explored. Low blood glucose concentrations stimulate the release of

glucagon which in turn stimulates the process of gluconeogenesis via enhancement of hepatic production of new glucose from non-carbohydrate precursors (102, 103, 104, 105). Compensation for a lack of glucose in fasting conditions is partially attributed to gluconeogenesis (106). This process of glucose production acts to maintain glucose homeostasis, however, increases in gluconeogenesis play a role in the resulting hyperglycemia of diabetes. Nearly 90% of the increased hepatic glucose production in type 2 diabetes is attributed to increases in gluconeogenesis (109). Due to the essential role that glucagon plays in gluconeogenesis, we hypothesized that leptin suppresses gluconeogenesis to lower blood glucose concentrations by inhibiting glucagon responsiveness.

Plasma glucagon concentrations were initially highly elevated in control blood samples of NDV and DL rats (Figure 11). This may be attributed to a stress response resulting from the prior surgical implantation of the two, jugular vein and carotid artery, cannulas. However, plasma glucagon levels of the DV rats were distinguishably lower in comparison. Glucagon concentration is speculated to be higher in diabetic rats than non-diabetic rats. Hyper-secretion of glucagon from α cells induces an elevation of cAMP levels stimulating hepatic glucose production, as well as glycogenolysis (90). The DV rats' response could be due, at least in part by enhanced sympathetic activity. Long-term enhancement of sympathetic activity within the diabetic condition may have down regulated adrenergic receptors resulting in a decrease of cAMP signaling and therefore low levels of plasma glucagon. The stress induced from the prior surgery elicited a sympathetic response in NDV rats resulting in an increase in glucagon as shown by the increased control values. Zhao et al. (2000) found that leptin acts to reduce both Ca^{2+}

levels as well as glucagon-stimulated cAMP accumulation, through a PI3K-dependent activation of PDE3B (90). This suggests that leptin treatment may block suppression of sympathetic activity (as seen in DV rats) resulting in increases in control plasma glucagon similar to that of NDV rats.

Next, a 3-hour ICV infusion of somatostatin was used in our current study in order to directly measure the rats' response to the administration of exogenous glucagon. Somatostatin knocks down both endogenous glucagon and insulin concentrations (91), and this effect was confirmed in both NDV and DL groups by our radioimmunoassay of plasma glucagon (Figure 11). Plasma glucagon concentrations after one-hour of continuous ICV infusion of somatostatin-glucagon were found to be highly elevated in all groups regardless of either leptin- or vehicle-treatment. An IP injection of pyruvate (2.0g/kg), one-hour amid the ICV infusion of glucagon-somatostatin (60 μ g at a rate of 0.33 μ g/min), produced an increase in blood glucose concentrations of all rats. The values were significantly greater than the blood glucose concentrations when the respective rats were given an injection of pyruvate in the absence of glucagon, so this increase is due to the effects of glucagon (Figures 4 & 8). Leptin-treatment of diabetic rats had no alteration on plasma glucagon concentrations after the infusion of glucagon, but rather a decrease in production of glucose in response to pyruvate was observed (Figure 7). No significant difference was found between control trial pyruvate challenges and pyruvate challenges in the presence of glucagon in DL rats, in contrast to the elevated responses of both DV and NDV rats (Figure 9). This suggests that the mechanism by which chronic central leptin lowered blood glucose concentrations was

not by decreasing plasma glucagon concentrations, but rather the data are consistent with leptin decreasing the responsiveness to glucagon.

Previous studies concluded that leptin-induced normalization of blood glucose in type 1 diabetic non-obese (NOD) mice (17, 23) and a rat model of type 2 diabetes (UCD-T2DM) (20) were through a leptin-mediated decrease in serum glucagon concentrations. Direct comparisons between these studies and the current study cannot be made due to differences in dose amount and method of administration of leptin. In the NOD mouse model, leptin was administered via an adenovirus, which produced high concentrations of peripheral leptin. Plasma glucagon concentrations may be affected by peripheral administration, whereas chronic central leptin administration, such as in the current study, may have an effect on the response to glucagon. This is supported by results from studies by Fujikawa et. al (2008), using T1D mice models, and German et al. (2011), using STZ-induced Wistar rats, where leptin was administered intracerebroventricularly. Considering we presently observed a leptin-induced normalization of blood glucose despite high plasma glucagon it is interesting to note that Fujikawa et al. (2008) found decreased pancreatic glucagon concentrations, but failed to find a statistical difference in plasma glucagon concentrations. Although, two notable differences between both of these studies and the current study was that we gave daily ICV bolus injections of leptin, whereas the study by Fujikawa et al. (2008) and German et al. (2011) gave ICV leptin by an osmotic mini-pump (7, 14), and we utilized somatostatin to block endogenous glucagon production. A daily bolus injection may more closely mimic a circadian rhythm of leptin across time (52), whereas an osmotic mini-pump or a large peripheral dose could potentially saturate leptin receptors

throughout the day. The use of somatostatin allows for the specific measure of response to glucagon in a leptin-treated model, whereas others may have interpreted a leptin-mediated decrease in hyperglycemia to be resultant from decreased concentrations of glucagon. In another study, a physiological dose of leptin given peripherally was able to improve insulin sensitivity and normalize plasma glucagon, but had only a modest effect at normalizing blood glucose concentration in STZ-induced diabetic rats (German 2010). This suggests that correcting high plasma glucagon concentrations doesn't necessarily lead to a normalization of blood glucose concentrations in diabetic rats.

A limitation of our present study is that we neglected to observe underlying factors of glucagon responsiveness such as the expression of hepatic genes or transcription factors under fasting conditions. It has previously been found that in rats with uncontrolled diabetes the gluconeogenic enzymes G6Pase (14), and PEPCK (14, 17, 19, 23) as well as the transcription factor PGC1 α (17) are elevated, but are reduced after leptin treatment. Intracellular cAMP, produced via glucagon enhancement, is typically elevated in diabetes, but a leptin-induced decrease in cAMP responsive element binding protein 1 (Creb1) phosphorylation of diabetic rats was observed. This suggests a lessened rate of cAMP signaling in the livers of leptin-treated rats (17, 23). Another limitation to consider is the presence and effect of having intact adrenal glands. Adrenal glands release hormones in response to stress through the synthesis of catecholamines such as epinephrine and norepinephrine and corticosteroids such as cortisol. These hormones can have several effects on blood glucose concentrations. In times of stress or decreased blood glucose concentrations, epinephrine stimulates the

conversion of glycogen to glucose in the liver and cortisol promotes mobilization of fatty acids.

Chapter IV

Conclusion

Leptin-treated diabetic rats were associated with normalized blood glucose levels despite high plasma glucagon concentrations. The plasma glucagon concentrations in DL rats were not different from DV rats, suggesting that central leptin treatment decreases the hepatic responsiveness to pyruvate in the presence of high levels of glucagon. In conclusion, the present study supports the hypothesis that chronic central leptin administration normalizes blood glucose concentrations of diabetic rats due to a leptin-mediated decrease in glucagon responsiveness.

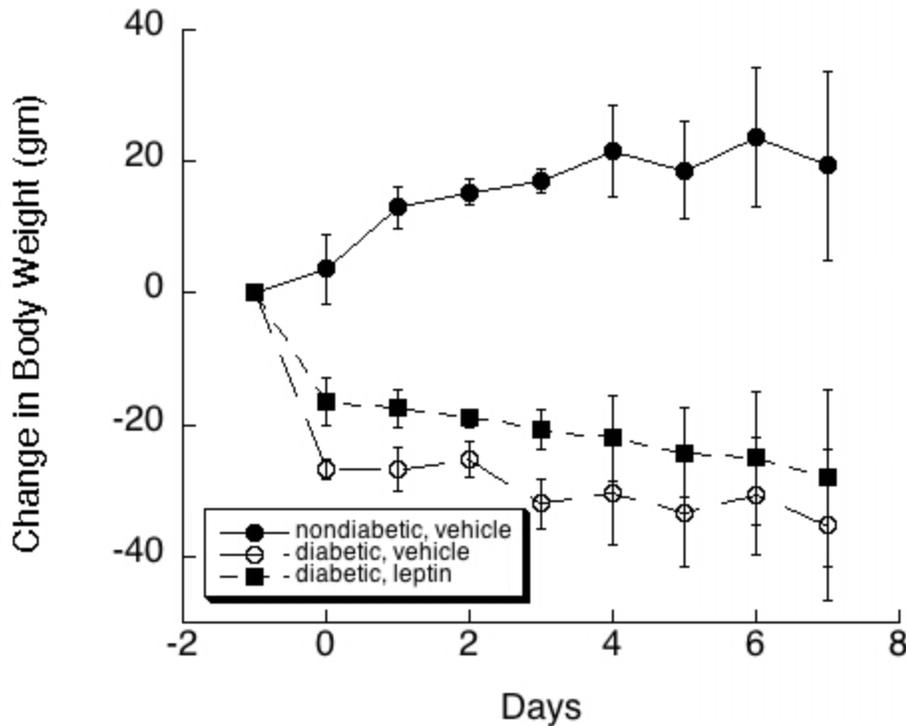


Figure 1: Changes in Body Weight of Non-Diabetic Vehicle-Treated, Diabetic Vehicle-Treated and Leptin-Treated Diabetic Rats

Body weights of non-diabetic vehicle-treated (NDV)(●), diabetic vehicle-treated (DV) (○), and diabetic leptin-treated (DL) (■) rats. Day -1 signifies STZ injection while day 0 refers to start of either leptin or vehicle treatment. No overall difference was found between the diabetic leptin-treated group and diabetic vehicle-treated group, whereas both leptin-treated and vehicle-treated diabetic rats had significantly lower body weight than non-diabetic rats ($p < 0.0001$).

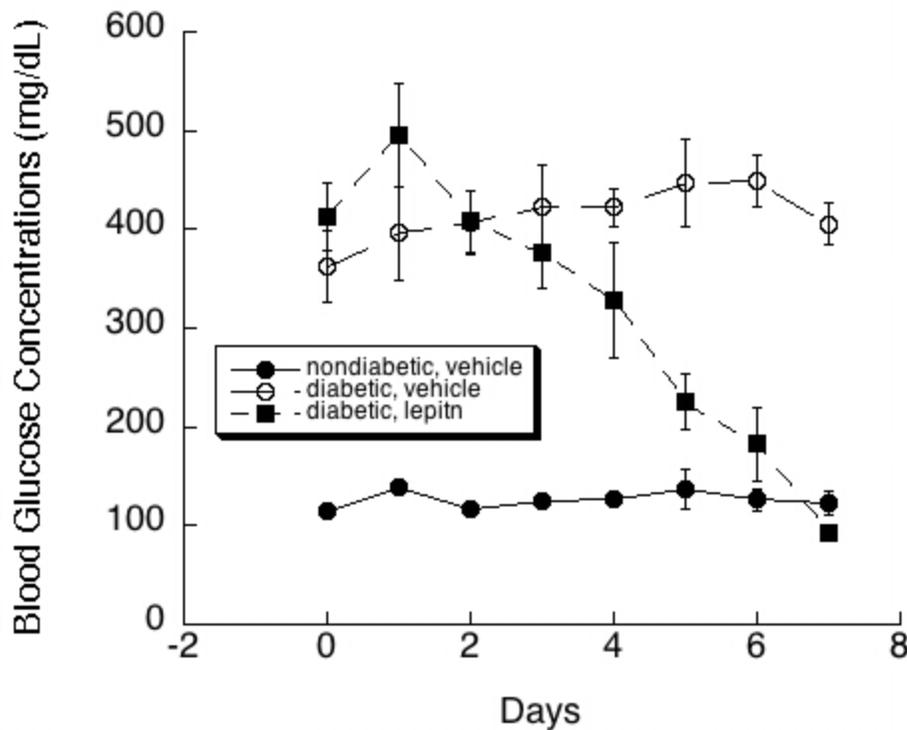


Figure 2: Daily Blood Glucose Concentrations of Non-Diabetic Vehicle-Treated, Diabetic Vehicle-Treated and Diabetic Leptin-Treated Rats

Daily blood glucose concentrations of NDV (●), DV (○), and DL (■) rats. At day 0, diabetic rats were greatly increased as compared to non-diabetic rats ($p < 0.0001$). Day 1 refers to start of either leptin or vehicle treatment. Five days after initiation of daily ICV leptin, blood glucose concentrations of DL rats was statistically lower ($p = 0.002$) than DV rats and is normalized (non-significant (NS)) to that of NDV rats.

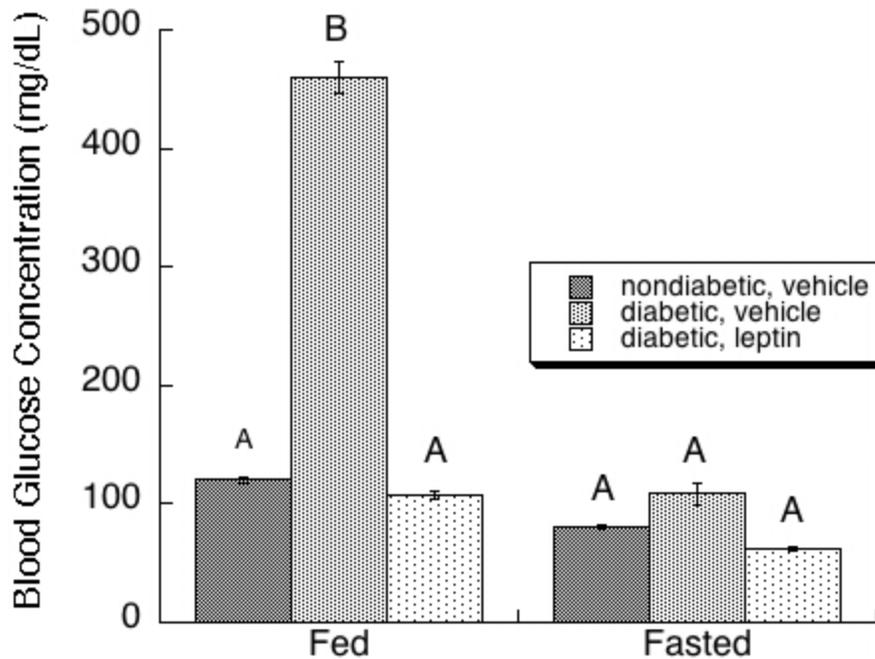


Figure 3: Blood Glucose Concentration Before and During a Fast

Blood glucose concentrations of NDV (●), DV (○), and DL (■) rats before (fed) and after a fast. Statistics were done on the logs in a factorial design 2-way analysis of variance looking at the groups' pre (fed) and post (fasted) values. Means are shown ± SEM. Each mean represents 2-4 observations. Within the fed and fasted states, means with different letters are statistically different. There was no significant difference between NDV and DL groups for fed or fasted blood glucose concentrations. Fed blood glucose of DV rats were significantly increased ($p < 0.0001$) as compared to leptin-treated and NDV rats. Fasting decreased blood glucose concentrations in NDV ($p = 0.0388$), DV ($p < 0.0001$), and DL ($p = 0.0335$) rats from fed levels. Fasting decreased blood glucose in all groups ($p < 0.0001$).

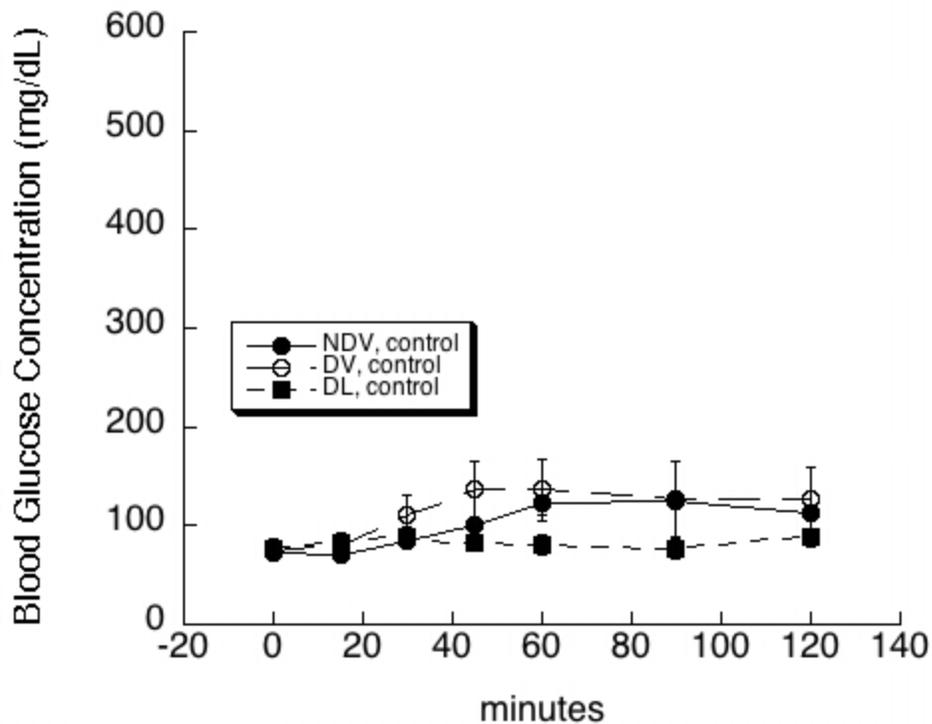


Figure 4: Blood Glucose Response to Pyruvate Challenge

Pyruvate challenge responses of NDV (●), DV (○), and DL (■) rats. Minute 0 refers to time of injection of 2.0g/kg pyruvate. No significant difference was found among groups. Means are shown ± SEM and represent 2-4 observations.

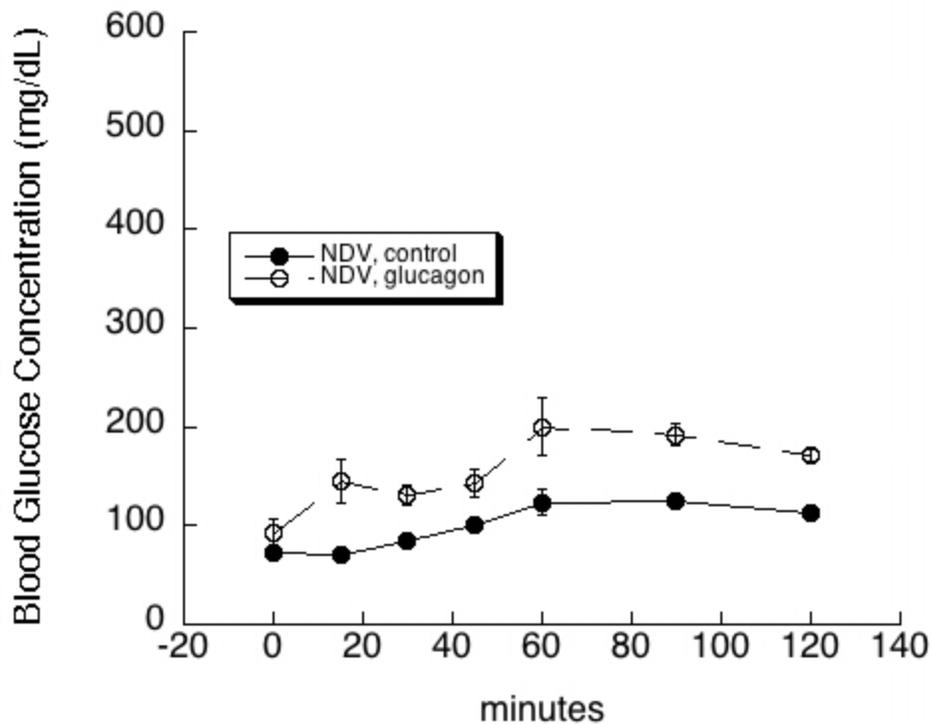


Figure 5: Effects of Glucagon in Non-Diabetic Vehicle-Treated Rats after an IP injection of Pyruvate

Blood glucose response of NDV rats to glucagon (o) compared to control values (●) from pyruvate challenge. Means are shown \pm SEM and represent 3 observations. NDV rats' overall response to a pyruvate challenge was significantly elevated ($p=0.0075$) with glucagon treatment relative to control values.

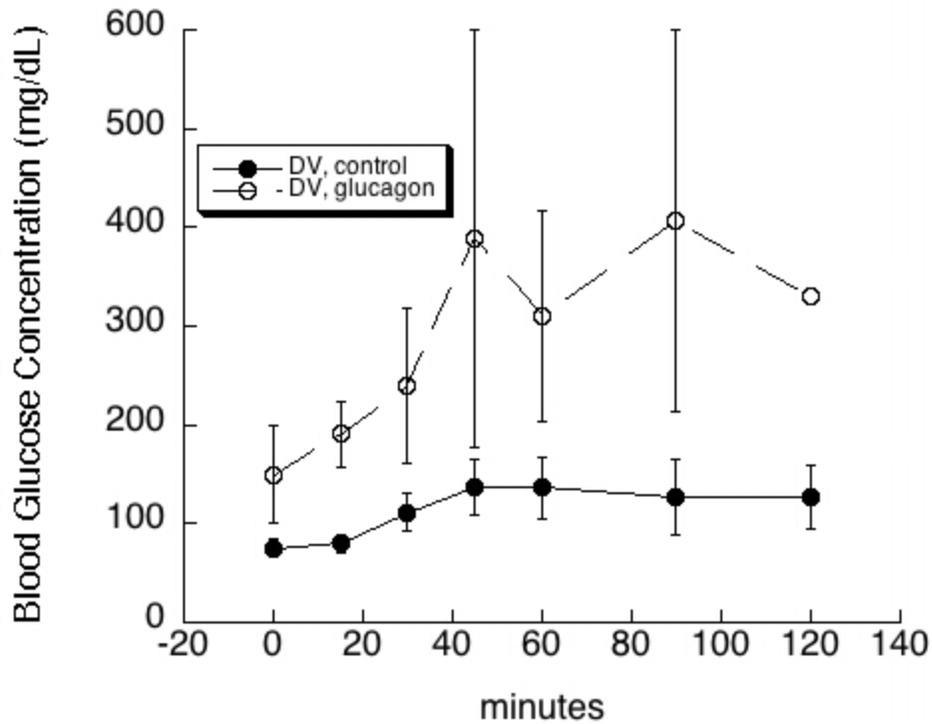


Figure 6: Effects of Glucagon in Diabetic Vehicle-Treated Rats after IP injection of Pyruvate

Blood glucose response of DV rats to glucagon (o) compared to control values (●) from the pyruvate challenge. Means are shown \pm SEM and represent 2-3 observations. DV rats' response to pyruvate challenge was significantly elevated ($p < 0.0001$) with glucagon treatment relative to control values.

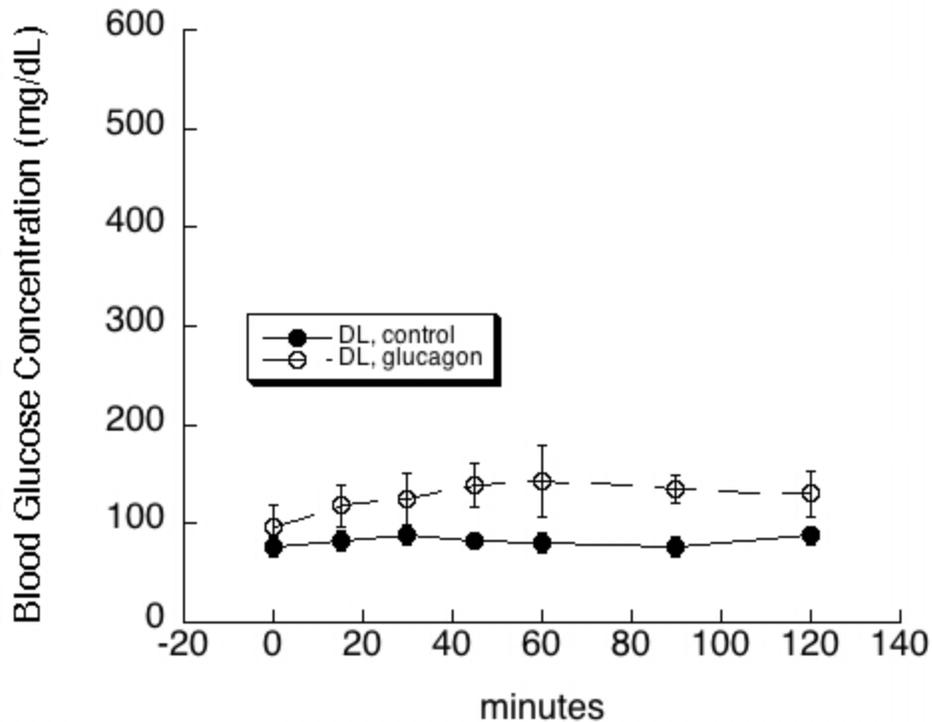


Figure 7: Effects of Glucagon in Diabetic Leptin-Treated Rats after IP injection of Pyruvate

Blood glucose response of DL rats to glucagon (o) compared to control values (●) from the pyruvate challenge. Means are shown \pm SEM and represent 4 observations. No overall significant difference was found between responses to pyruvate in the presence of glucagon or absence of glucagon (control) in DL rats.

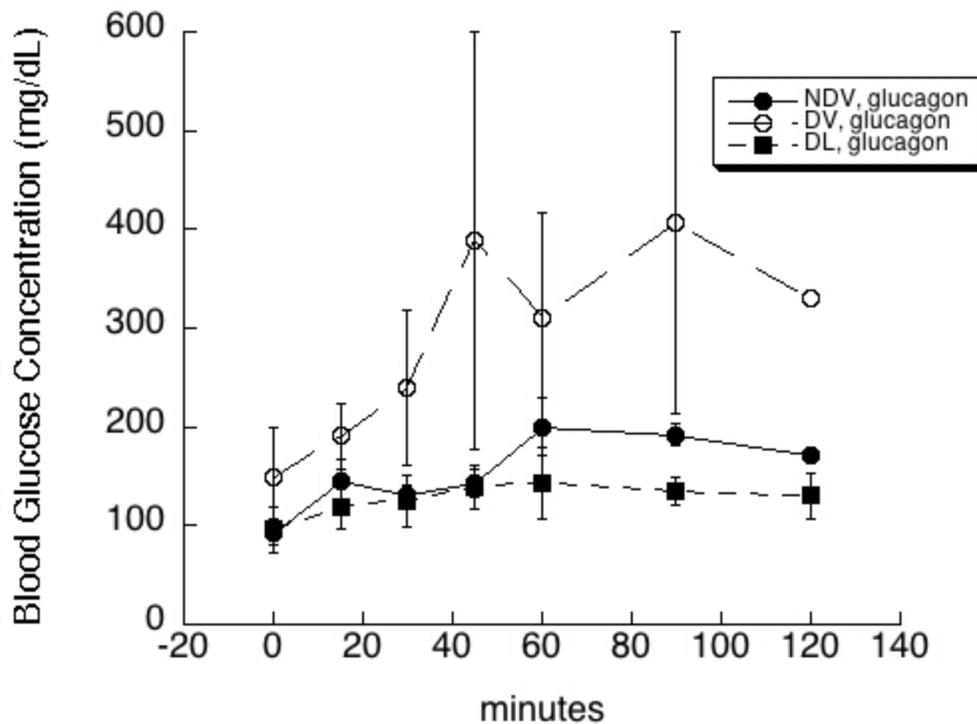


Figure 8: Comparison of Effects of Glucagon in Non-Diabetic Vehicle-Treated, Diabetic Vehicle-Treated, and Diabetic Leptin-Treated Rats

Blood glucose concentration responses to pyruvate after glucagon infusion of NDV (●), DV (○), and DL (■) rats. Means are shown ± SEM and represent 2-4 observations. DV values were significantly higher ($p=0.0013$) than the DL group in minutes 60-120. The NDV group was also significantly elevated ($p=0.0491$) in comparison to the DL group in minutes 60-120. NDV group values ($p=0.0021$) were significantly lower than DV.

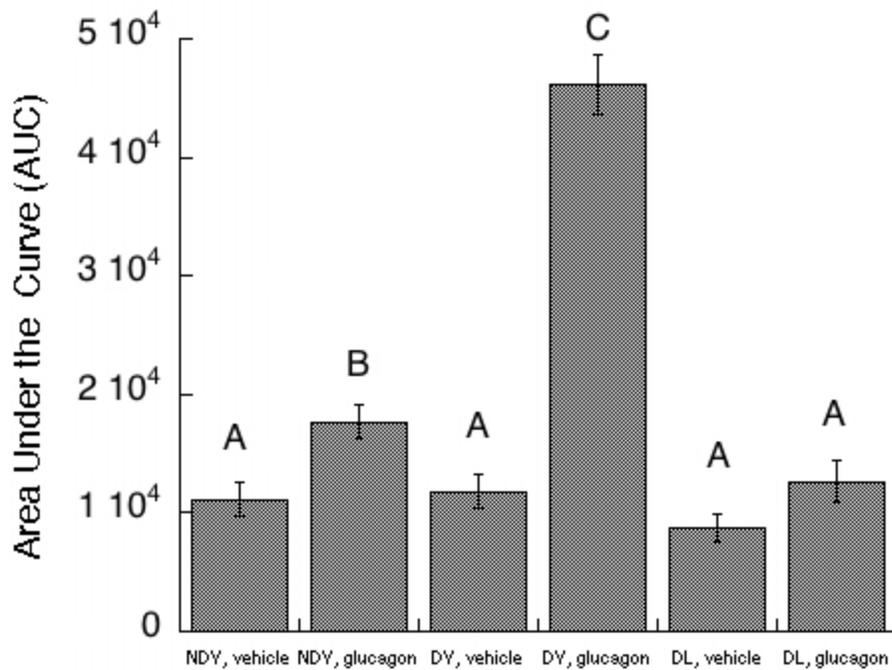


Figure 9: AUC for Pyruvate Challenge

Area under the curve (AUC) for a pyruvate challenge in absence (vehicle) or presence of glucagon in non-diabetic vehicle-treated (●), diabetic vehicle-treated (○), and diabetic leptin-treated (■) rats. Means are shown ± SEM. Each mean represents 2-4 observations. Means with different letters are statistically different. Glucagon increased the AUC in both NDV and DV rats, whereas DL rats response was non-significant.

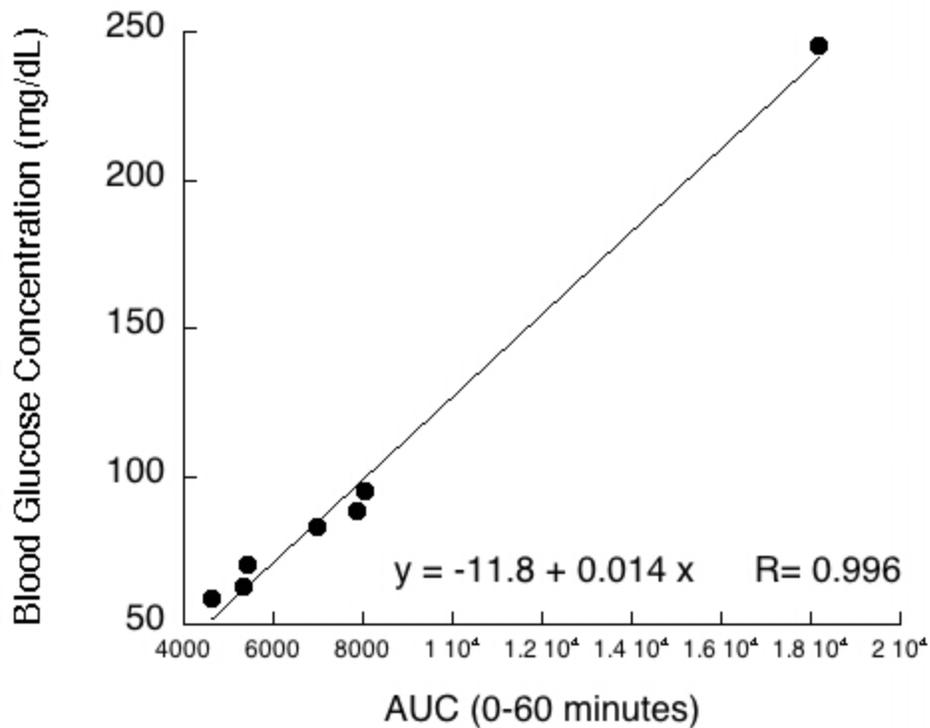


Figure 10: Blood Glucose Concentration during a Fast vs AUC of Pyruvate Challenge in the Presence of Glucagon

Blood Glucose Concentration during a fast vs the area under the curve of pyruvate challenge in the presence of glucagon. Blood glucose concentration and response to pyruvate after glucagon is positively correlated ($p < 0.0001$).

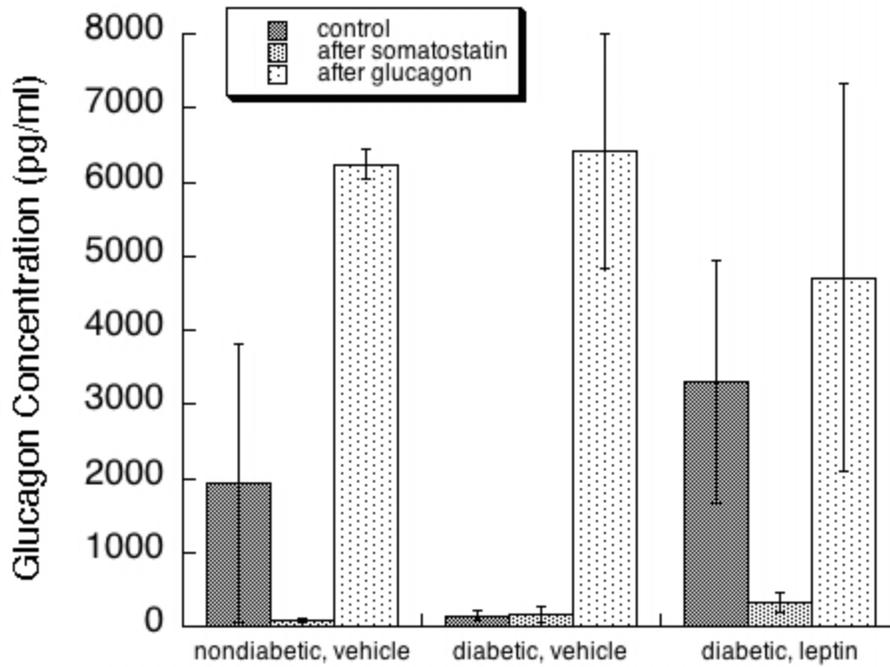


Figure 11: Plasma Glucagon Concentrations of Non-Diabetic Vehicle-Treated, Diabetic Vehicle-Treated, and Diabetic Leptin-Treated Rats

Plasma glucagon concentrations from (3) blood samples taken 1) post jugular vein and carotid artery cannulation (control), 2) post 3-hour ICV somatostatin injection, 3) and 1-hour post somatostatin-glucagon infusion. Means are shown \pm SEM. Each mean represents 2-4 observations.

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