

**Leptin Treatment in STZ-Induced Diabetic Rats Inhibits Glucagon Responsiveness
and Hepatic Gluconeogenic Gene Expression**

by

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Abstract

Central administration of leptin normalizes blood glucose concentrations of streptozotocin (STZ)-induced diabetic rats and dramatically decreases blood glucose concentrations in leptin-treated rats during a fast as compared vehicle-treated controls. We hypothesize that central leptin administration decreases blood glucose concentrations by either decreasing serum glucagon concentrations or glucagon responsiveness, which ultimately decreases gluconeogenesis.

Glucagon responsiveness was tested after fasting; a large dose of IP glucagon (750 ug/kg) did not increase the absolute blood glucose concentrations of leptin-treated diabetic rats back to levels observed in vehicle-treated diabetic rats. The expression of approximately thirty genes was determined in the liver by custom-made PCR arrays. Overall, the gene expression of several gluconeogenic enzymes, transcription factors, and coactivators, (G6pc3, Pck1, Ppargc1, Creb1, Mdh1 and IRS-2) appeared to increase in diabetic vehicle-treated rats compared to nondiabetic rats, and leptin treatment appeared to reverse this effect. This suggests that gluconeogenesis was suppressed in leptin-treated diabetic rats, despite the finding the serum glucagon concentrations were similar in vehicle-treated diabetic rats and leptin-treated diabetic rats. Therefore, it appears that leptin-treated diabetic rats were resistant to the effects of glucagon and that this contributed to lower daily blood glucose concentrations and decreased blood glucose concentrations during a fast.

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Table of Contents

Abstract.....	ii
Acknowledgments.....	iii
List of Tables	v
List of Figures.....	vi
CHAPTER I INTRODUCTION	1
CHAPTER II LITERATURE REVIEW	4
2.1 Obesity, Insulin Resistance and Diabetes Mellitus	4
2.1.1 Insulin resistance and Diabetes Mellitus	6
2.1.2 Obesity and Diabetes Mellitus	9
2. 2 Glucose Homeostasis	10
2.2.1 Reactions involve in regulating glucose metabolism	12
2. 2.2 Hormones involved in regulating glucose metabolism	19
2. 3 Research Hypothesis	28
CHAPTER III LEPTIN-TREATED STREPTOZOTOCIN (STZ)-INDUCED DIABETIC RATS ARE RESISTANT TO THE EFFECTS OF GLUCAGON	29
CHAPTER IV CONCLUSIONS	60
References	62

List of Tables

Table 1 Genes Examined in PCR arrays.....	54
Table 2 Hepatic gene expression of diabetic and leptin-treated rats following a fast.	59

List of Figures

Figure 3.1 Body weights of non-diabetic vehicle-treated, diabetic vehicle-treated and diabetic leptin-treated rats	47
Figure 3.2 Effects of daily leptin or vehicle treatment on blood glucose concentration of both diabetic and non-diabetic rats	48
Figure 3.3 Blood glucose concentrations of non-diabetic vehicle-treated rats, diabetic vehicle-treated rats, and diabetic leptin-treated rats in fed the fed and fasted states	49
Figure 3.4 Absolute and relative values of blood glucose concentration in non-diabetic vehicle-treated rats, diabetic vehicle-treated, and diabetic leptin-treated rats in response to IP glucagon injection after fasting	50
Figure 3.5 Absolute and relative values of blood glucose concentration in non-diabetic vehicle-treated rats, diabetic vehicle-treated, and diabetic leptin-treated rats in response to IP saline injection after fasting.	51
Figure 3.6 Relative blood glucose responses to IP glucagon vs saline in nondiabetic vehicle-treated, diabetic vehicle-treated, and diabetic leptin-treated rats.	52
Figure 3.7 Serum glucagon concentrations of non-diabetic vehicle-treated, diabetic vehicle-treated and diabetic leptin-treated rats after a fast	53
Figure 3.8 Gene expressions of gluconeogenic enzymes glucose 6-phosphatase (G6pc3) and PEPCK (Pck1) in the livers of non-diabetic vehicle-treated, diabetic vehicle-treated, and diabetic leptin-treated rats following a fast.	55
Figure 3.9 Gene expression of cytoplasmic malate dehydrogenase (Mdh1) in the livers of non-diabetic vehicle-treated, diabetic vehicle-treated, and diabetic leptin-treated rats.	56
Figure 3.10 Gene expression of transcription factors involved in the gluconeogenic pathway, PGC1a (Ppargc1) and Creb1 in the livers of non-diabetic vehicle-treated, diabetic vehicle-treated, and diabetic leptin-treated rats following a fast.	57

Figure 3.11 Gene expression of IRS-2 in liver of non-diabetic vehicle-treated, diabetic
vehicle-treated and diabetic leptin-treated rats following a fast 58

CHAPTER I

INTRODUCTION

The incidence of diabetes mellitus has become epidemic in the United States over the last several years, as well as in other parts of the world. According to data released in 2011 from the American Diabetes Association, 25.8 million Americans suffer from diabetes. This represents 8.3% of the population. The number of Americans with diabetes is predicted to increase by 30% over the next 20 years(1). Diabetes worldwide affects about 285 million people which is about 6.4% of the population (2). Heart disease and stroke, high blood pressure, blindness, kidney damage, neuropathy, and infections are common long-term complications of diabetes that seriously impair human health.

The major pathologic etiologies of diabetes are insufficient insulin secretion due to β cell dysfunction and/or insulin resistance of target tissues (3, 4). Insulin resistance (IR) refers to a physiologic state in which insulin is less effective than expected at enhancing glucose utilization in skeletal muscle and adipose tissue, and at repressing hepatic glucose output, leading to hyperglycemia and/or hyperinsulinemia and finally impairing glucose tolerance(5-7). Type 1 diabetes is characterized by a lack of insulin, related to a T-cell-mediated autoimmune attack of the insulin-producing beta cells. Type 2 diabetes is characterized by insulin resistance and a relative lack of insulin. Type 2 diabetes make up 90 -95% of all cases of diabetes. There is a positive correlation between the incidence of type 2 diabetes and obesity (8, 9), suggesting that obesity, in some way, may promote the development of insulin resistance and type 2 diabetes.

Leptin, a hormone that is primarily produced by white adipose tissue (10), interacts with its receptor (ObR-b) in the basomedial hypothalamus to decrease food intake and increase energy expenditure. This is thought to work through a leptin-mediated increase in alpha melanocyte-stimulating hormone (α MSH) secretion and a decrease in neuropeptide Y (NPY) secretion in the hypothalamus(11-13). Most obese people appear to be leptin resistant, in that they have high circulating leptin concentrations, despite being obese and hyperphagic (14, 15). This suggests the possibility that a lack of leptin signaling in the brain may contribute to insulin resistance and hyperglycemia. Subsequent studies have suggested that leptin, within the central nervous system (CNS), may play a role regulating glucose metabolism and homeostasis independent of changes in circulating insulin concentration (16, 17), and that insufficient central leptin signaling might result in insulin resistance (18). Indeed, several different studies have shown that intracerebroventricular (ICV) leptin treatment can normalize blood glucose concentrations of STZ-induced diabetic rats independent of any effects on insulin action, body weight, or food intake (17-20). Furthermore, fasting has been shown decrease blood glucose concentrations to a greater extent in leptin-treated rats as compared to vehicle-treated rats (18).

Contributing to fasting hyperglycemia, as seen in untreated diabetes, is the overproduction of hepatic glucose, which is caused by the increased rates of gluconeogenesis, the production of glucose from noncarbohydrate precursors, and glycogenolysis, the production of glucose from the breakdown of glycogen (21, 22). Because liver glycogen has a limited capacity to store and release glucose, gluconeogenesis is the primary source of glucose during a moderate to long-term fast.

Gluconeogenesis is enhanced by a lack of insulin, as well as the counterregulatory effects of glucagon (23, 24), Gluconeogenesis is regulated by certain key enzymes, including phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase which catalyzes the first and last step of gluconeogenesis, respectively (25, 26), as well as transcription factors, such as peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1alpha), cAMP response element-binding (CREB) and its coactivator CRTC2 (27, 28). Therefore, it is hypothesized that the normalization of the blood glucose concentrations in leptin-treated, STZ-induced diabetic rats and the decrease in blood glucose after a fast (20, 29), is due to a leptin-mediated decrease in circulating glucagon and/or a decrease in glucagon responsiveness. This would lead to suppressed gene expression of key gluconeogenic enzymes and transcription factors involved in gluconeogenesis (20, 23). To this end, we examined the blood glucose response to an IP injection of glucagon in STZ-induced diabetic rats treated daily with ICV leptin or vehicle. This was also compared to the response of nondiabetic rats treated with an IP injection of vehicle.

The goals of this study were to 1) determine whether chronic central leptin administration in streptozotocin-induced diabetic rats inhibits the glucose response to IP injected glucagon, 2) determine whether leptin alters the expression of hepatic genes that are potentially involved in gluconeogenesis, and 3) to find novel interactions between genes that are involved in gluconeogenesis. Ultimately, our objective was to determine the mechanism by which central leptin administration normalizes blood glucose concentrations in diabetic rats.

CHAPTER II

LITERATURE REVIEW

2.1 Obesity, Insulin Resistance and Diabetes Mellitus

In the United States, diabetes mellitus affects 25.8 million people, accounting for 8.3% of the American population, including 18.8 million diagnosed and 7.0 million undiagnosed cases (1). The number is still increasing and the Centers for Disease Control (CDC) predicts that the number of cases of diabetes will be double or triple by 2050 (30). Not only is diabetes a problem for the U.S, diabetes has become a global problem. Based on a report from the International Diabetes Federation, in 2011, 366 million people globally had diabetes and the number is expected to increase to 522 million by 2030 (31). The cost of diabetes is tremendous; for example, in 2007, total expenditures were \$174 billion and \$18 billion for people with diagnosed cases of diabetes and undiagnosed cases in the United States, Diabetes mellitus is described as a chronic metabolic disorder that is caused by either insulin secretion deficiency or a defect in insulin function, leading to high blood glucose concentrations (3, 4). Diabetes can bring about complications which can be separated into two types; acute and chronic (32, 33). Generally, acute diabetic complications include diabetic ketoacidosis (DKA), hyperglycemia hyperosmolar non-ketotic diabetic coma, as well as lactic acidosis (LA) and hypoglycemia during the

treatment of diabetic hypoglycemic coma (34). These severe acute complications may cause death without timely treatment.

On the other hand, complications can occur from the chronic effects of diabetes. Long-term high blood glucose concentrations induce vascular atherosclerosis in the vitals of the body; such like heart, brain, and this is the foundation for the pathogenesis of chronic diabetic complications (32). Damage to microvessels of the kidney causes nephropathy and even renal failure. Microvessel damage to the eyes causes retinopathy, resulting in eyesight degradation or even potential blindness. Microvessel damage to feet and skin can cause sores and infections of the feet and skin, leading to gangrene and the need for lower limb amputation. Damage to the macrovessels of heart and brain lead to cardiovascular disease and stroke (35). Diabetes and its complications seriously endanger patients' health and shorten their life spans. In 2011, diabetes globally contributed to 4.6 million deaths (31).

Traditionally, diabetes mellitus is classified into 2 types; type 1 and type 2 diabetes. Type 1 diabetes mellitus (T1DM) is an autoimmune disease in which the patient's immune system attacks and kills roughly 90% of pancreatic beta cells, which secrete insulin. This results in an absolute insulin production deficiency and subsequently leads to high blood glucose concentrations. T1DM is associated with some other autoimmune diseases, such as autoimmune thyroiditis (ATD), pernicious anemia (PA), autoimmune gastritis (AIG), etc. (36, 37). T1DM may be triggered by genetic susceptibility, diabetogenic environmental factors (38), as well as certain virus, such as coxsackievirus (39), and diet (40). Type 1 diabetes accounts for 5-10% of the cases of diabetes. The most common form of diabetes is type 2 diabetes mellitus (T2DM). It

accounts for 90-95% of the cases of diabetes. The major characteristic of T2DM is decreased insulin sensitivity with relative insufficient insulin production. Though patients with T2DM can still secrete insulin, their insulin-sensitive organs have a blunted response to insulin. This causes reduced glycogen storage in the liver, elevated hepatic glucose production, and decreased glucose uptake in skeletal muscle and adipose tissue. Together, the increased hepatic output and decreased glucose utilization by peripheral tissues result in elevated blood glucose concentrations (4, 41, 42). Initially, because of the insulin resistance, the normal concentration of insulin cannot maintain blood glucose concentrations within a normal range. At this point, insulin secretion may be increased by β -cells to compensate for insulin resistance. In other words, insulin resistance decreases insulin sensitivity and increases insulin production, which is known as hyperinsulinism. However, as diabetes progresses, the loss of β -cell function will progressively decrease the capacity of the pancreas to produce and secrete insulin. Eventually insufficient insulin release will be the result (43-45). If glucose intolerance and/or overt diabetes appears during pregnancy, but the diabetes abates after pregnancy, the pregnant woman is said to have gestational diabetes mellitus (GDM) – a special type of diabetes (46). Additionally, in recent years, researchers have speculated the existence of a ‘type 3 diabetes’ in which the brain reduces or stops to secrete insulin, leading to deterioration of brain cells. Some individuals believe that type 3 diabetes is actually Alzheimer's disease (AD) (47, 48).

2.1.1 Insulin resistance and Diabetes Mellitus

Insulin is a protein hormone containing 51 amino acids that is produced by pancreatic β -cells in the islets of Langerhans. Generally, high blood glucose levels stimulate the secretion of insulin, which promotes cellular glucose uptake from blood,

increases lipid and glycogen synthesis, and inhibits glycogen breakdown and gluconeogenesis, leading to decreased blood glucose concentrations. But during insulin resistance (IR), insulin efficiency in promoting glucose uptake and utilization becomes less effective, as does the ability of insulin to inhibit hepatic glucose output. In order to keep glucose levels within a normal range, pancreatic β -cells release additional insulin to compensate the insensitivity, leading to hyperinsulinemia. As insulin resistance increases over time, impaired glucose tolerance appears and compensatory insulin secretion fails, eventually developing type 2 diabetes mellitus (49, 50).

Inflammation is one of the most important factors to induce insulin resistance. Previous studies have suggested that there is a correlative relationship between insulin resistance and elevated inflammatory makers, such as C-reactive protein (CRP) (51, 52) and fibrinogen (53). In the early 1990s, scientists found that tumor necrosis factor-alpha (TNF- α), a proinflammatory cytokine, can induce insulin resistance. The inflammation interrupts insulin trans-membrane signaling by increasing serine/threonine phosphorylation instead of normal tyrosine phosphorylation of the insulin receptor and IRS-1 and IRS-2, the major insulin receptor substrates (54-56). Therefore, the binding capacity of insulin receptor and IRS decreases and phosphatidylinositol 3 – kinase (PI-3K) phosphorylation is weakened, because it is activated by IRS, this blocks insulin signaling transduction by the IR/IRS/PI3-K pathway (57). In addition, IRS serine/threonine phosphorylation triggers the degradation of IRS itself (58). All of these defects in insulin signaling are related to insulin resistance. Besides TNF- α , increased concentration of other key inflammatory mediators, such like interleukins-1 and -6 (IL-1,

and IL-6), also link to insulin resistance, indicating that chronic inflammation triggers chronic insulin insensitivity (59, 60).

GLUT4 is an insulin-sensitive glucose transporter found in fat tissues and skeletal and cardiac muscle. Without insulin stimulation, GLUT4 is stored in vesicles within the cell. However, when insulin combines to its receptor, a series of cascading effects are activated, resulting in the vesicles containing GLUT4 moving toward and fusing with the plasma membrane. Glucose binds to the translocated GLUT4 transporter on the plasma membrane causing a conformational change in the transporter, which allows glucose to move into the cell. After glucose is transported into the cell, the conformation of GLUT4 changes back to its original conformation, allowing it to bind and transport more glucose molecules. The process is reversed when plasma insulin concentration decreases: GLUT4 transporters are moved from the plasma membrane back into intracellular vesicles by endocytosis. Therefore, insulin-sensitive tissues can respond rapidly according to the fluctuation of circulating insulin levels, maintaining glucose homeostasis (61). It has been found that long-term exposure to high insulin levels decreases cellular GLUT4 content by about 40% and reduces its translocation (62, 63). Since hyperinsulinemia is found in the insulin resistance state because pancreatic β -cells release excessive insulin to compensate the insulin insensitivity, GLUT 4 concentration and activation are suppressed under insulin resistance which further enhances insulin resistance.

Some other factors also show a positive association with insulin resistance. Resistin, a protein secreted by adipose tissue, represses insulin-stimulated glucose uptake (64) and increased expression of resistin has been observed in diabetic patients (65).

Elevated triglycerides (TG) content in skeletal is also associated with insulin resistance because beta-cell dysfunction results from excessive TG accumulation (66, 67).

2.1.2 Obesity and Diabetes Mellitus

If energy intake goes beyond its metabolic need, excess calories will be stored as fat in adipose tissue, in the form of triglyceride. The storage of too much fat results in the person becoming overweight or obese. Weight and height calculation are used to determine body mass index (BMI) which is the assessment standard of obesity. If the BMI of an adult is between 25 and 29.9 kg/m², the person is considered to be overweight; if the BMI is over 30 kg/m², the person is considered as obese. The prevalence of obesity has steadily increased during the last two decades in U.S. Currently, over one-third of adults are obese (68). Based on the data from the 2007-2008 National Health and Examination Survey (NHANES), 33.8% of American adults and about 17% of children and teenagers are obese. In 2005, 1.1 billion adults worldwide and 10% of the children were overweight or obese. It has been widely accepted that there is a significant positive correlation between obesity and type 2 diabetes. Approximately 90% of type 2 diabetic patients are overweight or obese (69). In addition, obesity has been shown to diminish life span and increases the risk of type 2 diabetes, heart disease and atherosclerosis, dyslipidemia, several types of cancers, fatty liver, etc. (70, 71).

In obese people, increased adipose tissue mass elevates plasma free fat acid (FFA) concentrations which is related to enhance insulin resistance in skeletal muscle and increased endogenous glucose production. High FFA concentrations also impair pancreatic β -cell function so that insulin secretion decreases (72), leading to insufficient

circulating insulin concentrations, increasing the risk of developing diabetes. In addition, high levels of FFA also increase the release of other factors which are involved in insulin resistance, such as glycerol, hormones, pro-inflammatory cytokines (73).

When obesity occurs, adipose cells become hypertrophic which is negatively correlated with insulin sensitivity. Hypertrophic adipocytes have reduced IRS-1 (74), which leads to the decreased expression of GLUT4 (75). The enhanced concentration of inflammatory mediators, such like TNF- α , IL-6, in obese states might also inhibit insulin signal transduction (60). Obesity is also associated with endoplasmic reticulum (ER) stress, which also suppresses insulin sensitivity. The stress increases c-Jun N-terminal kinase (JNK) activation and serine phosphorylation of insulin receptor substrate-1 (IRS-1). This leads to the inhibition of insulin action in liver cells. X box-binding protein-1 (XBP-1) is a transcriptional factor which helps to relieve ER stress. Under insulin stimulation, free P85 breaks off and interacts with XBP-1, helping XBP-1 to move into the nucleus where it turns on the genes of chaperone that are necessary for ER function. But under obese conditions, insulin signaling is impaired so that concentration of free P85 is decreased and XBP-1 cannot get to the nucleus. This enhances ER stress and insulin resistance, and which could eventually contribute to the development of type 2 diabetes (76).

2.2 Glucose Homeostasis

Under normal fasting conditions, human blood glucose concentrations fluctuate in a state of dynamic equilibrium within a range of 80-120 mg/dL. Several internal systems coordinated by different biochemical and physiological processes maintain glucose

homeostasis. Blood glucose concentrations can increase by enhancing hepatic glucose production, through glycogen breakdown and/or gluconeogenesis, or by increasing absorption of carbohydrates from the gastrointestinal tract. On the other hand, blood glucose concentrations can decrease by enhancing glucose uptake into various cells and using glucose to produce energy, or transforming the glucose into fat, glycogen or other carbohydrates. Glucose imbalance causes a series of clinical symptoms: for example, if fasting plasma glucose concentration is less than 2.8 mmol/ L, the hypoglycemia causes adrenergic manifestations, including shakiness, nervousness, palpitations, etc., as well as neuroglycopenic manifestations, such like confusion, fatigue, nausea, dizziness, ataxia, blurred vision, stupor, coma and even death. On the other hand, if the fasting blood glucose concentration is beyond 160 – 180 mg/dl, which exceeds renal threshold of glucose (RTG), proximal tubule epithelial cells cannot resorb all of the glucose from the glomerular filtrate back into the blood, therefore, glucose appears in the urine (77). In addition, high concentrations of blood glucose can lead to several acute and chronic complications, which were discussed above.

The major pathogenesis of type 2 diabetes are pancreatic β -cell dysfunction, insufficient plasma insulin concentration, and blunted insulin responsiveness. The lack of insulin signaling disrupts normal glucose homeostasis, resulting in uncontrolled high hepatic output and reduced glucose utilization, both of which contribute to hyperglycemia. The former is the consequence of increased gluconeogenesis, glycogen breakdown, and impaired glycogen storage, while the later is the result of impaired glucose uptake in muscle and fat tissues (78).

2.2.1 Reactions involve in regulating glucose metabolism

Glucose balance is the net result of several antagonistic biochemical pathways. Increases in glycolysis and glycogen synthesis tend to remove glucose from the circulation, decreasing blood glucose concentrations. On the other hand, increases in gluconeogenesis and glycogenolysis tend to add glucose to the circulation, increasing blood glucose concentrations. Dysregulation of these biochemical pathways can disrupt glucose balance. Therefore, it is important to understanding these pathways and the factors that regulate their activity.

Glycolysis

Glycolysis refers to the process in which glucose, derived from the circulation (or from glycogen), is converted into two pyruvates, accompanied by the net generation of two ATP and two reducing equivalents, 2 NADH. This process occurs in the cytoplasm of the cell and each step is catalyzed by a specific enzyme, with a total of 10 enzymatic reactions in the pathway. Glycolysis can be divided to two stages: an initial ATP-utilizing stage, where two ATP are utilized, and a final ATP-generating stage, where four ATP and two NADH are generated. Thus, a net two new ATP are generated per glucose in glycolysis. All cells are able to utilize glucose for energy via glycolysis. It is a universal way of all glucose catabolism, where glucose provides energy for life activities and provides intermediates for other metabolic pathways. The net overall reaction for glycolysis is as follows: $\text{glucose} + 2 \text{ ADP} + 2 \text{ NAD}^+ + 2 \text{ Pi} \longrightarrow 2 \text{ pyruvate} + 2 \text{ ATP} + 2 \text{ NADH} + 2 \text{ H}^+$.

Though all cells can perform glycolysis to generate ATP, much more ATP can be generated under aerobic conditions and in the presence of mitochondria, which contain the enzymes and complexes of the pathways of the tricarboxylic cycle (TCA or Krebs's cycle), and the electron transport system coupled with oxidative phosphorylation. This sometime is referred to as aerobic glycolysis, as opposed to anaerobic glycolysis, which occurs under hypoxic conditions. Under hypoxic conditions, pyruvate is reduced to lactate by the enzyme lactate dehydrogenase. This regenerates cytoplasmic NAD^+ , which is necessarily in order for one of the glycolytic enzymes, glyceraldehyde 3-phosphate dehydrogenase, to continue to be active, allowing anaerobic glycolysis to continue. Thus, during anaerobic glycolysis, both pyruvate and NADH are utilized. This limits the production of ATP. However, under aerobic condition, much more ATP can be generated from glycolysis (79).

Aerobic glycolysis is an important way to produce energy for the cell's needs, which involves glucose being completely oxidized into carbon dioxide and water. Under aerobic conditions, cytoplasmic NAD^+ is regenerate by one of two shuttle systems, the malate-aspartate shuttle or the glycerol 3-phosphate shuttle. Therefore, under aerobic conditions, pyruvate is not utilized to make lactate and to regenerate NAD^+ and thus, can be used to generate additional ATP. The shuttle system themselves can make additional ATP. The malate-aspartate shuttle transfers the electrons of cytoplasmic NADH into the matrix of the mitochondria as malate, which is then converted to mitochondrial NADH . The electrons from NADH are then transferred to the electron transport system, where approximately 2.5 ATP are generated per NADH . The glycerol 3-phosphate shuttle transfers the electrons of cytoplasmic NADH into the matrix of the mitochondria as

mitochondrial FADH_2 , which transfers electrons to the electron transport system, where approximately 1.5 ATP are generated per FADH_2 .

Under aerobic conditions, pyruvate made by glycolysis is transported into the matrix of the mitochondria by a protein carrier. In the matrix, the enzyme, pyruvate dehydrogenase complex converts pyruvate into acetyl CoA, carbon dioxide, and NADH. The reaction is catalyzed by the enzyme, pyruvate dehydrogenase complex, and is an irreversible and committed step in metabolism. The carbons of acetyl CoA cannot be used to generate glucose, whereas the carbons of pyruvate can be used to make glucose. Thus, once acetyl CoA is made, those carbons are committed to be used to generate energy (ATP), fatty acids, cholesterol, or ketone bodies, but not glucose. Acetyl CoA is used by the enzymes of the TCA cycle to generate 3 NADHs 1 FADH_2 , and 1 GTP, which is similar to ATP. The reducing equivalents generated by the TCA cycle and by pyruvate dehydrogenase complex transport their electrons into the electron transport system, where oxygen is the final electron acceptor, with the production of water. Completely oxidized, aerobic glycolysis can generate 30-32 ATP/glucose, depending on which shuttle system was using to carry electrons from cytoplasmic NADH. Acetyl CoA can also be used to make intermediates of other metabolic pathways, depending on the tissue. For example, in liver, acetyl CoA can be used a precursor to make fatty acids or cholesterol, rather than being completely oxidized for energy production (79, 80).

Glycogen Synthesis

The production of glycogen is another way glucose can be “cleared” from the circulation, resulting in a decrease in blood glucose concentrations. Though glycogen is

probably stored to a small degree in many different cell types, including adipocytes (81, 82), the major quantitative sites are skeletal muscle and liver. About two thirds of the body's glycogen is stored in skeletal muscle, while about one third is stored in liver. Glycogen is a highly branched polymer of glucose. The donor molecule for glucose is not glucose itself, but rather UDP-glucose. UDP-glucose is made by the enzyme, UDP-glucose pyrophosphorylase, from glucose 1-phosphate and UTP. Glucose 1-phosphate is derived from the reversible conversion of glucose 6-phosphate by the enzyme, phosphoglucomutase. The enzyme, glycogen synthase acts at the nonreducing end of existing branches to elongate the branch. Once a branch is approximately 11 glucose residues in length, another enzyme, called branching enzyme, removes a block of 6-8 residues and attaches the block at a new site via an alpha 1,6 glycosidic bond, creating a new branch point. Glycogen synthase can then act again to elongate the existing branches. At the core of the glycogen molecule is a protein called glycogenin. Glycogenin is able to autoglycosylate itself, which serves as the original primer chain of glucose residues upon which glycogen synthase acts to further elongate the chain of glucose residues. Glycogen synthase is only active when in contact with glycogenin. Therefore, as individual glycogen molecules grow larger, glycogen synthase will eventually lose contact with glycogenin, halting further synthesis of that particular glycogen molecule. Fully formed glycogen molecules have 12 tiers of branches with approximately 95% of the glucose located in the outer 4 tiers (83).

Glycogenolysis

Glycogenolysis is a biochemical pathway that can add glucose to the circulation, helping to maintain or increase blood glucose concentrations. It does this by breaking the

glucosidic bonds that hold the glucose residues to the glycogen molecule. As discussed above, the major storage sites of glycogen are skeletal muscle and liver. There is a major metabolic difference between the glycogen that is stored in these two sites. Glycogen stored in skeletal muscle can only be used by the particular muscle cell in which it is stored. In other words, once glucose is stored as glycogen in a skeletal muscle cell, the glucose molecule is trapped by that cell. On the other hand, glucose stored as glycogen in liver can be used to produce glucose that can be released back into the circulation. This difference is due to the presence or absence of the enzyme, glucose 6-phosphatase, which remove the phosphate group from glucose 6-phosphate to form glucose. Because skeletal muscle doesn't have the glucose 6-phosphatase enzyme, it cannot form glucose, effectively trapping glucose within the cells. Liver cells do have glucose 6-phosphatase activity and well as GLUT2 transporters, so the liver can form glucose and transport it from within to outside the cell, where it can enter the circulation. The degradation of stored glycogen is catalyzed by the enzyme glycogen phosphorylase. Glycogen phosphorylase uses phosphorylytic cleavage to break the alpha 1,4 glucosidic bond of the glucose residue at the nonreducing end of a branch. The product of glycogen phosphorylase is therefore, glucose-1-phosphate, not glucose. Because of steric hinderance at the active site, glycogen phosphorylase can only degraded glucose up to four residues away from a branch point. At this point, debranching enzyme participates in the degradation process. Debranching enzyme possesses two activities; a 4:4 transferase and an alpha 1,6-glucosidase. The 4:4 transferase activity breaks an alpha 1,4 glucosidic bonds to remove a block of 3 glucosyl residues from one branch and moves it the another branch, creating another alpha 1,4 glucosidic bond. This exposes a lone

glucosyl residue that is attached by an alpha 1,6 glucosidic bond. The alpha 1,6 glucosidase activity of debranching enzyme catalyzes the hydrolytic cleavage of the lone glucosyl residue, forming glucose. Glycogen phosphorylase can now act again at the nonreducing end of branches to form more glucose 1-phosphate. Glucose 1-phosphate is converted to glucose 6-phosphate by phosphoglucomutase. There are several potential fates of glucose 6-phosphate, depending on the tissue and the physiological conditions. In skeletal muscle, the glycogen-derived glucose 6-phosphate most likely will enter glycolysis for generate ATP for the cell. In liver, the glycogen-derived glucose 6-phosphate will most likely have its phosphate group removed by glucose 6-phosphatase, forming glucose. As intracellular glucose concentrations increase, GLUT2 will transport glucose from within the cell to the circulation (84, 85).

Gluconeogenesis

Gluconeogenesis converts non-carbohydrate precursors, such as most amino acids, lactate, and glycerol, into glucose. The main site of gluconeogenesis is the liver, though up to 10% of gluconeogenesis can occur in the cortex of the kidney. Gluconeogenesis is an anabolic pathway. Therefore, it doesn't occur spontaneously without the input of free energy. This energy is supplied by beta-oxidation of fatty acid, which occurs under the same physiological conditions as gluconeogenesis. The physiological significance of gluconeogenesis is to help maintain blood glucose concentrations during a fast. Gluconeogenesis also plays an important role in other metabolic pathways. It is part of the Cori cycle, in which lactate produced by red blood cells or skeletal muscle is taken up by the liver and converted to glucose by gluconeogenesis. Glucose is released by the liver and taken up by the red blood cell or skeletal muscle, where lactate is produced again by

anaerobic glycolysis. Gluconeogenesis is also involved in preventing the occurrence of lactic acidosis (86), aiding amino acid metabolism (87, 88), and promoting ammonia secretion in renal tubules to counteract acidosis (89). Gluconeogenesis is regulated by several hormones, such as insulin, glucagon, epinephrine, and others. In addition, high plasma concentrations of the precursors of the gluconeogenesis, such as glycerol, lactic acid, and amino acid stimulate gluconeogenesis.

There are four important gluconeogenic enzymes of (Figure 1). These enzymes are used to bypass the irreversible enzymes of glycolysis. Pyruvate carboxylase (PC) which catalyzes the conversion of pyruvate to oxaloacetate (90) and phosphoenolpyruvate carboxylase kinase (PEPCK) which catalyzes conversion of oxaloacetate to phosphoenolpyruvate (PEP) are used to bypassed the glycolytic enzyme of pyruvate kinase. Fructose 1,6-biphosphatase catalyzes the conversion of fructose 1,6-bisphosphate to fructose 6-phosphate. This bypasses the glycolytic enzyme phosphofruktokinase-1. Glucose 6-phosphatase catalyzes the conversion of glucose 6-phosphate to glucose. This bypasses the glycolytic enzyme glucokinase (91). Part of the gluconeogenic pathway is shown in Figure 1. Lactate is converted to pyruvate by the enzyme lactate dehydrogenase. The amino acid alanine is converted to pyruvate by enzyme alanine transaminase. Glycerol enters gluconeogenesis at dihydroxyacetone phosphate.

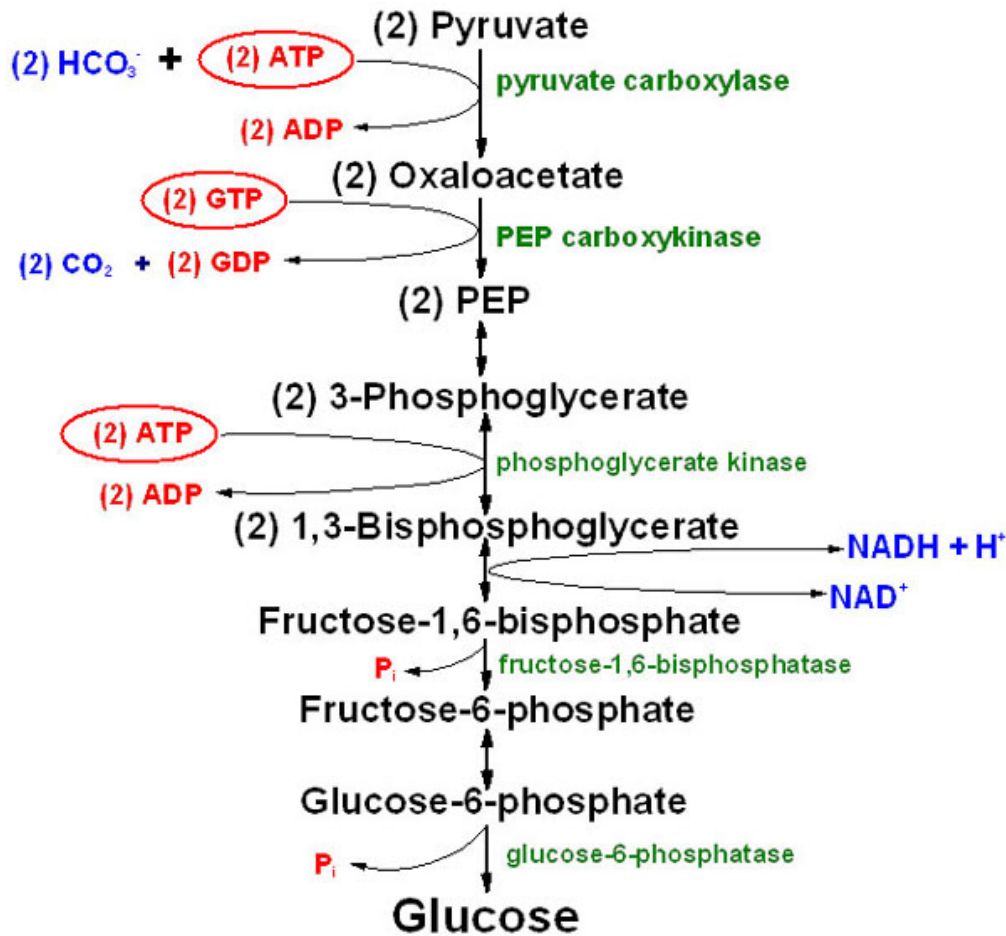


Figure 1. Gluconeogenesis pathway (80)

2.2.2 Hormones involved in regulating glucose metabolism

Insulin

Insulin is secreted by pancreatic β -cells and its biological function is to lower blood glucose concentrations. It accomplishes this in several different ways involving multiple biochemical pathways. 1) Insulin promotes the translocation of GLUT4 transporter from intracellular vesicles to the plasma membrane of target cells (92).

This enhances the uptake of glucose from circulation to skeletal muscle and adipose tissue. 2) Insulin increases the production of fructose 2,6, biphosphate, which is an allosteric stimulator of phosphofructokinase-1, the rate-controlling enzyme of glycolysis (93). 3) Insulin enhances phosphodiesterase activity through covalent modifications, reducing cAMP levels, increasing the concentration of cGMP to stimulate glycogen synthetase activity and suppresses glycogen phosphorylase activity. This accelerates glycogen synthesis and inhibits of glycogenolysis (94). 4) Insulin activates pyruvate dehydrogenase complex by stimulating pyruvate dehydrogenase phosphatase. This dephosphorylates the complex making it active. Increased activity of pyruvate dehydrogenase complex accelerates the conversion of pyruvate to acetyl CoA and the aerobic oxidation of glucose (95). 5) Insulin inhibits the synthesis of the gluconeogenic enzyme PEPCK and reduces the concentration of gluconeogenic precursors, leading to the inhibition of gluconeogenesis (96). 6) Insulin inhibits adipose triglyceride lipase in adipose tissue, slowing fat mobilization and beta-oxidation in the liver (97). This lowers the source of energy that powers gluconeogenesis.

Glucagon and Epinephrine

The effects of epinephrine and glucagon are counterregulatory to the effects of insulin.

As a result, these hormones lead to an increase in blood glucose concentrations.

Epinephrine is a catecholamine released primarily from the adrenal medulla during conditions of stress. It is part of the “flight or fight” response to stress and exercise, raising blood glucose concentrations to rapidly meet the body’s need under these conditions. Glucagon is a protein hormone produced by α -cells within the islets of the

pancreas. Glucagon is primarily released in response to low blood glucose concentrations, like what would occur during a fast. Glucagon and epinephrine stimulate glycogen phosphorylase in liver cells by activating the cAMP-protein kinase A system (98, 99). Activating cAMP also causes the inhibition of glycogen synthase. The net result is the stimulation of glycogenolysis and the inhibition of glycogen synthesis. Gluconeogenesis is increased by glucagon and epinephrine due to increasing the availability of gluconeogenic substrates and to increasing the activity or amount of gluconeogenic enzymes. Glucagon activates adipose triglyceride lipase, promoting lipolysis and enhancing fatty acid oxidation. The increase in lipolysis causes the enhanced release of glycerol into the circulation. Low insulin and high cortisol, another “stress” hormone, concentrations lead to the increased release of amino acids from muscle. Increase fatty acid oxidation in the liver increases acetyl CoA levels within the matrix of the mitochondria. Acetyl CoA is an allosteric stimulator of pyruvate carboxylase and an allosteric inhibitor of pyruvate dehydrogenase. This diverts carbons from pyruvate to be converted into oxaloacetate. The increase in cAMP caused by glucagon and epinephrine activates protein kinase A, which phosphorylates cAMP-response element binding protein (CREB) that stimulates the transcription of PEPCK. Glucagon also causes the phosphorylation of glycolytic enzyme protein kinase, inhibiting its activity, and blocking the conversion of phosphoenolpyruvate (PEP) to pyruvate. This prevents a futile cycle between PEP and pyruvate. Fructose 1,6-bisphosphatase is reciprocally regulated as compared the glycolytic enzyme phosphofruktokinase-1 (PFK-1). Fructose 2,6-bisphosphate stimulates PFK-1, which it inhibits fructose 1,6-bisphosphatase. The production of fructose 2,6-bisphosphate is determined by the ratio of insulin/glucagon,

which is ultimately dependent on the blood glucose concentrations. When blood glucose concentrations are low, glucagon secretion is increased and insulin secretion is decreased. This lowers the amount of fructose 2,6-bisphosphate produced, inhibiting the glycolysis and stimulating gluconeogenesis.

Hormones secreted by the anterior pituitary

Growth hormone, a peptide hormone secreted by the anterior pituitary, increases blood glucose concentrations by its antagonistic action to insulin by inhibiting glycogenolysis and preventing glucose transportation into cells for oxidation.

Corticotropin-releasing hormone (CRH) is secreted by the hypothalamus, where it enters the hypophyseal portal system to cause the anterior pituitary to synthesize and secrete adrenocorticotropic hormone (ACTH). ACTH spurs synthesis and secretion of glucocorticoids, which promote gluconeogenesis, glycogen synthesis in the liver, and inhibit glucose uptake and utilization in peripheral tissues. The net effect is an increase in blood glucose concentration. ACTH is derived from proopiomelanocortin (POMC) and serves as a precursor of α -melanocyte-stimulating hormone (α -MSH), an activator of melanocortin 4 receptor (MC4R), which are known to inhibit food intake and increase energy expenditure (100, 101).

Leptin

Leptin is an adipokine containing 167 amino acids encoded by the obese (ob) gene, which is located at chromosome 7q31.3 in humans (102). The amino end of the signal peptide of 21 amino acids is removed to form the mature leptin, which contains 146

amino acids, with a molecular weight of 16 KD. White adipose tissue (WAT) is the major source of leptin synthesis and secretion; however, other tissues have also been found to produce leptin to some degree, including brown adipose tissue (BAT), skeletal muscle, epithelial cells, gastric mucosa, placenta, pituitary, stomach and liver (103). Leptin reduces body weight by inhibiting feeding and increasing energy expenditure (104). Rodent models with ob gene mutations are obese and insulin resistance. These effects can be reversed by the administration of functional leptin. There is a positive correlation between body fat content and serum leptin levels, suggesting that leptin could be part of feedback signal informing the brain about the amount of fat that is in the body. When people overeat and gained 10% of their body weight, the serum leptin levels increased by 300% (105). However, the expected responsiveness didn't occur, despite the high circulating level of leptin, implying leptin resistance in obese people (106, 107).

The leptin receptor is encoded by the diabetes (db) gene, which is a member of the cytokine receptor superfamily (108) and has five different isoforms; Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, and Ob-Re. Among them, the long form of the receptor (Ob-Rb) is mainly distributed in hypothalamic neurons, T cells and vascular endothelial cells (109). It is the only receptor isoform that can mediate intracellular leptin signaling to regulate energy intake and expenditure by the Janus kinase family and signal transducers and activators of transcription (JAK-STAT), which is considered to be the major pathway for leptin signal transduction (110). After leptin combines to Ob-Rb, JAK2 is activated by binding to a specific site of Ob-Rb, which provides a tyrosine residue (Y1138) for STAT3 binding, leading to STAT 3 dimerization and translocation into nuclear, activating genes transcription (111). The phosphorylated tyrosine residues on JAKs can

be recognized by SH2 domains of SOCS, such as, SHC and IRS, which down-regulates cytokine receptor signaling (112). In addition, JAKs also activates the p85 subunit of phosphoinositide 3-kinase (PI 3-kinase) pathway (113), and Ras- MAPK (mitogen-activated protein kinase) pathway is activated through Src homology/collagen phosphorylation(108). The short receptor (Ob-Ra) isoform is located in fat, heart, lung, and other peripheral tissues that transport leptin across the blood-brain-barrier to active sites and degrades leptin to regulate free leptin levels (114). The function of Ob-Rc and Ob-Rd is to remove leptin from the circulation (11), while the soluble isoform Ob-Re is a binding protein. Overall, leptin combines with its receptor to provide signaling based on the nutritional status. The signal is received by hypothalamus and peripheral tissues, so that they can take part in adjusting physiological functions to a change in nutritional status, like feeding.

Leptin affects the central nervous system (CNS) and peripheral tissues. The hypothalamus is the major connection between the CNS and leptin action to regulate feeding behavior and energy balance. Several hypothalamic nuclei are rich in the Ob-Rb receptor isoform, including the ventromedial nucleus (VMN), dorsomedial nucleus (DMN), and arcuate nucleus (ARC). The arcuate nucleus has highest amount of leptin receptors (115, 116). Within the ARC, there are two subtypes of leptin-sensitive neurons; the first type of neuron is orexigenic, which synthesize neuropeptide-Y (NPY) and agouti-related protein (AgRP), and the second type of neuron is anorexigenic, which produces proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) (117). The binding of leptin to the NPY/AgRP-containing neurons causes a decrease in the release of NPY and AgRP. On the other hand, the binding of

leptin to the POMC/CART-containing neuron causes an increase in the release of alpha-MSH, the gene product of POMC. Thus, leptin inhibits food intake by both inhibiting the orexigenic neurons and stimulating the anorexigenic neurons.

NPY is a single neuronal polypeptide chain comprising by 36 amino acids, 'Y' refers to the tyrosine residues at both ends of the molecule. NPY participates numerous physiologic processes, including promoting feeding, regulation body temperature, and hormone secretion, etc. (118). In animal experiments, food intake increases significantly when NPY is injected into brain ventricles and paraventricular nucleus (119), indicating that NPY is a strong orexigenic agent. Leptin inhibits NPY function by negatively regulating the expression of NPY mRNA (120). After ICV leptin injection, hypothalamic NPY mRNA and NPY levels are significantly reduced, accompanied by reduced feeding and weight loss (121). Leptin may also reduce the intracellular Ca_2^{++} concentrations by inhibiting the cAMP-protein kinase A system in NPY neurons, which directly inhibits the activity of NPY neurons (122, 123). FoxO1 stimulates AgRP transcriptional activity, while STAT3 activation has the opposite effect, inhibiting the transcriptional level. Leptin regulates AgRP transcription by inhibiting FoxO1 and activating of STAT3, directly participating in the homeostatic regulation of body energy metabolism (124, 125). When leptin combines with its receptor, α -melanocyte-stimulating hormone (α -MSH), a hormone processed from POMC, is activated (117). The binding of α -MSH with melanocortin 4 receptor (MC4R) results in a decrease in feeding (126). Interestingly, AgRP is an endogenous antagonist to α -MSH for the binding to the melanocortin 4 receptor. Thus, activation of the orexigenic systems also inhibits the anorexic system.

Leptin can regulate insulin-mediated transduction by stimulating the IRS-phosphoinositide 3-kinase (PI3K) pathway. The PI3K pathway decreases cAMP concentration (127), and in liver, it stimulates glycogen synthesis by inhibiting glycogen synthase kinase-3 phosphorylation by protein kinase B (PKB) (128).

Leptin plays a crucial role in helping to maintain glucose homeostasis via its direct action on central nervous system (CNS). It has been shown that intravenous infusion of leptin increases glucose turnover and glucose uptake and inhibits hepatic glucose production in wild-type mice, without changing plasma insulin concentration (16). In addition, animal models that don't naturally produce functional leptin, (*ob/ob*, lipodystrophy) are insulin resistant or even diabetic, while leptin administration in both *ob/ob* (129) and insulin-resistant congenital lipodystrophic mice (130) modulates glucose disposal, improving insulin sensitivity and glucose regulation.

Several studies demonstrate that leptin administration can restore blood glucose concentration in STZ-induced diabetic rats to normal levels. Chinookoswong et al. (1999) showed that large doses of leptin given peripherally over time would normalize blood glucose concentrations in a model of type 1 diabetes (the streptozotocin (STZ)-induced diabetic rat) (131). Subsequently, our laboratory (18) and a group from Japan (19) extended this finding by showing that small daily doses of leptin given directly in the ventricle of the brain would, over several days, also normalize blood glucose concentrations in these rats. This finding has since been observed by others (17, 132). It therefore appears that the site of action for leptin is within the central nervous system (CNS). This suggests that a lack of central leptin signaling might result in insulin resistance (18). The mechanism by which leptin helps to maintain glucose homeostasis is not known. It has been shown that

peripheral alpha1-, beta1-, beta2-, and beta3-adrenergic activity blockade does not stop central leptin administration from normalizing blood glucose concentration in diabetic rats (17, 132). In support of this, chemical sympathectomy with the use of guanethidine also does not attenuate the leptin-induced normalization blood glucose in STZ-induced diabetic rats (133). Therefore, it appears unlikely that central leptin administration normalizes blood glucose concentrations in diabetic rats via the sympathetic nervous system. It is known that the leptin-induced normalization of blood glucose concentrations in STZ-diabetic rats is not due to through decreasing food intake, enhancing urinary glucose excretion or insulin secretion (16, 19, 131, 134). In fact, decreased serum insulin concentrations were observed in both diabetic and non-diabetic rats under leptin treatment (18). It has been widely accepted that leptin doesn't change peripheral insulin action; instead, it changes the gene expression of hepatic metabolic enzymes and results in redistribution of glucose fluxes in the liver (135, 136). Actually, severely insulin-deficient rats can thrive if they are given an adenovirus that peripherally expresses large amounts of leptin (137). This suggests that leptin affects glucose homeostasis independent of insulin.

Since hyperglucagonemia has been observed in insulin-deficient rodents (138), Yu et al. (2008) suggest that leptin might inhibit serum glucagon concentrations to control blood glucose levels. Leptin suppresses gluconeogenesis, which is manifested by reduction of hepatic cAMP response element-binding protein, phosphoenolpyruvate carboxykinase, and peroxisome proliferator-activated receptor- γ -coactivator-1 α . In skeletal muscle, increased phosphorylated-insulin receptor substrate-1 (P-IRS-1), phosphatidylinositol-3-kinase (PI3K), phosphorylated-extracellular signal-regulated

kinase (P-ERK) increased to 65%, 30% and 44%, respectively, which might respond to enhanced insulin-like growth factor-1 (IGF-1) expression and IGF-1 receptor phosphorylation (137).

2.3 Research Hypothesis

We have previously found that chronic intracerebroventricular (ICV) leptin treatment can normalize blood glucose concentrations of STZ-induced diabetic rats. Furthermore, we have found that the blood glucose concentrations of leptin-treated rats dramatically decreases during a fast as compared to vehicle-treated animals, regardless of whether the rat were diabetic or nondiabetic. We hypothesize that chronic ICV leptin administration either inhibits the concentration of circulating serum glucagon or the responsiveness to glucagon in STZ-induced diabetic rats, contributing to the suppression of gluconeogenesis, resulting in normalization of blood glucose concentration.

CHAPTER III

LEPTIN-TREATED STREPTOZOTOCIN (STZ)-INDUCED DIABETIC RATS ARE RESISTANT TO THE EFFECTS OF GLUCAGON

Abstract Leptin has been shown to have an effect on glucose homeostasis. Our laboratory and others have found that chronic central administration of leptin can normalize blood glucose concentrations in streptozotocin (STZ)-induced diabetic rats. Since insulin concentrations remain low in these rats, we hypothesized that central leptin administration inhibits the responsiveness to glucagon, leading to the normalization of blood glucose concentrations. Therefore, we examined the blood glucose response to an IP injection of glucagon in STZ-induced diabetic rats treated daily with ICV leptin or vehicle. This was also compared to the response of nondiabetic rats treated with an IP injection of vehicle. Thirty male Wistar rats were implanted with an ICV cannula directed into the lateral ventricle. Twenty rats were given an IP injection of STZ (50 mg/kg) to induce diabetes, while ten rats were given control injections. After hyperglycemia was confirmed in the diabetic rats, one group of diabetic rats received daily injections of leptin (5 ug), while the other diabetic group and the nondiabetic group received daily injections of vehicle. Blood glucose concentrations were determined daily. After blood glucose concentrations were normalized in diabetic rats receiving daily leptin injections, rats were fasted (leptin-treated rats for 6 hours, while others for 21 hours). Baseline blood

glucose concentrations were determined from the tail vein, and rats received either an IP injection of glucagon (750 ug/kg) or vehicle. Blood samples from the tail vein were taken every 30 minutes for 2.5 hours and blood glucose concentrations determined. After several days, the rats were fasted as before and each rat was given an IP injection of glucagon or vehicle in a crossover fashion and blood glucose concentration determined as before. 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. There were differences in how the groups responded to glucagon. In nondiabetic rats, blood glucose concentrations in response to glucagon initially decreased by about 10%, followed by an increase of about 20% above baseline values. In both sets of diabetic rats, there was no initial decrease in blood glucose in response to glucagon, rather blood glucose concentrations initially increased by about 10%. Over the 2.5-hour post-injection period, blood glucose concentrations in response to glucagon of leptin-treated diabetic rats decreased to about 70% of baseline values. In vehicle-treated diabetic rats, the blood glucose in response to glucagon maintained baseline values. Despite these somewhat subtle changes, the large dose of IP glucagon did not increase the absolute blood glucose concentrations of leptin-treated diabetic rats back to levels observed in vehicle-treated diabetic rats. Therefore, it appears that leptin-treated diabetic rats are resistant to the effects of glucagon.

3.1 Introduction

Current statistics from the American Diabetes Association indicate that 25.8 million people have diabetes in the United States. Type 2 diabetes is the most common form of diabetes, accounting for 90-95% of the cases (1). It is this form of the disease that

is associated with obesity and is increasing in prevalence. Diabetes is not only a problem in the United States, it has become a global epidemic, with 346 million people worldwide having diabetes in 2011 (139).

Insulin is a hormone produced and secreted by pancreatic beta cells. It centrally regulates glucose metabolism and helps control blood glucose concentration. Insulin promotes the uptake of glucose into cells from the blood stream, and then the glucose is either used to produce energy or is stored in the form of glycogen (140-142). This helps to lower blood glucose concentrations. Type 2 diabetes is characterized by insulin resistance. With insulin resistance, insulin-sensitive tissues cannot lower blood glucose concentrations appropriately for a given amount of insulin, resulting in an increase in glucose production and release by the liver, and a decrease in glucose uptake in muscle and fat tissues leading to high level of blood glucose concentration (4, 41, 42, 143). Since normal levels of insulin are not adequate to regulate blood glucose levels during insulin resistance, patients with insulin resistance are sometimes also considered to be relatively insulin deficient. Thus, the traditional therapy for type 2 diabetes has centered on the secretion of additional insulin and increasing the sensitivity to existing insulin (44, 144, 145). Obese people are at high risk for type 2 diabetes because obesity might be the most important factor to develop insulin resistance (73, 146).

On the other hand, type 2 diabetes can possibly be controlled by regulating blood glucose concentration independent of insulin. Leptin, a hormone secreted primarily from white adipose tissue, plays a key role in the regulation of food intake and body weight. Leptin combines with leptin receptors in the hypothalamus to help maintain energy homeostasis by inhibiting energy intake and increasing energy expenditure, or by

preventing decreasing energy expenditure coupled with decreasing food intake (147, 148). Most obese people have high circulating levels of leptin, suggesting they are leptin resistant, which would contribute to both elevated feeding and weight gain, and eventually obesity (14, 15). Several previous studies have shown that leptin can regulate blood glucose concentrations. 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 serum insulin level (18, 136, 149). This suggests that leptin, acting independent of insulin, can normalize blood glucose conditions in diabetic rats. We have also shown that leptin-treated rats cannot maintain normal blood glucose concentrations during a fast as compared to vehicle-treated rats. Glucagon is thought to help maintain blood glucose concentrations during a fast and to increase blood glucose concentrations during type 1 diabetes. Therefore, it is possible that leptin mediates these effects by either decreasing the concentrations of circulating glucagon or decreasing the responsiveness to glucagon.

The release of glucagon is stimulated by a decrease in blood glucose concentrations. Glucagon breaks down liver glycogen into glucose by enhancing glycogenolysis, which is then releases glucose into the blood. In addition, glucagon enhances the hepatic production of new glucose from noncarbohydrate precursors by stimulating gluconeogenesis (150-153). Generally, gluconeogenesis is the major pathway to maintain blood glucose concentration during a moderate to long-term fast (154). Therefore, since blood glucose concentration decreases significantly in leptin-treated rats after a fast, the aim of this study was to examine the effect of glucagon in chronically leptin-treated, STZ-induced diabetic rats. We hypothesized that chronic central leptin

administration into the lateral ventricle of brain will either decrease the concentration of circulating glucagon or decrease the responsiveness to glucagon.

3.2 Materials and methods

Animals: Thirty male Wistar rats (~200g; Harlan, Indianapolis, IN) were housed in individual hanging wire-bottomed cages and kept in a temperature- ($23^{\circ}\text{C}\pm 3$) 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).

Experiment design: After ketamine anesthesia treatment, all of the rats were implanted with an intracerebroventricular (ICV) cannula into the lateral ventricle of the brain. Twenty rats were made diabetic by an intraperitoneal injection of streptozotocin (STZ) (50 mg/kg), while the other ten rats were given control injections of vehicle and were therefore nondiabetic. After hyperglycemia was confirmed in the twenty diabetic rats, half of the diabetic rats received daily ICV injections of leptin (5 ug/day), while the other half received daily ICV vehicle injections. The nondiabetic rats were also given daily ICV injections of vehicle. Body weights were determined daily, as were blood glucose concentrations from the tail vein via a handheld glucometer. When the blood glucose concentration of the leptin-treated diabetic rats normalized, rats were fasted for the determination of glucagon responsiveness. Vehicle-treated rats were fasted for twenty-one hours, while leptin-treated diabetic rats fasted for six hours. The reason why leptin-treated rats were fasted for a shorter time was to prevent hypoglycemia from

occurring. A previous study from our laboratory showed that the blood glucose concentration of leptin-treated rats decreased by 50% after six hours of fasting (12). We determined baseline blood glucose concentrations from the tail vein, and then rats were given an intraperitoneal injection of either glucagon (750 ug/kg) or vehicle and the blood glucose concentrations were determined every thirty minutes for two and a half hours. Several days later, all animals were fasted as before and the glucagon responsiveness test was repeated, except this time the rats previously injected with glucagon were injected with vehicle and those rats that were previously injected with vehicle were injected with glucagon (750 ug/kg), in a crossover fashion. As before, blood glucose concentrations were determined via the tail vein every thirty minutes for two and a half hours.

To better determine the hormonal, enzymatic, and intracellular signaling environment of the three groups of rats (i.e., diabetic vehicle-treated, diabetic leptin-treated, and nondiabetic vehicle-treated) in which the exogenous glucagon was introduced, after several days the rats were fasted as described above. Blood glucose concentrations in the fed state were determined prior to the initiation of the fast, while fasted blood glucose concentrations were also determined after the rats fasted for the appropriate period of time. Rats were euthanized by decapitation. Trunk blood was collected with a protease inhibitor to determine serum glucagon concentrations. The liver was removed and a sample collected and stored in RNAlater for subsequent analysis via custom-made PCR arrays (SABiosciences, Valencia, CA) to determine the expression of approximately 30 hepatic genes of interest (Table 1).

Surgical procedures: Thirty male Wistar 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. Rats were given an intraperitoneal (IP) injection of ketamine-xylazine (100mg/kg ketamine and 1mg/kg xylazine) to anesthetize the animals before surgery. 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. Rats were removed from stereotaxic apparatus and placed in a warm shoebox cage. After recovering from anesthesia, all animals were transferred to individual cages. An angiotensin II drinking test was used to verify the correct placement of the ICV cannula after several days of recovery from surgery. Each rat was given an ICV injection of angiotensin II (40ng/4 ul) 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 included in the experiment. After the drinking test, 27 of 30 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.

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

Hepatic gene expression determination: Hepatic tissue samples were homogenized in Trizol (Invitrogen, Carlsbad, CA) to isolate RNA, followed by cleaning the isolated RNA by an RNAeasy Mini Kit (SABiosciences, Valencia, CA). An iScript cDNA synthesis Kit (BioRad, Hercules, CA) was used to synthesize cDNA. PCR was performed using an RT² real time SYBR Green Mastermix kit (SABiosciences, Valencia, CA) to determine the expression of approximately thirty hepatic genes of interest (Table 1) on custom-made PCR arrays (SA Bioscience, Valencia, CA). The relative quantification of gene expression between groups was calculated using the delta delta Ct method with actin used as a reference gene (155).

Statistical analysis: Statistical analyses of the results were performed by using statistical computer program JMP 8.0. 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. Hormonal, fed and fasted blood glucose data, and gene expression data were determined by ANOVA without repeated measure. Again, if necessary, contrast statements were used to determine differences between specific groups. For gene expression data, statistics were performed on the delta delta Ct. However, the data were then expressed as a fold-change. P values of 0.05 or less were considered to be statistically different.

3.3 Results

Body Weight: Body weight of non-diabetic vehicle-treated rats increased gradually during the experiment, ultimately reaching ~375g compared with ~275g, the baseline body weight that was measured on the day before STZ or vehicle treatment began. Based on treatment over time, the difference in body weight between the non-diabetic vehicle-treated group and the diabetic vehicle-treated group was significant (Figure 3.1, $p < 0.001$). Body weights of diabetic leptin-treated rats were significantly less than non-diabetic vehicle-treated rats ($p < 0.01$), whereas there was no difference between the leptin-treated diabetic group and the vehicle-treated diabetic group (Figure 3.1).

Blood glucose concentrations: Daily blood glucose concentrations are shown in Figure 3.2. As expected, diabetes (STZ treatment) greatly increased the daily blood glucose concentrations as compared to nondiabetic rats ($p < 0.0001$) (~ 450 mg/dL vs ~ 125 mg/dL). Two days after initiating daily ICV leptin injections, blood glucose concentrations began to decrease in leptin-treated diabetic rats. This continued until about

day 10 at which time the blood glucose concentration leveled off at approximately 200 mg/dL. This concentration was significantly greater than the blood glucose concentrations of nondiabetic rats ($p=0.0007$), but was significantly less than the glucose concentrations of diabetic vehicle-treated rats ($p<0.0001$).

Similar to the daily blood glucose values, blood sampled prior to the fast (i.e., in the fed state) showed that blood glucose concentrations of diabetic leptin-treated rats (~275 mg/dL) were greater ($p=0.0005$) than that of nondiabetic rats (~120 mg/dL), but significantly less than diabetic vehicle-treated rats (~500 mg/dL) ($p=0.0006$) (Figure 3.3A). After fasting, the blood glucose concentration of diabetic leptin-treated rats (~95 mg/dL) was similar to that of the nondiabetic rats (~95 mg/dL). The blood glucose concentration of both the diabetic leptin-treated rats ($p=0.0012$) and nondiabetic vehicle-treated rats ($p=0.0015$) were significantly less than the diabetic vehicle-treated rats (Figure 3.3B).

Glucagon Test: In response to a large dose of IP glucagon injection (750 $\mu\text{g}/\text{kg}$), absolute blood glucose concentrations of diabetic leptin-treated rats kept in a normal range and did not increase as high as glucose concentrations of diabetic vehicle-treated rats (Figure 3.4A). Initially, blood glucose concentration of nondiabetic rats decreased by about 10% and then increased about 20% above baseline values, while blood glucose concentrations of both diabetic leptin- or vehicle-treated rats increased initially by 10-20% (Figure 3.4B). In the 150 min post injection period, blood glucose concentration of diabetic leptin-treated rats decreased by about 20% of baseline values compared with diabetic vehicle-treated rats, which remained around baseline values without significant change.

In response to IP saline injection, absolute blood glucose levels of diabetic vehicle-treated rats was around 300 mg/dl; the value decreased from about 110 mg/dl to 50 mg/dL for the diabetic leptin-treated group, while non-diabetic vehicle-treated rats was around 100 mg/dL (Figure 3.5A). The plasma glucose decreased to 60% of baseline value in the diabetic leptin-treated group, while the value of vehicle-treated groups was stable around baseline value or slightly below (Figure 3.5B).

In the non-diabetic group, glucagon injection initially decreased blood glucose concentrations by about 10%, and then increased 20% above baseline value by 90 minutes post-injection. The blood glucose concentrations remained at this level for the remainder of the study. Blood glucose concentrations of non-diabetic rats that received IP saline injections remained around the baseline value or slightly lower. (Figure 3.6A). In the diabetic vehicle-treated group, glucose values after glucagon injection increased about 10% at first, but then decreased to around baseline value. Saline injection in this group of rats resulted in a slight decrease in blood glucose concentration as compared to baseline values (Figure 3.6B). In the diabetic leptin-treated group, though blood glucose level increased about 20% after IP glucagon injection, it decreased to 70% of baseline values by the end of the study. A parallel decrease in blood glucose concentrations was seen in rats that received an IP saline injection (Figure 3.6C).

Serum glucagon concentration: Diabetic rats had greater concentrations of serum glucagon than did nondiabetic rats, regardless of whether the diabetic rats were treated with vehicle (p=0.02) or leptin (p=0.002). Interestingly, leptin treatment of diabetic rat did not alter the concentration of serum glucagon as compared diabetic vehicle-treated rats (Figure 3.7).

Hepatic gene expression: Table 1 shows the genes of interest that were determined in the nondiabetic vehicle-treated, diabetic vehicle-treated, and diabetic leptin-treated rats. Two housekeeping genes were determined: actin and ribosomal protein P1. Six genes had an expression pattern similar to the pattern of the fasting blood glucose concentrations. Specifically, the gene expression increased in diabetic vehicle-treated rats as compared to nondiabetic vehicle-treated rats, and that the increase was negated in diabetic rats treated with leptin.

The gene expression of two gluconeogenic enzymes, glucose 6-phosphatase (G6pc3) and phosphoenolpyruate carboxykinase (PEPCK) (Pck1) appeared to be increased in diabetic vehicle-treated rats as compared to nondiabetic vehicle-treated rats, while the effect was negated in diabetic rats treated with leptin (Figure 3.8). Though the gene expression of Pck1 in diabetic vehicle-treated rats was not statistically greater than that of nondiabetic vehicle-treated rats ($p > 0.05$), the average gene expression was approximately 2.5-fold greater in the diabetic vehicle-treated rats than the nondiabetic rats. A similar pattern was observed with the gene expression of the soluble form of malate dehydrogenase (Mdh1) (Figure 3.9). Although malate dehydrogenase is not exclusively used in gluconeogenesis, it can be part of the gluconeogenic pathway between pyruvate carboxylase and PEPCK. The gene expression of two transcription factors known to be involved in intracellular signaling of the gluconeogenic pathway, peroxisome proliferator-activated receptor gamma coactivator 1 alpha (Ppargc1a) (PGC1a) and cAMP responsive element binding protein 1 (Creb1) appeared to be increased in diabetic vehicle-treated rats as compared to nondiabetic vehicle-treated rats, while the effect appeared to be negated in diabetic rats treated with leptin (Figure 3.10A and 3.10B,

respectively). Interestingly, the gene expression for insulin receptor substrate-2 (IRS-2) also followed the same pattern. Hepatic IRS-2 gene expression was increased in diabetic vehicle-treated rats as compared to nondiabetic vehicle-treated rats. The increase in IRS-2 gene expression was negated in diabetic rats by leptin treatment (Figure 3.11).

The fold change for the remainder of the genes examined in the three groups of rats is shown in Table 2. For most of these genes, there were no statistical differences between groups. Four genes did show group differences, though the pattern was different than the genes discussed above. The gene expression of hepatocyte nuclear factor 2 alpha (HNF4a) was increased in diabetic vehicle-treated rats as compared to nondiabetic vehicle-treated ($p < 0.03$), however, the increase in gene expression was not negated by leptin treatment. The gene expression of pyruvate carboxylase (Pc) was decreased in diabetic vehicle-treated rats as compared to nondiabetic vehicle-treated rats ($p < 0.02$). Leptin treatment of diabetic rats negated this inhibition bringing the gene expression back to the level of nondiabetic vehicle-treated rats. The gene expression of inhibitor of kappa light polypeptide gene enhancer in β -cells, kinase beta (Ikk β) and nuclear factor of kappa light polypeptide gene enhancer in β -cells 1 (Nfkb1) were increased in diabetic rats as compared to nondiabetic rats. When the two groups of diabetic rats (vehicle- and leptin-treated) were combined, the gene expression was significantly increased as compared to that of nondiabetic rats for both Ikk β ($p < 0.017$) and Nfkb1 ($p < 0.015$).

3.4 Discussion

In the present study, we confirmed that chronic central leptin administration normalizes blood glucose concentrations in diabetic rats. This has previously been

observed in our laboratory (18, 133), as well by others (19, 132). While daily blood glucose concentrations of diabetic leptin-treated rats (in the fed state) did not quite decrease to the level of nondiabetic rats, they were significantly decreased compared to diabetic vehicle-treated rats. However, leptin treatment decreased fasting blood glucose concentrations of diabetic rats to the level of nondiabetic rats (Figure 3.3). Our laboratory has previously shown that leptin treatment, in both diabetic and nondiabetic rats, has an enhanced effect to reduce blood glucose concentrations after a 6-hour fast as compared to vehicle-treated rats (18). In that study, blood glucose concentrations of leptin-treated fasted rats were about 50% of that of the same rats in the fed state, while the blood glucose concentrations of vehicle-treated rats were not significantly changed from that of the fed state. Interestingly, in the present study, after the leptin-treated diabetic rats fasted for 6 hours, blood glucose concentrations also decreased by about 50%. (The difference in fasting times between leptin- and vehicle-treated rats was used to prevent hypoglycemia from potentially developing in the leptin-treated rats).

Normally, gluconeogenesis is increased during fasting to compensate for the lack of glucose absorption from the GI tract. Gluconeogenesis is partially responsible for the secretion of glucose into the circulation during a fast to help maintain glucose homeostasis. In addition to its effect during fasting, an increase in gluconeogenesis also appears to help drive hyperglycemia during diabetes. An increase in gluconeogenesis appears to account for about 90% of the increased hepatic glucose production in type 2 diabetic humans (156). Since glucagon plays a crucial role in gluconeogenesis, we hypothesized that leptin suppresses gluconeogenesis to lower blood glucose

concentrations by either inhibiting serum glucagon concentrations or inhibiting glucagon responsiveness.

A large dose of intraperitoneal (IP) glucagon (750 ug/kg) caused an initial (30 minute post-injection) decrease in blood glucose concentration in the nondiabetic rats. The decrease was most likely due to a compensatory release of insulin. Rao (1995) demonstrated that plasma glucose levels of fasted rats began to increase within several minutes after an IV injection of glucagon (20 ug/kg), but started to return to normal after 15 minutes (157). In Rao's study, plasma insulin levels increased within minutes in response to the early rise in blood glucose, which then helped to bring glucose levels back to normal. Therefore, it is likely we missed an early increase in blood glucose due to the glucagon injection, and that this caused a rapid increase in the release of insulin, which countered the effect of the glucagon, driving the blood glucose concentration lower. This is supported by the findings that the initial decrease in blood glucose was absent in both of the diabetic groups, which would not be able to increase the release of insulin. Instead in the diabetic rats, glucagon injection initially increased blood glucose by about 10-20% at 30 minutes post-injection. These values were significantly greater than the blood glucose concentrations when the respective rats were given an injection of saline, so this increase is due to the effects of glucagon (Figure 3.6). As time past, the blood glucose response of the three groups of rats injected with glucagon varied. The percentage increase in blood glucose concentrations was greatest in the nondiabetic rats. These rats were 20% above baseline from 90-150 post-injection. The change in blood glucose response to glucagon in diabetic vehicle-treated rats was rather flat, with the exception of during the first 30 minutes post-injection. In contrast, the change in blood

glucose in diabetic leptin-treated rats injected with glucagon decreased during the 2.5-hour post-injection period, with the exception of during the first 30 minutes. A similar rate of decrease in blood glucose concentration was also observed in the leptin-treated diabetic rats that received IP saline. This suggests that leptin treatment induced a metabolic environment that decreases blood glucose concentration during a fast, even after a large dose of glucagon.

Serum glucagon concentrations were not different between diabetic vehicle-treated and diabetic leptin-treated rats and both groups of diabetic rats had serum glucagon concentrations that were significantly greater than the nondiabetic vehicle-treated rats (Figure 3.7). This suggests that the mechanism by which leptin lowered blood glucose concentrations was not by decreasing serum glucagon concentrations, but rather the data are consistent with leptin decreasing the responsiveness to glucagon. Others have found leptin treatment in type 1 diabetic NOD mice to be associated with normalization of hyperglycemia and with a decrease in serum glucagon concentrations (137, 158). In these studies, leptin was delivered by an adenovirus, which produced very high concentrations of peripheral leptin. It is possible that peripheral administered leptin may have an effect on serum glucagon concentrations, whereas central leptin administration has an effect on glucagon responsiveness. However, similar results were also found using STZ-induced diabetic Wistar rats, in which leptin was given intracerebroventricularly (German et al. 2011), as was the case in the present study. One difference from the present study was that we gave daily ICV bolus injections of leptin, whereas ICV leptin was given by an osmotic minipump in the study by German et al. (2011) (132). A daily bolus injection may more closely mimic a circadian rhythm of leptin across time, whereas an osmotic

minipump or a large peripheral dose could potentially saturate leptin receptors throughout the day. In other 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 (159). This suggests that correcting high plasma glucagon concentrations doesn't necessarily lead to a normalization of blood glucose concentrations in diabetic rats.

To better understand the metabolic environment under which glucagon was administered, we determined the expression of several hepatic genes of interest under the same fasting conditions that glucagon administered. While not always statistically significant, the overall tendency was for gluconeogenic enzymes, transcription factors, and coactivators to be increased in diabetic vehicle-treated rats as compared to nondiabetic vehicle-treated rats, and for leptin treatment of diabetic rats to bring these back to the levels of the nondiabetic controls. This was true for the gluconeogenic enzymes glucose 6 phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) and the gluconeogenic transcription factors PGC1a and Creb1. Others have found similar results with PEPCK (Yu et al. 2008, German et al. 2010, Wang et al. 2010, German et al. 2011), G6Pase (German et al. 2011), and PGC1a (Yu et al. 2008) in that they are increased in uncontrolled diabetic rats and reduced with leptin treatment (132, 137, 158). Unger's group has found a leptin-induced decrease in Creb1 phosphorylation in diabetic rats, suggesting that there is less cAMP signaling in the livers of leptin-treated rats (137, 158). Glucagon is normally thought to enhance the intracellular production of cAMP. We have also found similar pattern with the gene expression of the cytoplasmic form of malate dehydrogenase. Though this is not exclusively a gluconeogenic enzyme, it

can act between pyruvate carboxylase and PEPCK to convert malate to oxaloacetate, which is then converted to phosphoenolpyruvate by PEPCK. Interestingly, the gene expression of IRS-2, which is part of the insulin-signaling pathway, followed the same pattern of being increased in diabetic vehicle-treated rats and normalized in diabetic leptin-treated rats. IRS-2 is stimulated during a fast by Creb1 and its coactivator Crtc2 (160). Though this initially seems counterintuitive, the induction of IRS-2 during times of increased glucose production is thought to be critical for glucose homeostasis, which limits glucose output from the liver during a fast. In this light, the induction of IRS-2 may be a good indicator of the activation of the gluconeogenic program. Interestingly, there was not a significant change in the gene expression of Crtc2 itself (Table 2).

In conclusion, the present study supported the hypothesis that chronic central leptin administration normalizes blood glucose concentrations of diabetic rats at least in part by inhibiting gluconeogenesis. Leptin-treated diabetic rats were associated with normalized levels of several gluconeogenic genes, transcription factors, and coactivators. Previous studies have shown the effect of leptin is not related to decreased feeding, enhanced insulin secretion, or increased urinary glucose excretion (132). Despite the leptin-induced normalization in these gluconeogenic factors, serum glucagon concentrations were not different from diabetic vehicle-treated rats, suggesting that central leptin treatment decreases the hepatic responsiveness to glucagon. This could explain why blood glucose concentrations decreased over time in leptin-treated diabetic rats even after being given a large dose of exogenous glucagon. If central leptin is able to have these effects on glucose homeostasis, there may also be implications for glucose homeostasis under conditions of leptin resistance as is thought to occur during obesity.

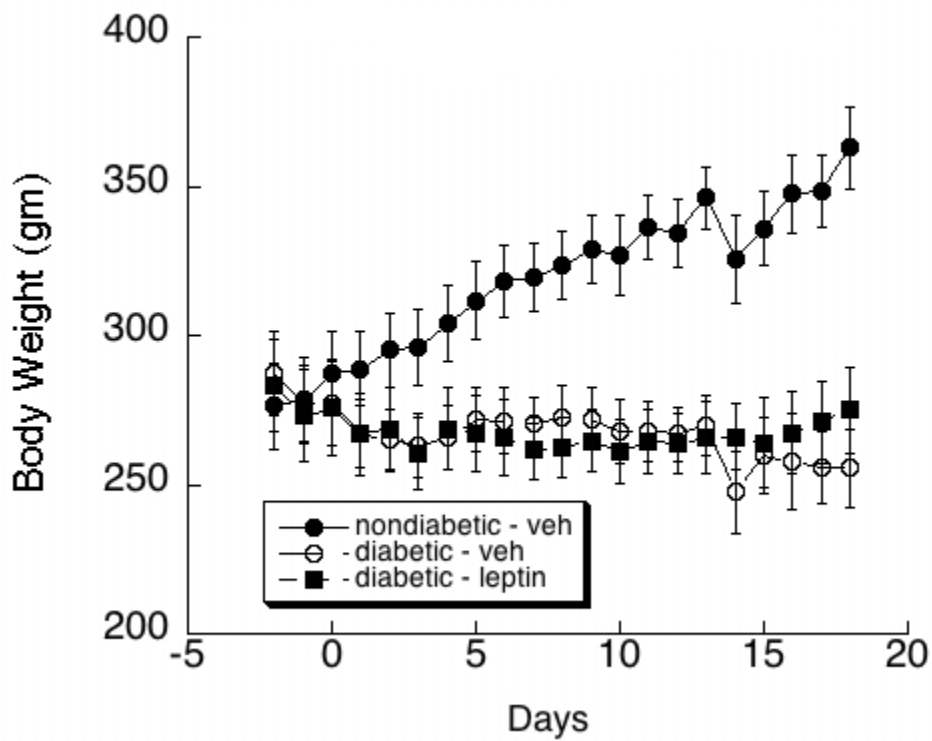


Figure 3.1 Body weights of non-diabetic vehicle-treated(●), diabetic vehicle-treated(○), diabetic leptin-treated rats(■). Day -2 stands for STZ injection while day 0 refers to begin ICV leptin treatment. Within group, no difference was found between diabetic leptin-treated group and diabetic vehicle-treated group, whereas leptin-treated diabetic rats had significant lower body weight than non-diabetic rats ($p < 0.01$).

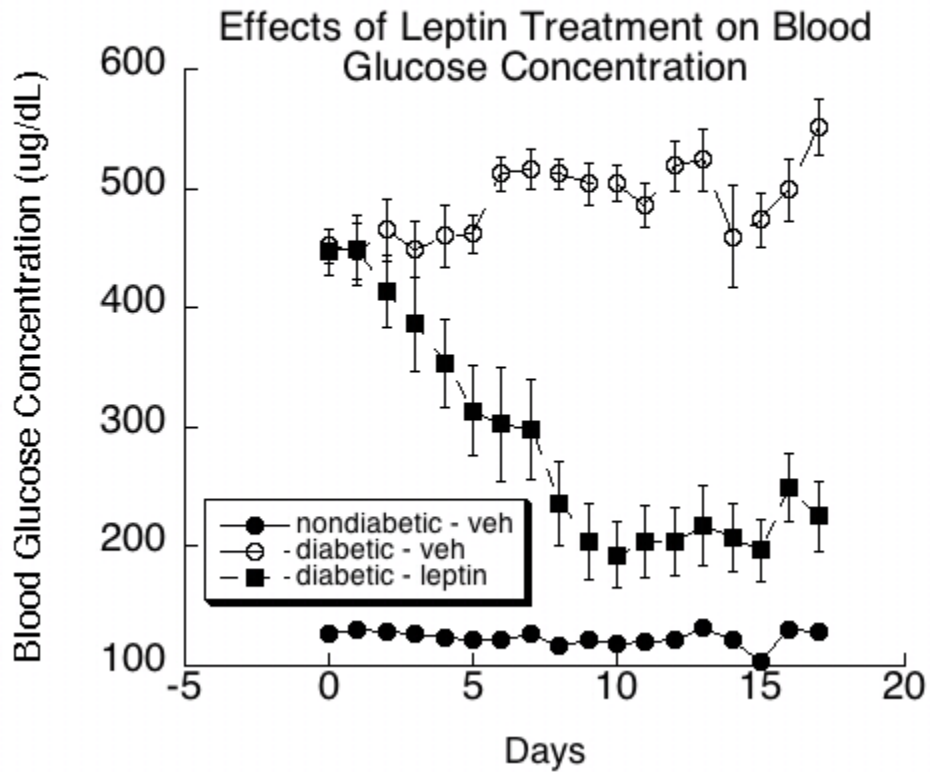
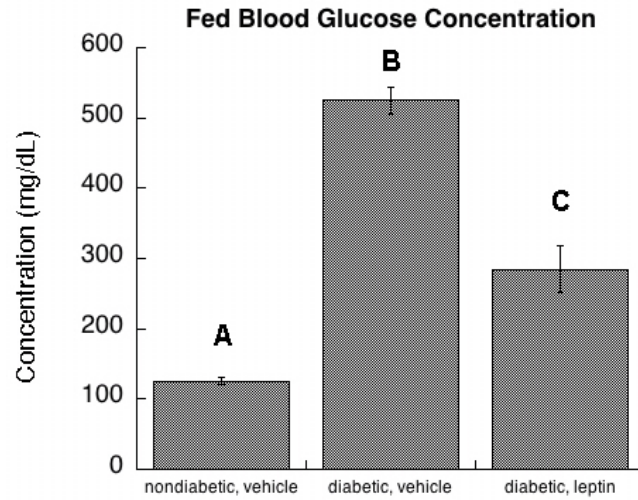


Figure 3.2 Effects of daily leptin or vehicle treatment on blood glucose concentration of both diabetic and non-diabetic rats, including non-diabetic vehicle-treated rats (●), diabetic vehicle-treated (○), diabetic leptin-treated rats (■). Day 0 began to inject leptin or vehicle. Within group over time, blood glucose concentrations were significantly different between diabetic vehicle-treated rats and diabetic leptin-treated rats ($p < .0001$). There was a significant difference between the non-diabetic vehicle-treated group and the diabetic vehicle-treated group ($p < .05$).

A



B

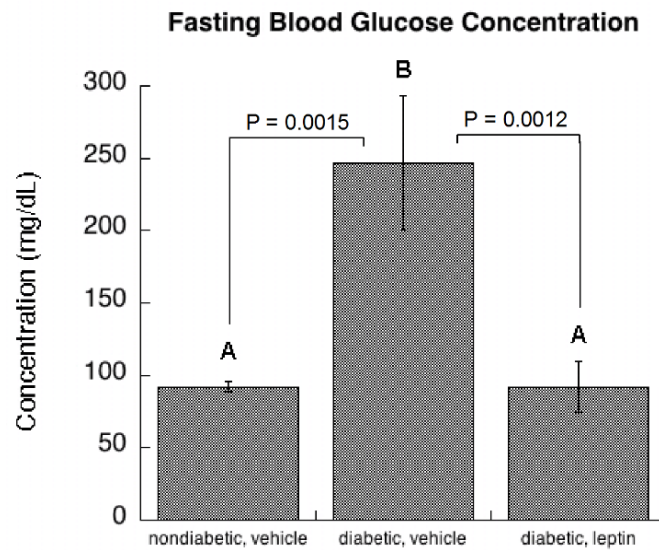
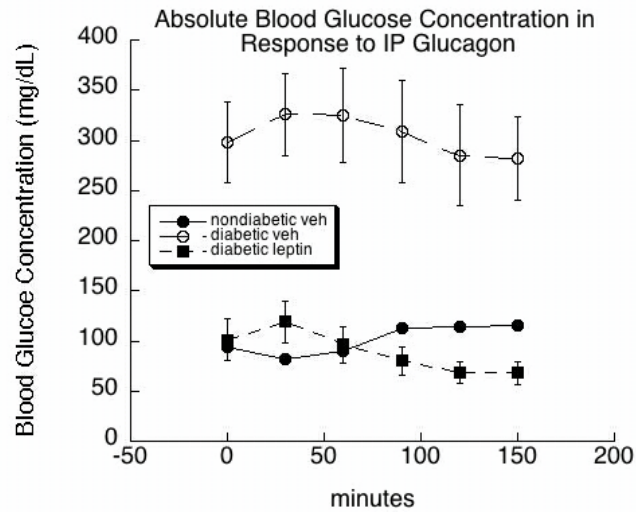


Figure 3.3 Blood glucose concentrations of non-diabetic vehicle-treated rats, diabetic vehicle-treated rats, and diabetic leptin-treated rats in fed the fed (A) and fasted (B) states. Mean are shown \pm SEM. Each mean represents 8-10 observations. Means with different letters are statistically different. Diabetic vehicle-treated rats had greater blood glucose

concentrations than nondiabetic vehicle-treated rats. Leptin treatment decreased blood glucose concentrations in diabetic rats.

A



B

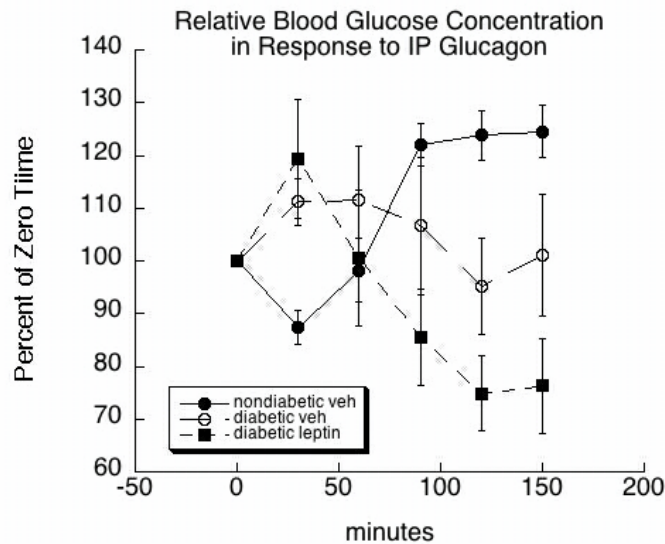
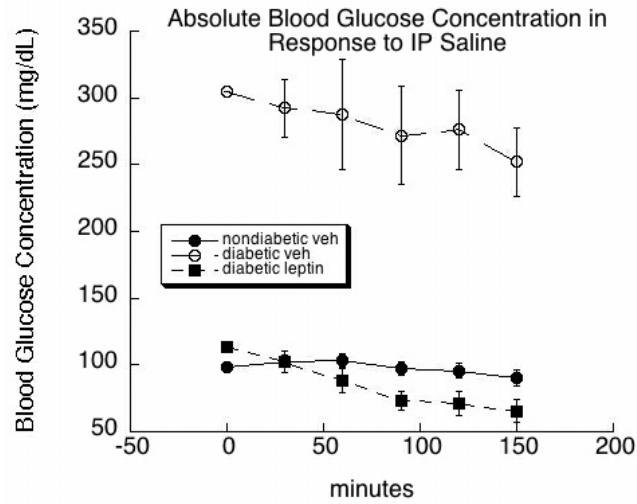


Figure 3.4 Absolute (A) and relative (B) values of blood glucose concentration in non-diabetic vehicle-treated rats (●), diabetic vehicle-treated (○), diabetic leptin-treated rats (■) in response to IP glucagon injection after fasting. Minutes 0 shows the baseline value of blood glucose concentration. Means are shown \pm SEM and represent 8-10 observations.

A



B

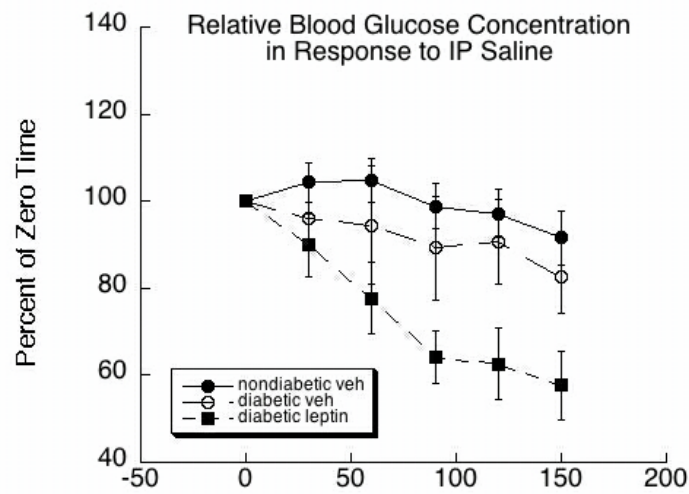
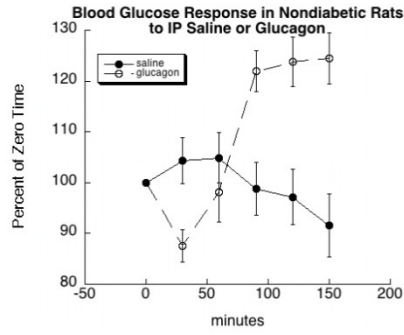


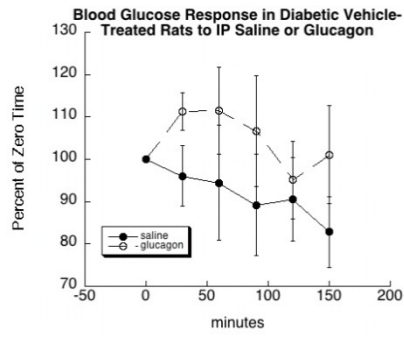
Figure 3.5 4 Absolute (A) and relative (B) values of blood glucose concentration in non-diabetic vehicle-treated rats (●), diabetic vehicle-treated (○), diabetic leptin-treated rats (■) in response to IP saline injection after fasting. Minutes 0 shows the baseline value

of blood glucose concentration. Means are shown \pm SEM and represent 8-10 observations.

A



B



C

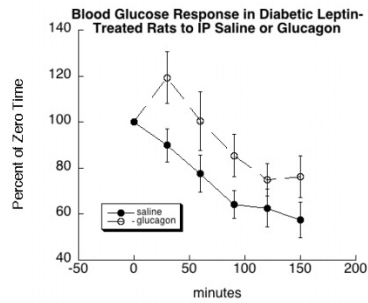


Figure 3.6 Relative blood glucose response to IP glucagon vs saline in A) nondiabetic vehicle-treated, B) diabetic vehicle-treated, and C) diabetic leptin-treated rats. Means are shown \pm SEM. Each mean represents the average of 8-10 observations.

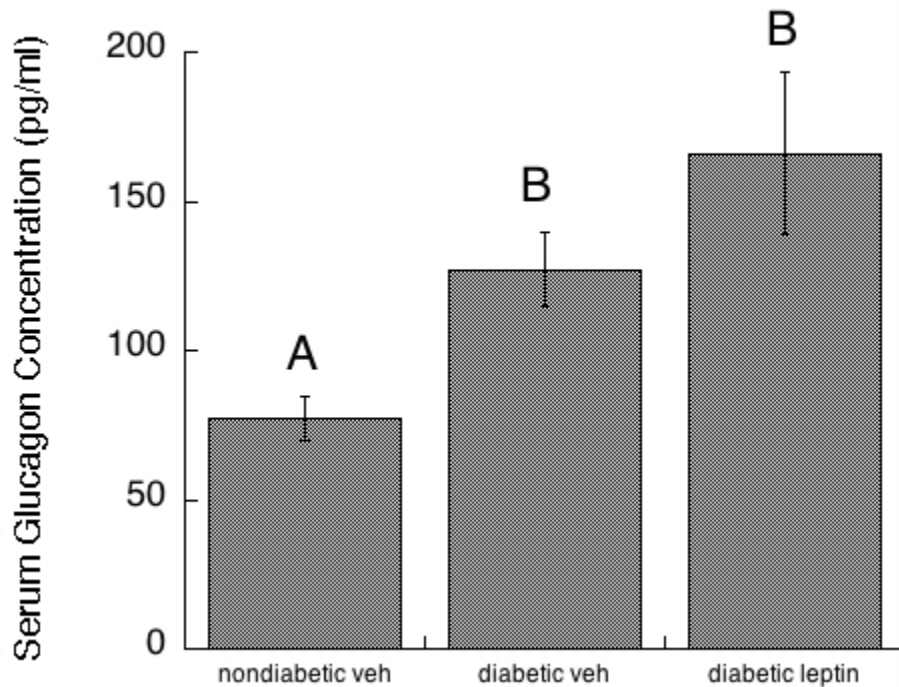
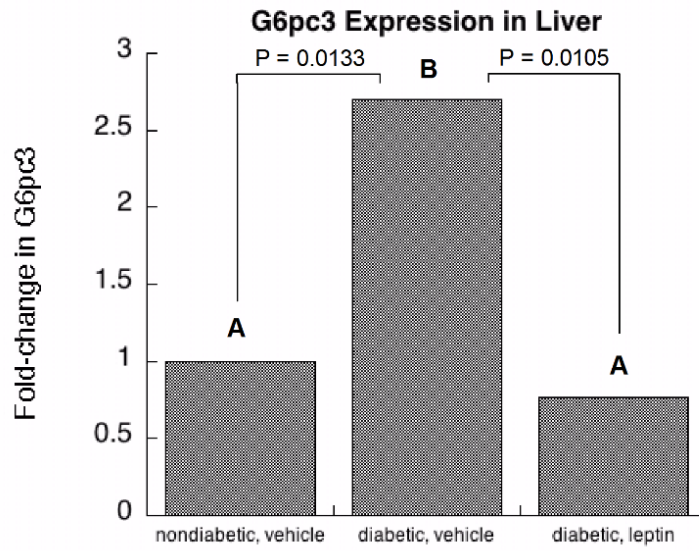


Figure 3.7 Serum glucagon concentrations of non-diabetic vehicle-treated, diabetic vehicle-treated, and diabetic leptin-treated rats after a fast. Means are shown \pm SEM. Each mean represents the average of 8-10 observations. Serum glucagon concentrations of diabetic vehicle-treated rats were greater than that of non-diabetic vehicle-treated rats. Leptin treatment of diabetic rats did not decrease the concentration of serum glucagon.

Table 1: Genes Examined in PCR arrays

Symbol	Gene
Rab4a	RAB4A,member RAN oncogene family
Icam1	Intercellular adhesion molecule 1
Gcgr	Glucagon receptor
Dpp4	Dipeptidylpeptidase 4
G6pc3	Glucose 6 phosphatase, catalytic, 3
Gck	Glucokinase
Nos3	Nitric oxide synthase 3, endothelial cell
Il12b	Interleukin 12b
Ikbkb	Inhibitor of kappa light polypeptide gene enhancer in B-cells, Kinase beta
Srebf1	Sterol regulatory element binding transcription factor 1
Hnf4a	Hepatocyte nuclear factor 4, alpha
Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
Ppargc1a	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha
Creb1	CAMP responsive element binding protein 1
Crtc2	CREB regulated transcription coactivator 2
Fbp1	Fructose-1,6 biphosphatase 1
Pc	Pyruvate carboxylase
Pck1	Phosphoenolpyruvate carboxykinase 1(soluble)
Pck2	Phosphoenolpyruvate carboxykinase 2(mitochondrial)
Gsk3a	Glycogen synthase kinase 3 alpha
Gsk3b	Glycogen synthase kinase 3 beta
Irs2	Insulin receptor substrate 2
Pcaf	P300/CBP-associated factor
Mdh1	Malate dehydrogenase 1, NAD(soluble)
Mdh2	Malate dehydrogenase 2, NAD(mitochondrial)
Pygl	Phosphorylase, glycogen, liver
Gys2	Glycogen synthase 2
Actb	Actin, beta
Rplp1	Ribosomal protein, large, P1

A



B

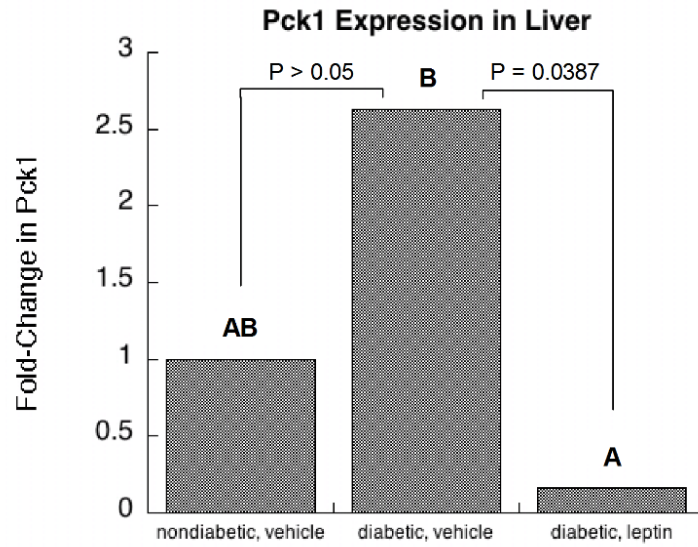


Figure 3.8 Gene expression of gluconeogenic enzymes glucose 6-phosphatase (G6pc3) (A) and PEPCK (Pck1) (B) in the livers of non-diabetic vehicle-treated, diabetic vehicle-treated, and diabetic leptin-treated rats following a fast. Means are expressed as the fold-change relative to the mean of the nondiabetic vehicle-treated group. Each mean represents 8-9 observations. Means with different letters are statistically different. Overall, the gene expression seemed to increase in diabetic vehicle-treated rats compared to nondiabetic rats, and leptin treatment appeared to reverse this effect.

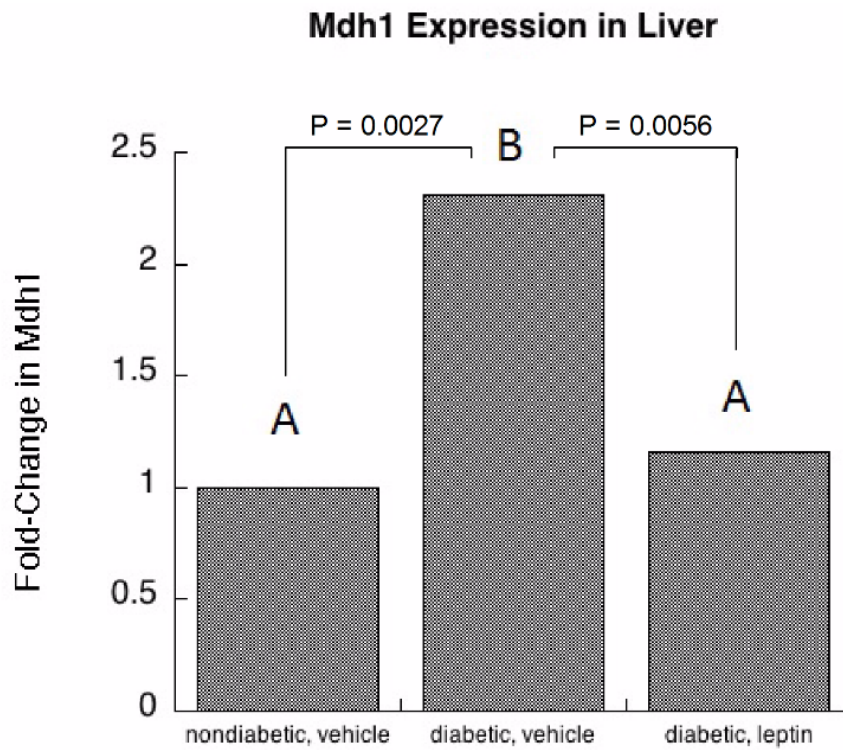
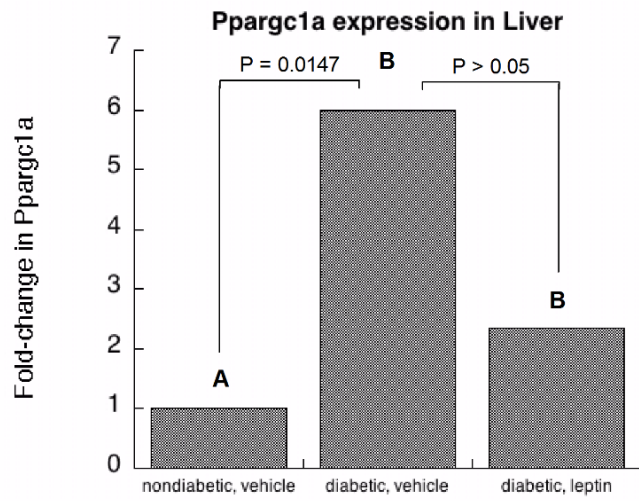


Figure 3.9 Gene expression of cytoplasmic malate dehydrogenase (Mdh1) in the livers of non-diabetic vehicle-treated, diabetic vehicle-treated, and diabetic leptin-treated rats. Means are expressed as the fold-change relative to the mean of the nondiabetic vehicle-treated group. Each mean represents 8-9 observations. Means with different letters are statistically different. The gene expression increased in diabetic vehicle-treated rats compared to nondiabetic rats, and leptin treatment reversed this effect. While the enzyme is not exclusively used in gluconeogenesis, it is used in the pathway of gluconeogenesis between pyruvate carboxylase and PEPCK.

A.



B.

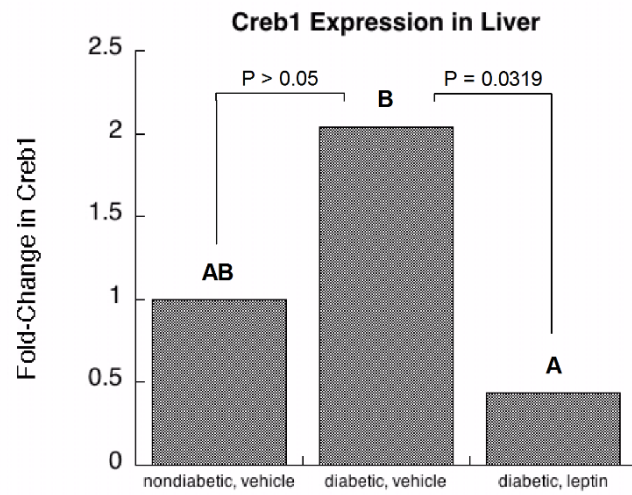


Figure 3.10 Gene expression of transcription factors involved in the gluconeogenic pathway, PGC1a (Pparg1) (A) and Creb1 (B) in the livers of non-diabetic vehicle-treated, diabetic vehicle-treated, and diabetic leptin-treated rats following a fast. Means are expressed as the fold-change relative to the mean of the nondiabetic vehicle-treated group. Each mean represents 8-9 observations. Means with different letters are statistically different. Overall, the gene expression seemed to increase in diabetic vehicle-treated rats compared to nondiabetic rats, and leptin treatment appeared to reverse this effect.

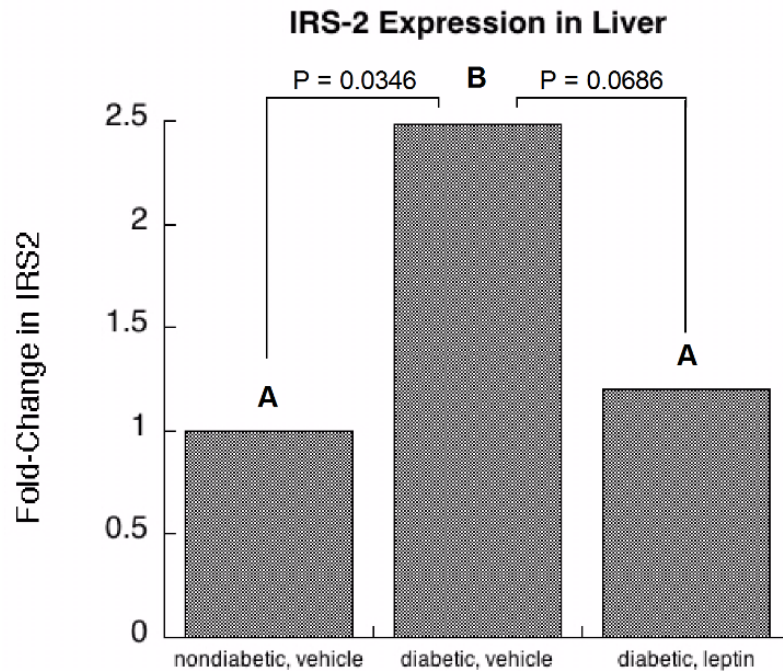


Figure 3.11 Gene expression of IRS-2 in liver of non-diabetic vehicle-treated, diabetic vehicle-treated and diabetic leptin-treated rats following a fast. Means are expressed as the fold-change relative to the mean of the nondiabetic vehicle-treated group. Each mean represents 8-9 observations. Means with different letters are statistically different. The gene expression increased in diabetic vehicle-treated rats compared to nondiabetic rats, and leptin treatment appeared to reverse this effect.

Table 2: Hepatic gene expression of diabetic and leptin-treated rats following a fast.

Gene	Nondiabetic, vehicle	Diabetic, vehicle	Diabetic, leptin
Crtc2	1.000 ^A	1.526 ^A	1.956 ^A
Dpp4	1.000 ^A	1.441 ^A	1.483 ^A
Fbp1	1.000 ^A	1.425 ^A	1.413 ^A
Gcgr	1.000 ^A	0.983 ^A	1.184 ^A
Gck	1.000 ^A	0.597 ^A	0.946 ^A
Gsk3a	1.000 ^A	1.219 ^A	1.233 ^A
Gsk3b	1.000 ^A	0.913 ^A	1.055 ^A
Gys2	1.000 ^A	1.808 ^A	1.162 ^A
Icam	1.000 ^A	1.103 ^A	0.931 ^A
Il12b	1.000 ^A	1.001 ^A	2.379 ^A
Mdh2	1.000 ^A	1.108 ^A	0.987 ^A
Nos3	1.000 ^A	0.508 ^A	0.545 ^A
Pcaf	1.000 ^A	0.582 ^A	0.574 ^A
Pck2	1.000 ^A	0.596 ^A	0.384 ^A
Pyg1	1.000 ^A	1.052 ^A	1.371 ^A
Rab4	1.000 ^A	1.188 ^A	0.867 ^A
Srebf1	1.000 ^A	1.811 ^A	0.416 ^A
Hnf4a	1.000 ^A	1.901 ^B	1.464 ^{A,B}
Pc	1.000 ^A	0.660 ^B	1.108 ^A

Ikkbb	1.000 ^A	2.550 ^B	1.760 ^{A,B}
Nfkb1	1.000 ^A	1.526 ^{A,B}	1.956 ^B

Values are mean fold-change relative to the nondiabetic vehicle-treated rats. Each value represents the average of 8-9 observations. Statistical comparisons were performed on the delta delta Ct values. Within rows, means with different superscripts are statistically different ($p < 0.05$).

CHAPTER IV

CONCLUSIONS

Chronic intracerebroventricular leptin administration has been shown to normalize blood glucose concentrations in STZ-induced diabetic rats (18, 19, 133). In the present study, daily blood glucose concentrations of diabetic leptin-treated rats decreased significantly compared to diabetic vehicle-treated rats. Furthermore, blood glucose concentrations of diabetic leptin-treated rats were completely normalized to the level nondiabetic following a fast. Generally, gluconeogenesis is stimulated in response to low blood glucose levels and is considered to be the major metabolic source of glucose during a moderate fast. This suggests that that leptin could lower blood glucose concentration by inhibiting gluconeogenesis. Since glucagon plays a key role in gluconeogenesis activation, leptin might either decreased serum glucagon concentrations or inhibit the responsiveness to glucagon, resulting in suppression of gluconeogenesis. Serum glucagon concentrations were determined in diabetic rats treated with either vehicle or leptin, and in nondiabetic rats. While serum glucagon concentrations were increased in diabetic rats as compared to nondiabetic, leptin treatment did not decrease serum glucagon concentrations in the diabetic rats. Therefore, under our experimental conditions, it appears that chronic central leptin treatment does not inhibit serum glucagon

concentrations to inhibit gluconeogenesis and blood glucose concentrations. In vivo administration of a high dose of serum glucagon did not increase the blood glucose concentration of diabetic leptin-treated rats to the level of diabetic vehicle-treated rats, suggesting the effect of glucagon may have been blunted in the leptin-treated rats. The expression of various genes of interest was determined in the three groups of rats after a fast to determine the enzymatic and intracellular signaling background upon which the exogenous glucagon was administered. Leptin treatment prevented the increased gene expression of Pck1 and G6pc3 in diabetic rats, which encode the key gluconeogenic enzymes of PEPCK and glucose-6-phosphatase, respectively. Leptin also prevented the increased gene expression of Mdh1, which encodes for the soluble form of malate dehydrogenase. Although malate dehydrogenase is not exclusively used in gluconeogenesis, it is involved in the interconversion of oxaloacetate and malate, which can be used to convert lactate and some amino acids into phosphoenolpyruvate via gluconeogenesis. Similar results were found for the expression of Creb1 and Pgc1a, two transcription factors involved in the signal transduction of the gluconeogenic pathway. The gene expression of IRS-2 followed the same pattern. Evidence suggests that the gene expression of IRS-2 reflects the activation of Crtc2, a coactivator of Creb1 (160-162). This is important in transducing the signal from glucagon-stimulated intracellular cAMP production to activate gluconeogenesis. The present study provides several examples where enzymes, transcription factors, and coactivators of the gluconeogenic pathway that are normally activated by glucagon are inhibited leptin treatment. Therefore, the present results suggest that chronic central leptin administration normalizes blood glucose by suppressing the responsiveness to circulating glucagon to

inhibit the gluconeogenic pathway and suppressing hepatic glucose output, without changing serum glucagon levels.

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