**Blood Glucose Concentrations Are Not Increased by Chronic IP Glucagon Administration in Leptin-Treated Type 1 Diabetic Rats**

by

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## Abstract

The overall objective of this research is to increase our understanding of how central leptin administration normalizes blood glucose concentrations independent of insulin in type 1 diabetic rats. This research may lead to new insulin-independent treatments for diabetes. It is widely accepted that leptin administration into the brain of previously uncontrolled diabetic animals can normalize blood glucose concentrations, independent of insulin. The mechanism by which central leptin administration normalizes blood glucose concentrations in diabetic animals is not understood. Some studies have suggested that leptin acts by decreasing the serum concentration of glucagon. However, we hypothesize that leptin decreases the responsiveness of glucagon by inhibiting cyclic adenosine monophosphate (cAMP) signaling in the liver. This would decrease gluconeogenesis in the liver, resulting in a reduction in hepatic glucose output, normalizing blood glucose concentrations.

To test this hypothesis, we performed a study to directly examine whether intracerebroventricular (ICV) leptin administration blocks the ability of chronically high doses of glucagon (delivered via an intraperitoneal (IP) osmotic pump) to increase blood glucose concentrations in streptozotocin (STZ)-induced diabetic rats. Two cohorts of rats were used. Four groups of diabetic rats were examined within each cohort: 1) leptin-treated (ICV), glucagon-treated (IP), 2) leptin-treated (ICV), vehicle-treated (IP), 3) vehicle-treated (ICV), glucagon-treated (IP), and 4) vehicle-treated (ICV), vehicle-treated (IP). The change in blood glucose concentration of the four groups was determined on a daily basis and during three

different conditions. The three conditions were 1) at various times over the circadian cycle, 2) during an 8-hour fast, and 3) following the IP injection of pyruvate (to determine the rat's gluconeogenic capacity).

As we have seen previously, leptin treatment normalized blood glucose concentrations in diabetic rats. Our new findings showed that chronic glucagon treatment did not increase blood glucose concentrations of leptin-treated diabetic rats. This was true whether based on the concentration of daily blood glucose, the blood glucose concentration around the circadian cycle, the blood glucose concentration during an 8-hour fast, or the blood glucose concentration in response to an injection of pyruvate. This lack of difference was observed despite the fact that serum glucagon concentrations were 4-9-fold greater in glucagon-treated rats as compared to vehicle-treated rats. Leptin treatment decreased phosphoenolpyruvate carboxykinase (PEPCK)/beta-actin content in the liver. We also saw a decrease in hepatic total cAMP-response element binding protein (CREB) with leptin treatment, but not in phospho-CREB (Ser133). This suggests that chronic leptin treatment decreases the cAMP signaling pathway in the liver, resulting in a decrease in gluconeogenesis. Our data also suggests that the serum glucagon concentration does not have to be reduced in order for leptin to normalize blood glucose concentrations. This supports the hypothesis that leptin treatment acts by decreasing the responsiveness to glucagon, rather than by decreasing circulating glucagon concentrations.

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## List of Abbreviations

| | |
|---|---|
| ALT | Alanine transaminase |
| AgRP | Agouti-related peptide |
| ANOVA | One-way analysis of variance |
| cAMP | Cyclic adenosine monophosphate |
| CRE | Cyclic adenosine monophosphate-response element |
| CREB | Cyclic adenosine monophosphate response element binding protein |
| DG | Diglyceride |
| DKA | Diabetic ketoacidosis |
| EE | Energy expenditure |
| eIF2B | Guanine nucleotide exchange factor for eukaryotic initiation factor 2 |
| F2, 6BP | Fructose-2, 6-bisphosphate |
| G6Pase | Glucose 6-phosphatase |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GLUT2 | Glucose transporter 2 |
| GLUT4 | Glucose transporter 4 |
| GPCR | G protein-coupled receptor |
| GPD1 | Glycerol-3-Phosphate Dehydrogenase 1 |
| GSK3 | Glycogen synthase kinase 3 |
| HPA | Hypothalamic-pituitary-adrenal |

| | |
|---|---|
| HS-Lipase | Hormone-sensitive lipase |
| ICV | Intracerebroventricular |
| IP | Intraperitoneal |
| IRS | Insulin receptor substrate family |
| JAK/STAT3 | Janus Kinase-Signal Transducer and Activator of Transcription-3 |
| LDH | Lactate dehydrogenase |
| mTOR | Mechanistic target of rapamycin |
| NPY | Neuropeptide Y |
| NTS | Nucleus tractus solitarius |
| ObRa | Short leptin receptor isoform |
| ObRb | Long leptin receptor isoform |
| p-CREB | Phospho-cyclic adenosine monophosphate response element binding protein |
| PARP | Poly-ADP-ribose polymerase |
| PDK1 | Protein kinase 3-phosphoinositide dependent protein kinase 1 |
| PEPCK | Phosphoenolpyruvate carboxykinase |
| PGAM1 | Platinum Grove Asset Management; Phosphoglyceric Acid Mutase 1 |
| PGI | Phosphoglucose isomerase |
| PGK1 | Phosphoglycerate Kinase 1 |
| PI3K | Phosphoinositide-3 kinase |
| PIP3 | Phosphatidylinositol (3, 4, 5)-triphosphate |
| PKA | Protein kinase A |
| PKB | Protein Kinase B |
| PMSF | Phenylmethanesulfonyl fluoride |

| | |
|---|---|
| PTB | Phosphotyrosine-binding |
| SDS | Sodium dodecyl sulfate |
| SEM | Standard error of the mean |
| SREBP | Sterol regulatory element-binding protein |
| STZ | Streptozotocin |
| T1D | Type 1 diabetes |
| T2D | Type 2 diabete |
| TBS | Tris-buffered saline |
| TCA | Tricarboxylic acid |
| TG | Triglyceride |
| α-MSH | Alpha melanocyte-stimulating hormone |

## Chapter 1: Introduction

Diabetes is a problem that causes blood glucose concentrations to increase more than normal. This is also called hyperglycemia (1). Diabetes is classified into 3 types: type 1 diabetes (T1D), type 2 diabetes (T2D), and gestational diabetes (2). T1D and T2D have high blood glucose concentrations that can cause serious health problems, including ketoacidosis, kidney failure, heart disease, stroke, and blindness (3). According to the data from the National Diabetes Statistics Report released in 2014, 29.1 million Americans had diabetes in 2012, which is 9.3% of the population (4). Diabetes affects about 285 million people worldwide, which is about 6.4% of the population (5). T1D incidence has been rising globally during the past decades by 5.3% and it accounts for 5-10% of the cases of diabetes (6). If the trend continues, predicted new cases of T1D in children younger than 5 years would double between 2005 and 2020 (7). T1D is also associated with some other autoimmune diseases, such as autoimmune thyroiditis, pernicious anemia, autoimmune gastritis, etc. which does harm to public health (8).

To achieve glucose homeostasis, different hormones are involved. Insulin is an anabolic hormone secreted by β-cells in pancreas in response to high concentrations of blood glucose, those achieved after a meal. Its major function is to control glucose, protein, and lipids metabolism by triggering the uptake of glucose, amino acids, and fatty acids into liver, muscle, and adipose tissue for the production of proteins and storage of energy in the form of glycogen and triglycerides (9–12). Insulin needs to bind to the insulin receptor located in the cell membrane to send messages into the cells. Insulin receptor is a transmembrane receptor that is

activated by high concentrations of circulating insulin. Insulin receptors consist of two α subunits and two β subunits (12,13). When insulin binds to the extracellular α subunit, a conformational change is induced and it results in the phosphorylation of tyrosine residues in the β subunit. These residues can be recognized by phosphotyrosine-binding (PTB) domains of insulin receptor substrate family (IRS) proteins (14). The phosphorylation of tyrosine residues on IRS proteins can then be recognized by phosphoinositide-3 kinase (PI3K) (15). The catalytic subunit of PI3K then phosphorylates phosphatidylinositol-4,5-bisphosphate, which leads to the formation of phosphatidylinositol (3,4,5)-triphosphate (PIP3) (16). When the protein kinase 3-phosphoinositid-dependent protein kinase 1 (PDK1) combines with a kinase to phosphorylate protein kinase B (PKB) (also known as Akt), PKB enters the cytoplasm to phosphorylate and inactivate glycogen synthase kinase 3 (GSK3) (17–20). GSK3 can inhibit glycogen synthesis by phosphorylating glycogen synthase (21). Therefore, when GSK3 is inactivated, glycogen synthesis is promoted, which leads to the storage of glucose as glycogen. In addition to promoting glycogen synthesis, insulin inhibits the output of liver glucose by inhibiting gluconeogenesis and glycogenolysis. Insulin regulates the activities of metabolic enzymes by promoting dephosphorylation (22). Insulin can also control the expression of genes to regulate gluconeogenesis (23). Insulin stimulates glucose uptake into cells by inducing translocation of the glucose transporter 4 (GLUT4) from intracellular storage to the plasma membrane (24–26).

Glucagon, a hormone secreted from α-cells in the pancreas, is secreted under fasting conditions and is characterized as having antagonistic effects of insulin. Glucagon generally elevates the concentration of blood glucose in the blood by promoting glycogenolysis and gluconeogenesis, as well as increasing lipolysis (27–29). Glucagon regulates the gene expression of phosphoenolpyruvate carboxykinase (PEPCK) by regulating the activation of the transcription

factor, cAMP-response element-binding protein (CREB) (30,31). When glucagon binds to its receptor, the intracellular production of cAMP is increased by the enzyme adenylate cyclase. An increased level of cAMP leads to the phosphorylation of the transcription factor CREB at Ser133 (32). Phosphorylated CREB (p-CREB) then translocates to the nucleus and binds to the cAMP-response element (CRE) in the PEPCK genes to activate transcription and PEPCK production, which will promote gluconeogenesis (33).

Patients with type 1 diabetes do not secrete an adequate amount of insulin to maintain normal concentrations of blood glucose, resulting in hyperglycemia. This is believed to be due to damage to β-cells caused by an inappropriate autoimmune response to a virus (8). Conversely, type 2 diabetic patients, for reasons not completely understood, become resistant to the effects of insulin, which may result in hyperglycemia in the long-term without treatment. Interestingly, during the early period of developing T2D, the patient may develop hyperinsulinemia as the body responds to the insulin resistance by secreting increasing concentrations of insulin. To treat T1D, exogenous insulin needs to be injected to replace the missing insulin. However, in the early stages of T2D, diet and exercise may be effective at reversing insulin resistance. This is usually accompanied by some type of oral hypoglycemic agent, whose mechanism of action is either to increase endogenous insulin secretion, or to increase insulin action. One class of oral hypoglycemic agent (the alpha glucosidase inhibitors) is designed to inhibit the digestion and absorption of starches. Eventually, endogenous insulin secretion may become suppressed in type 2 diabetics, and at this point, the patients may need to be treated with insulin, like the type 1 diabetic patients.

Streptozotocin (STZ)-induced diabetes is commonly used as an experimental animal model of type 1 diabetes (34). STZ is a glucosamine-nitrosourea compound synthesized by

Streptomycetes achromogenes. It is a naturally occurring chemical that is particularly toxic to the insulin-producing beta cells of the pancreas in mammals (34). STZ is quite similar to glucose. It enters the pancreatic beta cell through the Glut-2 transporter (GLUT2) and causes alkylation of the DNA, but is not recognized by the other glucose transporters (35). Glut-2 transporter (GLUT2) is a transmembrane carrier protein that enables glucose to move across cell membranes. It is the principal transporter for transfer of glucose between liver and blood, as well as pancreas and the blood. GLUT2 has a high capacity for glucose, but a low affinity, and acts as part of the "glucose sensor" in beta cells of pancreas. It is a very efficient in carrying glucose. Subsequent activation of poly-ADP-ribose polymerase (PARP) leads to $NAD^+$ depletion, a reduction in cellular ATP and subsequent inhibition of insulin production (36).

Leptin, a hormone that is primarily produced and secreted by white adipose tissue, interacts with its receptor in the basomedial hypothalamus to decrease food intake and increase energy expenditure (37). It has been found that leptin administration into the brain of streptozotocin (STZ)-induced diabetic rats will lead to a normalization of blood glucose concentrations when rats are in the fed state and a decrease in blood glucose concentrations when rats are in the fasting state (37–40). This suggests that leptin administration into the brain can lower blood glucose concentrations of diabetic rats independent of insulin. This effect of leptin appears to be independent of insulin. However, leptin-treated diabetic rats cannot maintain blood glucose concentrations during a fast, while blood glucose levels can be maintained in vehicle-treated animals. Glucagon helps maintain blood glucose concentrations during a fast and contributes to the elevated hepatic glucose output during diabetes. Some researchers have suggested that leptin normalizes blood glucose concentrations by reducing the concentration of glucagon (41,42). However, we hypothesize that leptin decreases the responsiveness of glucagon

by inhibiting cAMP signaling in the liver. This would decrease gluconeogenesis in the liver, resulting in a reduction in hepatic glucose output, normalizing blood glucose concentrations. In the study, we will determine the effects of central leptin administration to normalize blood glucose concentrations in diabetic rats that are chronically supplemented with high concentrations of glucagon. If high levels of glucagon negate the effect of leptin to normalize blood glucose concentrations, this would suggest that leptin is affecting blood glucose by inhibiting glucagon concentrations. However, if high levels glucose do not negate the effect of leptin to normalize blood glucose, this would suggest leptin is reducing blood glucose by some means other than by reducing glucagon concentrations.

# Chapter 2: Literature Review

## 2.1 Diabetes Mellitus

Diabetes mellitus is a metabolic disorder that causes blood glucose concentrations to increase more than normal (3,43). The chronic hyperglycemia results from insulin deficiency, insulin resistance, or both. Clinically, diabetes mellitus is diagnosed by the two continuous fasting blood glucose concentrations higher than 126 mg/dl or a random blood glucose concentration greater than 200 mg/dl (3). The characteristic symptoms of diabetes mellitus are polydipsia, polyphagia, polyuria, blurry vision, emaciation, and weakness (44,45). The long-term effects of diabetes include blindness, foot ulcers, renal failure, heart failure, stroke, and hypertension (46–49). People with the most severe forms of diabetes can develop ketoacidosis, which may lead to coma or even death, if it is not treated effectively (50).

Diabetes mellitus is a prevalent disease worldwide, especially in developed countries. It affects about 285 million people in the world, which is about 6.4% of the world's population in 2010. This is expected to increase to 7.7% of the world's population by 2030 (51,52). According to the National Diabetes Statistics Report released in 2014, 29.1 million Americans had diabetes in 2012, which is 9.3% of the population(53). Among the 29.1 million diabetic cases, 21.0 million were diagnosed, while 8.1 million were undiagnosed (54). In 2013, 382 million people suffer from diabetes globally, and account for 8.3% of the population (55–57).

There are three main types of diabetes: type 1 diabetes (T1D), type 2 diabetes (T2D) and gestational diabetes. T1D and T2D will be discussed as below:

### 2.1.1 Type 1 Diabetes

Insulin is a peptide hormone produced by β-cells in the pancreas which regulates the metabolism of carbohydrates, proteins, and fats. Beta cells are a type of cells found in the islets of the pancreas. The primary function of a β-cell is to store and release insulin. Beta cells can respond quickly to spikes in blood glucose concentrations by secreting some of their stored insulin, while simultaneously producing more.

T1D is a chronic disease characterized by insulin deficiency and hyperglycemia due to destruction or damaging of 90% of the β-cells in the pancreas (3). T1D is usually associated with an inappropriate immune response (8). When the body is invaded by some viruses like coxsackie B virus and cytomegalovirus, which have the same antigens as β-cells, T cells from the immune system will mistakenly attack and destroy β-cells in the islets of Langerhans when responding against these viruses (58). The autoimmune T1D can also be associated with some other autoimmune diseases, such as autoimmune thyroiditis, pernicious anemia, autoimmune gastritis, and others (59,60). T1D can also be triggered by genetic susceptibility and it is associated with family inheritance. People with family history of T1D are more likely to develop it. Diabetogenic environmental factors, such as foods, early infant diet, exposure to toxins, and geography play a role in the development of T1D, as well (61–63). The symptoms of T1D usually can be developed within a short time, even though the destruction of β-cells usually takes years. T1D can happen at any age, but it occurs in childhood and young adulthood more frequently.

T1D incidence has been rising globally during the past decades by 5.3% and it accounts for 5-10% of the cases of diabetes. If the trend continues, new cases of T1D in children younger than 5 years to be predicted would be doubled between 2005 and 2020 (7,64). To treat T1D,

patients can take insulin injection or use insulin pumps to compensate for the deficiency of insulin. According to Tamborlan *et al.* (2010), continuous glucose monitoring is associated with the improvement of blood glucose level control among T1D patients (65). While pancreas transplantation and islet transplantation would be proposed cures for T1D (66–68), currently they are still somewhat experimental and not widely used.

Rodent models are usually used in diabetes research. As mentioned above, T1D may be triggered by genetic susceptibility, diabetogenic environmental factors (63), as well as exposure to certain viruses. In rodent models, insulin deficiency can also be achieved by chemical ablation of β-cells. There are some primary factors that directly result in beta-cell death, such as autoreactive, cytotoxic (islet-antigen specific) T-lymphocytes (CTL), and inflammatory cytokines(69). A significant number of viruses have been associated with T1D, including enteroviruses such as Coxsackie B virus(58), but also rotavirus (70–73), mumps virus (74), and cytomegalovirus (74–76). Streptozotocin is a glucosamine-nitrosourea compound synthesized by Streptomycetes achromogenes. It is a naturally occurring chemical that is particularly toxic to the insulin-producing beta cells of the pancreas in mammals. STZ is quite similar to glucose. It enters the pancreatic beta cell through the Glut-2 transporter (GLUT2)(77) and causes alkylation of the DNA, but is not recognized by the other glucose transporters. Glut-2 transporter (GLUT2) is a transmembrane carrier protein that enables glucose moves across cell membranes. It is the main transporter for transferring glucose between blood and liver. GLUT2 has a high capacity for glucose, but a low affinity and acts as part of the glucose sensor in beta cells of pancreas. It is a very efficient in carrying glucose. Subsequent activation of PARP leads to $NAD^+$ depletion, a reduction in cellular ATP and subsequent inhibition of insulin production. STZ treatment is a commonly used experimental animal model of type 1 diabetes (34).

### 2.1.2 Type 2 Diabetes

Type 2 diabetes is the most common form of diabetes and it accounts for nearly 90% of the cases (78). It is characterized by insulin resistance and relative insulin deficiency caused by β-cell dysfunctions. The prevalence of T2D is higher in adults than in children (79). The frequency of this form of diabetes varies in different racial subgroups. Native Americans, Hispanic Americans, African Americans, and Asian Americans have a higher risk of getting T2D (80,81), especially among people who have a strong family history of diabetes (82). Although the genetics of this form of diabetes are complex and not clarified, a strong genetic predisposition is associated with a high frequency of T2D (83).

Most patients with T2D are obese, for obesity can cause a decrease in insulin sensitivity. Increasing the amount of body fat distributed in the abdominal region may cause T2D in a normal weight person (84). The onset of T2D is usually latent, for hyperglycemia is frequently developed gradually (79). Therefore, many patients can remain undiagnosed for years (85). Patients with this form of diabetes develop macrovascular and microvascular complications (86,87). The cause of T2D is still not clear (88). At least initially, the function of β-cells is normal in T2D patients, and insulin levels are normal or even elevated. Some patients can be hyperinsulinmia at the very beginning of developing T2D to compensate the low sensitivity or resistance to insulin (3). When this condition continues uncontrolled and β-cells can no longer secrete high amounts of insulin, an increase in the blood glucose concentration will occur. T2D is reversible at the very beginning by changing diets and losing weight (89,90).

## 2.2 Regulation of Glucose Metabolism

Glucose is the only energy source for some organs in our body such as brain and red blood cells (91,92). Glucose stored in our body in the form of liver glycogen is an important site for storing glycogen. Glucose levels are measured in terms of milligrams per deciliter (mg/dl). The normal concentrations range is from 70 to 110 mg/dl (93). When blood glucose concentrations are low, glycogen will convert to glucose through the process of glycogenolysis. Glucose can also be generated from non-carbohydrate precursors like pyruvate, amino acids and glycerol through the process of gluconeogenesis (94). During fasting, starvation, and intense exercise, blood glucose concentrations are maintained by gluconeogenesis. Glucose homeostasis involves multiple hormones, including pancreatic hormones (insulin, amylin, somatostatin, and glucagon) (95), gut hormone (incretin hormones, like glucagon-like peptide-1 (GLP-1)) (96), adrenal hormones (epinephrine and cortisol) (97), thyroid hormones (thyroxin and triiodothyronine) (98) and adipocyte hormones (leptin and adiponectin) (99).

### 2.2.1 Insulin

The pancreas, as an endocrine organ, consists of four different cell types: alpha cells, beta cells, delta cells, and F cells (100). The major function of pancreas is to produce and release insulin and glucagon, which are the hormones responsible for the endocrine control of glucose metabolism (27,101). Alpha cells in the pancreas produce glucagon and beta cells produce the inactive form of insulin (proinsulin) (102). Proinsulin is transported to the Golgi apparatus where it is processed to form the mature insulin released to the circulation (103). Insulin and glucagon play an important role in glucose homeostasis in vivo (104).

Insulin is an anabolic hormone secreted by β-cells in pancreas. One insulin molecule has 51 amino acids (105). Its major function is to control glucose, protein, and lipids metabolism by triggering the uptake of glucose and fatty acids into the liver for storage in the form of glycogen and lipids, respectively.

Insulin receptors consist of two α subunits and two β subunits (12,105). When insulin binds to the extracellular α subunit, a conformational change is induced and it results in the phosphorylation of tyrosine residues in the β subunit. These residues are recognized by phosphotyrosine-binding (PTB) domains of the insulin-receptor-substrate family (IRS) of proteins (13,106). The phosphorylation of tyrosine residues on IRS proteins can then be recognized by phosphoinositide-3 kinase (PI3K) (14,15). The catalytic subunit of PI3K then phosphorylates phosphatidylinositol-4,5-bisphosphate, which leads to the formation of phosphatidylinositol (3,4,5)-triphosphate (PIP3) (16). When the protein kinase 3-phosphoinositide-dependent protein kinase 1 (PDK1) combines with a kinase to phosphorylate protein kinase B (PKB, also known a Akt), PKB enters the cytoplasm to phosphorylate and inactivate glycogen synthase kinase-3 (GSK3) (17–20). GSK3 can inhibit glycogen synthesis by phosphorylating glycogen synthase (21). Therefore, when GSK3 is inactivated by PKB, glycogen synthesis is promoted, which leads to the storage of glucose as glycogen. In addition to promote glycogen synthesis, insulin inhibits the output of liver glucose by inhibiting gluconeogenesis and glycogenolysis. Insulin regulates the activities of metabolic enzymes by promoting dephosphorylation (22). Insulin can also inhibit the expression of genes to regulate gluconeogenesis (23). Insulin stimulates glucose uptake into cells by inducing translocation of the glucose transporter 4 (GLUT4) from intracellular storage to the plasma membrane (24–26).

High levels of insulin stimulate cell amino acid uptake, inhibits protein degradation, and promotes protein synthesis (107,108). When insulin is in the basal level, GSK3 phosphorylates the guanine nucleotide exchange factor eIF2B, which stimulates protein translation (109). When insulin levels are high, GSK3 is inactivated by PKB, and then eIF2B is dephosphorylated, which leads to a promotion of protein synthesis and increases amino acids storage. PKB can also promote protein synthesis by activating mechanistic target of rapamycin (mTOR) (110,111).

Increased transcription factor sterol regulatory element-binding proteins (SREBPs) stimulated by insulin promote the uptake of fatty acids and the lipids synthesis. Insulin activates cAMP-specific phosphodiesterase in adipocytes to decrease cellular cAMP levels, which leads to an inhibition of lipolysis (112–114).

### 2.2.2 Glucagon

Glucagon is a peptide hormone produced by alpha cells of pancreas that maintains the concentration of glucose in the bloodstream during fasting. Characterized as having antagonistic effects of insulin, glucagon generally elevates the concentration of blood glucose in the blood by promoting glycogenolysis and gluconeogenesis, as well as lipolysis (27–29). Glucagon needs to bind to its receptor to work. Glucagon receptor belongs to the superfamily of G protein-coupled receptors (GPCRs) and is activated by glucagon (115). Glucagon maintains blood glucose levels by stimulating the breakdown of glycogen into glucose through the process of glycogenolysis, stimulating glucose production from amino acids, glycerol, and lactate through the process of gluconeogenesis, and stimulating the release of glucose from the liver.

#### 2.2.2.1 Glucagon in Gluconeogenesis

Gluconeogenesis is a metabolic pathway for the generation of glucose which can be created from non-carbohydrate carbon substrates, such as lactate, glycerol, pyruvate, and glucogenic amino acids (94). It is one of the two primary mechanisms to maintain blood glucose levels and to avoid low blood glucose concentrations (hypoglycemia) in humans and many other animals.

Phosphoenolpyruvate carboxykinase (PEPCK) is an enzyme found both in cytosolic and mitochondrial forms to convert oxaloacetate into phosphoenolpyruvate and carbon dioxide in gluconeogenesis (116). Glucagon regulates the gene expression of PEPCK by regulating the activation of the transcription factor, cAMP -response element-binding protein (CREB) (117). When glucagon binds to its receptor, the production of cAMP is increased (104). An increased level of cAMP signal leads to a phosphorylation of the transcription factor CREB at Ser133. When phosphorylated CREB (p-CREB) then translocate to the nucleus and binds to the cAMP-response element (CRE) in the PEPCK genes to activate the transcription, which will promote gluconeogenesis pathway (31,118).

During a short-term fast, high level of glucagon will decrease the hepatocyte fructose-2, 6-bisphosphate level by activating cAMP-dependent protein kinase A (PKA). Phosphofructokinase 2/fructose 2, 6 bisphosphatase (PFK-2/Fruc 2, 6 BisPtase) is an enzyme, which is responsible for both the synthesis and hydrolysis reactions of fructose-2, 6-bisphosphate (F2, 6BP). When PFK-2/Fruc 2,6 BisPtase is phosphorylated by PKA, the phosphatase part is active to dephosphorylate F2,6BP. Lower F2,6BP also causes an increase in the activity of fructose 1,6-bisphosphatase (F1,6BPase), which will promote the process of gluconeogenesis pathway to generate glucose (119).

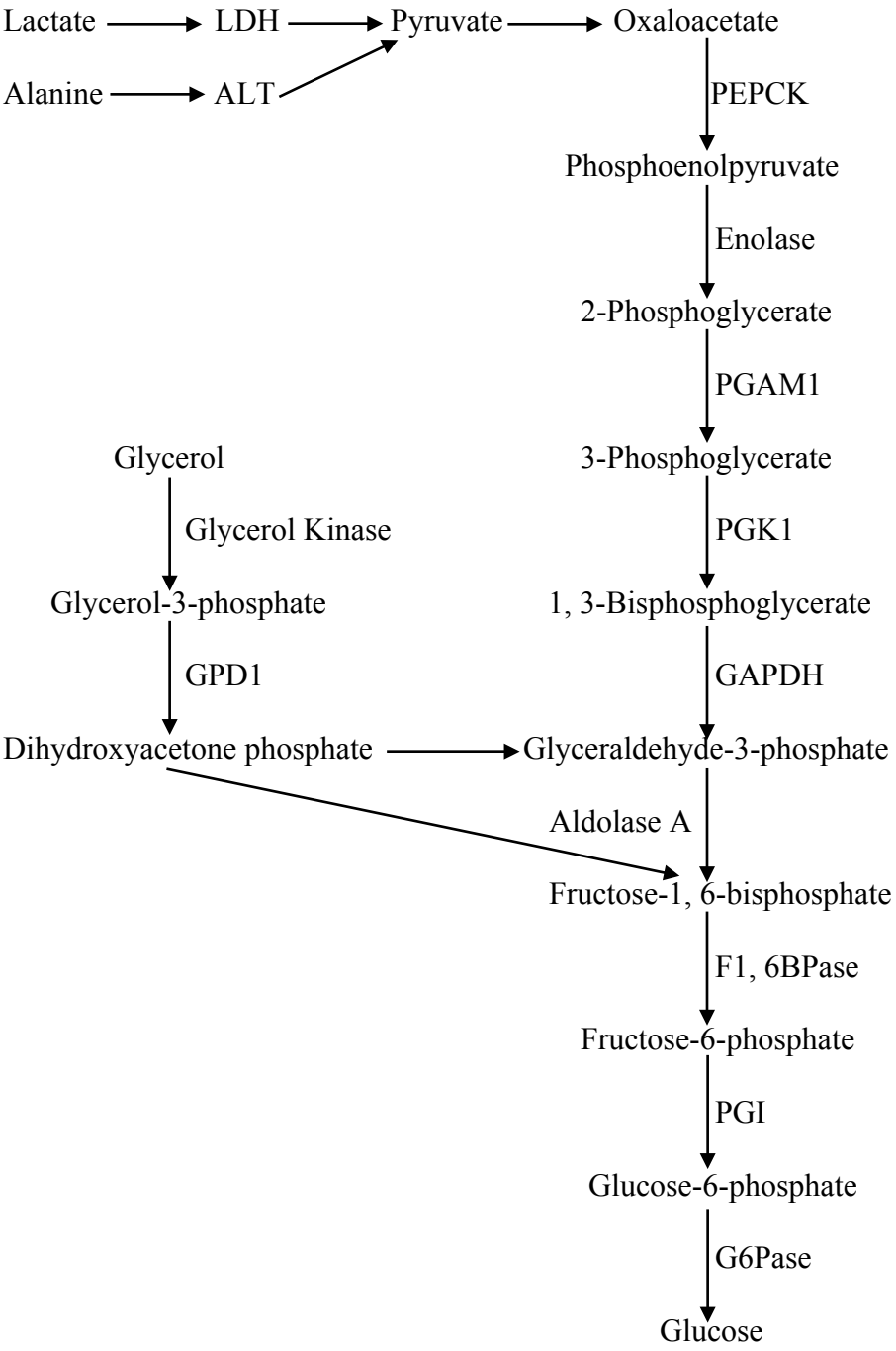


**Figure 1. Effect of Glucagon on Gluconeogenesis pathway* (120)**

*Full name of abbreviations in Figure 1 is listed in the List of Abbreviations (Page X).

#### 2.2.2.2 Glucagon in Glycogenolysis

Glycogen is the principal glucose storage form for animal cells. Glycogen molecules is a large, branched polymer of glucose (121). In fed states, glycogen is made from glucose through the action of glycogen synthase and glycogen branching enzyme. The major storage sites for glycogen in the body are in the liver and in muscle. During a short-term fast or muscle activity, glycogen is readily mobilized as an energy source through glycogenolysis to increase the glucose amount immediately available for the body. Some organs such as brain rely on glucose as the preferred fuel; therefore, the ability to maintain a steady supply of glucose in the circulation is critical for survival.

Glycogenolysis in the liver plays an important role in the regulation of glucose levels in the blood by breaking down glycogen to glucose. Early during fasting, glucagon stimulates glycogenolysis to convert liver glycogen to glucose that enters into the bloodstream when blood glucose level is low. Muscle cells do not have glucose 6-phosphatase or the GLUT 2 transporter; therefore, glucose can not be released by muscle cells. Glucagon stimulates glycogenolysis by binding to glucagon receptor that in turn activates the membrane localized enzyme adenylate cyclase. Adenylate cyclase converts ATP to cAMP which activates PKA, which in turn phosphorylates and activates glycogen phosphorylase (122).

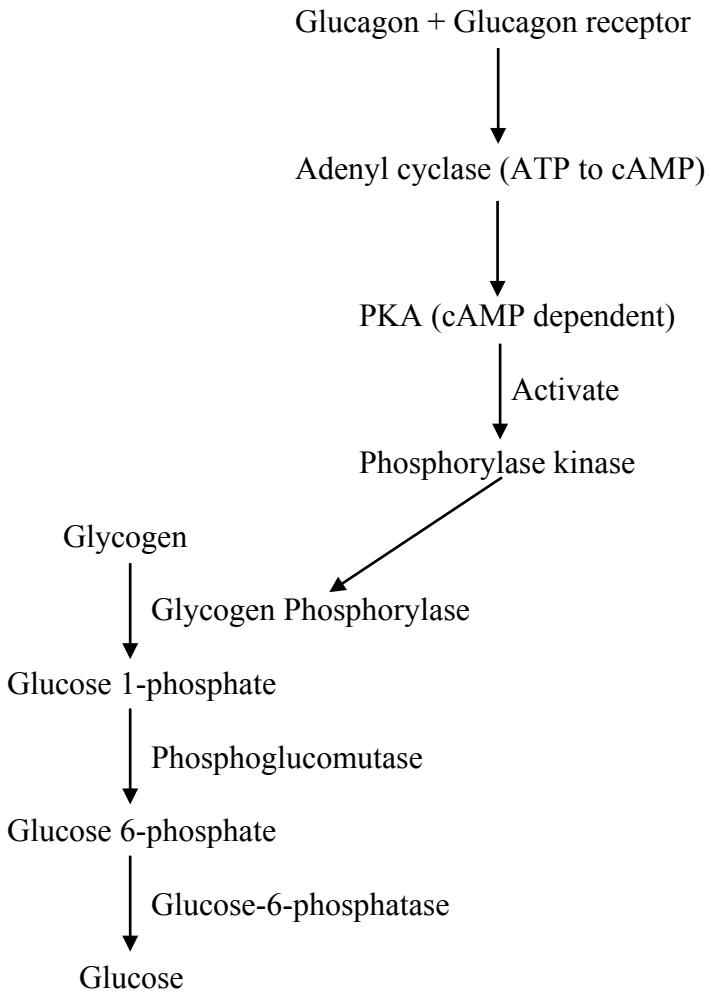


**Figure 2. Effect of Glucagon on Glycogenolysis pathway (122)**

### 2.2.2.3 Glucagon in Lipolysis

Gluconeogenesis can be affected by lipolysis (123). Energy from lipolysis enhances hepatic glucose output. Precursors of gluconeogenesis can be generated from triglycerides (124). Lipolysis can be regulated by glucagon (125,126). Glucagon induces lipolysis in humans under conditions of insulin suppression such as T1D (115,127). Glucagon promotes lipolysis by stimulating cAMP. Cyclic AMP phosphorylates the inactive form of adipose triglyceride lipase to active phosphorylated form. Under the effect of phosphorylated adipose triglyceride lipase, triglyceride (TG) converts to diglyceride (DG) and lipolysis is promoted (128).

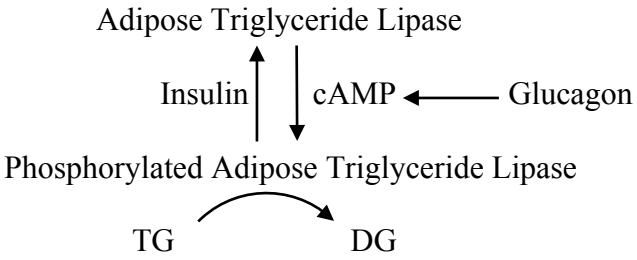


**Figure 3. Effect of Glucagon on Lipolysis Pathway(128)**

### 2.2.3 Leptin

Leptin, a 167-amino-acid protein hormone of human leptin gene, was first discovered from ob/ob mice at Rockefeller University (129). It is primarily produced by white adipose tissue and the levels are proportional to the amount of body fat (130). Leptin interacts with the long form of the leptin receptor (ObRb) in the basomedial hypothalamus (37) to decrease food intake and increase energy expenditure (131). Specific types of leptin receptors are found in the brain and in peripheral tissues. There are several isoforms of leptin receptors. The short leptin receptor

isoform (ObRa isoform) plays an important role in the transportation of leptin to across blood-brain barrier. The long leptin receptor isoform (ObRb isoform) mediates signal transduction (132). It is strongly expressed in the hypothalamus, which plays an important role in the regulation of energy and neuroendocrine function(133).

Several signal transduction pathways are activated by the leptin receptor when bound to leptin, including the energy homeostasis regulation pathway Janus Kinase-Signal Transducer and Activator of Transcription-3 (JAK/STAT3) (134), and the food intake and glucose homeostasis regulation pathway phosphatidylinositol 3-Kinase (PI3K) (135). The short form of the leptin receptor appears to activate the MAP kinase pathway.

Leptin has been suggested to regulate blood glucose concentrations by affecting the hypothalamic-pituitary-adrenal (HPA) axis independent of insulin (136). Hypothalamic-pituitary-adrenal (HPA) axis is a complex set of direct influences and feedback interactions among three endocrine glands: the hypothalamus, the anterior pituitary gland, and the adrenal cortex (137). It works through a leptin-mediated increase in alpha melanocyte-stimulating hormone (α-MSH) secretion and a decrease in neuropeptide Y (NPY) secretion in the hypothalamus (138). Leptin binds to NPY neurons in the arcuate nucleus in such a way as to decrease the activity of these neurons. Leptin receptor activation inhibits NPY and agouti-related peptide (AgRP), and activates α-MSH. States of energy excess are related to hyperleptinemia. However, the hypothalamus appears resistant to the increase in leptin. When energy is deficient, it will lead to hypoleptinemia and an increase in food intake due to suppression of α-MSH and stimulation of NPY and AgRP (139). The long isoform of leptin receptor (ObRb) is also located in the nucleus tractus solitarius (NTS) of the brain stem and hypothalamus. When leptin binds to

ObRb in the hypothalamus, food intake and body weight are decreased (140). In addition, when NTS of the brain stem is activated by leptin, a feeling of satiety is increased (141).

It has been found that central leptin administration into the brain of streptozotocin (STZ)-induced diabetic rats (type 1 diabetic rats) will lead to a normalization of blood glucose concentrations when rats are in the fed state and a decrease in blood glucose concentrations when rats are in the fasting state. This suggests that leptin administration into the brain can lower blood glucose concentrations of diabetic rats independent of insulin. Research found that leptin-treated diabetic rats could not maintain blood glucose concentrations during a fast, while blood glucose levels can be maintained in vehicle-treated animals. During a fast, the catabolic protein hormone, glucagon, helps maintain blood glucose concentrations and contributes to the elevated hepatic glucose output during diabetes. Some researchers have suggested that leptin normalizes blood glucose concentrations by reducing the concentration of glucagon.

### 2.2.4 Ketone bodies and leptin

Ketone bodies are produced from acetyl CoA through ketogenesis in the mitochondrial matrix when carbohydrates are scarce to meet the need of energy during fasting state or when carbohydrate in the diet is scarce (142). High levels of ketone bodies build up in the blood; pH of the blood is substantially decreased, which leads to ketoacidosis. In diabetic ketoacidosis (DKA), low levels of insulin with increased counter-regulatory hormones result in producing high levels of ketones (143,144).

It has been found that both insulin and leptin can prevent ketoacidosis, cachexia, and death in T1D rodent model (145). An increased ketones level is only detected in pair-fed rats instead of leptin-treated rats (146). Leptin reduces circulating levels of free fatty acids and

ketones, increases fat oxidation, and prevents the fall in resting energy expenditure (EE) (146) that normally occurs with reduced caloric intake, which helps to prevent ketoacidosis, cachexia, and death in T1D rodent model(145).

### 2.2.5 Phosphoenolpyruvate carboxykinase (PEPCK)

Phosphoenolpyruvate carboxykinase (PEPCK) belongs to the lyase family used to convert oxaloacetate into phosphoenolpyruvate and carbon dioxide in the gluconeogenesis pathway. As the rate-controlling step in gluconeogenesis, PEPCK is the junction between glycolysis and tricarboxylic acid (TCA) cycle (147). There are two isoforms of PEPCK, cytosolic form and mitochondrial isoform in humans and the cytosolic form is important in gluconeogenesis to convert oxaloacetate into phosphoenolpyruvate and carbon dioxide. Transcription of the PEPCK gene is promoted by glucagon, glucocorticoids, retinoic acid, and cAMP (148).

Glucagon regulates the gene expression of PEPCK by regulating the activation of the transcription factor, cAMP-response element-binding protein (CREB) (30,31). When glucagon binds to its receptor, the production of cAMP is increased. An increased level of cAMP signal leads to a phosphorylation of the transcription factor CREB at Ser133(32). Phosphorylated CREB (p-CREB) translocates to the nucleus and binds to the cAMP-response element (CRE) in the PEPCK genes to activate the transcription, which will increase the amount of PEPCK protein, enhancing the gluconeogenic pathway (33).

### 2.2.6 Cyclic AMP response element-binding protein (CREB)

As the cellular transcription factor of PEPCK, cAMP response element-binding protein (CREB) was first described as a cAMP-responsive transcription factor that can regulate somatostatin gene (149). It binds to cAMP response elements (CRE) on DNA to increase or decrease the transcription of the downstream genes (150). CREB proteins are expressed in humans (151) and many other higher animals (152). CREB has a variety of functions in different organs, and some of the functions are related to the brain (153). CREB plays an important role in the formation of long-term memory in the brain (154). The phosphorylation of CREB by PKA and $Ca^{2+}$-dependent protein kinases is usually at the serine 133 residue (155).

When glucagon binds to its receptor, the production of cAMP is increased. An increased level of cAMP signal leads to a phosphorylation of the transcription factor CREB at Ser133. Phosphorylated CREB (p-CREB) translocates to the nucleus and binds to the cAMP-response element (CRE) in the PEPCK genes to activate the transcription of the PEPCK protein.

## 2.3 Objectives

The overall objective of this research is to increase our understanding of how central leptin administration normalizes blood glucose concentrations independent of insulin in type 1 diabetic rats. In the study, we will determine the effects of central leptin administration to normalize blood glucose concentrations in diabetic rats that are chronically supplemented with high concentrations of glucagon. If high levels of glucagon negate the effect of leptin to normalize blood glucose concentrations, this would suggest that leptin reduces blood glucose by inhibiting glucagon concentrations. However, if high levels glucose do not negate the effect of leptin to normalize blood glucose, this would suggest leptin reduces blood glucose by some

means other than by reducing glucagon concentrations. Our specific aims are to: 1) examine the effect of high doses of glucagon on blood glucose concentrations in leptin-treated diabetic rats; and 2) determine changes in biochemical markers of liver gluconeogenesis in leptin- and glucagon-treated diabetic rats.

### 2.4 Hypothesis

We hypothesized that blood glucose concentrations are regulated in T1D by leptin treatment. The delivery of glucagon will not increase daily blood glucose concentrations or blood glucose concentration during a fast despite dramatic increases in serum glucagon levels. Chronic leptin treatment into the brain of type 1 diabetic rats will normalize blood glucose concentrations by decreasing the responsiveness to glucagon.

# Chapter 3: Blood Glucose Concentrations Are Not Increased by Chronic IP Glucagon Administration in Leptin-Treated Type 1 Diabetic Rats

## 3.1 Abstract

The overall objective of this research is to increase our understanding of how central leptin administration normalizes blood glucose concentrations independent of insulin in type 1 diabetic rats. This research may lead to new insulin-independent treatments for diabetes. It is widely accepted that leptin administration into the brain of previously uncontrolled diabetic animals can normalize blood glucose concentrations, independent of insulin. The mechanism by which central leptin administration normalizes blood glucose concentrations in diabetic animals is not understood. Some studies have suggested that leptin acts by decreasing the serum concentration of glucagon. However, we hypothesize that leptin decreases the responsiveness of glucagon by inhibiting cyclic adenosine monophosphate (cAMP) signaling in the liver. This would decrease gluconeogenesis in the liver, resulting in a reduction in hepatic glucose output, normalizing blood glucose concentrations.

To test this hypothesis, we performed a study to directly examine whether chronic high doses of glucagon (delivered via an intraperitoneal (IP) osmotic pump) blocked or attenuated the ability of intracerebroventricular (ICV) leptin administration to normalize glucose concentrations in streptozotocin (STZ)-induced diabetic rats. Two cohorts of rats were used. Four groups of diabetic rats were examined within each cohort: 1) leptin-treated (ICV), glucagon-treated (IP), 2) leptin-treated (ICV), vehicle-treated (IP), 3) vehicle-treated (ICV), glucagon-treated (IP), and 4) vehicle-treated (ICV), vehicle-treated (IP). The change in blood glucose concentration of the four groups was determined on a daily basis and during three different conditions. The three

conditions were 1) at various times over the circadian cycle, 2) during an 8-hour fast, and 3) following an IP injection of pyruvate (to determine the rat's gluconeogenic capacity).

As we have seen previously, leptin treatment normalized blood glucose concentrations in diabetic rats. Our new findings showed that chronic glucagon treatment did not block or attenuate the normalization of blood glucose concentrations of leptin-treated diabetic rats whether based on the blood glucose concentration around the circadian cycle, the blood glucose concentration during an 8-hour fast, or the blood glucose concentration in response to an injection of pyruvate. There was an indication that glucagon slightly attenuated daily concentration of blood glucose in leptin-treated rats. Overall, this lack of difference was observed despite the fact that serum glucagon concentrations were 4-9-fold greater in glucagon-treated rats as compared to vehicle-treated rats. Leptin treatment decreased PEPCK/beta-actin in the liver. In addition, the hepatic CREB protein content was decreased by leptin treatment, but the phosphorylated CREB content was not. This suggests that the serum glucagon concentration does not have to be reduced in order for leptin to normalize blood glucose concentrations. It further suggests that central leptin administration can inhibit the cAMP signaling pathway without decreasing serum glucagon concentrations. This supports the hypothesis that leptin treatment acts to normalize blood glucose concentrations of type 1 diabetic rats by decreasing the responsiveness to glucagon.

### 3.2 Introduction

How central leptin administration normalizes blood glucose concentrations in type 1 diabetic rats has been the subject of many studies (38,156–158), but the mechanism is not fully understood. Leptin is a hormone primarily produced and secreted by white adipose tissue (129).

The concentration of circulating leptin is related to the size of the body fat mass (159). It interacts with leptin receptors in the basomedial hypothalamus to decrease food intake, increase energy expenditure and regulate glucose homeostasis (37). Previous studies have demonstrated that leptin administration into the brain of uncontrolled diabetic animals can normalize blood glucose concentrations (158,160). They have suggested that leptin treatment enhances insulin sensitivity, as indicated by increased insulin-stimulated glucose utilization in peripheral tissues (160).

Streptozotocin (STZ) is a glucosamine-nitrosourea compound synthesized by Streptomycetes achromogenes. It is a naturally occurring chemical that is particularly toxic to the insulin-producing beta cells of the pancreas in mammals (161). STZ-induced diabetes is commonly used as an experimental animal model of type 1 diabetes (162). It has been found that leptin administration into the brain of STZ-induced diabetic rats leads to the normalization of blood glucose concentrations when rats are in the fed state and a decrease in blood glucose concentrations when rats are in the fasting state (160). This suggests that the effect of leptin administration into the brain to lower blood glucose concentrations of diabetic rats is independent of serum insulin levels. Leptin is known to reduce food intake and decrease body weight (132). However, blood glucose concentrations were not normalized in rats pair-fed to the level of the leptin-treated. This indicates that the leptin-induced normalization of blood glucose is not related to the decrease in food intake (163). Leptin-treated diabetic rats cannot maintain blood glucose concentrations during a fast, while blood glucose levels can be maintained in vehicle-treated animals. This may provide a clue to the mechanism by which leptin acts to normalize blood glucose. Glucagon helps maintain blood glucose concentrations during a fast and contributes to the elevated hepatic glucose output during diabetes (27–29). Previous studies

have suggested that leptin normalizes blood glucose levels in type 1 diabetic, non-obese mice (164,165) and that the mechanism could be a reduction in the concentration of circulating glucagon (41,164,165). However, in a preliminary study from our laboratory, we found no alteration in plasma glucagon concentrations in leptin-treated diabetic rats when compared to the control group (166). A recent study suggests that short-term peripheral leptin administration to uncontrolled diabetic rats suppresses gluconeogenesis (167).

Therefore, we hypothesize that leptin decreases the responsiveness of glucagon by inhibiting cAMP signaling in the liver. This would decrease gluconeogenesis in the liver, resulting in a reduction in hepatic glucose output, normalizing blood glucose concentrations. In the study, we will determine the effects of central leptin administration to normalize blood glucose concentrations in diabetic rats that are chronically supplemented with high concentrations of glucagon. If high levels of glucagon negate the effect of leptin to normalize blood glucose concentrations, this would suggest that leptin reduces blood glucose by inhibiting glucagon concentrations. However, if high levels of glucose do not negate the effect of leptin to normalize blood glucose, this would suggest leptin reduces blood glucose by some means other than by reducing glucagon concentrations.

Results suggested that high concentrations of glucagon did not negate the effect of leptin to normalize blood glucose concentrations in leptin-treated diabetic rats. Leptin treatment was also shown to decrease the hepatic gluconeogenic enzyme, phosphenolpyruvate carboxykinase (PEPCK), as well as a transcription factor that mediates cAMP signaling in the liver (cAMP-response element binding protein (CREB). These results are consistent with the hypothesis that leptin administration in the brain leads to a decrease in the responsiveness of glucagon in the liver.

## 3.3 Material and Methods

### 3.3.1 Animals

This study was performed using two cohorts of 48 male Wistar rats (200-300g; Harlan, Indianapolis, IN), which were housed in individual shoebox cages. The temperature was 23 ± 3℃ and the environment was humidity-controlled with a 12-hour light and dark cycle. Animals were allowed free access to tap water and standard rat chow (Prolab RMH 300 meal, Purina Mills, Richmond, Indiana) ad libitum. All procedures involving animals were approved by Auburn University's Institutional Animal Care and Use Committee (IACUC).

### 3.3.2 Cannula Implantation

After one week of acclimatization, each rat was anesthetized by isoflurane and placed in a stereotaxic apparatus. Then a 22 gauge, stainless steel guide cannula (Plastics One, Roanoke, Virginia) was implanted into the lateral ventricle of the brain (0.8mm posterior to bregma, 1.4mm lateral to the midline, and 3.5mm ventral to the surface of the skull). Four stainless steel screws and dental cement were used to fix the cannula to the skull under aseptic conditions. A removable dummy cannula extending 1mm beyond the guide cannula was inserted into the guide cannula until the time of infusions.

### 3.3.3 Angiotensin II Drinking Test

After 2 days of post-surgical recovery, the placement of the cannula was confirmed by observing a positive drinking response to an intracerebroventricular (ICV) infusion of angiotensin II (Sigma, St. Louis, Missouri). All rats were given an ICV injection of angiotensin II (40ng/4μl) and placed in a hanging cage with a graduated tube containing water. The amount

of water consumed in 15 minutes was determined. Rats consuming 3 ml of water or greater were considered to have the correct cannula placement.

### 3.3.4 Induction of Diabetes

All rats were made diabetic by an injection of streptozotocin (STZ) (Sigma, St. Louis, Missouri). The STZ solution (50 mg/kg) was made immediately before use (in 0.05M citrate buffer at pH 4.5) and was administered through an intraperitoneal (IP) injection. After twenty-four hours, blood was sampled from the tail vein and the blood glucose concentration was determined by a handheld glucometer (Walgreens, Trueresult Blood Glucose Monitoring System). The induction of diabetes was verified by hyperglycemia (blood glucose > 300 mg/dl). Diabetic rats that did not reach 300 mg/dl were given a second injection of STZ. All rats with STZ injections were confirmed diabetic by the third day.

### 3.3.5 Intracerebroventricular (ICV) leptin/vehicle administration

After confirmation of hyperglycemia, half of the diabetic rats received daily ICV injections of leptin (5μg/day) (R&D Systems, Minneapolis, Minnesota) and the other half received vehicle injections. ICV infusions were delivered from a motorized syringed pump over a minute period.

### 3.3.6 Blood glucose concentrations and body weight determination

Blood glucose concentrations were monitored from the tail vein every day using a handheld glucometer, while body weight measurements were obtained every day during the light period between 2:00 pm to 4:00 pm.

### 3.3.7 Osmotic glucagon pump implantation

When blood glucose concentrations returned to normal in the leptin-treated rats (~120 mg/dl), half of the rats in each group were implanted (IP) with a two-week osmotic pump containing glucagon (4 mg/ml); the remainder of the rats were implanted with a pump containing vehicle. Rats were anesthetized with isoflurane and the abdomen hair shaved. A small incision was made through the skin and muscle wall of the abdomen under aseptic condition. The pump was placed in the peritoneal cavity. The muscle wall was sutured with absorbable suture material and then the skin was stapled.

### 3.3.8 Circadian cycle blood glucose

After several days, blood glucose concentrations were determined via the tail vein throughout the 24-hour day. Blood glucose concentrations were determined by a handheld glucometer beginning at 8:00 am and thereafter every 4 hours for 24 hours. Therefore, blood glucose concentrations were determined at 8:00 am, 12:00 pm, 4:00 pm, 8:00 pm, 12:00 am, 4:00 am, and 8:00 am. Therefore, a total of seven blood samples were taken in 24 hours. A red light was used in the animal room during the dark phase.

### 3.3.9 Short-term fast blood glucose

Several days after determining blood glucose concentration across the 24-hour day, all animals were fasted for 8 hours from 8:00 AM to 4:00 PM. All animals had ad libitum access to water during this time. Blood glucose concentrations were determined from the tail vein at 8:00 am and every 2 hours after that until 4:00 pm. At the completion of the 8-hour fast, free access to food was returned to all animals.

### 3.3.10 Pyruvate challenge

Rats were fasted for six hours between 8:00 am – 2:00 pm. This was to remove liver glycogen and to ensure no food remained in the GI tract that could contribute to an increase in blood glucose concentration. The blood glucose concentration was determined via the tail vein prior to fasting, and again (time 0) just prior to an IP injection of pyruvate (2.0 g/kg body weight). During the challenge, rats had free access water, but not food. Blood glucose concentrations were determined by a handheld glucometer at 15, 30, 60, 90, 120, and 180 minutes post-injection. After the 180-minute time point, free access to food was restored to the animals.

### 3.3.11 Suspension of leptin treatment

Several days after the pyruvate challenge, leptin-treated rats were switched to vehicle treatment for several days to determine whether blood glucose concentrations would increase faster in glucagon-treated rats, as compared to vehicle-treated rats.

### 3.3.12 Resumption of leptin treatment

Rats originally in the leptin group were retreated with ICV leptin (5μg/day). Rats receiving daily vehicle injection, continued to receive vehicle injections. Daily blood glucose concentrations were determined.

### 3.3.13 Blood sample and liver tissue preparation

Rats were euthanized with a euthanizing dose of pentobarbital. As the heart stopped beating, blood was collected from a cardiac puncture in a heparinized tube containing protease

inhibitors. Blood was centrifuged for 20 minutes; plasma was collected into aliquots, and frozen and stored at -80 ° C. The livers were collected, weighed, and frozen in liquid nitrogen immediately after being harvested. Liver were then stored in -80℃.

### 3.3.14 Determination of plasma glucagon concentration

Determination of plasma glucagon concentrations was done by the radioimmunoassay kit (EMD Millipore Corporation, Billerica, MA).

### 3.3.15 Liver sample preparation for Western blots

Rat livers were taken out of frozen storage and put on ice. Approximately, 50 mg of each liver tissue was removed and the exact sample weight was recorded. Each sample was homogenized with 1mL of lysis butter until no tissue could be seen. The lysis buffer contained 50 mM HEPES (pH=7.6), 150 mM NaCl, 20 mM sodium pyrophosphate, 10 mM NaF, 1% Triton, 10µg/ml Leupeptin, 10µg/ml Aprotinin, 1mM $Na_3VO_4$, and 1mM Phenylmethanesulfonyl fluoride (PMSF). The homogenate was transferred to a microcentrifuge tube, kept on ice for 10 minutes. The sample was then centrifuged for 10 minutes at 14,000 RPM, and the supernatant was transferred to a new tube.

A Pierce® 660nm protein assay (Pierce, Thermo Scientific, Rockford, IL) was performed on the supernatant sample for the determination of the liver protein concentration. This was done by spectrophotometric comparison of the liver homogenate supernatant to a bovin serum albumin standard. The Pierce microtiter plate protocols were used and absorbance was measured at 660nm.

### 3.3.16 Liver PEPCK Determination

Ten micrograms of liver protein were analyzed by SDS-gel electrophoresis using Criterion gel system (Thermo Scientific, Rockford, IL). Proteins were transferred to nitrocellulose. The nitrocellulose sheets were blocked for 1 hour in 5% skinned milk (5 g skinned milk powder/100ml Tris-buffered saline, 0.1% Tween 20, TBST). The primary antibody used was rabbit antibody PEPCK H-300: sc-32879 with a 1:1000 dilution (Sigma, St. Louis, Missouri) and the secondary antibody was AMDEX goat anti-rabbit IgG-HRP (Amersham, Piscataway, NJ). The results were visualized using film developer.

### 3.3.17 Liver CREB-1 and p-CREB-1 Determination

Twenty micrograms of liver protein were analyzed by SDS-gel electrophoresis using Criterion gel system (Thermo Scientific, Rockford, IL). Proteins were transferred to nitrocellulose. The nitrocellulose sheets were blocked for 1 hour in 5% skinned milk (5g skinned milk powder/100ml TBST). The primary antibody used was rabbit antibody CREB-1 (C-21): sc-186 and p-CREB-1 (Ser 133): sc-7978 with a 1:1000 of dilution (Sigma, St. Louis, Missouri) and the secondary antibody was AMDEX goat anti-rabbit IgG-HRP (Amersham, Piscataway, NJ). The results were visualized using film developer.

### 3.3.18 Glycogen Determination

Hepatic glycogen content was determined using Glycogen Assay Kit (Item No. 700480, Cayman Chemical, Ann Arbor, MI). Three-hundred micrograms of frozen liver tissue were homogenized in glycogen assay buffer (Item No. 700482, Cayman Chemical, Ann Arbor, MI) containing 200 μM PMSF and 10 μg/ml Leupeptin. Supernatant after centrifuge (800g, 10min,

4℃) was transferred to another tube and stored on ice. This was done by fluorometric comparison of the liver homogenate supernatant to the glycogen standard (Item No. 700481, Cayman Chemical, Ann Arbor, MI). Fluorescence was monitored with an excitation wavelength of 530nm and an emission wavelength of 590nm by using a FLUOstar Optima fluorescence microplate reader (BMG Labtech, Durham, NC, USA).

### 3.3.19 Statistical Analysis

All results were presented as means ± standard error of the mean (SEM). Statistical analyses were performed by using SSPS 12.0 and JMP 12. Two-way analysis of variance (ANOVA) with repeated measures was utilized to analyze ICV injections (leptin vs vehicle) and IP infusions (glucagon vs vehicle) and their interaction on body weight and blood glucose concentrations. Plasma glucagon, liver glycogen, and hepatic PEPCK and CREB content were analyzed using a two-way ANOVA. Statistical significance among the groups was determined with a one-way analysis of variance (ANOVA). Simple linear regression was performed between blood glucose concentrations and liver CREB content. A difference of $P < 0.05$ was considered statistically significant.

## 3.4 Results and Discussion

### 3.4.1 Blood glucose concentrations

As expected, STZ administration caused a large and rapid increase in the blood glucose concentration in the rats of this study (**Figure 4**). In one day, blood glucose concentrations under fed conditions increased from approximately 150 mg/dl to approximately 425-450 mg/dl. The increase in blood glucose concentrations indicated that the rats had become diabetic. Specifically, due to a lack of insulin (168), theses rats had become a model of type 1 diabetes. When leptin infusion started in the leptin-treated group, blood glucose concentrations started to decrease in the diabetic rats as we have seen previously and has been seen in other studies (165,169). There are several possible mechanisms by which leptin could normalize blood glucose levels. Some researchers suggested that the decrease of blood glucose concentrations in T1D rats is due to a decreased appetite caused by leptin-induced satiety (170). However, this seems to be unlikely because the pair-feeding experiments does not normalize the blood glucose concentrations in the diabetic rats (163). Some researchers have suggested that leptin might have an interaction with insulin, and leptin administration might increase the sensitivity to insulin (160). But this seems to be unlikely since blood glucose continued to drop in the fasted state when insulin levels are very low (26). In our study, type 1 diabetes was induced in rats, which means insulin levels were extremely low. However, we still see a significantly drop in blood glucose in rats treated with leptin, which confirms that the effect of leptin treatment is independent of insulin (41,164,165).

In the fasting state, glucagon contributes to elevated hepatic glucose output and helps maintain blood glucose concentrations (122). Therefore, it is possible that the normalization of blood glucose concentration in diabetic rats is due to an interaction between leptin and glucagon.

Some researchers have suggested that leptin normalizes blood glucose concentrations by reducing the concentration of glucagon (41,164,165). From previous experiments (166,167), we found that the levels of glucagon are not significantly changed due to central leptin administration. In the present study, when blood glucose concentrations returned to normal in the leptin-treated rats, half of the rats in each group were implanted (IP) with a two-week osmotic pump containing glucagon (4 mg/ml); the remainder of the rats were implanted with a pump containing vehicle. Chronic glucagon infusion via the osmotic pump resulted in very high concentrations of plasma glucagon concentrations, almost ten times greater than normal concentrations (**Figure 5**). However, blood glucose concentrations in the leptin-treated, glucagon-treated rats were still significantly lower than rats not treated with leptin. (**Figure 6**), though glucagon treatment did appear to have increase blood glucose concentration above that of the leptin-treated, vehicle group. This suggested that high levels of glucagon treatment did not reverse the effects of leptin, though it did appear to attenuate the glucose-lowering effect of leptin on daily blood glucose concentrations.

Rats are nocturnal; therefore they are more activity at night and eat a greater proportion of their food during the night. Blood glucose concentrations would then be expected to increase after they eat. When blood glucose concentrations were determined throughout the 24-hour day, there was no significant change in blood glucose concentrations in rats with leptin treatment (**Figure 7**). This suggests that glucagon even at high doses did not increase blood glucose concentration across the circadian cycle in leptin-treated rats.

During a fast, the decrease in blood glucose concentration will cause glucagon to be secreted from the pancreas, enhancing gluconeogenesis and glycogenolysis, increasing hepatic glucose output (122). Normally, with an increased concentration of glucagon, blood glucose

concentrations will be maintained to a larger degree. When our T1D rats underwent a short-term fast (8 hours), Blood glucose concentrations were significantly lower in the leptin-treated group compared to the non-leptin-treated rats (**Figure 8**). This suggests that leptin-treated rats need to eat in order to maintain blood glucose concentration. Furthermore, the administration of high doses of exogenous glucagon in these leptin-treated rats did not increase blood glucose concentrations. This could indicate that exogenous glucagon is not functional in leptin-treated rats during a fast.

Increasing concentrations of glucagon are known to increase gluconeogenesis (122). During gluconeogenesis, glucose is generated from non-carbohydrate carbon substrates. One of the precursors in the gluconeogenic pathway is pyruvate. When pyruvate transports from the cytosol to mitochondria, it is converted into oxaloacetate by the enzyme, pyruvate carboxylase. Either malate dehydrogenase then converts oxaloacetate into malate or a transaminase converts oxaloacetate into aspartate and these compounds are translocated from mitochondria to the cytosol and converted back into oxaloacetate. Under the effect of PEPCK, oxaloacetate is converted into phosphoenolpyruvate. This bypasses the irreversible glycolytic hormone, pyruvate kinase. Using the reversible enzymes of glycolysis, along with fructose 1, 6 – bisphosphatase, and glucose 6-phosphatase, phosphoenolpyruvate can be converted into glucose. Blood glucose levels for all rats increased 30 minutes after the IP injection of pyruvate and returned to basal levels within 180 minutes. There was no differences between glucagon-treated rats to non-glucagon treated rats (**Figure 9**). These observations suggest that glucagon did not increase blood glucose concentrations in response to pyruvate in leptin-treated rats.

### 3.4.2 Liver PEPCK, CREB and p-CREB Content

During gluconeogenesis, glucose is generated from non-carbohydrate carbon substrates such as pyruvate. Under the effect of PEPCK, oxaloacetate turns into phosphoenolpyruvate, bypassing an irreversible step of glycolysis. The regulation of PEPCK is through alterations in transcription, resulting in changes in the amount of the PEPCK protein (171). Glucagon is known to stimulate PEPCK through the cAMP signaling pathway (172). Western blots of PEPCK, CREB and p-CREB are shown in **Figure 10**. Beta-actin was chosen as the housekeeping protein to normalize for the slight differences in the loading of protein. As the rate-controlling step in gluconeogenesis, the amount of PEPCK can reflect the flux through the gluconeogenic pathway (116). Leptin treatment decreased the amount of PEPCK/beta-actin (**Figure 11)**, which suggests that leptin may normalize blood glucose in type 1 diabetics rats by decreasing liver PEPCK protein and inhibiting gluconeogenesis, even when glucagon levels are high.

As the cellular transcription factor of PEPCK, cAMP response element-binding protein (CREB) was first described as a cAMP-responsive transcription factor that regulated the somatostatin gene (117). It binds to certain sequences called cAMP-response elements (CRE) on DNA to increase or decrease the transcription of the downstream genes. When glucagon binds to its receptor, the production of cAMP is increased. An increased level of cAMP signal leads to a phosphorylation of the transcription factor CREB at Ser133. Phosphorylated CREB (p-CREB) then translocates to the nucleus and binds to the cAMP-response element (CRE) in the PEPCK genes to activate transcription. Like PEPCK, total liver CREB protein was decreased in leptin-treated rats as compared to rats not treated with leptin (**Figure 13**), however, no differences between groups were found in p-CREB (**Figure 12**). This might be because the alteration of total

CREB needs a longer time to change and reflects long-term regulation, whereas the phosphorylation of CREB may be more reflective of short-term regulation.. For our study, it is a long-term trial which takes 7 weeks. This suggests that the total amount of CREB protein may reflect long-term changes in the cAMP pathway, and that it may be inhibited by leptin treatment. This is supported by the strong correlation between total CREB protein in the liver and blood glucose concentrations at the time the rats were euthanized (**Figure 14**). Changes in CREB protein in the liver accounted for 54% of the variation in blood glucose concentration.

### 3.4.3 Liver Glycogen Content

Liver glycogen, as the principal glucose storage form for animal cells, is readily mobilized as an energy source through glycogenolysis to increase the glucose amount immediately available for the organism during a short time fasting. Glucagon stimulates glycogenolysis by binding to glucagon receptor, which in turn activates the membrane localized enzyme adenylate cyclase. Adenylate cyclase converts ATP to cAMP which activates PKA which in turn phosphorylates and activates glycogen phosphorylase. The amount of glycogen left in the liver can be an indicator of the efficiency of glucagon on gluconeogenesis. The amount of liver glycogen in the fed state was not different between the various groups. It would be having interesting to determine liver glycogen levels in the fasted state. In our study, glycogen contents are not significantly different between groups. The rats in this study were not fasted before being euthanized, which could be a reason for the non-significant difference in glycogen content. It is possible that leptin treatment suppressed glycogenolysis, even in the presence of high level of glucagon, resulting in the retention of liver glycogen.

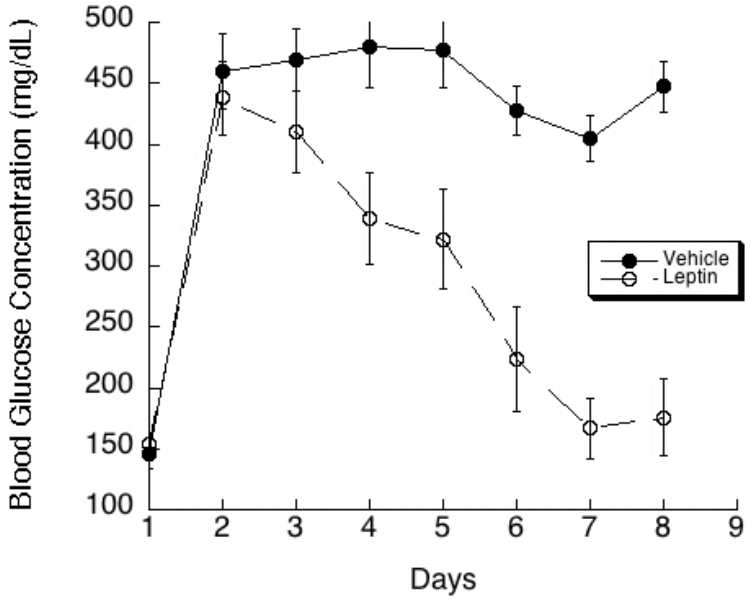


**Figure 4. Daily blood glucose concentrations in type 1 diabetic rats treated daily with ICV leptin or vehicle. Each point is the mean of 9-11 rats ± SEM.** Leptin normalized blood glucose concentrations in type 1 diabetic rats.

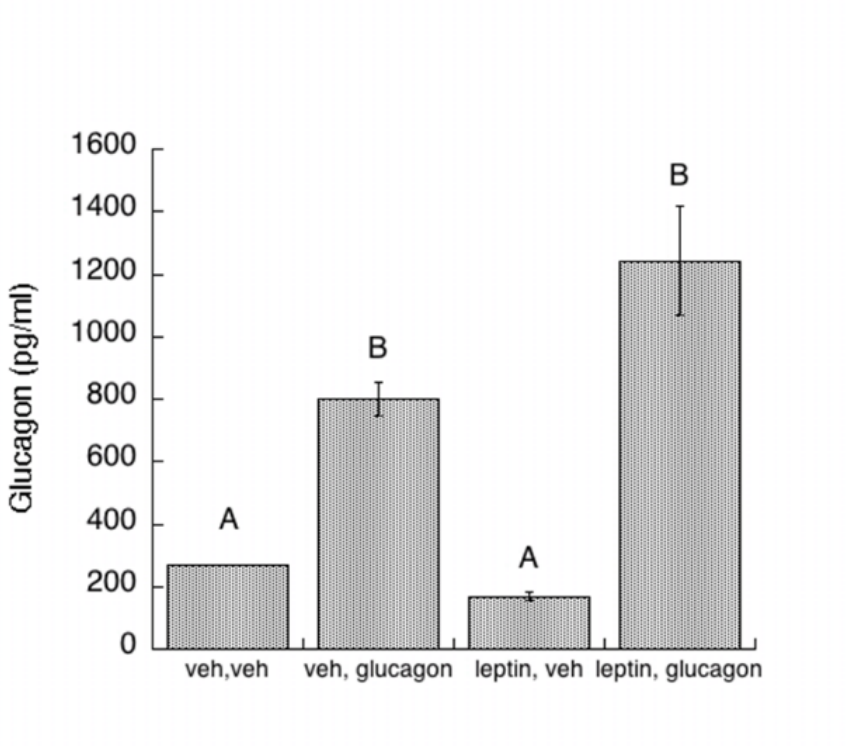


**Figure 5. Plasma glucagon concentrations in type 1 diabetic rats treated with leptin/glucagon. Each bar is the mean of 4-6 rats. Group means with different letters indicate a significant difference ($p < 0.05$).** Chronic glucagon infusion via an osmotic pump resulted in very high concentrations of plasma glucagon.

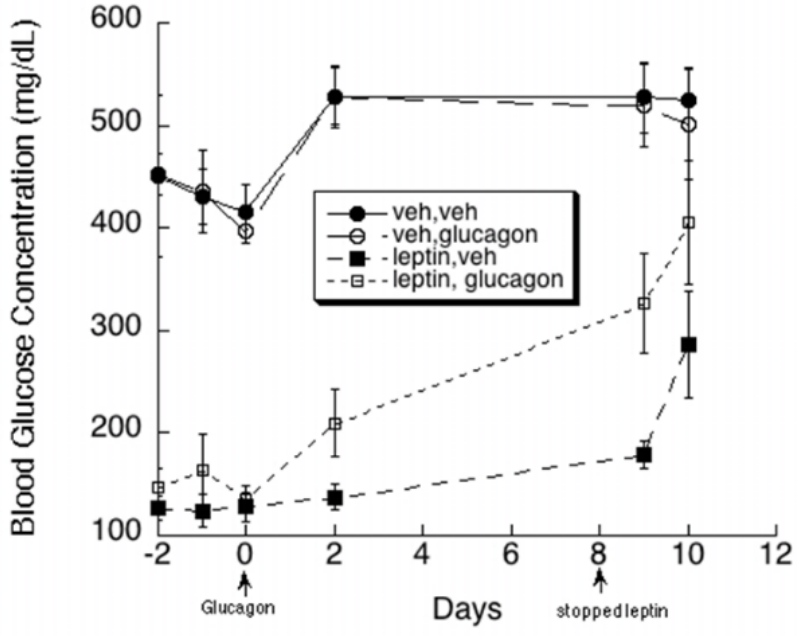


**Figure 6. Effects of chronic glucagon via osmotic pump on blood glucose concentrations in leptin-treated diabetic rats. Each point is the mean of 4-6 rats ± SEM.** High levels of glucagon treatment did not reverse the effects of leptin, though it did increase blood glucose concentrations on day 2 as compared to the leptin/vehicle-treated rats ($p>0.05$).

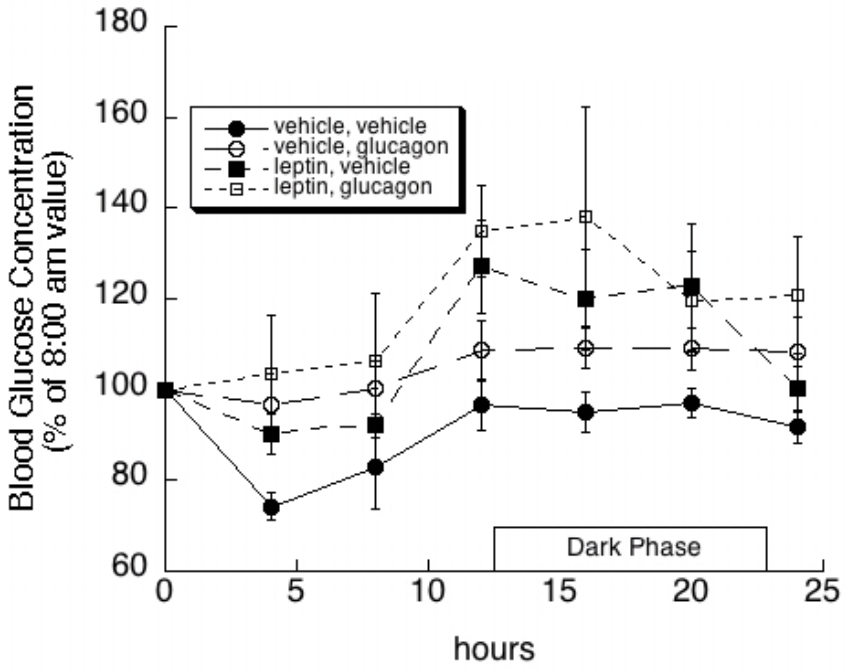


**Figure 7. Percent change in blood glucose concentrations across the 24-hour day in type 1 diabetic rats treated with leptin/glucagon. Each point is the mean of 4-6 rats ($p > 0.05$ with repeated measures).** Glucagon did not increase blood glucose concentration across the 24-hour day in leptin-treated rats.

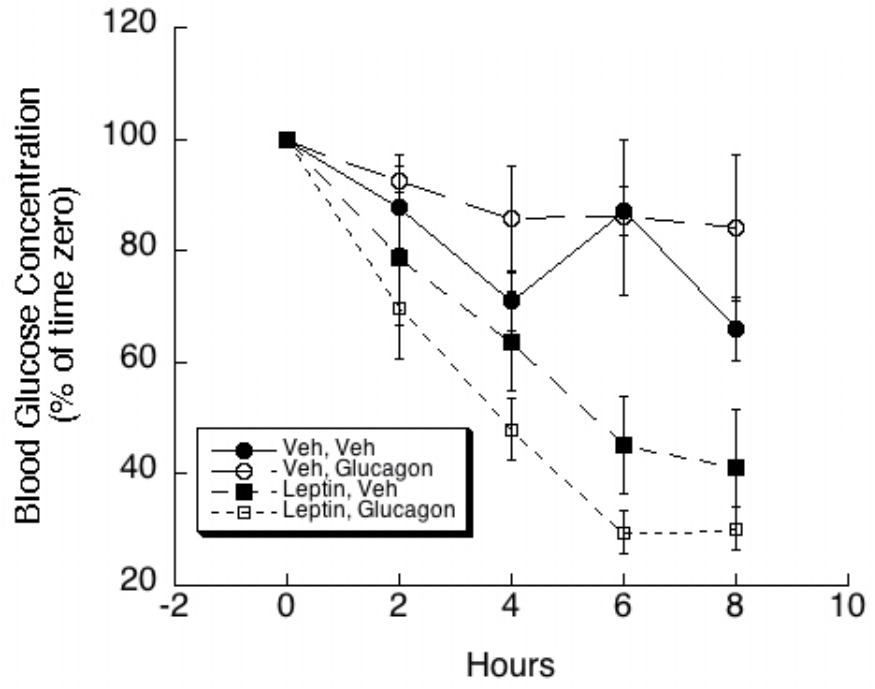


**Figure 8. Percent change in blood glucose concentrations during an 8-hour fast in type 1 diabetic rats treated with leptin/glucagon. Each point is the mean of 4-6 rats ± SEM.** Blood glucose during the fast was lower in leptin-treated than vehicle-treated rats ($P < 0.05$). Glucagon did not increase the blood glucose concentrations in leptin-treated rats during a short-term fast.

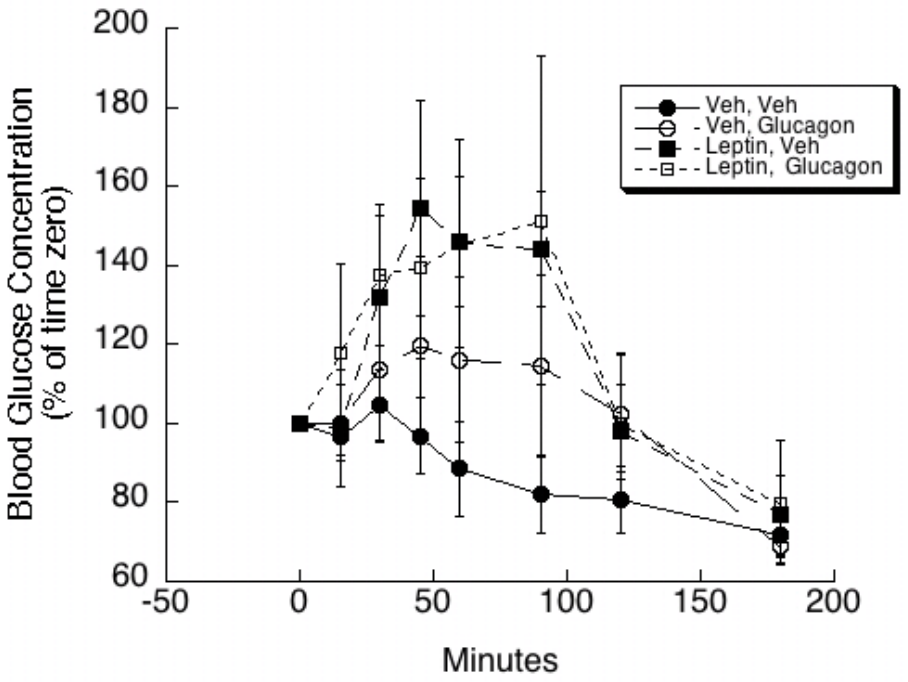


**Figure 9. Percent change in blood glucose concentrations in response to an IP pyruvate challenge (2mg/kg) in type 1 diabetic rats treated with leptin/glucagon. Each point is the mean of 4-6 rats ± SEM.** Blood glucose concentrations increases in response to pyruvate in leptin-treated rats compared to the non-leptin treated rats (30min, 45min, 60min by repeated measures, $p < 0.05$).

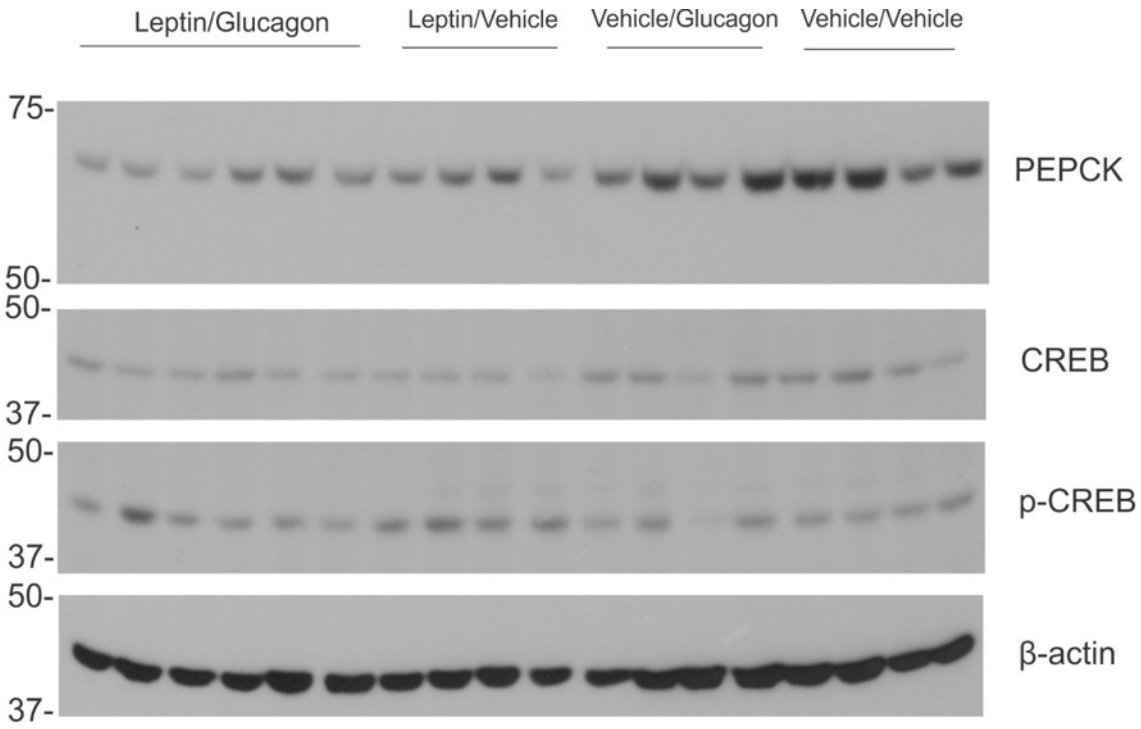


**Figure 10. Expression of gluconeogenic genes in liver: Protein levels of PEPCK, CREB and p-CREB.** Total protein was made from liver tissue and used in a Western blot.

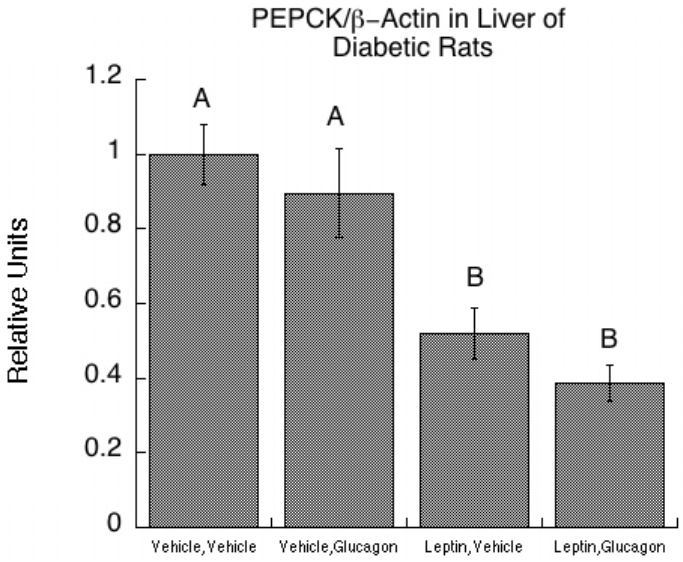


**Figure 11. Relative intensity of PEPCK/β-Actin in livers of type 1 diabetic rats treated with leptin/glucagon. Each bar is the mean of 4-6 rats ± SEM. Means with different letters represent a statistical difference ($p<0.05$).** Leptin treatment decreased liver phosphoenolpyruvate carboxykinase (PEPCK)/beta-actin.

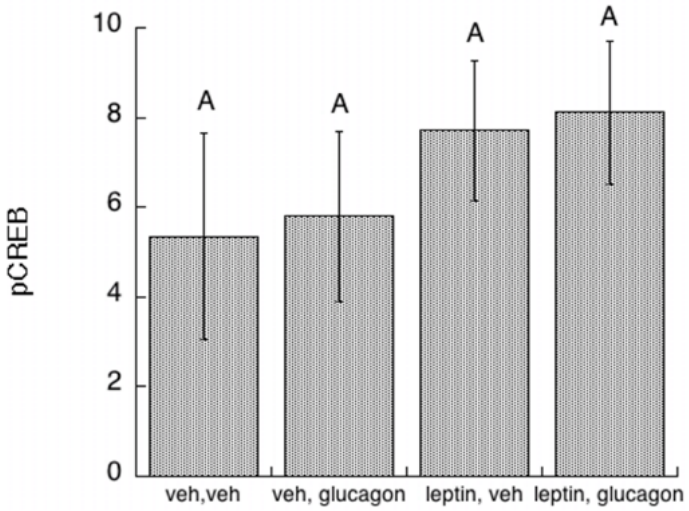


**Figure 12. Relative intensity of p-CREB protein in livers of type 1 diabetic rats treated with leptin/glucagon. Each bar is the mean of 4-6 rats ± SEM. Group means with the same letter indicate no statistically different ($p > 0.05$).** There were no statistical significant differences between groups.

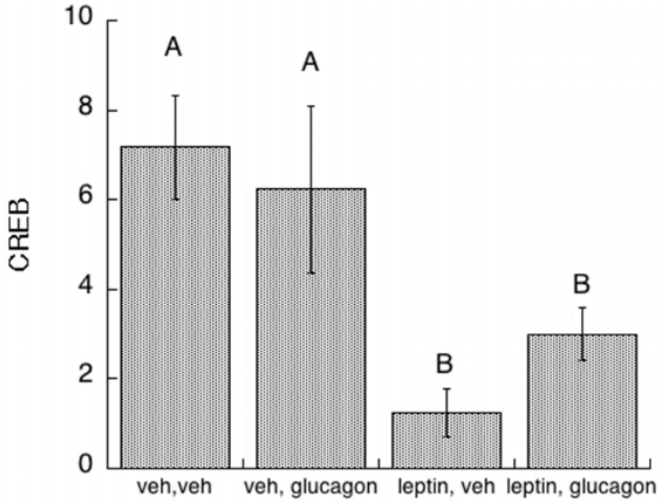


**Figure 13. Relative Intensity of CREB protein in livers of type 1 diabetic rats treated with leptin/glucagon. Each bar is the mean of 4-6 rats ± SEM. Group means with different letters indicate a significant difference ($p < 0.05$).** CREB protein was decreased in the livers of leptin-treated rats.

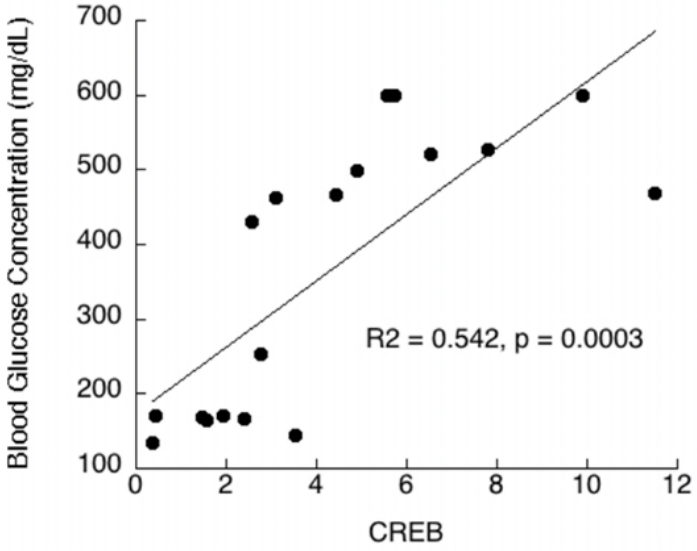


**Figure 14. Regression between CREB protein in the livers and blood glucose concentrations at the time the rats were euthanized. Differences in** CREB protein in the liver accounted for 54% of the variation in blood glucose concentration.

### 3.5 Summary and Conclusion

Daily ICV leptin treatment normalized blood glucose concentrations in type 1 diabetic rats. Despite large increases in plasma glucagon concentrations, blood glucose concentrations in leptin-treated diabetic rats across the 24-hour day, during a short-term fast, or in response to a pyruvate challenge were not returned to the level of vehicle-treated diabetic rats. Therefore, it seems unlikely that a reduction in glucagon concentration could be responsible for the leptin-induced normalization of blood glucose concentration observed in type 1 diabetic rats, as has been suggested by others (41,164,165). Rather, the data is consistent with a leptin-induced decrease in glucagon responsiveness. Liver PEPCK protein was decreased in rats with leptin treatment, which indicates that central leptin treatment may have an effect to inhibit glucagon responsiveness and blocking gluconeogenesis. Liver p-CREB and CREB proteins were examined as an index of activation of the cAMP pathway. CREB protein was decreased in leptin-treated rats, suggesting that chronic leptin treatment may reduce the cAMP pathway in the liver.

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