ASSESSMENT OF THE GLUCONEOGENIC CAPABILITIES OF LEPTIN-TREATED DIABETIC RATS BY FEEDING ALBUMIN AND FRUCTOSE DIETS

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THESIS ABSTRACT

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Uncovering the effects of the adipocyte hormone leptin will significantly increase our understanding of glucose regulation in the body. Leptin's effects on hepatic glucose homeostasis were assessed in this study. Diabetic rats and controls were given chronic central leptin or vehicle injections and fed purified gluconeogenic diets. The rats' ability to convert these precursors into glucose was evaluated by determining blood glucose levels and PEPCK content in the liver.

The gluconeogenic precursors enter gluconeogenesis at the level of specific enzymes known to regulate the process. Therefore, the glucogenic response to the different gluconeogenic diets should provide information about the key regulatory enzymes of gluconeogenesis. When provided as the sole food source for leptin-treated rats, the gluconeogenic precursors showed varying abilities to be converted into glucose. Data suggests that a defect exists at the level of PEPCK in diabetic rats given chronic central leptin treatment.

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CHAPTER I

INTRODUCTION

The prevalence of obesity is rising throughout the industrialized world at a substantial pace (1). Already the World Health Organization reports more than one billion people are overweight worldwide (2). The co-morbidities resulting from the obese condition such as type 2 diabetes, hypertension, heart disease, kidney disease, and dyslipidemia are rising as well (2, 3). These disorders add to the 36-39% increase in health care expenditures in obese adults compared to healthy adults (3). The findings of a European study determined that 80-95% of the increase in diabetics in Europe was attributed to obesity (4). Moreover, diabetes can lead to renal failure, amputations, and blindness which can greatly diminish quality of life (5, 6).

This link between diet-induced obesity and type 2 diabetes has focused attention on leptin, an adipocyte hormone associated with both obesity and diabetes. Leptin is known to induce satiety through the hypothalamus, but is also implicated in a host of metabolic regulatory mechanisms throughout the body (7). Leptin is released in proportion to the amount of fat storage and has therefore been determined to be a signal for high energy stores (7). This is similar to the process in which insulin responds to a high carbohydrate intake. Insulin signals the body to store energy by limiting food intake, regulating glucose homeostasis, and through other processes throughout the body (8). Leptin has also been shown to have many of the same effects (9). In fact, the two

hormones share many of the same intracellular pathways to control processes in the brain and tissues and therefore, have many overlapping functions (8). Both hormones have been shown to activate the PIP3 (phosphatidylinositol 3,4,5-triphosphate) and AMPK (AMP-activated protein kinase)pathways and therefore, share many of the same effects both peripherally and centrally (8).

In the liver, insulin has been shown to regulate glucose homeostasis through the regulation of the gluconeogenic enzymes PEPCK and G6Pase (10). It is thought that the lack of insulin caused by diabetes prevents the down regulation of gluconeogenesis during the fed state and results in the inappropriate production and release of glucose into the blood stream (10). The failure of insulin to down-regulate this process results in the elevation of blood glucose concentrations already raised by the digestion and absorption of food.

The similarities between the intracellular pathways used by leptin and insulin also suggest that leptin could have the same effect on hepatic glucose regulation (11). Short term leptin treatment appears to cause a decrease in gluconeogenesis, but there is no accepted consensus (12). The overlapping mechanisms of action of insulin and leptin as well as each of the hormone's regulatory effects on the other complicate these experiments (8). Moreover, acute treatment with leptin does not reflect physiological conditions in which leptin is always in circulation. Therefore, the most appropriate experiment to test the effect of leptin treatment involves chronic treatment of leptin in an environment lacking endogenous leptin and insulin.

In our experiments, we used STZ-induced diabetic rats to provide a background of low levels of endogenous insulin and leptin. These rats were treated with daily leptin

infusions ICV and provided pure diets of fructose and albumin which enter the gluconeogenic process at two different points. We hypothesized that gluconeogenesis would be significantly inhibited by leptin, resulting in leptin-treated animals having a decreased ability to convert these gluconeogenic precursors into glucose. This was measured through hourly blood glucose measurements during administration of the diets. The analysis of blood glucose resulting from the diets isolated to the gluconeogenic precursors suggests that gluconeogenesis is decreased. The significantly different blood glucose levels from the two experimental diets further indicate that the repression occurs at PEPCK.

CHAPTER II

LITERATURE REVIEW

OBESITY

Obesity in America has become a crisis. Health care expenditures resulting from the obese condition have become the most expensive disorder in American health care, costing more than any other medical disorder (13). An estimated 75 billion dollars is spent per year on treatment in the U.S., while another 139 billion is thought to be lost through indirect costs such as lost productivity (13). Complicating matters, obesity has reached epidemic levels as 66.3% of American adults over 20 years old are overweight (BMI > 25 kg/m^2), with half of that group classified as obese (BMI > 30 kg/m^2). In addition, one in twenty Americans are considered extremely obese (BMI > 40 kg/m^2) (13). This also underscores a greater than 50% increase in obese Americans in the last 2 decades with a much larger gain in the number of the extremely obese (13). The significance of these figures is relevant because of the considerable increase in health risks caused by obesity. Serious co-morbidities resulting from obesity depreciate quality of life and can increase the rate of premature death. These co-morbidities include cardiovascular disease, osteoarthritis, respiratory disease, and type 2 diabetes mellitus. For example, recent data indicate that there is an 18-fold increase in type 2 diabetes in extremely obese men (13). Type 2 diabetes has increased across all racial, sex, age, and

educational categories (13). Consequently, the prevalence of diabetes worldwide is expected to reach 5.4% by the year 2025 (14). Additionally, the number of people with impaired glucose tolerance is expected to reach 420 million people by the same year, mostly caused by obesity (1).

TYPE 2 DIABETES: DIET AND LIFESTYLE

The rapid increase in diabetes, especially in the industrialized world suggests that extrinsic factors are responsible for the epidemic rather than gene defects. The correlation between obesity and diabetes implies a common etiology: diet and lifestyle. The Center of Disease Control reports that the average daily caloric intake in the United States has risen during the period from 1971 to 2000 by 168 kcal per day in men and 335 kcal per day in women (15). This factor is exacerbated by a sedentary lifestyle and correlates to the rapidly rising incidence of type 2 diabetes in America. With this growing epidemic, scientists are focused on determining the link between nutrition and diabetes. Hopefully this will aid in providing better methods of prevention and treatment.

This research has revealed a very specific relationship of dietary intake to increased risks for diabetes. Many animal studies have examined the link between diet and decreased insulin sensitivity, a significant characteristic of type 2 diabetes. A high fat diet (~60% of energy supplied by fat) leads to decreased insulin sensitivity in rats (14). Long-term studies in humans have also revealed that the type of fat matters rather than simple fat content. A 3-month diet intervention study of 162 healthy subjects demonstrated that a diet high in saturated fat decreased insulin sensitivity compared to a diet similarly high in mono-unsaturated fats (16). This finding has been corroborated by

animal studies that propose benefits from diets high in mono-unsaturated and poly-unsaturated fats on insulin sensitivity (14). Similarly, epidemiological studies of trans-fat intake in humans have also been linked to an increased incidence of diabetes (14).

Fat content, however, is not the only element of diet associated with diabetes. Carbohydrate intake has similarly been associated with increased risk for the disease. Numerous studies presented in a review by Hu et al. have concentrated on the glycemic response which is a measure of the body's increase in blood glucose concentrations after consuming various types of carbohydrates (14). This response is measured by the glycemic index (GI), an attempt to quantify the rate of digestion and speed of absorption of different forms of carbohydrates (14). Many of these studies have linked diets with a high GI to the increased incidence of diabetes and decreased insulin sensitivity (14). The results of these studies illustrate that certain macronutrients can cause unique pathological consequences in the system. The whole body effects of certain dietary factors suggest that they modify homeostasis through hormonal regulation. The understanding that diet causes changes in the hormonal control of metabolism suggests the importance of studying this relationship.

HORMONAL REGULATION OF GLUCOSE HOMEOSTASIS

As discussed previously, high fat and high glycemic index diets can lead to decreased insulin sensitivity, a characteristic of type 2 diabetes. Insulin secretion is the normal response of the pancreatic β cells to increased blood glucose concentrations. Insulin is the body's response to energy intake and functions to induce anabolic effects throughout the body for the storage of energy. Insulin causes the uptake of plasma

glucose into tissues by inducing transcription and the translocation to cell membranes of GLUT 4 transporters (17). It also stimulates protein, glycogen, and fat synthesis while inhibiting many processes throughout the body that produce energy from storage molecules such as fat and glycogen (18). In contrast to the effects of storing energy throughout the body, insulin has also been shown to regulate the intake of energy by inhibiting feeding (8). The insulin receptor is present in many parts of the brain including the hypothalamus, an important regulatory center for energy homeostasis (8). Insulin appears to cross the blood brain barrier, but through a saturatable mechanism (8).

Insulin resistance is a decrease in sensitivity to insulin and the effects are found in many tissues throughout the body. The body recognizes elevated blood glucose and releases insulin in increasing amounts to maintain glucose homeostasis. When the insulin released is unable to normalize blood glucose levels, the body releases more insulin to regulate blood glucose. As the body decreases its sensitivity to insulin, more is released to regulate glucose levels. The chronic additive effect of the decrease in sensitivity and consequential overproduction of insulin eventually leads to pancreatic β cell dysfunction and diabetes (19). This process leads to specific, pathological causes of β cell demise including glucotoxicity, mitochondrial dysfunction, dysfunctional triglyceride/free fatty acid cycling, and oxidative stress (19).

In the case of glucotoxicity, research suggests that it is induced by an improper increase in gluconeogenesis in the liver (20, 21). This results in chronically high plasma glucose levels which freely enter the pancreatic β cells to stimulate increased insulin release. Insulin plays an important role in the down regulation of endogenous hepatic glucose production during periods following high carbohydrate intake (21). It is

responsible for the suppression of expression of key gluconeogenic enzymes that control endogenous glucose production such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) (10). A decrease in insulin action prevents the hepatic response to high blood glucose which normally inhibits the formation of glucose by gluconeogenesis. This is commonly a result of decreased insulin sensitivity in the pre-diabetic state as well as a consequence of the lack of insulin production from diabetes induced β cells failure (10). In this situation, the liver exacerbates high plasma glucose levels despite already having high plasma glucose concentrations. The resulting glucotoxicity is then related to further impairment of β cells in a debilitating cycle leading to diabetes (21). The investigation into this self-perpetuating cycle and the body's regulation of gluconeogenesis is an important topic in the search to understand the genesis of type 2 diabetes and is the target of interest in this study.

Before the diabetic state presents itself, the body naturally attempts to regulate glucose homeostasis through a multitude of hormone feed-back mechanisms in addition to insulin release. For example, when the stomach is full, the brain is signaled to not eat anymore. Additionally, as people gain fat stores in adipose tissue, their resting metabolic rate increases, requiring a greater caloric intake to further gain weight (13). Not all of these processes can be attributed to insulin alone and the mechanisms involved in these processes are the subject of extensive study.

The discovery of hormones released from adipose tissue, adipocytokines, has revitalized our understanding of how the body regulates energy homeostasis. These hormones have been found to have roles in thermogenesis, immune function, neuro-endocrine function, and feeding behavior (22). Insulin has been found to be just one

facet in the total regulation of energy balance. In contrast to insulin, adipocytokines are released in response to fat storage levels rather than glucose levels (22). However, many of the pathways and effects of adipocytokines overlap with insulin and each other (22). Research is beginning to paint a picture that metabolic regulation contains multiple, redundant feedback mechanisms. The hormones respond to specific markers of energy status and can act through shared pathways to maintain homeostasis (23). The adipocytokines and insulin also combine their metabolic regulatory duties with those of glucagon, catecholamines, and glucocorticoids to manage glucose homeostasis. These hormones can even directly regulate the production of each other in further collaboration (23).

LEPTIN

One such adipocytokine, leptin, has been implicated in processes throughout the body and is vital in metabolic regulation and reproduction (24, 25). This recently discovered hormone has been implicated in appetite regulation and in signaling when energy reserves are sufficient (7). Many of the effects have been found to be centrally regulated (much like insulin), but direct effects at the peripheral tissues are also important.

Serum levels of the leptin protein reflect a chronic release of the peptide by adipocytes in relation to body fat mass and nutritional status (10, 26). Expression and secretion is regulated by several factors such as estrogen, insulin, glucocorticoids, β3-adrenergic activity, and growth hormone (26). Food intake does not acutely regulate serum leptin concentrations, but leptin levels will vary over periods of hours in response

to fasting or overeating (24). This variation is thought to be possibly stimulated by insulin-induced variations of leptin secretion (24). However, dietary fat and fructose lead to decreased leptin production (with no effects on insulin) resulting in decreased metabolic rate and increased energy storage (7). It is believed that these other factors have a minor role in regulation of leptin concentration, but the most important factor is body fat mass (24).

The 16KDa peptide hormone is produced by the *ob* gene in mice and binds a receptor which is encoded by the *db* gene (24). Mutation of either gene results in a severe obese phenotype and diabetes in both mice and humans (24). When initially cloned, leptin therapy was thought to be the key to inducing satiety and weight loss. In genetically leptin deficient humans and mice, leptin treatment induced hypophagia and caused weight loss (24). Interestingly, the weight lost by animal subjects was accounted for by a decrease in fat with no effect on lean muscle mass (27). This contrasts with a calorie restricted diet, which results in a loss of both fat and lean muscle mass.

Unfortunately for the pharmaceutical industry, most obese patients are found to be in a state of leptin resistance. An increase in adipose tissue causes the body to release increased amounts of leptin in a negative feedback loop to induce satiety and signal sufficient energy stores (7, 26). Obese individuals have high circulating levels of the hormone, but do not produce the response expected from these high levels (24). The individuals gain fat mass and produce more leptin in an attempt to achieve the expected effects. This is probably due to the saturation of the transportation system for leptin across the blood brain barrier, as well as abnormalities in receptor activation or signal transduction (7, 24).

Leptin is unable to cross the blood brain barrier by diffusion, but is known to enter the brain through a saturatable transport mechanism (24). This transportation system likely involves one of the short forms of the leptin receptor. The long form of the receptor (Ob-Rb) consists of 1162 amino acids and is the isoform capable of signal transduction in the brain (24). The short isoforms (Ob-Ra and Ob-Rc) have a truncated intracellular domain and are unable to generate the signal induced by the long form. Their presence in the choroids plexus and brain microvessels suggests that they have a role in transport across the blood brain barrier (24). A limited presence of these transport receptors explains the ability of the system to become saturated. Additionally, obesity itself has been shown to decrease the transport of leptin into the brain (7).

In many experiments, leptin is infused directly into the ventricles of the brain [intracerebroventricular (ICV)] to bypass the need to cross the blood brain barrier. The effects of central leptin injections are seen throughout the body and require very small doses in comparison to peripherally injected leptin. Although many of leptin's effects are mediated centrally, other studies have found direct effects of leptin binding peripherally. This result is supported by evidence that the long form of the receptor has been found in a host of tissues throughout the body including the hypothalamus, pituitary, liver, kidney, adrenal cortex, spleen testes, adipose tissue, lung, and others (25). The ubiquitous distribution of the receptor suggests a multitude of physiological responses to leptin, many of which have not yet been identified.

PHYSIOLOGY OF LEPTIN IN THE BRAIN

The high level of expression of the Ob-Rb in the hypothalamus has lead to a significant number of studies on this region of the brain. Leptin is thought to act directly on the hypothalamus to suppress food intake and increase energy expenditure (24). An analysis of the receptor distribution in this part of the brain has revealed a concentration of the receptors in the hypothalamus, including the arcuate and paraventricular nuclei (7). Within these nuclei, the leptin receptor is found in two separate populations of neurons (figure 1).

The first set of neurons is orexigenic and produce neuropeptide Y (NPY) and agouti-related peptide (AgRP) (7). NPY is a neuropeptide that stimulates appetite when released and is involved in the regulation of several pituitary hormones: suppression of growth hormone, suppression of gonadotropins, and a stimulation of the pituitary-adrenal axis (7, 24). AgRP is in an inhibitor of melanocortin-3 and melanocortin-4 receptors, responsible for regulation of food intake, body weight, energy expenditure, and insulin action (28, 29, 30). Melanocortin-4 receptors (MC4Rs) are known to suppress food intake, prevent weight gain (possibly by increasing energy expenditure), and regulate sexual function (7, 29). Much of the mechanism of action of MC4Rs is still unknown. Leptin acts on this set of neurons through its receptor to inhibit the activity of NPY/AgRP neurons which suppresses their orexigenic effects (7).

The second group of neurons known to express the leptin receptor in the hypothalamus synthesizes POMC (pro-opiomelanocortin) and CART (cocaine and amphetamine-regulated transcript) (7, 31). In these neurons, POMC is processed to α-melanocyte-stimulating hormone (αMSH), an activator of downstream MC4Rs that

causes an anorexic response (29). CART, when activated, also produces an anorexic response to leptin signaling (31). In these neurons, leptin has a positive influence on the synthesis and processing of POMC, resulting in an amplification of its effects (7).

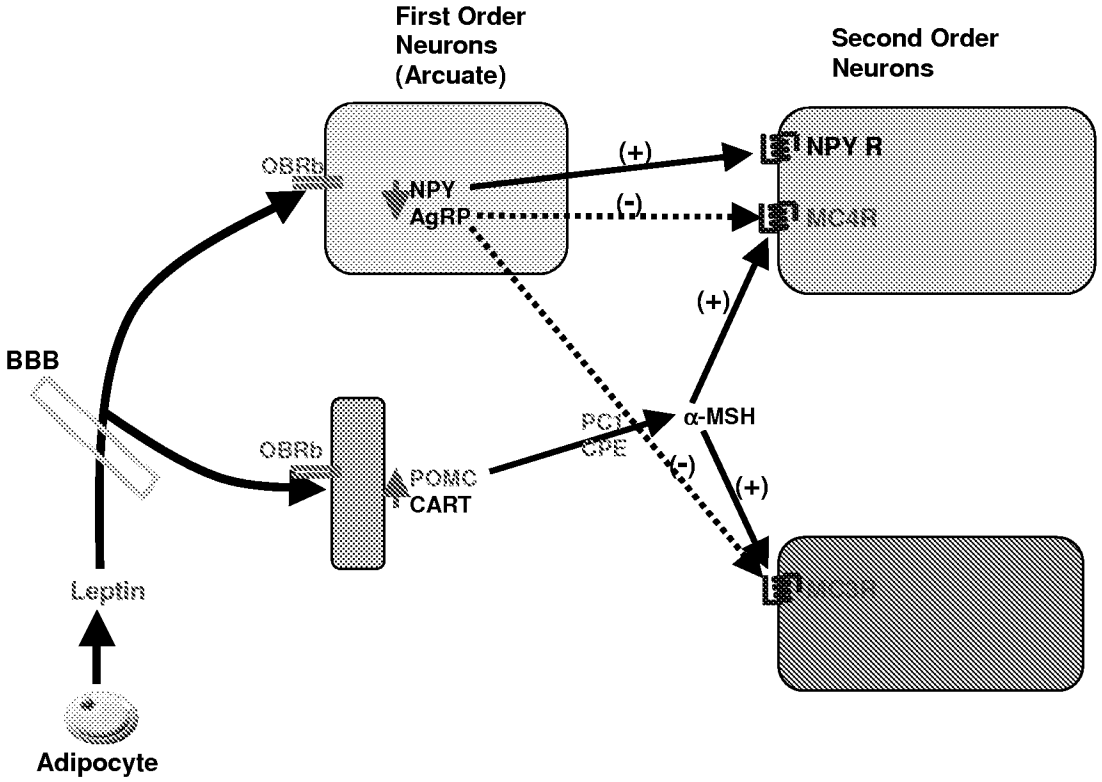


Figure 1 (32)

The leptin receptor appears to induce these neuronal effects in the hypothalamus through activation of two pathways. Regulation of PI3K (phosphatidylinositol 3,4,5-triphosphate kinase) is the same pathway used by insulin to regulate many of its activities (8). In the brain, it appears that both insulin and leptin induce satiety through phosphorylation of PI3K which leads to transcriptional regulation in these cells (8). This pathway of leptin action is not limited to neuronal cells and is active throughout the body.

Leptin also acts through a second pathway independent of insulin by activating STAT3, a transcription factor that also affects multiple cell types (8).

LEPTIN ACTION IN THE PERIPHERY

Although the intermediate mechanisms are unclear, the downstream effects of leptin acting in the hypothalamus are registered throughout the body. Variations in leptin levels affect the release of several factors including TNF-α/IL-1, catecholamines, T3/T4 (Thyroid hormones), cAMP (cyclic AMP), and androgens (7). Increased leptin stimulates fatty acid oxidation, insulin release, and peripheral insulin action (22). A reduction in leptin levels decreases thermogenesis, decreases thyroid and reproductive hormones, and increases glucocorticoid release (23). These effects have been seen in the pancreas (regulating insulin secretion), in skeletal muscle (varying glucose uptake and metabolism), and in the liver (modifying hepatic glucose production) (7).

In these peripheral tissues, leptin is thought to act through endocrine signaling, direct leptin receptor mediated action, and possibly through sympathetic nerve transduction initiated in the brain. A study by Pocai et al. has linked activity in the vagus nerve to a substantial decrease in hepatic glucose production through a decrease in regulatory enzymes in gluconeogenesis (33). This decrease has been linked to potassium dependant (K_{ATP}) channels in the mediobasal hypothalamus (34). Consequently, a transaction of the hepatic branch of the vagus nerve or addition of a K_{ATP} blocker within the mediobasal hypothalamus blocks this phenomenon (34). Central administration of long-chain fatty acyl CoAs and insulin has been connected to this effect suggesting it is a response to energy sufficiency (33, 34). Although no direct link has yet been established,

it is possible that in the brain, leptin follows the same pathway and decreases gluconeogenesis.

LEPTIN AND GLUCOSE HOMEOSTASIS

Although the mechanisms are unclear, leptin has a profound peripheral effect on glucose homeostasis. These effects are often observed in type 1 models of diabetes mellitus induced by streptozotocin (STZ). This drug destroys most of the β cells of the pancreas, severely limiting the synthesis and release of insulin (23). The effect of insulin on the release of leptin is noticeable in this model because it greatly diminished circulating leptin levels (23). This is because the release of leptin is controlled by intracellular glucose levels in the adipocyte which are significantly decreased due to the lack of insulin. The STZ model serves as an excellent background to study leptin effects because it greatly decreased endogenous serum insulin and leptin concentrations which may confound the effects of exogenously administered leptin. This "blank slate" ensures that the results are from the experimental treatment rather than potentially interfering endogenous hormones. Interestingly, the chronic hyperglycemia produced by diabetes is completely normalized by chronic leptin infusion in this model (35). The mechanism of action is not known, but the hormone may affect insulin sensitivity or may act through an insulin-independent route.

In ob/ob mouse models, mice lacking a functioning leptin gene are profoundly obese and diabetic. Their inability to normalize blood glucose concentrations provides a model to study the effects of leptin. In these mice, acute ICV infusion of leptin increases glucose turnover in an insulin-independent manner (35). This may be accomplished by

increasing glucose uptake in brown adipose tissue (BAT), brain, cardiac tissue, and possibly skeletal muscle and WAT (23, 35). Although significantly increased, leptin-induced glucose turnover is not near the levels induced by insulin (23). Interestingly, serum levels of glucagon, thyroid hormones, and growth hormone are unaffected (35). Hepatic effects on glucose turnover have also been studied in this model and others with conflicting results.

There is a consensus in the literature that glucose homeostasis is significantly affected by leptin, although the mechanism of action is not entirely clear. In our own experiments, blood glucose concentrations in diabetic rats are completely normalized by chronic ICV leptin treatment. These rats were made diabetic by STZ injection and therefore have extremely low endogenous insulin and leptin levels. This suggests that the effects on glucose homeostasis are mediated centrally. Additionally, neither circulating glucagon levels nor the insulin/glucagon ratio seemed to be altered. In these animals and their nondiabetic counterparts treated with chronic leptin, we have witnessed that a short-term fast caused a severe drop in blood glucose to near lethal levels. These effects are significant and the dramatic changes in blood glucose levels have not yet been explained.

On the cellular level, AMPK is thought to be important in the pathway of leptin-induced glucose homeostasis (35). Activation of the kinase by leptin stimulates glucose uptake and lipid oxidation and decreases glucose and lipid production in many of the tissues that regulate metabolism (9). These tissues include skeletal muscle, adipose tissue, the pancreas, and liver (9). Glucose production in the liver is thought to be inhibited through a decrease in PEPCK transcription caused by AMPK, possibly induced by leptin or adiponectin (9, 31). In previous experiments in our laboratory, a decrease in

gluconeogenesis by leptin would explain the loss of fat induced by leptin without any corresponding loss in lean muscle mass. This is because the muscle proteins would not be catabolized as a source of carbons to maintain blood glucose levels as is the case in rats calorically restricted. In this model, glycogenolysis would have to maintain blood sugar levels during a fast; however, glycogen storage seems to be disrupted in our studies. A lack of stored glycogen and diminished gluconeogenesis could potentially explain the diminished blood glucose levels seen in our leptin-treated rats during a fast.

Moreover, acute IV leptin has been shown to induce fatty acid oxidation in skeletal muscle by direct activation of AMPK in mice. This response is sustained through the hypothalamic-sympathetic nervous system via activation of α-adrenergic receptors without an increase in AMP levels (9, 31). In longer term studies, chronic leptin treatment resulted in an increase in AMPK protein expression and phosphorylation (31). This activity could also explain the paucity of fat tissue found in experiments on our chronic leptin treated rats and in other research (31).

As stated previously, leptin has also been linked to the regulation of PI3K, a pathway shared with insulin in cells (8). Barthel et al. suggests that the activation of PI3K in the liver leads to a decrease in transcription of the gluconeogenic enzymes PEPCK and G6Pase (36). This is another possible mechanism in which leptin may regulate glucose homeostasis.

LEPTIN IN THE PANCREAS

The pancreas is an important location for leptin action because of its role in insulin synthesis and release. Leptin receptors are expressed in pancreatic β cells and directly inhibit glucose-stimulated insulin secretion (23). Unfortunately, the mechanism by which leptin acts and the degree that this is physiological is unknown due to varied data from different studies. However, a predominant theory is that the failure of leptin to regulate insulin secretion in pancreatic β cells of obese humans may contribute to chronic hyperinsulinemia (21, 23). This is the result of the pancreas attempting to maintain homeostasis despite decreasing insulin sensitivity. This period before the diagnosis of type 2 diabetes is known as pre-diabetes and characterized by the initial stages of insulin resistance (23). The consequence of chronic overproduction of insulin is β cell hyperplasia, the eventual β cell death, and the induction of diabetes mellitus (21).

The pathology of pancreatic β cells and the induction of diabetes mellitus is also a contested issue. Studies suggest that a mechanism of β cell death is linked to a lack of leptin signaling (19). Leptin resistance appears to exacerbate β cell pathology through triglyceride accumulation (19, 23). Leptin is implicated in the regulation of cellular triglyceride levels and leptin resistance is thought to lead to lipotoxicity (37). These outcomes appear to be induced by leptin resistance developed under the obese condition.

LEPTIN IN ADIPOSE TISSUE

Leptin production and release occurs in the adipose tissue. In models of lipodystrophy, a condition deficient in white adipose tissue (WAT), patients are found to be hypoleptinemic (23). These subjects exhibit hyperinsulinemia, hyperglycemia, insulin

resistance, and an enlarged fatty liver (23). The lack of WAT limits the synthesis of leptin and exogenous leptin treatment reverses the symptoms, including insulin resistance (7, 21, 23, 37). This illustrates the importance of leptin in the regulation of insulin sensitivity, rather than obesity per se. However, the lipodystrophic model is a rare condition and leptin is not the sole contributor to the induction of insulin resistance (38).

Moreover, adipose tissue not only produces leptin, but is also a target of the hormone. Leptin is released corresponding to fat stores and is an indicator of adequate energy levels. Consequently, leptin appears to act through AMPK to decrease lipogenesis in adipose tissue and to increase its oxidation in other tissues (19).

EFFECTS OF ACUTE LEPTIN IN THE LIVER

Many facets of the relationship between the liver and leptin have been examined with confounding and paradoxical results. It does appear likely however, that leptin's effects on glucose homeostasis may partially be mediated through the liver, possibly through regulation of gluconeogenesis and glycogenolysis. ICV leptin experiments on this relationship have suggested that the primary effects of leptin are a result of central control, rather than direct tissue binding (39). Many studies use a variety of research methods, but have produced no consensus.

In hepatic cell line experiments, leptin treatment must be the result of direct action rather than central regulation. In one such experiment, high dose leptin seems to attenuate insulin-induced activities such as tyrosine phosphorylation of the insulin receptor substrate-1 (IRS-1) (40). This activity diminishes many of the effects of insulin and amplifies the downstream response of decreased insulin secretion from the pancreas.

Consequently, a down regulation of gluconeogenesis was attributed to the decrease in insulin activity (40, 41). In other cell models, gluconeogenesis also seems to be a target of leptin action, as the release of glucose from several gluconeogenic precursors (glycerol, L-lactate, L-alanine, and L-glutamine) were reduced following leptin treatment (42). In perfused rat livers, glucagon- and epinephrine-stimulated glycogenolysis was also significantly reduced by leptin treatment, probably through the reduction of cAMP levels (9, 23).

In mouse models, the effects of leptin can be attributed to both direct hormone action and hypothalamic response. In ob/ob mice, the rate of glycogenolysis was increased by chronic subcutaneous leptin administration; in perfused rat livers, similar results were obtained (43). On the other hand, another study by the same group using adenoviral leptin therapy showed no effect on glycogenolysis (43). Short-term IV and ICV administration of leptin in rats, however, suppresses hepatic glycogenolysis and increased gluconeogenesis as measured by increased PEPCK mRNA (39). In this study, intrahepatic glucose flux was markedly altered by acute ICV leptin, possibly by decreasing insulin's repressive effects on gene expression of PEPCK (39). Contradicting evidence reported by Burcelin et al. suggested that PEPCK activity was diminished in acute IV leptin-treated ob/ob mice (35). This group also reported that leptin stimulated hepatic glucose production was associated with elevated G6Pase and IRS-1 markers (35).

In an attempt to clarify leptin's role in hepatic glucose regulation, Gutierrez-Juarez et al. examined the ability of the melanocortin system to mediate leptin's affect on hepatic glucose metabolism. Acute ICV leptin resulted in increased gluconeogenesis and a concurrent decrease in glycogenolysis (44). Using a melanocortin antagonist, they

revealed that leptin increased PEPCK and G6Pase expression via central activation of melanocortin receptors (44). Conversely, glycogenolysis was decreased, but through a melanocortin-independent mechanism (44). The above studies produced conflicting results of acute leptin treatment, which are further complicated by studies using chronic infusion of the hormone.

THE EFFECT OF CHRONIC LEPTIN IN THE LIVER

Due to the conflicting nature of results produced by these experiments, further research must continue to examine the mechanism of leptin action in the body. Using STZ-induced diabetic rat models, our laboratory is seeking to determine the role of leptin in one facet of its role in glucose homeostasis. Our experiments have focused on leptin's function in regulating plasma glucose via the hypothalamus. As previously stated, chronic ICV leptin treatment has profound effects on blood glucose levels in a background almost completely absent of endogenous leptin and insulin (STZ-induced) (11). In these experiments, blood glucose concentrations of diabetic rats are normalized by leptin treatment through an unknown mechanism, with no change in insulin levels (11).

In additional experiments, diabetic and nondiabetic rats treated with a daily leptin bolus (5 μg) have extremely low blood glucose levels during a short-term fast. Under normal conditions, a significant drop in blood glucose would be countered by a decrease in insulin's repressive effects on gluconeogenesis and a concurrent increase in glucagon induced PEPCK expression (39). However, in diabetic rats, there is little insulin present to inhibit gluconeogenesis. With chronic leptin infusions, the rats are unable to maintain

normal blood glucose unless they consume food. During a relatively short-term fast, their blood glucose concentrations can fall to near fatal levels (< 20 mg/dL). Correspondingly, we have witnessed the animals feeding during periods of low blood glucose when food is provided. This feeding behavior occurs even during periods when the rats would not normally eat. The feeding response is a life saving reaction by the rats when their blood glucose drops below normal levels. The inability of the leptin treated rats to regulate blood glucose levels is an unexpected and unexplained finding. The most likely explanation is a down-regulation by the hypothalamus of gluconeogenesis and/or glycogenolysis, preventing normalization of blood glucose levels during a fast. This corresponds to the finding by Rossetti et al. which demonstrated that short term leptin treatment results in a decrease in gluconeogenesis (12).

Preliminary experiments to test this hypothesis with chronic leptin treatment in our laboratory have determined that a reduction in gluconeogenesis is a likely possibility. A gastric gavage of lactate (150 mg/100 g of body weight) in diabetic rats receiving daily ICV vehicle injections produced an expected increase in blood glucose and PEPCK mRNA. In the rats treated with ICV leptin, there was no increase in blood glucose or PEPCK, suggesting a decrease in gluconeogenesis. These results were supported by the finding that nondiabetic rats given chronic leptin treatment resulted in a 3-fold reduction in PEPCK mRNA. However, these results have not been published and require further study.

The decrease in gluconeogenesis due to leptin has also been witnessed by Hidaka et al. (11). They discovered that GLUT2 and G6Pase mRNA were up-regulated in diabetic rats while glucokinase (GK) mRNA was significantly down-regulated. GLUT2

is a glucose transporter required for the export of glucose from the liver and the induction of GLUT2 and G6Pase with the suppression of GK indicates an increase in gluconeogenesis. Following six days of central leptin treatment, the expression of each of these genes returned to the level of nondiabetic rats (11). The change in expression of these enzymes suggests that chronic ICV leptin decreases gluconeogenesis through these enzymes. Interestingly, Hidaka et al. discovered an induction of PEPCK mRNA in diabetic rats, but leptin did not reduce the enzyme expression as it did the others. (11).

Although gluconeogenesis was discernibly decreased in these experiments, the effect on glycogenolysis was not as easily detected. Results have varied in our preliminary experiments, but for the most part the data has suggested that there is at least some interference by leptin in the process. The chronic treatment of leptin appears to disturb glycogen storage in the liver, resulting in a decrease in available glycogen during a fast. Moreover, the fat stores in our rats chronically treated with leptin are also severely diminished, decreasing this form of energy storage.

RESEARCH HYPOTHESIS

1) We hypothesized that the normalization of blood glucose concentrations in leptin-treated diabetic rats and the decrease in blood glucose concentrations in leptin-treated fasted rats is due to a decrease in gluconeogenesis.
2) The decrease in gluconeogenesis in leptin-treated fasted rats is due to a defect at the level of PEPCK.

THE EXPERIMENTAL DESIGN

In the following experiment, we treated STZ-induced diabetic and nondiabetic rats with chronic ICV leptin. During a 5-hour test period we limited their food intake to 4 diets (chow, fructose, albumin, or no food). When in the fasted state, rats were observed for a drop in blood glucose which served as the positive control. The chow diet served as the negative control as its diverse mixture of macronutrients could be metabolized in several pathways. A diet of fructose, however, could only be metabolized by the liver into glucose by entering gluconeogenesis. Specifically, the hexose sugar could only enter the gluconeogenic pathway through the aldolase enzyme before the regulatory enzymes fructose1,6 bisphosphatase (F1,6BPase) and glucose-6-phosphatase (G6Pase) [figure 2]. In contrast, amino acids from the albumin diet could only form glucose by entering the gluconeogenic pathway prior to PEPCK. In this case, the precursors had to pass through both the regulatory enzymes PEPCK, F1,6BPase, and G6Pase. By analyzing the blood glucose of the animals at regular intervals, we attempted to determine the effects of chronic leptin treatment on gluconeogenesis and the location of these effects. Following the experiment, we removed the livers and analyzed the liver content of PEPCK.

Gluconeogenesis

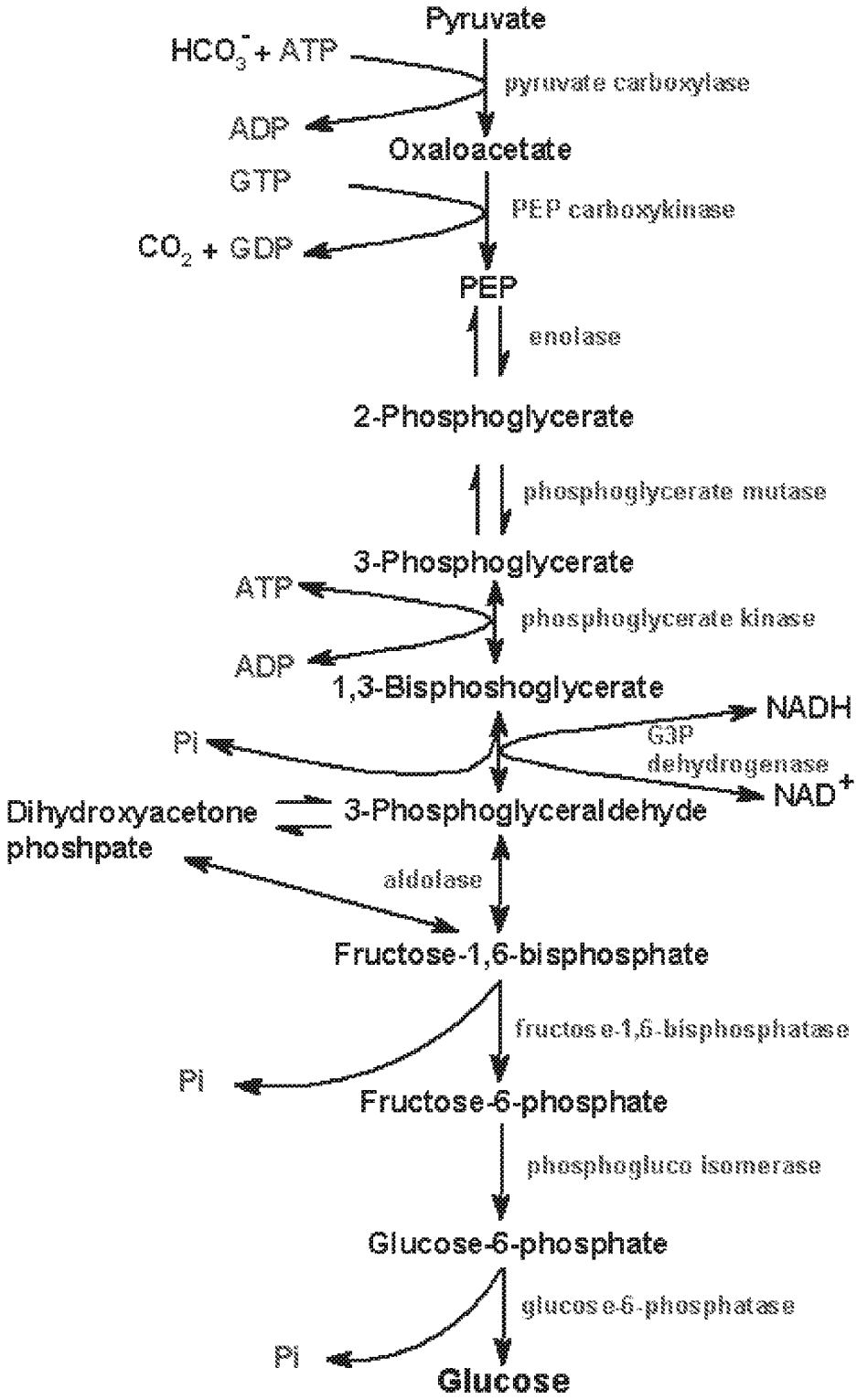


Figure 2 (45)

CHAPTER III

ASSESSMENT OF THE GLUCONEOGENIC CAPABILITIES OF LEPTIN-TREATED DIABETIC RATS BY FEEDING ALBUMIN AND FRUCTOSE DIETS

ABSTRACT

The role of leptin in glucose homeostasis is not clearly defined. Chronic intracerebroventricular (ICV) leptin treatment in diabetic rats leads to a normalization of blood glucose. Additionally, short-term fasting of the same animals leads to a significant drop in blood glucose concentrations. However, when provided food ad libitum, leptin-treated rats regulate blood glucose levels at normal levels. Apparently, glucose from the diet helps to maintain glucose concentrations at normal physiological levels. In fasted animals not treated with leptin, gluconeogenesis and glycogenolysis maintain blood glucose levels in a normal physiological range. We propose that central leptin administration causes a reduction in hepatic glucose output, leading to the inability of the animals to maintain blood glucose concentrations during a fast.

In this experiment, we used purified diets of albumin and fructose, which are known to be processed through gluconeogenesis to form glucose, to assess the gluconeogenic capability of leptin-treated rats. The inability of leptin-treated diabetic rats to use either precursor to successfully normalize fasted blood glucose levels suggests that gluconeogenesis is suppressed by leptin. The partial recovery of fructose toward normal blood glucose concentrations further suggests that the primary point of inhibition

is through the repression of PEPCK. Additionally, lower stores of glycogen in leptin-treated diabetic rats after a fast suggest that leptin-treated rats are able to derive glucose from glycogen stores, but that these stores may be insufficient to maintain blood glucose concentrations during the fast.

INTRODUCTION

Diabetes has become epidemic in the United States. According to the American Diabetes Association, 20.8 million Americans have diabetes (46). This represents 7% of the population. If current trends continue, one out of every three Americans born after the year 2000 will develop diabetes sometime in their lifetime (46). Diabetes is also becoming a world-wide problem. Estimates from the year 2000 suggest that world-wide 171 million people have diabetes. This number is projected to increase to 366 million by the year 2030 (5). Complications resulting from diabetes include cardiovascular disease and stroke, kidney disease, blindness, and non-traumatic amputation (47).

Evidence suggests that the fat-derived hormone, leptin, acting in the brain, has effects on blood glucose regulation in the body. Leptin has the ability to normalize blood glucose concentrations in type 1 diabetic rats (11). This suggests that the effects of leptin may be independent of insulin. Large doses of leptin (4 mg/kg/day) administered peripherally over a period of 14 days restore glucose concentrations to normal in streptozotocin (STZ)-induced diabetic rats (48). This is associated with a decrease in glucose production under both basal and glucose clamp conditions. Leptin treatment greatly increases glucose utilization during a glucose clamp. These effects are largely independent of the leptin-induced decrease in food intake. Several laboratories, including

ours, have extended this finding by demonstrating that chronic administration of much smaller doses of leptin into the ventricles of the brain also normalizes blood glucose in STZ-induced diabetic rats (11, 49, 53). This effect was not associated with an increase in serum insulin concentrations and again was found to be independent of leptin's effect on food intake. These studies demonstrate that leptin is acting in the brain to normalize blood glucose concentrations in diabetic rats. The feeding of high fat diets leads to insulin resistance and has been used as a model of insulin resistance in humans. Central leptin infused acutely (hours) into the third ventricle during a hyperinsulinemic-euglycemic clamp reverses the increase in hepatic glucose production rate induced by 3 days of feeding a high-fat diet (50). In addition, peripheral and central leptin administration improves insulin resistance and hyperglycemia in MKR mice, a mouse model of type 2 diabetes (41, 51).

Recently our laboratory has found that leptin-treated diabetic rats cannot maintain normal blood glucose concentrations during relatively short-term fast (52). A daytime fast of 3-5-hours leads to a 50% reduction in blood glucose levels, while an 8-hour fast leads to an 80% reduction in blood glucose. We suspect that the leptin-induced normalization of blood glucose concentrations in diabetic rats and the decrease in blood glucose concentration in leptin-treated fasted diabetic rats result from a common cause. The maintenance of blood glucose concentrations during a fast is associated with increased gluconeogenesis. Therefore, we hypothesize that chronic administration of leptin into the brain impairs gluconeogenesis in diabetic rats.

This study examined the gluconeogenic capabilities of diabetic and nondiabetic Wistar rats chronically treated with either leptin or vehicle. The gluconeogenic capability

of the animals was determined by the change in blood glucose concentrations in response to a diet of albumin or fructose. Results indicated that leptin-treated diabetic rats could not maintain normal blood glucose concentrations when fed albumin, whereas they could partially normalize their blood glucose concentration when fed a fructose diet. This suggests that the gluconeogenic enzyme, PEPCK may be impaired by chronic central leptin treatment. Additionally, decreased stores of glycogen in leptin-treated diabetic rats explain why glycogenolysis is not able return low blood glucose levels to normal in these rats.

METHODS & MATERIALS

Animal procedures

Thirty-two male Wistar rats (Harlan, Indianapolis, Indiana) between 250-275g were obtained and placed in individual hanging wire mesh cages. The environment was controlled to provide a 12-hour light and dark cycle and a constant temperature. The rats were fed a powdered chow diet (Prolab RMH 300 meal, Purina Mills, Richmond, Indiana) ad libitum and weighed daily. Water in excess was also provided at all times throughout the study. Food consumption was determined each day by determining the difference in food cup weights and accounting for spillage.

After 2 days, each rat was anesthetized using a mixture of ketamine and xylazine at 100 mg per kg and 1 mg per kg respectively. Each rat was placed in a stereotaxic apparatus and a 22 gauge, stainless steel guide cannula (Plastics One, Roanoke, Virginia) was then inserted into the right lateral ventricle of the brain. Under aseptic conditions, the cannula was secured by placing four stainless steel screws around the cannula and

secured with dental cement. The guide cannula was placed 0.8 mm posterior to the bregma, 1.4 mm lateral to the midline, and 3.5 mm ventral to the surface of the skull. A removable dummy cannula was inserted into the guide cannula until the time of infusions. Post surgery, the animals were given several days to recover and return to normal activity levels.

Placement of the cannula was verified by an angiotensin II (Sigma, St. Louis, Missouri) test. Each rat was infused with 40 ng of angiotensin II in 5 µl solution through the cannula via a syringe pump. A positive response to the test involved greater than 5 ml water consumption in 15 minutes after the infusion. This response confirmed the position of the cannula in the ventricle due to the interaction of angiotensin II and the subfornical organ which regulates the thirst response.

Sixteen of the rats were then made diabetic by injections of STZ (Sigma, St. Louis, Missouri), while the remaining rats received a vehicle only infusion (0.05 M citrate buffer at pH 4.5). The STZ was administered at 30 mg/kg (in 0.05 M citrate buffer at pH 4.5) through an intravenous tail injection. The induction of diabetes was verified by hyperglycemia (blood glucose > 350 mg/dL). Those rats given STZ that did not reach this blood glucose concentration were given a second dose after 2 days. All rats given STZ injections were confirmed diabetic by the fourth day. All blood glucose determinations were made using an Accu-chek simplicity glucometer (Boeringer Mannheim, Indianapolis, Indiana).

Half of the rats in each treatment group (diabetic and nondiabetic) were assigned to groups that received either leptin or vehicle injection. Each rat then began receiving daily 5µg/5µl infusions of either leptin (R&D Systems, Minneapolis, Minnesota) or

vehicle via the ICV cannula. Infusions were delivered from a syringed pump over a one minute period. Blood glucose concentrations were determined periodically throughout the study. The blood glucose levels of the diabetic rats were then allowed to normalize to levels near the nondiabetic animals for four days before the feeding trials began.

Feeding trials

The animals were randomly selected for one of two trial groups and each group alternated daily between the feeding trial and rest. Each animal in the feeding trial was randomly given one of the 4 trial diets every other day until they had been tested on all 4 diets (chow, fructose, albumin derived from egg source, or no food; Sigma, St. Louis, Missouri). The diets were weighed prior to the beginning of each trial. The trials consisted of the transfer of each animal to a cage with one of the four diets for a period of 6 hours. This process was performed during daylight hours each day beginning at approximately 9 AM. Blood glucose concentrations were determined at time zero (transfer of the animal) and at each hour of the trial. Food consumption was measured by the difference in the weights of the food cups while accounting for spillage. Following measurements at the fifth hour, each animal's food was replaced with chow for one hour, after which blood glucose concentrations and food consumption were determined. The rats were then returned to their normal cages for a day of recovery. Food consumption and blood glucose concentrations were determined during the recovery day.

After each group had completed the trial diets they were given 3 days to recover. During this period, food consumption measurements, blood glucose measurements, and leptin/vehicle infusions continued. The animals were then placed in a separate cage

without food for 5 hours. Following this fast, they were euthanized by decapitation. Trunk blood was collected from each specimen, placed in ice, and the serum collected. Livers were also removed from the body and flash frozen in liquid nitrogen and later in a -80ºC freezer.

Liver sample preparation

Approximately 50 mg of each liver sample frozen in the -80ºC freezer were removed, thawed, homogenized, and added to 300µl of 1x cell lysis buffer (Cell Signaling Technologies, Danvers, Massachusetts). The cell lysis buffer contained protease inhibitors, chelating agents, phosphatase inhibitors, and detergent to solublize the protein. 1.4 ml distilled water and additional protease inhibitors were added: leupeptin (1µg/ml), aprotinin (1µg/ml), protease inhibitor cocktails 1 and 2 (0.002 ml each), and PMSF (0.1 ml of 1 mM solution) (Sigma, St Louis, Missouri). The mixture was then kept in ice during sonication for 10 seconds to break up the cells. The product was then vortexed and re-sonicated for 10 seconds before being mixed again and left on ice for 1 hour. The mixture was then centrifuged for 20 minutes and the supernant removed and stored at -20 ºC.

A Bradford assay (Bio-Rad protein assay; Bio-Rad, Hercules, California) was then performed on the supernant sample to determine protein concentration in the liver homogenates. This was done by spectrophotometric comparison of the sample homogenates to a bovine serum albumin standard. The Bio-Rad microtiter plate protocols were used and absorbance was measured at 595 nm.

PEPCK determination

Fifty-micrograms of liver protein were analyzed by SDS-gel electrophoresis using the Criterion gel system (Bio-Rad, Hercules, CA). Proteins were transferred to nitrocellulose using standard conditions and the nitrocellulose sheets were blocked for 1 hour with SuperBlock (Pierce, Rockford, IL). The primary antibody used was rabbit anti-rat PEPCK (Cayman Chemical, Ann Arbor, MI) or rabbit anti-rat B-actin (Cell Signaling Technology, Danvers, MA) and secondary antibody was AMDEX goat anti-rabbit IgG-HRP (Amersham, Piscataway, NJ). The blots were treated with Pierce ECL Western Blotting Substrate ((Pierce, Rockford, IL), the results were visualized using the UVP EC3 Bioimaging System (UVP, Upland, CA) and quantitated using LabWorks software (UVP, Upland, CA).

Glycogen Determination

Hepatic glycogen content was determined by the procedures of Lo et al. 1970 (54). Briefly, approximately 50 mg of liver was digested in 30% potassium hydroxide saturated with sodium sulfate. Samples were boiled for 10 minutes, then rapidly cooled on ice. Glycogen was precipitated by the addition of 95% ethanol and pelleted by centrifugation. The pellet was dissolved with water and a standard volume (100 ul) transferred to glass tubes in triplicate. Glycogen standards were prepared and the sugar content of standards and unknowns determined by the addition of 0.1 ml 5% phenol and 0.5 ml (96-98%) sulfuric acid, vortexing gently, incubating in a 30 ° C water bath for 20 minutes, and determining the absorbance at 490 nm. The glycogen content of the

unknowns was determined from the generated standard curve. Glycogen content was expressed both as mg glycogen/mg protein and mg glycogen/liver.

Statistical analysis

Statistical analyses were performed using JMP version 5.1.2 (SAS Institute, Inc. Cary, NJ). Repeated Measures Analyses of Variance were used to determine the effects of diabetes and leptin on daily food intake, body weight, and blood glucose concentrations. Repeated Measures Analysis of Variance was also used to determine differences in the blood glucose concentration and diet consumed between diet groups across time within each treatment group (i.e., nondiabetic, vehicle-treated; nondiabetic, leptin-treated; diabetic, vehicle-treated; diabetic, leptin-treated). The effect of diabetes and leptin on hepatic PEPCK and glycogen content were determined by a Least Squares Analysis of Variance using a factorial design. Differences between individual groups were determined either by orthogonal contrasts or Least Square Student's t test as a follow-up test. Differences were deemed statistically significant at a P value equal to or less the 0.05.

RESULTS

Food intake, weight gain, and daily blood glucose concentrations

Daily food intake and body weight determinations began when the rats arrived, four days prior to STZ injections. The day of administration of STZ was designated as day zero, while leptin/vehicle injections were initiated on day 5. Body weights are shown in figure 3. Across days (day 5-day 22), there was a significant effect of diabetes (P =

0.001) and a significant effect of leptin ($p = 0.03$). Nondiabetic, vehicle-treated rats gained body weight throughout the experiment. Most of the main effect of leptin was attributable to the difference in body weights between nondiabetic, vehicle-treated rats and nondiabetic, leptin-treated rat ($P= 0.02$), since leptin did not cause a further decrease in body weights in diabetic rats.

Daily food intake is shown in figure 4. Across days (day 5-day 22), diabetes significantly increased daily food intake ($P = 0.005$), while leptin resulted in a significant decrease in daily food intake ($P < 0.0001$). There was a significant interaction between the effect of diabetes and leptin ($P = 0.003$). The effect of diabetes was solely attributable to the increase in food intake in the diabetic, vehicle-treated rats. Diabetic, leptin-treated rats had the same lowered food intake as nondiabetic, leptin-treated rats. This accounted for the significant interaction between the effects of diabetes and leptin.

Daily blood glucose concentrations are shown in figure 5. Across days (day 5 – day 22), diabetic rats had significantly greater blood glucose concentrations than nondiabetic rats ($P < 0.0001$). There was a significant effect of leptin to decrease blood glucose concentrations ($P < 0.0001$). A significant interaction between the effects of diabetes and the effects of leptin ($P = 0.0001$) was also observed. The interaction was attributable to the finding that leptin was only effective in decreasing blood glucose concentrations in the diabetic rats. Leptin treatment had no effect in nondiabetic rats. Chronic central leptin administration normalized blood glucose concentration in diabetic rats in about three days after ICV injections began. Normalized levels of blood glucose were maintained in the diabetic rats throughout the study.

Feeding trials

Blood glucose concentrations with the corresponding food intakes of the various diets are shown in figures 6, 7, 8, and 9. The top panels show the blood glucose concentrations each hour during the trial period, while the bottom panels show the hourly food intake of the rats fed the various diets. Figure 6 shows the results from the nondiabetic, vehicle-treated rats. Across time, there was no effect of diet on blood glucose concentrations. There was also no significant effect of time, indicating that blood glucose concentrations remain constant across time. Across time, chow- and fructose-fed rats ate similar amounts of food. The amount of food consumed by the albumin-fed rats was not significantly different from fasting. In other words, the rats did not eat a significant amount greater than zero.

Figure 7 shows the results from the nondiabetic, leptin-treated rats. Across time, there was no effect of diet on blood glucose concentrations, however, there was a significant overall effect of time ($P = 0.006$). This indicates that overall, blood glucose concentrations decreased over time in these leptin-treated animals. Across time, chow-fed rats ate more than fructose-fed rats ($P = 0.01$). The fructose-fed rats ate more than the albumin-fed rats ($P = 0.001$), however, in these leptin-treated rats, the albumin-fed animals ate significantly more than the fasted rats ($P = 0.001$).

Figure 8 shows the results of the diabetic, vehicle-treated rats. Across time, there was a significant effect of diet on blood glucose concentrations ($P = 0.002$) and a significant effect of time ($P = 0.002$). While blood glucose concentrations of the fasted rats were not statistically different from the chow- or fructose-fed animals, there was a tendency towards a difference (chow vs. fasted, $P = 0.06$; fructose vs. fasted $P = 0.13$).

The albumin-fed rats had lower blood glucose concentrations than the fasted rats ($P = 0.04$), fructose-fed rats ($P = 0.001$), and the chow-fed rats ($P = 0.0004$). Across time, chow-fed rats ate more than the other groups, while fructose-fed rats ate more the albumin-fed rats ($P = 0.04$). Interestingly, despite the changes in blood glucose concentration with albumin feeding, the albumin-fed rats did not eat significantly more than the fasted rats.

Figure 9 shows the results of the diabetic, leptin-treated rats. Across time, there was a significant effect of diet on blood glucose concentrations ($P < 0.0001$), as well as a significant effect of time ($P < 0.0001$). Fasting decreased blood glucose concentrations relative to the chow-fed rats ($P < 0.0001$). Blood glucose concentrations of albumin-fed rats were virtually superimposable to that of the fasted rats. Fructose-fed rats had greater blood glucose concentrations that the fasted ($P = 0.03$) or albumin-fed rats ($P = 0.04$), but they were still lower than the chow-fed rats ($P = 0.005$). Across time, there were differences in the amounts of diet consumed ($P = 0.0001$). The chow-fed rats ate more than the fructose-fed ($P = 0.002$) or albumin-fed rats ($P < 0.0001$), while the fructose-fed rats ate more than the albumin-fed rats ($P = 0.006$). The albumin-fed rats ate significantly more than the fasted rats ($P = 0.003$).

Liver PEPCK content

PEPCK protein content in the liver of the 5-hour fasted rats is shown in figure 10. The main effects of diabetes and leptin were not statistically significant, however, there was a tendency for an interaction between these effects ($p = 0.07$). Tests on each group showed that central leptin treatment significantly increased hepatic PEPCK content in

nondiabetic rats (P = 0.03), but had no effect diabetic rats. In addition, hepatic PEPCK content was greater in diabetic, vehicle-treated rats as compared to nondiabetic vehicle-treated rats (P = 0.03).

Liver glycogen content

The liver glycogen content of the 5-hour fasted rats is shown in figure 11. The top panel shows liver glycogen expressed as micrograms per milligram protein. The bottom panel shows liver glycogen expressed as milligrams per liver. On a per milligram protein basis, glycogen content was decreased both by the effects of diabetes (P = 0.02) and leptin (P = .008). There was no significant interaction between the effects. Though not statistically significant, there was a strong tendency for leptin to decrease glycogen in diabetic rats (P = 0.051). On a per liver basis, glycogen content was not affected by diabetes, however, there was a significant overall effect of leptin treatment to decrease glycogen per liver (P = 0.04). The latter effect was mostly attributable to the effect of leptin in diabetic rats, since leptin did not significantly decrease glycogen per liver in nondiabetic rats.

DISCUSSION

The reduction in body weight with central leptin administration seen in other studies was repeated in this experiment (figure 3) [11]. The use of chronic central leptin infusions likely acted through the hypothalamus to increase satiety in the animals (7, 28, 29, 30). This was corroborated by the food intake data which demonstrated a decrease in consumption in both leptin groups (figure 4).

As expected from previous experiments and other studies, central leptin administration normalized hyperglycemia in diabetic rats (11). In other experiments the same amount of leptin injected subcutaneously did not have the same effect; suggesting that leptin is acting centrally to affect blood glucose homeostasis (11). There are several possible mechanisms by which leptin could restore blood glucose levels to the normal range. One possibility is an increase in glucose uptake into muscle tissue, but this does not appear to occur according to Burcelin et al. (35). A decrease in energy intake caused by leptin-induced satiety is another possibility, but this seems unlikely since pair-feeding does not normalize blood glucose concentrations in diabetic rats (11). Likewise, a change in insulin secretion is not the cause as there is no increase in insulin levels in diabetic rats resulting from central leptin infusion (11). The most likely possibility is a down-regulation of glucose producing processes in the liver: glycogenolysis and gluconeogenesis.

The drop in blood glucose during a fast in leptin-treated diabetic animals also points to a disruption of normal glucose metabolism. When fed chow, the rats appear to prevent a drop in blood glucose by eating. However, when fasted, the rats appear to have impaired regulation of blood glucose concentrations, which drop significantly. The normal processes that recover blood glucose concentrations during a fast, i.e. gluconeogenesis and glycogenolysis, appear to be disrupted.

The low levels of insulin in type 1 diabetics are implicated in the genesis of hyperglycemia because insulin cannot suppress gluconeogenesis as it does in nondiabetic animals (39). Gluconeogenesis is therefore, a likely target of leptin action. The feeding trials attempted to ascertain this by placing the animals in a controlled environment with

specific diets. Diabetic rats treated with leptin were unable to maintain their blood glucose during a fast (figure 9). When given chow, the animals maintained their blood glucose at normal physiological levels. When consuming albumin however, the animals were unable to normalize blood glucose levels. This suggests that leptin-treated diabetic rats were unable to convert the amino acids from albumin into glucose. When fed fructose, these rats were able to increase their blood glucose concentrations compared to fasted rats, however, not to the level of the chow-fed rats. This suggests that the fructose is able to be converted to glucose, however, perhaps not to a normal degree. Both fructose and the amino acids derived from albumin are only able to be metabolized in the liver to produce glucose through gluconeogenesis. The animals' inability to regulate blood glucose levels when fed these diets suggests that gluconeogenesis may be inhibited.

The diabetic, vehicle-treated rats also demonstrated an inability to increase blood glucose in response to albumin feeding (figure 8a), in fact, blood glucose concentrations of the albumin-fed rats were decreased compared to the fasted rats. Interestingly, this occurred even though very little albumin was actually consumed by the rats. The relatively constant glucose levels in these animals followed by the rapid decrease in the second hour suggest that the diet induced the change. Although not receiving leptin infusions, gluconeogenesis could have possibly been inhibited by other means. In a study by Garlick, high intakes of leucine caused a significant increase in insulin which down-regulates gluconeogenesis (55). However, diabetic animals do not have the ability to produce large amounts of insulin due to the β-cell death induced by STZ. Other studies suggest that the high intake of leucine in the absence of carbohydrates affects the insulin

signaling pathway rather than insulin directly (56). The insulin signaling pathway is known to directly regulate gluconeogenesis and may cause this response.

The lack of decrease in blood glucose concentrations in the nondiabetic, leptin-treated animals was a surprising finding in comparison to some of our previous results (Figure 5). During the fasting period prior to sacrificing the animals, three rats in this group did not decrease their blood glucose concentration in response to fasting, while four rats decreased their blood glucose concentrations to the level of fasting diabetic, leptin-treated rats. This suggests that three of the animals may not have responded to the leptin treatment and the data may improperly represent what is occurring. When these animals are not considered, the data closely resembles previous experiments in which nondiabetic, leptin-treated animals had significantly decreased blood glucose concentrations during a fast.

The feeding trials in this experiment were also designed to distinguish which gluconeogenic enzymes could be down-regulated by leptin. The significantly decreased blood glucose concentrations in response to the albumin diet in diabetic, leptin-treated animals imply that the PEPCK enzyme may be suppressed. In these rats, fructose was also unable to return blood glucose levels to normal, but to a lesser extent than albumin. This would suggest that either G6Pase or fructose 1,6-bisphosphatase may also be partially repressed. This is contrary to the findings of Hidaka et al. who found a repression of G6Pase, but not PEPCK mRNA in chronically leptin-treated diabetic rats (11).

Similar to the findings of Hidaka et al., liver PEPCK protein content was increased in diabetic rats in our study (11). Also similarly, we found no decrease in

PEPCK protein by leptin treatment. It is possible that PEPCK is not repressed and translation occurs normally, but post-translational modification is decreasing enzyme activity. A PEPCK activity assay may clarify this hypothesis, however, there is no research suggesting that the PEPCK enzyme is modified in the regulation of gluconeogenesis.

There may also be regulation of the enzymes downstream to PEPCK. This could include the glucose transporters associated with the G6Pase complex or GLUT2 which exports glucose from gluconeogenesis and glycogenolysis out of the liver (59). This hypothesis, however, is not supported by the results of the feeding trials which had a greater drop in blood glucose from albumin than fructose. This finding supports the hypothesis that the point of regulation is at PEPCK.

In previous experiments we have observed a decrease of hepatic glycogen in rats given chronic leptin injections, even in the fed state (57, 58). In the present study, there was a decrease in total glycogen per liver in leptin-treated diabetic animals compared to the diabetic, vehicle-treated rats. This suggests that leptin-treated diabetic rats are able to utilize their hepatic glycogen stores in the production of glucose, but that these store are insufficient to maintain blood glucose concentration over the 5-hour fast. If this is so, it would be expected that glucose produced from gluconeogenesis would help maintain the glucose output from the liver. However, if leptin impairs gluconeogenesis as was suggested above, hepatic glucose cannot be maintained, resulting in a decrease in the blood glucose concentrations.

From previous studies, we did expect a significant decrease in hepatic glycogen in all leptin-treated animals compared to the control groups. This did not occur in the

leptin-treated nondiabetic group which was not significantly different from the vehicle-treated, nondiabetic group. It was possible that three animals in the leptin-treated group (n = 7) did not fully respond to leptin treatment. Their blood glucose levels remained comparable to the vehicle-treated animals group during the fast. The nondiabetic, leptin-treated animals that did not decrease their blood glucose concentrations in response to fasting, had much greater liver glycogen levels after the fast. The glycogen stores of these three animals were several fold greater than their counterparts and raised the average value. Discounting these animals, the glycogen content in the liver and blood glucose concentrations during the fast would have been comparable between the nondiabetic leptin-treated rats and the diabetic, leptin-treated rats. This suggests that the significantly diminished glycogen stores found in leptin-treated rats is an important factor in the inability to maintain glucose homeostasis during a fast.

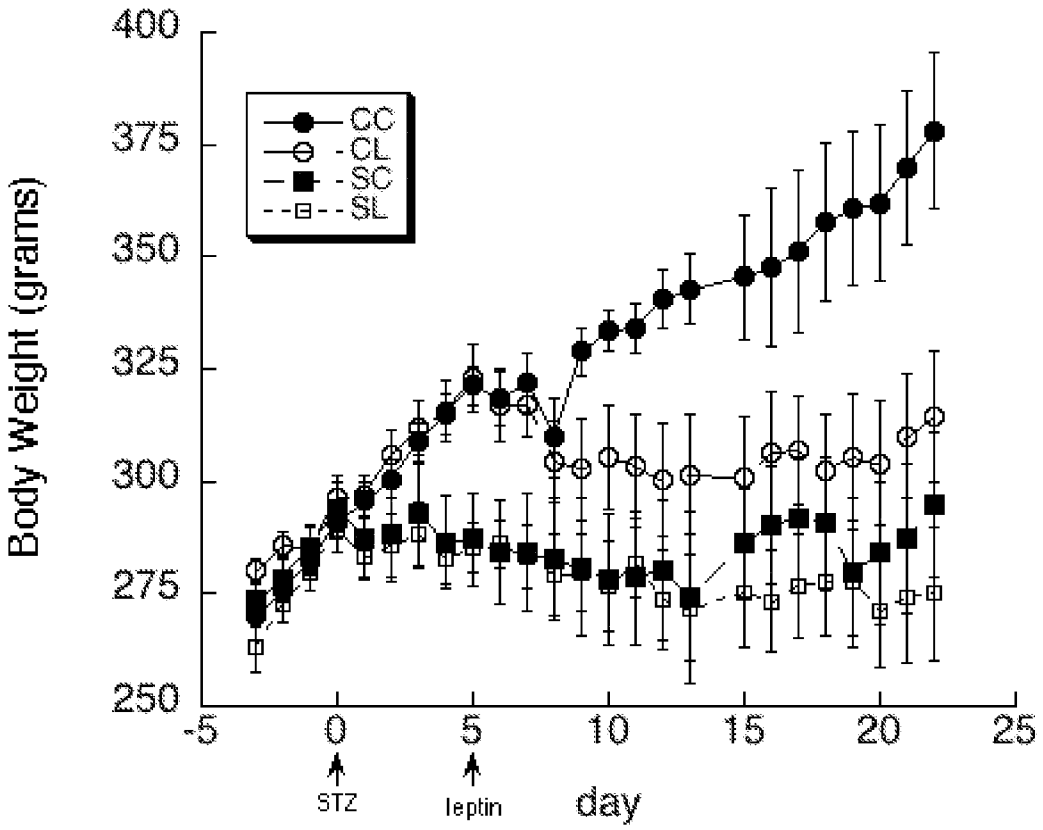


Figure 3. Daily body weight. Means are shown +/- standard error. Vehicle-treated nondiabetic group (CC - ●), leptin-treated nondiabetic group (CL - ○), vehicle-treated diabetic group (SC - ■), leptin-treated diabetic group (SL - □). There was a significant decrease in body weight due to the induction of type1 diabetes. There was also a significant decrease in body weight following the administration of leptin.

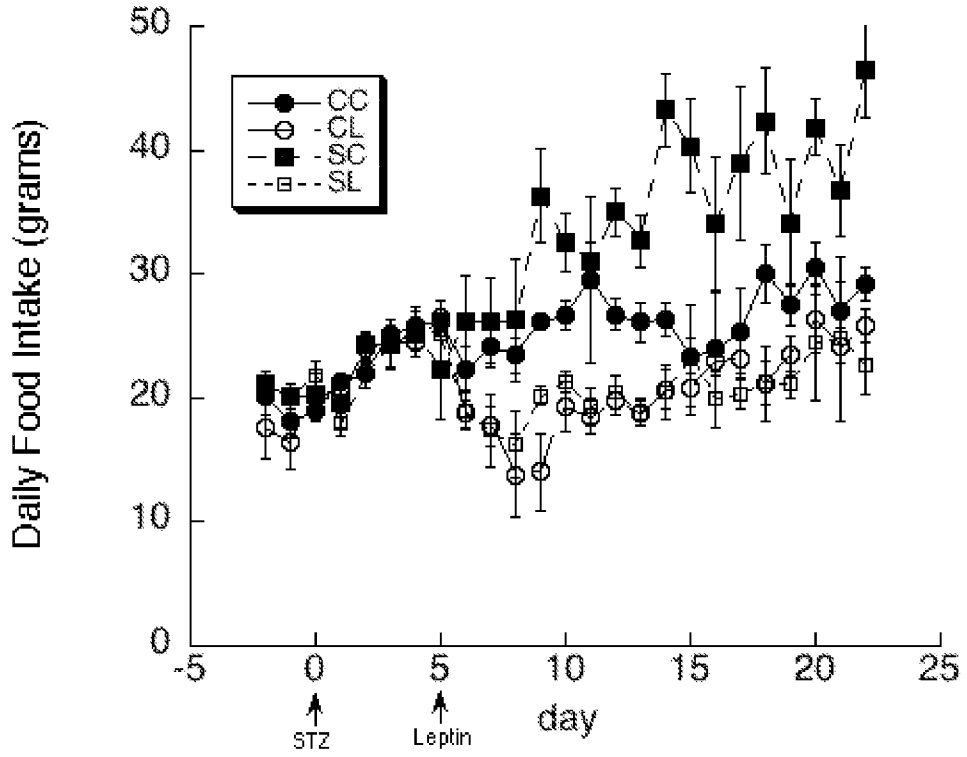


Figure 4. Daily food intake. Means are shown +/- standard error. Vehicle-treated nondiabetic group (CC - ●), leptin-treated nondiabetic group (CL - ○), vehicle-treated diabetic group (SC - ■), leptin-treated diabetic group (SL - □).
The induction of type 1 diabetes induced a significant increase in food intake which is negated by central leptin infusion.

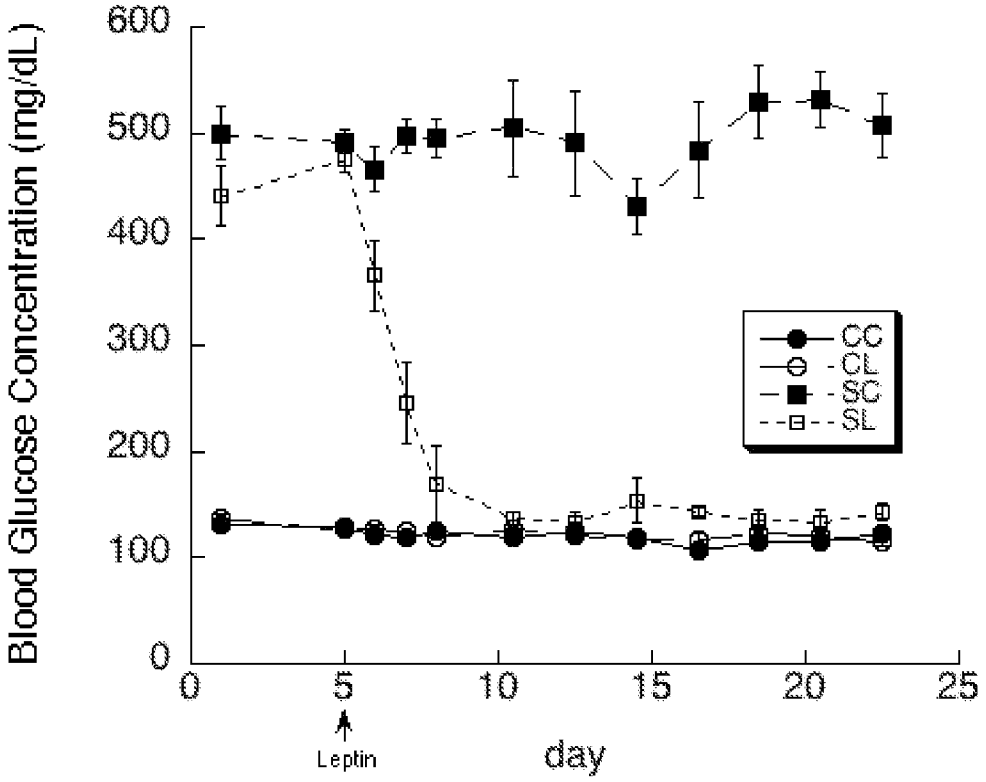


Figure 5. Daily blood glucose concentrations. Means are shown +/- standard error. Vehicle-treated nondiabetic group (CC - ●), leptin-treated nondiabetic group (CL - ○), vehicle-treated diabetic group (SC - ■), leptin-treated diabetic group (SL - □). The induction of type 1 diabetes led to a significant increase in blood glucose concentrations. The administration of central leptin caused a decrease in blood glucose concentrations to levels equal to nondiabetic animals.

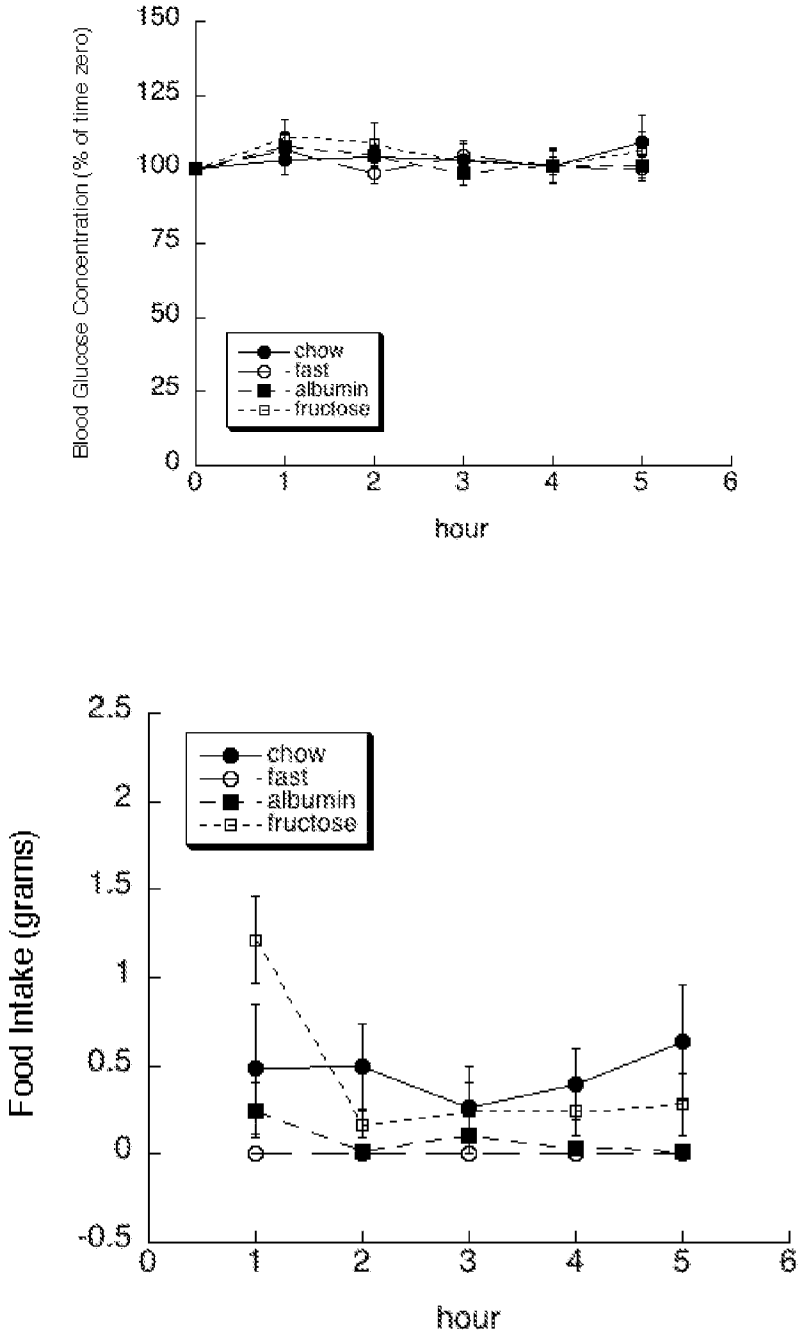


Figure 6. Nondiabetic vehicle-treated hourly blood glucose concentrations and food intake. Means are shown +/- standard error. Blood glucose concentrations expressed as a percent of time zero (upper panel). Food intake measured in grams (lower panel). Chow diet – (●), fast – (○), albumin diet – (■), and fructose diet – (□).
These animals maintained blood glucose concentrations within tight parameters with no variation due to diet.

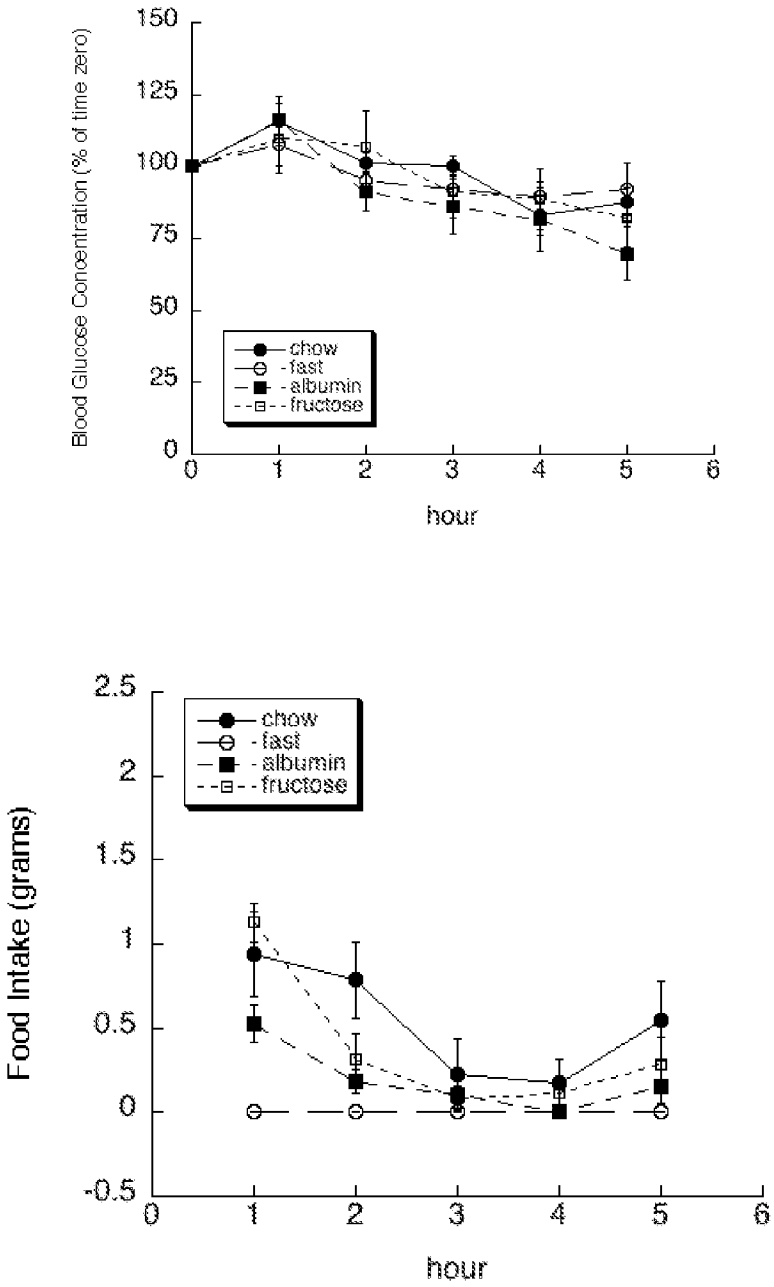


Figure 7. Nondiabetic leptin-treated hourly blood glucose concentrations and food intake. Means are shown +/- standard error. Blood glucose concentrations expressed as a percent of time zero (upper panel). Food intake measured in grams (lower panel). Chow diet – (●), fast – (○), albumin diet – (■), and fructose diet – (□). There was a significant decrease in blood glucose over time in all groups; however, there was no significant difference in blood glucose levels between animals fed the different diets.

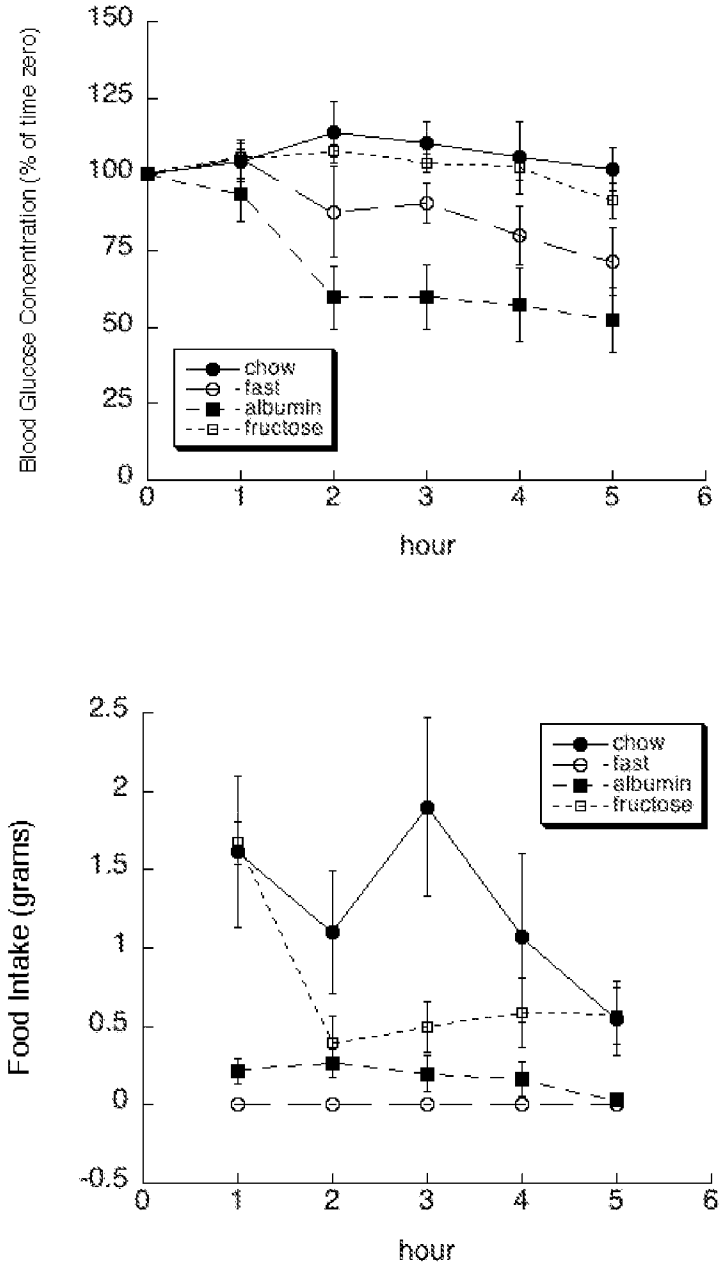
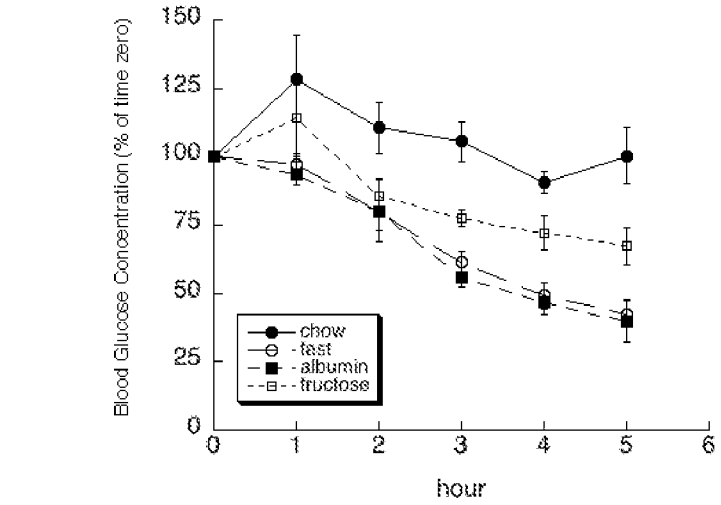


Figure 8. Diabetic vehicle-treated hourly blood glucose concentrations and food intake. Means are shown +/- standard error. Blood glucose concentrations expressed as a percent of time zero (upper panel). Food intake measured in grams (lower panel). Chow diet – (●), fast – (○), albumin diet – (■), and fructose diet – (□). There was a trend toward a decrease in blood glucose in the fasted rats when compared to the chow-fed rats (P=0.06). There was a significant decrease in the blood glucose concentrations of animals fed albumin when compared to all other groups. Notably, this group did not statistically consume more food than the fasted rats.



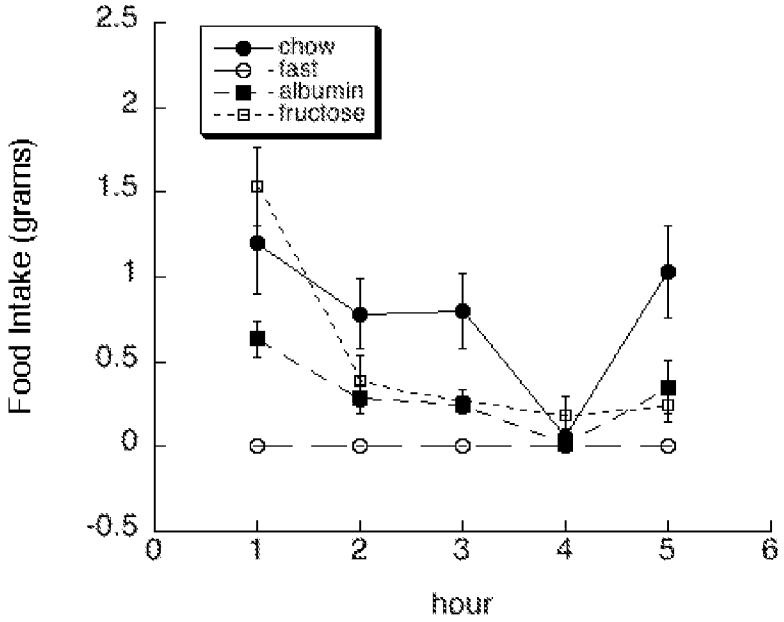


Figure 9. Diabetic leptin-treated hourly blood glucose concentrations and food intake. Mean are show +/- standard error. Blood glucose concentrations expressed as a percent of time zero (upper panel). Food intake measured in grams (lower panel). Chow diet – (●), fast – (○), albumin diet – (■), and fructose diet – (□). There was a significant difference in the blood glucose concentrations in the animals fed fructose from the other groups. The blood glucose concentrations of animals fed albumin and those who were fasted were very similar and were significantly decreased from the other groups.

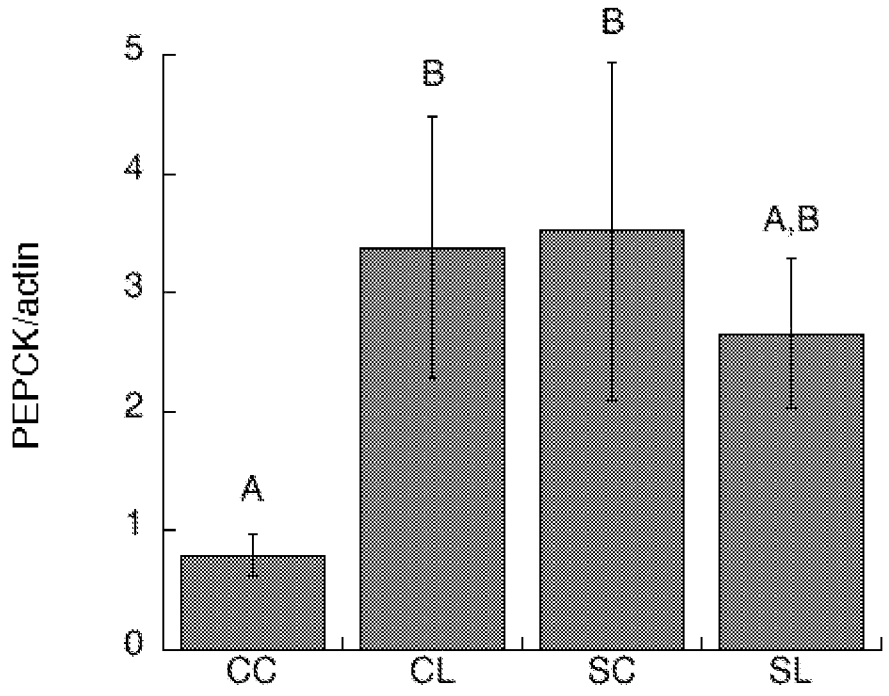


Figure 10. PEPCK protein content in liver of fasted rats. Means are show +/- standard error. Vehicle-treated nondiabetic group (CC), leptin-treated nondiabetic group (CL), vehicle-treated diabetic group (SC), leptin-treated diabetic group (SL). Different letters represent a statistical difference between groups ($P < 0.05$). There was a significant increase in liver PEPCK content caused by both leptin and diabetes. However, this difference was not significant in the SL group.

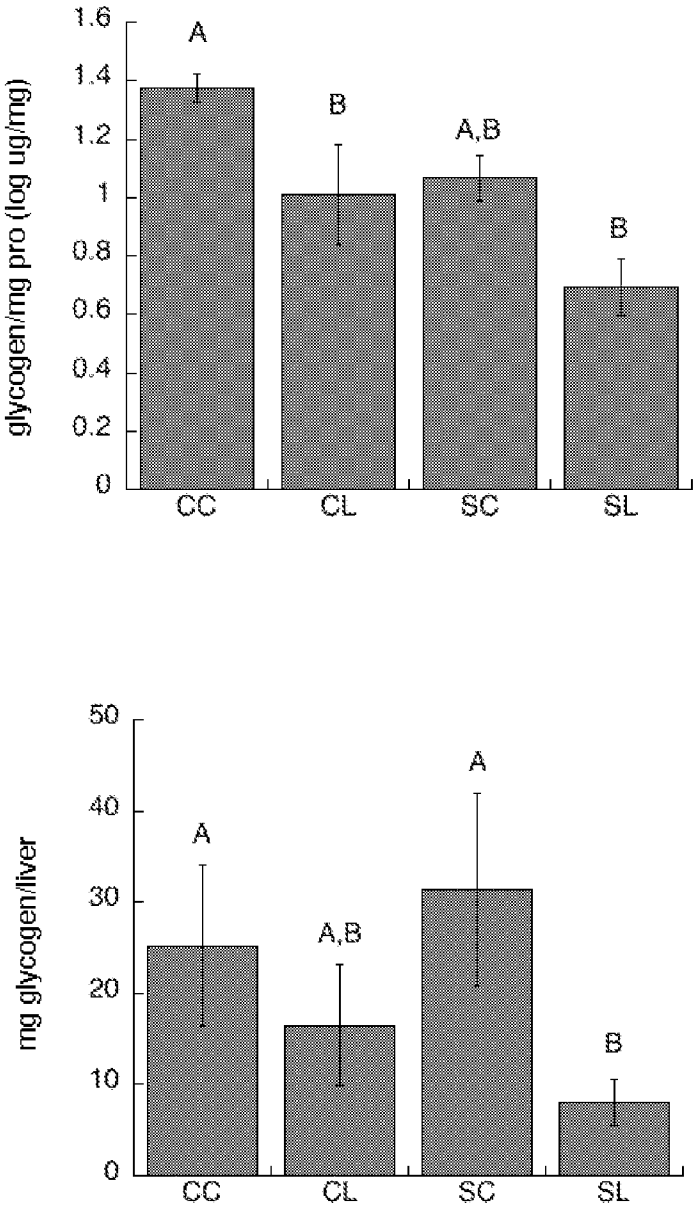


Figure 11. Hepatic glycogen content. Mean are shown +/- standard error. Glycogen is expressed as micrograms per milligram protein (upper panel) and as milligrams glycogen per liver (lower panel). Vehicle-treated nondiabetic group (CC), leptin-treated nondiabetic group (CL), vehicle-treated diabetic group (SC), leptin-treated diabetic group (SL). Different letters represent a statistical difference between groups ($P < 0.05$). Chronic leptin treatment resulted in a decrease in liver glycogen content.

CHAPTER IV

SUMMARY

Gluconeogenesis appears to be down-regulated by leptin in these experiments. This explains the inability of diabetic animals to maintain normal physiological blood glucose concentrations during a fast. It would also explain the mechanism by which leptin reduces diabetic hyperglycemia to normal physiological levels. The decreased amount of stored hepatic glycogen because of leptin also seems to play a role in the inability to maintain blood glucose.

Leptin seems to decrease gluconeogenesis through the suppression of PEPCK, although this conclusion is not supported by all of the data. More research is needed to explain the disparity between our results and gene expression data from other studies (11). An analysis of PEPCK activity may illuminate this difference.

The inability to properly regulate glucose homeostasis through the control of gluconeogenesis is a hallmark of type 2 diabetes (10). The result of this research implies that type 2 diabetes is a result of the inability of both insulin and leptin to inhibit post-prandial hepatic glucose output. However, the exact mechanism by which leptin acts is still debatable.

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