THE APPARENT INCREASE IN INSULIN SENSITIVITY OF LEPTIN-TREATED

RATS APPEARS TO BE DUE TO A DECREASE IN BLOOD GLUCOSE

CONCENTRATIONS IN RESPONSE TO FASTING

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THESIS ABSTRACT

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RATS APPEARS TO BE DUE TO A DECREASE IN BLOOD GLUCOSE

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To distinguish between the effects of insulin stimulation and fasting on blood glucose homeostasis in leptin-treated animals, streptozotocin(STZ)-induced diabetic (STZ-D) and non-diabetic rats given chronic intracerebroventricular (icv) injections of leptin (5 µg/5 µl) or vehicle (5 µl) underwent insulin tolerance tests (ITT) with either intraperitoneal (ip) injection of insulin (1 U/kg) or saline (0.9% NaCl) in a cross-over design study. Regardless of whether the rats were diabetic or non-diabetic, the insulin-mediated decrease in blood glucose tended to be greater in leptin-treated rats than in vehicle-treated animals, indicating enhanced insulin sensitivity. However, leptin treatment also resulted in a significant decline in blood glucose after saline injection ($P < 0.005$), which served as a control for the change in blood glucose due to fasting sustained during the ITT testing period (90 min). This decrease due to the fast during the ITT accounted for 92 % of the difference in response between the leptin-treated and

vehicle-treated rats given insulin. This suggests that most of the difference between leptin-treated and vehicle-treated rats given insulin was due to a leptin-induced decrease in blood glucose due to fasting and not to enhanced insulin sensitivity. In addition, leptin-treated rats maintain normoglycemia in the fed state, while blood glucose is markedly diminished during an acute (3.5 h) fast relative to vehicle-treated animals ($P < 0.0001$). Thus, without access to food, leptin-treated animals appear unable to maintain proper blood glucose levels. Liver glycogen levels were markedly decreased in leptin-treated animals compared to vehicle-treated controls during fasting ($P < 0.0005$). Short-term (3 h) fasting also caused a significant increase in mRNA expression levels of both phosphoenolpyruvate-carboxykinase (PEPCK) and glucose-6-phosphate (G6Pase), two key gluconeogenic enzymes, while the mRNA expression of glucokinase (GCK), a glycolytic enzyme, was decreased. Together, these results suggest that the stimulation of either glycogenolysis or gluconeogenesis was not effected during fasting in leptin treated rats. Nevertheless, a corresponding increase in glucose-6-phosphatase (G6Pase) activity and elevation in hepatic glucose output were not observed, which indicates leptin may reduce blood glucose levels via alternative regulatory mechanisms. Overall, these results demonstrate a potent blood glucose-lowering effect of leptin independently of insulin action that inhibits leptin-treated rats from adequately raising blood glucose concentration during a fast and normalizes blood glucose concentrations in diabetic rats in the fed state.

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CHAPTER 1: INTRODUCTION

As the rates of obesity and the associated onset of type 2 diabetes (T2D) continue to accelerate in the United States and around the world, an abundance of research has emerged investigating the pathogenesis of these disorders. Accordingly, the discovery of leptin, a hormone secreted primarily by adipocytes, has provided greater insight into the complex interplay between peripheral and central signaling in the regulation of metabolic homeostasis (1). Experiments involving leptin-deficient *ob/ob* and leptin receptor-deficient *db/db* mice, which are both grossly obese and exhibit hyperglycemia and hyperinsulinemia, revealed leptin suppresses appetite and adiposity and stimulates energy expenditure mainly through activation of the long form of its receptor (ObR-b) in the hypothalamus (2-4). Numerous studies have also established leptin's ability to normalize elevated blood glucose levels independently of weight loss using a variety of insulin-resistant and diabetic animal models (4-7).

In addition, this effect may be attributable, at least in part, to the enhanced insulin sensitivity observed in both insulin-deficient streptozocin(STZ)-induced diabetic (STZ-D) and normal animals (7-12). Likewise, previous research in our lab revealed a greater overall decrease in blood glucose in response to exogenous insulin during an ITT in leptin-treated rats than in vehicle-treated animals (unpublished data). The markedly reduced blood glucose levels in both STZ-D and non-diabetic animals treated with leptin during an acute fast, however, indicate leptin may have profound effects on blood

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glucose homeostasis independently of insulin action as well. As insulin tolerance tests typically require fasting prior to and during the procedure, our lab hypothesizes that the previously observed heightened response to insulin following chronic leptin treatment may actually be due to this fasting effect. The decrease in blood glucose observed during short-term fasting in leptin-treated animals also suggests central leptin signaling may initiate events that lead to inhibition of gluconeogenesis and/or glycogenolysis, thereby preventing leptin-treated animals from maintaining euglycemia when denied access to food.

Thus, in the present study, the effects of fasting or insulin on blood glucose levels were monitored in chronic icv leptin-treated STZ-diabetic and vehicle-treated non-diabetic rats to evaluate the potential role of fasting in mediating the glucose-lowering effects of leptin during an ITT. To investigate possible mechanisms by which leptin may lower blood glucose, this study also assessed liver glycogen content and the regulation of key hepatic enzymes involved in hepatic glucose flux at gene expression and activity levels in both the fed and fasted states.

Our results demonstrate chronic central leptin treatment normalizes blood glucose in STZ-diabetic rats and significantly reduces blood glucose concentration during an acute fast. In addition, this fasting effect, rather than an enhanced response to insulin, accounts for the greater decline in blood glucose levels observed in both STZ-D and non-diabetic leptin-treated rats during the ITT. Leptin treatment, moreover, diminishes hepatic glycogen stores, suggesting augmented glycogenolysis, while the up-regulation of gluconeogenic enzyme expression in response to fasting was not inhibited by leptin treatment. In contrast, a corresponding increase in glucose-6-phosphatase activity and

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elevation in blood glucose concentration were not detected in leptin-treated animals. Thus, the blood glucose-lowering effects of central leptin may be mediated by additional regulatory mechanisms. Overall, the results from this experiment may contribute to a greater understanding of how leptin signaling in the brain leads to lower blood glucose levels independently of insulin action.

CHAPTER 2: REVIEW OF LITERATURE

Obesity and Diabetes

During the past two decades, the incidence of overweight and obesity has risen dramatically. Over half of the U.S. adult population is overweight according to Body Mass Index (BMI) standards (BMI between 25-29.9 kg/m^2), while approximately one-third is considered obese (BMI of 30 kg/m^2 or higher), or as having an exceptionally high proportion of body fat. Moreover, the prevalence of overweight and obesity in children aged 2-5 and 6-11 years has tripled since 1980 to 14 and 19 %, respectively, and in adolescents aged 12-19 years to 17 % (13). With a 70 % probability of an overweight youth becoming an overweight adult, the overall prevalence of excess body fat is only expected to increase (14).

Although once confined to "western," industrialized countries, these trends have also begun to spread worldwide as increased urbanization and economic growth in developing countries have been accompanied by greater consumption of energy-dense, processed foods and declining physical activity levels. Current global estimates of overweight adults are at one billion, with 300 million of these classified as clinically obese (15). Of greater concern, however, is the increased risk of a myriad of chronic diseases and health complications associated with overweight and obesity, including: type 2 diabetes (T2D), cardiovascular disease, hypertension, osteoarthritis, and some

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cancers (16). Not surprisingly, obesity has been ranked as the second leading cause of preventable death in the U.S. (17). Increased diagnoses of T2D, in particular, have paralleled the acceleration in overweight and obesity rates around the globe, with approximately 60% of all diabetes associated with obesity worldwide. In America, 90% of all T2D cases reported in the nurse's health study were related to obesity (18), and it is estimated that one in six overweight adolescents are "pre-diabetic" (19).

Type 2 diabetes is a disease characterized by elevated blood glucose levels due to insulin resistance with insufficient insulin secretion, and it is often accompanied by an array of debilitating and potentially fatal health problems, such as cardiovascular disease, blindness, kidney disease and failure, nerve damage, and limb amputation (20). Accordingly, the American Diabetes Association reports that diabetic patients cost insurance companies over 3 times as much as non-diabetic clients, and national health care and loss of production costs associated with diabetes are expected to increase by 70% to 192 billion dollars by 2020.

With such ominous implications for the future, an extensive amount of research has focused on characterizing the pathogenesis of T2D and its relationship to obesity in an effort to determine effective treatment and prevention measures for this and other weight-related disorders.

Energy Balance

Obesity results primarily from an imbalance between energy (i.e. caloric) intake (EI) and energy expenditure (EE) according to the following equation:

$$\text{Energy Balance} = \text{EI-EE}$$

Moreover, the ability to maintain energy balance is influenced by polygenetic and environmental factors (21) and, in rare cases, is directly attributable to genetic mutation (22). In general, greater EI than EE creates a positive energy balance, which leads to energy storage (weight gain), while increased EE relative to EI results in negative energy balance and subsequent weight loss. Intrinsic mechanisms directed by the central nervous system (CNS) make appropriate adjustments in EI and EE to balance these parameters in response to numerous signals related to nutrient stores and energy demands (23). Accordingly, these changes can influence appetite or total energy output, which is comprised of: 1) the basal metabolic rate, or energy required to perform biochemical functions necessary for survival; 2) voluntary physical activity; 3) the thermic effect of food, or energy used for digestion of nutrients; and 4) adaptive thermogenesis, or heat generated in response to external stimuli (24,25). In fact, the CNS integrates effects on physical and feeding behavior, autonomic nervous system activity, and the neuroendocrine system to coordinate energy homeostasis among multiple tissues (24).

Leptin

Leptin, a hormone secreted predominantly by adipocytes, was first identified as the protein encoded by the obese (*ob*) gene in 1994, and it plays an integral role in maintaining energy balance through regulation of appetite, body weight, and energy expenditure (1). Leptin generally helps establish satiety by enabling the CNS to gauge long-term energy availability versus short-term satiety signals, such as cholecystokinin, that regulate individual meals (26). Leptin deficiency, however, prevents the immediate satiety response to meals, which suggests long-term and short-term signals intersect to regulate overall food intake (23,24). Moreover, due to the direct correlation between

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plasma leptin levels and the amount of fat mass in both rodents and humans (27-29), leptin signaling appears to form a negative feedback loop between adipose stores and feeding and energy regulatory centers in the CNS (3,30). Indeed, the dramatic 20-fold increase in leptin mRNA expression and plasma leptin levels in mice with ventromedial hypothalamic (VMH) lesions (28,31), along with the lack of response to either intravenous (iv) or intracerebroventricular (icv) leptin treatment in these animals, indicates the CNS is a major target of leptin action (31).

ob/ob and *db/db* mice

Leptin's physiological functions are dramatically demonstrated in *ob/ob* mice, which are leptin-deficient due to a mutation in the *ob* gene, and in *db/db* mice, which are leptin-resistant due to a defect in the long form of the leptin-receptor (*db*) gene. As a result of either deficiency, both mouse strains exhibit a similar phenotype of hyperphagia, extreme obesity, hyperinsulinemia, insulin resistance, and a tendency to develop type 2 diabetes (T2D) (1,32). Peripheral or central administration of leptin to *ob/ob* mice significantly reduces food intake and adiposity (3,4,33), while leptin infusion in lean, wild-type (WT) mice also lowers total body weight, especially fat tissue, with only modest changes in food intake (33,34). Subcutaneous injections of leptin in the low, physiological range (0.01–0.04 mg/kg) daily for eighteen months also effectively ameliorates severe obesity in human subjects with rare conditions of congenital leptin deficiency (22).

Meanwhile, the reduced metabolic rate, activity level, and body temperature characteristic of *ob/ob* mice are restored to the levels of lean controls following daily intraperitoneal (ip) leptin injection for 4 weeks (4). Accordingly, total energy

expenditure, as measured by total oxygen consumption, is markedly increased in leptin-treated *ob/ob* mice (33), while peripheral or central leptin treatment in lean, WT mice prevents the decrease in energy expenditure that normally accompanies weight loss (2).

A separate study by Cusin *et al.* using lean rats reported similar findings regarding adaptive thermogenesis (9). In adaptive thermogenesis, energy produced from fuel metabolism is dissipated as heat rather than utilized for biological work. For example, uncoupling proteins (UCPs) can facilitate a proton leak across the inner mitochondrial membrane, which disrupts the proton electrochemical gradient established by the electron transport chain used to generate ATP. Cusin *et al.* found lean rats pair-fed to animals treated with continuous icv leptin infusion ($12\mu g \cdot day^{-1}$ for 4 days) had significantly decreased mRNA expression of UCP-1, UCP-2 and, UCP-3 in thermogenic brown adipose tissue (BAT), along with decreased white adipose tissue UCP-2 and muscle UCP-3 mRNA. Leptin-infused rats, meanwhile, maintained high UCP mRNA levels in all these tissues, despite reductions in body weight similar to those of pair-fed controls (9).

This enhanced UCP gene expression is also associated with a leptin-mediated increase in sympathetic nervous system (SNS) activity and β-adrenoreceptor stimulation in thermogenic target tissues (35), which indicates leptin initiates efferent signals from the CNS that modulate adaptive thermogenesis. In fact, β-less mice, which lack β-adrenoreceptor subtypes 1, 2, and 3 in brown tissue adipocytes, gain significantly more weight on either a standard chow or a high fat diet versus WT controls, despite having food intake levels indistinguishable from those of control animals (36). The increase in average metabolic rate (~16%), as measured by total oxygen consumption, in WT mice

after 5 days of high-fat feeding is not observed in β-less mice. This difference, moreover, is attributable solely to a lack of diet-induced thermogenesis since physical activity and thyroid hormone levels are similar in both WT and β-less mice (36). Thus, stimulation of thermogenesis appears to be an important regulatory mechanism by which leptin prevents excess energy storage.

Overall, elevated circulating leptin levels lead to appetite suppression and enhanced energy expenditure in times of energy excess, while reduced leptin signaling induces a fasting or starvation response that stimulates hunger and energy conservation (37). Therefore, leptin effectively influences metabolic homeostasis by modulating both feeding behavior and energy utilization.

Leptin Receptor (ObR-b) Activity

Although direct activation of leptin receptors has been documented in a variety of tissues involved in metabolism, including pancreatic islets, liver, adipose, skeletal muscle, and multiple regions in the brain (38-43), leptin appears to exert its regulatory effects on metabolism primarily through stimulation of the long form of the leptin receptor (ObR-b) in the arcuate nucleus (ARC) of the hypothalamus (44-47).

ObR-b receptors have been located in two classes of arcuate neurons: one type co-expressing orexigenic stimulants neuropeptide Y (NPY) and Agouti-related protein (AgRP), and a second type expressing anorexigenic neuropeptides proopiomelanocortin (POMC) and cocaine-and amphetamine-regulated transcript (CART) (48). Previous research has associated leptin-mediated appetite suppression with increased POMC (49) and CART (50) mRNA levels, along with stimulation of action potentials of POMC neurons in the ARC (49). These effects are accompanied by the simultaneous

suppression of both NPY and AgRP mRNA expression (23). Accordingly, leptin-deficient (*ob/ob*) and leptin-resistant (*db/db*) mice have significantly enhanced NPY levels and reduced POMC gene expression relative to WT animals (51). Systemic leptin treatment (150μg·day^{-1} for 5 days) significantly elevated levels of NPY mRNA in the ARC of *ob/ob* mice by 42.3% compared to saline-treated controls (5). Furthermore, this observation was directly attributable to leptin action versus a result of weight loss, as NPY mRNA levels remained unchanged in *ob/ob* mice pair-fed to leptin-treated animals, despite comparable reductions in body weight of both treatment groups (5).

Neither iv nor icv leptin treatment, meanwhile, ameliorates obesity in *db/db* mice (52), which have deficient leptin signaling due to a mutated leptin-receptor. However, expression of neuron-specific leptin receptor-b (*Lepr^b*) transgenes in *db/db* mice reverses obesity (53). Injections of an adenoviral vector expressing *Lepr^b* into the ARC of Koletsky (*fa^k/fa^k*) rats, which have mutated fatty (*fa*) leptin-receptor genes, also reduces food intake and body weight gain (54). Stimulation of signaling pathways down-stream of the leptin receptor also alleviates obesity in a manner similar to leptin administration. Genetically suppressed NPY expression, for example, markedly lowers body weight in *ob/ob* mice (55). Meanwhile, enhanced melanocortin receptor (MCR) signaling induced by overexpression of adenoviral POMC, the precursor for the MCR agonist melanocyte stimulating hormone (α-MSH), in the ARC of leptin receptor-deficient Zucker fatty (*fa/fa*) rats also significantly reduces weight gain and decreases visceral adiposity by 24% compared to controls (56). Together, these findings demonstrate that intact hypothalamic neuron signaling is essential for leptin's regulatory effects on appetite and body weight.

Intracellular Signaling

In fact, central administration of leptin directly into the VMH of *ob/ob* mice improves food intake, adiposity, and energy expenditure to an even greater extent than peripheral leptin given at much higher doses (2,3). Serum leptin levels also remain unchanged following icv leptin infusion, thereby demonstrating central leptin signaling is sufficient to regulate energy metabolism in peripheral tissues. Moreover, neuron-specific leptin receptor knockout mice exhibit extreme obesity and hepatic steatosis, while peripheral (i.e. hepatocyte) leptin receptor knockout mice have no apparent metabolic abnormalities (57). Thus, much research has focused on delineating the mechanisms by which leptin influences peripheral activity through central signaling.

The leptin receptor belongs to a family of cytokine receptors known to modulate gene transcription via activation of cytosolic signal transducer and activator of transcription (STAT) proteins (58). Accordingly, central intracellular leptin signaling is primarily mediated by association of the ObR-b receptor with the Janus tyrosine kinase 2 (JAK2), which leads to phosphorylation of STAT3 (59). Activation of this pathway is believed to occur exclusively through the long form of the leptin receptor; thus, dose-dependent activation of STAT3 in the hypothalamus via exogenous leptin treatment has been detected in WT and *ob/ob* mice, but not in *db/db* mice (60).

Furthermore, mice homozygous (*s/s*) for a mutant leptin receptor incapable of binding STAT3, have disrupted ObR-b/STAT3 signaling and as a result are hyperphagic, obese, hyperglycemic, and have reduced energy expenditure (35,61). Leptin treatment in *s/s* mice (61), or in mice with selective reduction of STAT3 protein levels in the CNS (62), fails to ameliorate the pronounced hyperphagia and obesity in these animals.

Dual icv injection of leptin (1.5 μg) and a STAT3 inhibitor likewise negates the significant acute drop in food intake (63%) observed in leptin-treated control animals (63). Although these studies demonstrate the importance of intact STAT3 signaling in mediation of leptin's anorectic effects, additional pathways downstream of JAK2, including mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) are also activated by leptin and may, therefore, contribute to regulation of food intake or other leptin-mediated events (39,64). Overall, leptin appears to employ a complex network of intracellular signaling in the CNS to exert multiple effects in the regulation of metabolic homeostasis.

Glucose Homeostasis

Maintaining adequate availability and proper utilization of glucose, a major source of energy and under normal conditions the only form of energy used by certain cells types, is a critical component of proper energy balance. In fact, plasma blood glucose levels are rigorously kept within a narrow range of levels by a complex network of physiological signals (65). Insulin, a hormone secreted by the beta cells of the pancreatic islets of Langerhans, is one of the most well characterized regulators of glucose homeostasis.

In the postprandial state, i.e. when blood glucose levels are elevated, augmented insulin signaling increases glucose uptake in peripheral tissues, such as skeletal muscle, cardiac muscle, and fat, while simultaneously inhibiting hepatic glucose production via suppression of both glycogenolysis (the breakdown of liver glycogen) and gluconeogenesis (synthesis of glucose) (66). Accordingly, insulin down-regulates expression of key gluconeogenic enzymes in the liver, including phosphoenolpyruvate

carboxykinase (PEPCK), which catalyzes a rate-limiting reaction converting oxaloacetate acid to phosphoenolpyruvate, and glucose-6-phosphatase (G6Pase), which catalyzes the final step involving de-phosphorylation of glucose-6-phosphate to glucose (67). Insulin also enhances intracellular translocation of glucose transporters, such as GLUT4 in the skeletal muscle, which aid in glucose uptake across the plasma membranes of cells in the periphery (68). The resulting combination of reduced hepatic glucose output and enhanced cellular glucose utilization effectively lowers plasma glucose levels and increases energy supply to cells.

Leptin and Glucose Metabolism

Several studies involving insulin resistant and diabetic animal models have demonstrated a prominent role for leptin in the regulation of glucose homeostasis as well. Elevated plasma insulin and glucose levels are normalized in *ob/ob* mice following peripheral leptin treatment (4,5); however, this effect may be secondary to parallel reductions in food intake and fat mass also observed in these animals. In fact, the excessive accumulation of visceral fat (VF) characteristic of obesity has been implicated in the development of insulin resistance and T2D (69,70), and even moderate weight loss (~8% of body weight) in patients with T2D restores euglycemia and lowers basal hepatic glucose production (HGP) (71). Similar degrees of improved hepatic insulin action (i.e. reduced HGP) in rats during euglycemic-hyperinsulinemic clamp studies were achieved following the selective reduction of VF mass (~60%) with chronic leptin treatment, a β_3-adrenoreceptor agonist, or calorie restriction (72). However, only leptin-treated animals in this study demonstrated markedly enhanced glucose uptake, glycolysis, and glycogen synthesis, thereby indicating leptin also affects changes in peripheral glucose metabolism

independently of reduced VF. In addition, normal rats given continual subcutaneous leptin infusion over a 48-hour period exhibited significantly lower fasting glucose and insulin levels, as well as enhanced insulin response during hyperinsulinemic-euglycemic clamp, compared to vehicle-treated controls (12). Systemic leptin administration also corrects hyperglycemia and hyperinsulinemia in *ob/ob* mice prior to the reversal of obesity by reducing serum glucose and insulin levels by ~3-fold and 2-fold, respectively, which further demonstrates a direct role for leptin in glucose metabolism (5).

In contrast to *ob/ob* mice, neither iv nor icv leptin treatment affects blood glucose in *db/db* mice (52); however, as with obesity, expression of neuron-specific leptin receptor-b (*Lepr^b*) transgenes in *db/db* mice reverses diabetes (53). Interestingly, unilateral reactivation of the leptin receptor in the ARC of leptin receptor null mice (*Lepr$^{neo/neo}$*), which are obese, hyperinsulinemic, and hyperglycemic, significantly improves plasma insulin levels and restores euglycemia, while only modestly decreasing body fat mass (73). These findings therefore suggest that, along with the aforementioned improvements in body weight regulation, re-establishing central leptin signaling is sufficient to stabilize glucose homeostasis.

Lipodystrophy

Meanwhile, the overt insulin resistance and diabetes displayed by a congenital lipodystrophic mouse model, which lacks adequate circulating leptin due to an inability to properly store fat, is reversed with either systemic leptin treatment (6) or transgenic overexpression of leptin (74). Fat accumulation in both the liver and skeletal muscle of these animals, which has been demonstrated to contribute to insulin resistance (75,76), was also reduced by exogenous leptin (6,74). Likewise, insulin resistance,

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hyperglycemia, hypertriglyceridemia, and hepatic steatosis were all ameliorated in female lipodystrophic patients given twice-daily subcutaneous leptin injections for four months (77).

Indeed, leptin has been found to increase fatty acid oxidation in muscle (58) and liver tissue (71), thereby possibly reversing the lipotoxicity associated with insulin resistance in these tissues. Enhanced fatty acid oxidation resulting from leptin treatment may, therefore, contribute to improved glucose regulation as well. Nevertheless, Shimomura *et al.* found calorie-restricted lipodystrophic mice remained hyperinsulinemic and hyperglycemic, despite significantly reduced body weights and liver triglyceride content (6). In addition, although *ob/ob* mice lost a greater percentage of body weight while retaining greater liver triglyceride content than lipodystrophic mice, both animal models displayed similar improvements in serum insulin and glucose levels following leptin treatment (6). Meanwhile, transplantation of white adipose tissue (WAT) from normal mice, but not leptin-deficient *ob/ob* animals, also significantly improved insulin response and plasma glucose levels in lipodystrophic animals (78,79). These findings as a whole demonstrate that leptin participates in the regulation of glucose homeostasis, at least in part, independently of modified feeding behavior, reduced adiposity, or increased fatty acid oxidation. Furthermore, the reversal of insulin resistance and hyperglycemia following restored leptin signaling suggests leptin's glucoregulatory effects may be mediated through improved insulin action.

Leptin and Insulin Interactions

Indeed, Pocai *et al.* found that even acute icv leptin infusion (1.5 μg/6h) significantly enhances insulin-stimulated inhibition of HGP via decreased glycogenolysis

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in rats after the development of insulin resistance in these animals through short-term overfeeding (80). Insulin tolerance tests (ITTs) also revealed a greater response to insulin in the livers of leptin-treated MKR mice, an animal model of T2D that lacks insulin-like growth factor-1 (IGF-1) and insulin receptor signaling in the skeletal muscle, compared to controls. Accordingly, hyperinsulinemic-euglycemic clamp studies showed an approximate 4-fold increase in glucose infusion rate in leptin-treated MKR mice versus controls (81).

Meanwhile, several lines of evidence suggest overall regulation of glucose homeostasis may involve "cross-talk" between leptin and insulin. Similar to the effects of central leptin activity, insulin signaling in the hypothalamus is also essential for its associated glucoregulatory actions in the periphery (66). In addition, insulin and leptin signaling are terminated by common mechanisms, such as protein tyrosine phosphatase-1B (PTP-1B), which de-phosphorylates insulin and leptin receptors (82,83), and suppressor of cytokine signaling 3 (SOCS3), which blocks both leptin and insulin signaling (83). Mice deficient in either PTP-1B or SOCS3, meanwhile, exhibit increased leptin and insulin sensitivity and protection against diet-induced obesity (84,85). Insulin and leptin also activate some common signaling pathways in the hypothalamus, such as the insulin receptor substrate-phosphatidylinositol-3-OH kinase (IRS-PI3K) pathway (64,86), while dual administration has an additive effect on JAK-2/STAT-5b activation in the liver (87). Insulin and leptin, moreover, each influence glucose-responsive hypothalamic neuron activity by modulating ATP-sensitive potassium (K_{ATP}) channels in these cells via a PI3K-dependent mechanism (88,89); however, these neurons are unresponsive to either hormone in Zucker fatty (*fa/fa*) rats (83). Interestingly, both leptin

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and insulin resistance are evident as early as three days after overfeeding in Sprague-Dawley rats given a palatable diet (33% fat) versus animals fed a standard chow diet (21% fat) (90). Specifically, insulin-stimulated inhibition of HGP is significantly blunted in overfed rats during insulin clamp studies, while glucose uptake in peripheral tissues is diminished in these animals after seven days of ad libitum feeding. Systemic leptin infusion, meanwhile, has no effect on gluconeogenesis in overfed rats, while increased circulating leptin causes a 2-fold increase in the contribution of gluconeogenesis to hepatic glucose output in control animals (90). Thus, both leptin and insulin appear to play key roles in maintaining glucose homeostasis, and their actions may be related in such a way that the proper functioning of one hormone may require intact signaling of the other.

Streptozotocin (STZ)-Diabetic Model

While studies involving leptin-treated streptozotocin-diabetic (STZ-D) rats have also indicated an association between improved glucose homeostasis and enhanced insulin action following leptin administration (7,8), these studies raise the possibility of significant insulin-independent effects as well. In this animal model, diabetes is chemically induced via intravenous administration of the cytotoxin STZ, which selectively destroys pancreatic beta cells and renders the animal moderately to severely insulin-deficient and insulin-resistant depending on dosage (91). As a result, these animals also display hyperglycemia, polydipsia, hyperphagia without increased weight gain, and additional chronic complications associated with human diabetes (92).

Despite severely reduced circulating insulin levels (as much as 10% of controls), either chronic subcutaneous (7) or chronic central leptin treatment (8,93) normalizes

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blood glucose levels in STZ-D rats. Hidaka *et al.*, moreover, found central leptin's

restorative effects on glucose metabolism in STZ-D animals correlate with alterations in

the expression of genes involved in HGP and glucose uptake and energy expenditure in

peripheral tissues (93). Specifically, the elevated mRNA expression of hepatic G6Pase in

STZ-D animals was decreased to the levels of control animals following chronic (6 days)

icv leptin infusion, which indicates a decline in hepatic glucose output. Meanwhile, the

reduced UCP-1, UCP-3, and GLUT4 mRNA levels in the BAT of STZ-D rats were up-

regulated to normal levels with leptin treatment, which suggests leptin restores

thermogenesis capabilities in STZ-D animals. Leptin treatment also corrected the up-

regulation of genes involved in fatty acid metabolism and β-oxidation in skeletal muscle

characteristic of STZ-induced diabetes, thereby implying improved glucose utilization in

these tissues. In addition, although leptin treatment reversed the hyperphagia of STZ-D

rats, vehicle-treated STZ-D rats pair-fed to these animals did not exhibit improved

hyperglycemia (8,93). Thus, as in other animal studies, leptin's effects on improved

glycemic control also appear to be independent of altered feeding behavior in STZ-D rats.

Meanwhile, chronic peripheral leptin infusion (12-14 days) in STZ-D rats

significantly diminishes HGP rate during both basal and hyperinsulinemic states

compared to vehicle-treated STZ-D animals, which suggests both insulin-independent

and insulin-sensitizing actions of leptin (7). In addition, the superior glycemic control

observed in *s/s* mice compared to *db/db* mice, which have partial and total obliteration of

ObR-b signaling, respectively, was initially attributed to greater insulin sensitivity based

on lower serum insulin levels in *s/s* animals relative to *db/db* counterparts (94).

However, a similar study conducted by Buettner *et al.* determined significant insulin

resistance in calorie-restricted *s/s* mice at levels comparable to those of PF *db/db* animals during euglycemic-hyperinsulinemic clamp studies (63), which also indicates central leptin signaling exerts glucoregulatory effects in part independently of insulin action.

Recently, our lab observed a phenomenon that further indicates central leptin may affect blood glucose homeostasis directly. As in previous experiments (8,93), elevated blood glucose levels in STZ-diabetic rats were normalized and thereafter maintained at steady levels with chronic, central leptin treatment, while blood glucose levels of leptin-treated non-diabetic animals, likewise, did not vary from those of controls throughout the study. However, both groups of leptin-treated rats (diabetics and controls) exhibited striking, and potentially fatal, reductions in blood glucose during a short-term fast. These data indicate leptin-treated animals may regulate blood glucose levels by ingesting food. Furthermore, since insulin tolerance tests typically require fasting the animals before testing, as well as denying access to food during the assessment, the apparent enhanced response to insulin previously detected in these animals may actually be due to a leptin-mediated effect of fasting. Given this phenomenon occurred regardless of an induced insulin-deficiency, central leptin may exert potent glucoregulatory effects independently of insulin action. Accordingly, the alterations in glucose metabolism in the liver (7) and corresponding changes in the regulation of gluconeogenic genes (93) resulting from chronic leptin administration may, in fact, be mechanisms by which leptin indirectly influences plasma glucose homeostasis.

Objectives and Hypotheses

Although several studies have demonstrated increased insulin sensitivity in association with central leptin treatment, these analyses have not been performed while

taking into account the potentially significant effects of chronic leptin treatment on blood glucose levels during fasting. Results from this study may, therefore, help clarify the mechanism(s) by which central leptin treatment restores euglycemia. Thus, the primary goals of this study are: (1) To assess whether the marked decrease in blood glucose levels of central leptin-treated STZ-diabetic and non-diabetic rats during ITT is due to enhanced insulin sensitivity or is an effect of fasting, and (2) To evaluate central leptin's effects on hepatic glycogen storage and gluconeogenesis as possible mechanisms for the lowering of blood glucose levels in leptin-treated animals during a short-term fast. Accordingly, we hypothesize that (1) The reduced blood glucose in leptin-treated rats (STZ-diabetic and non-diabetic) during ITT is due to a fasting effect rather than increased insulin sensitivity, and (2) The inability of leptin-treated animals to maintain euglycemia while fasting is due to leptin-mediated effects on hepatic gluconeogenesis and/or glycogen storage.

CHAPTER 3: MATERIALS AND METHODS

Animals. This study was performed using thirty-five male Wistar rats (250-300 g; Harlan, Indianapolis, IN), which were housed in individual wire mesh cages in a temperature- ($23 \pm 3°$ C) and humidity-controlled environment with a 12:12 h light:dark cycle. They were allowed free access to tap water and standard Purina rat chow ad libitum. All procedures involving animals were approved by Auburn University's Institutional Animal Care and Use Committee (IACUC) prior to initiation of the study.

Cannula implantation. All animals underwent surgery for implantation of a 22-gauge stainless steel guide cannula (Plastics One, Roanoke, VA) into the lateral ventricle of the brain (0.8 mm posterior to bregma, 1.4 mm lateral to the midline, and 3.5 mm ventral to the surface of the skull) using a stereotaxic apparatus. Animals were anesthetized via intraperitoneal (ip) injection of ketamine-xylazine (100 mg/kg ketamine and 1 mg/kg xylazine) prior to surgery. Four stainless steel screws and dental cement were used to fix the cannula to the skull, and a removable 'dummy' cannula extending 1 mm beyond the guide cannula was inserted to prevent obstruction. After 2 days of post-surgical recovery, proper placement of the cannula was confirmed by observing a positive drinking response (> 3 ml water in 15 min) following intracerebroventricular (icv) administration of angiotensin II (40 ng/5 μl).

Induction of diabetes. Insulin-deficient diabetes was induced in half the animals via intravenous tail-vein injections of streptozotocin (STZ 30 mg/kg; Sigma-Aldrich, St. Louis, MO) freshly prepared in 0.05 M citrate buffer (pH 4.5). Control animals received injections of citrate buffer vehicle. The onset of diabetes in STZ-treated rats was confirmed 2 days later by blood glucose concentrations of $\geq$ 300 mg/dl.

Intracerebroventricular (icv) leptin administration. Following induction of STZ-diabetes, half of the diabetic and half of the non-diabetic rats received daily icv bolus injections of 5 μl leptin (1μg/1μl) for 12-15 days using a syringe pump, while the remaining animals from each group received 5 μl of vehicle (15 mM HCl and 7.5 mM NaOH in a 5:3 ratio, respectively). This yielded a total of 4 treatment groups: 1) STZ-diabetic-leptin (SL) (n=7), control-leptin (CL) (n=9), STZ-diabetic-control (SC) (n=10), and control-control (CC) (n=9).

Blood glucose and body weight determination. Blood glucose levels were monitored approximately every other day using an Accu-Chek simplicity glucometer (Boehringer Mannheim, Indianapolis, IN), while body weight measurements were obtained daily during the experiment. After blood glucose levels were normalized in SL rats (~ 120 mg/dl), animals underwent the following experimental assessments.

Short-term fast. Animals were fasted after 9 days of leptin treatment for 3.5 hours, with blood glucose measurements obtained from tail-vein blood samples at 0, 30, 60, 90, 150, 180, and 210 minutes.

Insulin tolerance test. Insulin tolerance tests (ITTs) were begun on day 5/6 of icv leptin treatment using a cross-over design in which half of the animals in each treatment group were given an ip injection of regular insulin (1 U/kg), while the remaining animals

received an ip saline (0.9% NaCl) vehicle injection. The next day, ITTs were repeated by giving saline to animals previously treated with insulin and vice versa. Food was removed, and baseline blood glucose concentrations were measured as previously described just prior to injection (i.e. at time zero) and at 15, 30, 60, and 90 minutes post-injection.

Several days following the insulin tolerance test, animals were divided into fed and fasted groups and sacrificed by decapitation. Food was removed from the cages of fasted animals 3 hours prior to sacrifice, while fed animals had continuous access to food. Animals were not given icv injections the day of sacrifice. Therefore, they were killed approximately 24 h after their last icv injection. Whole livers were removed and weighed. Approximately 50 mg of liver was placed in RNA*Later®* (Invitrogen, Carlsbad, CA) for gene expression analysis and stored at 4°C. Additional portions of liver (50-100 mg) were removed for protein and glycogen content quantification and stored at -20°C, while the remaining liver tissue was rapidly frozen in liquid nitrogen and stored at -80°C for microsome preparation.

Liver glycogen assessment. Liver glycogen levels were measured using a colorimetric assay adapted from Lo *et al*. (95). Briefly, 0.5 ml of a 30% KOH solution saturated with Na_2SO_4 was added to liver tissue samples. After heating at 100°C for 10 min, this mixture was combined with 0.6 ml 95% ethanol and centrifuged at 10,000 rpm for 10 min. The supernatant was removed, and the pellet was dissolved in 330 μl deionized water. Appropriate amounts (1-100 μl) of this solution were then transferred to 12 x 75 mm borosilicate glass tubes, with final volumes adjusted to 100 μl as necessary. Next, 0.1 ml 5% phenol was added to each tube and mixed. Then, 0.5 ml sulfuric acid was

added to each tube, and all tubes were vortexed and incubated for 30 min in a 30°C water bath. The absorbance of each sample was measured at 490 nm using a Bio-Rad Smartspec™ Plus Spectrophotometer (Bio-Rad, Hercules, CA). Rabbit liver glycogen (Sigma, 12.5-500 µg/ml) was used as the standard.

Hepatic microsomal fraction. Liver tissue samples (~3 g) were homogenized manually using a Dounce homogenizer in Buffer A (2 ml/g liver tissue): 0.25 M sucrose, 0.005 M HEPES, 1µg/ml each leupeptin and aprotinin, and 2 ml protease inhibitor cocktail (Sigma; Cat. No. P2714) per 100 ml buffer. Volumes were normalized by weight with Buffer A, and centrifuged at 8000 rpm (4°C) for 10 min. The supernatants were transferred to new tubes and volumes were again adjusted by weight with Buffer A. After centrifugation at 46,000 rpm (4°C) for 1 h, the resulting pellets were re-suspended with 2 ml Buffer A containing 40% glycerol and stored at -20°C.

Glucose-6-phosphatase (G6Pase) enzyme activity. Enzymatic activity of hepatic G6Pase was measured using a colorimetric assay developed by Burchell *et al*. (96). All reactions were prepared on ice. Briefly, 10 µl phosphate standard (Sigma: 0.04, 0.08, 0.16, 0.32, 0.65 mM) or 10 µl liver microsomal fraction sample (1:20 dilution) were added to individual glass tubes containing 90 µl of a reaction buffer containing 50 mM sodium cacodylate (pH 6.5), 2mM EDTA, and 10 mM glucose-6-phosphate. Reactions were incubated in a 30°C water bath for 10 min. The reaction tubes were placed on ice and 0.4 ml of freshly prepared stop solution (3.4 mM ammonium molybdate in 0.5 M sulfuric acid, 0.52 M sodium dodecyl sulphate, and 0.6 M ascorbic acid in a 6:2:1 ratio) was added to each tube. The tubes were then placed in a 45°C water bath for 20 min. Enzyme activity, defined as units of inorganic phosphate (P_i) released per milligram liver

protein per minute (U $P_i \cdot mg^{-1} \cdot min^{-1}$), was calculated from sample absorbance readings at 660 nm. Sample G6Pase activity measurements were corrected for non-specific phosphatase activity, as assessed using 10 mM β-2-glycerophosphate substrate.

Quantitative real-time RT-PCR liver mRNA. Quantitative RT-PCR was conducted using fresh liver tissue samples (50-100 mg) stored in RNAlater® (Invitrogen). Total RNA was isolated with Trizol reagent (Invitrogen), followed by purification using RNAeasy mini-kits (Qiagen, Valencia, CA). Synthesis of cDNA was performed using the iScript^{TM} cDNA Synthesis kit (Bio-Rad, Hercules, CA). RT-PCR analyses were run on individual samples of purified mRNA from rat liver (n ~ 3-5 per group). Reaction mixtures contained 1x iQ^{TM} SYBR Green Supermix (50 mM KCl, 20 mM Tris-HCl, pH 8.4, 0.2 mM of each dATP, dCTP, dGTP, and dTTP) iTaq DNA polymerase (25 units/ml), 3 mM $MgCl_2$, SYBR Green I, 10 nM each fluorescein and stabilizer, and 100 nM of one primer set with 1μl of cDNA in a total volume of 25μl. RT-PCR was performed with the MyiQ^{TM} Real-Time PCR Detection System (Bio-Rad) as follows: 1) 1 cycle at 95°C for 5 min; 2) 40 cycles at 94°C for 15 s, 56°C for 30 s, and 72°C for 30 s; and 3) 80 cycles starting at 55°C for 10 s, where the set- point temperature was increased by 0.5°C after cycle 2. The DNA sequences of primers (Invitrogen) used were: PEPCK, 5′-ACAGGCAAGGTCATCATGCA-3′ (forward) and 5′-TGCCGAAGTTGTAGCC-AAAGA-3′ (reverse); G6Pase 5′-ACCCTGGTAGCCCT-GTCTTT-3′ (forward) and G6Pase 5′-GGGCTTTCTCTTCTGTGTCG-3′ (reverse); GCK 5′-TCGTGTCACAAG-TGGAGAGC-3′ (forward) and 5′-GCGATTTATGACCC-CAGCTA-3′ (reverse); PGC-1 5′-ATGTGTCGCCTTCTTGCTCT-3′ (forward) and 5′-CGAGAAAAGGATCTCG-AACG-3′ (reverse); and β-actin 5′-CCTCTATGCCAACACAGTGC-3′ (forward) and

5′-CATCGTACTCCTGCTTGCTG-3′ (reverse).

The ratio (i.e. fold-change) of gene expression levels in experimental treatment groups relative to CC-fed rats was calculated using the formula, $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T$ values represent the difference between treatment group and CC-fed ΔC_T values determined for individual target genes relative to the housekeeping control gene β-actin.

Statistical analyses. Statistical analyses were performed using JMP IN 5.1.2 software (SAS Institute, Inc., Cary, NC) and SPSS 12.0 for Windows software (Aspire Software International, Ashburn, VA). Data were analyzed using multiple analysis of variance (MANOVA) with repeated measures or analysis of variance (ANOVA). These analyses were followed by studentized *t* tests or one-way ANOVA to determine statistical differences between individual treatment groups. Statistical analysis for real-time RT-PCR gene expression data was performed using $\Delta\Delta C_T$ values. Data were transformed by obtaining log calculations prior to statistical analysis as needed. Values were expressed as means ± SE, and statistical significance was designated at $P < 0.05$.

CHAPTER 4: RESULTS

Daily body weight. Body weights gradually increased in control vehicle-treated (CC) rats throughout the experiment, ultimately reaching levels ~ 20 % higher than basal measurements (Fig. 1). Overall, leptin treatment caused a marked reduction in body weight in both non-diabetic and STZ-D rats ($P < 0.02$). Although the main effect of diabetes was not significantly different, vehicle-treated non-diabetic rats had larger body weights than vehicle-treated diabetic rats ($P < 0.05$).   There was also significant weight-loss over time with leptin treatment ($P < 0.0001$), and this effect was similar in both non-diabetic and STZ-D animals (i.e. CL and SL groups, respectively) (Fig.1).

Daily blood glucose. There were main effects of both STZ-diabetes and leptin on overall blood glucose levels ($P < 0.0001$), and these effects were also significant over time ($P < 0.05$ and $P < 0.01$, respectively). However, these effects were mainly observed in leptin-treated STZ-D (SL) rats, whose nearly 2-fold elevation in blood glucose concentration was significantly reduced relative to vehicle-treated STZ-D (SC) rats as early as the second day of leptin treatment ($P < 0.05$) and subsequently restored to the level of control (CC) animals by ~ day 3 (Fig. 2). Meanwhile, blood glucose concentrations of both SC and CL rats remained relatively stable throughout the experiment (i.e. either at severely hyperglycemic (≥ 300 mg/dl) or euglycemic (~ 120 mg/dl) concentrations, respectively) (Fig. 2). Thus, there was a significant interaction between the effects of diabetes and leptin ($P < 0.0005$).

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Short-term fasting blood glucose. Leptin-treated groups (CL and SL) exhibited significantly reduced blood glucose levels during a short-term (3.5 h) fast relative to vehicle-treated animals ($P < 0.0001$), with each group ultimately reaching ~ % 50 of starting levels (Fig. 3). The blood glucose-lowering effect of leptin was also significant over time ($P < 0.0001$). In contrast, there was no significant effect of diabetes on fasting blood glucose levels.

Insulin tolerance tests. There was no significant effect of STZ-induced diabetes on blood glucose levels during testing with either insulin or saline injection (repeated measures MANOVA); consequently, data were pooled into two groups to examine the main effects of treatment (i.e. icv leptin or vehicle) on blood glucose during testing. Across treatment groups, insulin injection markedly reduced blood glucose levels compared to saline ($P < 0.0001$) (Fig. 4). Leptin treatment, meanwhile, had a significant overall blood-glucose lowering effect relative to vehicle treatment ($P < 0.01$). Whereas blood glucose concentrations varied significantly between leptin- and vehicle-treated animals given saline at 30, 60, and 90 min post-injection, such differences were only detected at 15 and 30 min after insulin injection (Fig. 4A). Thus, the overall effect of leptin was statistically significant following saline, but not insulin, injection ($P = 0.005$ and $P = 0.06$, respectively) (Fig. 4B). Nevertheless, the decrease in blood glucose with saline injection in leptin-treated rats relative to controls was nearly identical to the insulin-induced decrease in leptin-treated rats versus controls—with the reduction in blood glucose after saline accounting for ~ 92 % of the response to insulin in leptin-treated animals (Fig. 4B).

Liver glycogen. There was no overall effect of diabetes on total glycogen content (mg) per liver (Fig. 5). Liver glycogen stores in leptin-treated rats (CL and SL), meanwhile, were markedly reduced compared to vehicle-treated (CC and SC) rats ($P < 0.0001$). In addition, while liver glycogen levels did not vary between the fed and fasted states within the vehicle-treated groups, fasting tended to lower liver glycogen in leptin-treated groups. Thus, the enhanced effect of fasting with leptin treatment produced a significant interaction ($P < 0.05$). Similar results were found when glycogen was expressed per milligram protein, with a substantial hepatic glycogen-lowering effect in leptin-treated rats compared to controls ($P < 0.0005$) that was more pronounced during fasting (Fig. 6).

Glucose-6-Phosphatase enzymatic activity. Fed animals generally exhibited elevated G6Pase activity relative to fasted animals ($P < 0.05$); however, this trend was not observed in CC rats, whose enzymatic activity did not vary significantly between the fed and fasted states (Fig. 7). Meanwhile, enzymatic activity was markedly elevated by either leptin treatment ($P < 0.01$) or diabetes ($P < 0.001$). Interestingly, G6Pase activity was reduced in fasted CL and SC, but not SL, rats to levels comparable to those of controls (Fig. 7). Overall, diabetes and leptin treatment, alone, but not in combination, enhanced G6Pase activity in the fed state relative to fasted animals, thereby resulting in a three-way interaction between diabetes, leptin, and feeding ($P < 0.001$).

Hepatic gene expression. Across all groups, fasting had a significant stimulatory effect on the induction of both PEPCK and G6Pase mRNA expression ($P < 0.001$ and $P < 0.002$, respectively), resulting in as much as ~ 6- and 4-fold increases in expression, respectively (Table 1). Neither STZ-diabetes nor leptin, however, had a significant effect on the expression of either gene.

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Meanwhile, GCK mRNA expression was significantly down-regulated in fasted animals relative to fed animals ($P < 0.02$) (Table 1). In addition, decreased GCK expression in the fed state was evident in diabetic groups (SC and SL), reaching only ~ 20 and 25 % of control levels, respectively. Nevertheless, these reductions were not statistically significant (Table 1).

Whereas leptin treatment had no significant effect on GCK expression, leptin-treated rats had markedly elevated PGC-1 mRNA expression levels compared to vehicle-treated animals ($P < 0.02$). There was also a trend towards increased PGC-1 mRNA expression after fasting in all experimental groups relative to fed animals; however, this increase did not reach statistical significance ($P = 0.07$) (Table 1).

CHAPTER 5: DISCUSSION

In the present study, we demonstrate that the greater reduction in blood glucose during an ITT in leptin versus vehicle-treated rats is due to an effect of fasting, rather than enhanced insulin sensitivity. Indeed, the blood glucose response to saline injection, which essentially represented the changes in blood glucose that occurred while animals were fasted during the ITT procedure, indicates fasting has a substantial blood glucose-lowering effect in leptin-treated rats apart from insulin action. Accordingly, there was a considerable decline in the blood glucose levels of icv leptin-treated rats during a brief (3.5 h) fast, while blood glucose levels in icv vehicle-treated animals remained relatively unaltered. This is consistent with previous experiments in our lab (unpublished data), as well as a study by Lin *et al.* that found chronic icv leptin treatment significantly reduced blood glucose in STZ-D and non-diabetic rats after a 6 h fast, while vehicle-treated animals exhibited no change in blood glucose concentration (8). The decrease in blood glucose after saline injection (i.e. due to fasting), moreover, almost completely accounted for the difference between the insulin-mediated blood glucose responses of leptin and vehicle-treated rats, which indicates insulin sensitivity does not vary between the two treatment groups. In addition, nearly identical responses to acute food deprivation were detected in leptin-treated animals regardless of insulin deficiency (i.e. STZ-diabetes), which further suggests leptin exerts potent hypoglycemic effects independently of insulin.

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Furthermore, since blood glucose concentration remained relatively stable during acute fasting in vehicle-treated rats as compared to leptin-treated rats, leptin treatment may prevent the intrinsic processes by which animals raise blood glucose levels during food deprivation. This is supported by the fact that average daily blood glucose levels obtained while animals were given access to food did not vary between controls and leptin-treated rats, which suggests the latter animals may use feeding to regulate blood glucose.

Animals normally maintain euglycemia during a fast by increasing hepatic glucose output (HGO) via glycogenolysis (i.e. the breakdown of liver glycogen stores) and gluconeogenesis (i.e. the endogenous production of glucose from non-carbohydrate precursors). Several lines of evidence suggest that leptin administration alters hepatic glucose production (HGP) through changes in gene expression, as well as hepatic glucose fluxes (7, 97-99), and these effects, in turn, lead to normalization of blood glucose levels in diabetic animals, as elevated HGP is a major contributor to hyperglycemia in diabetes (100). Meanwhile, leptin levels are normally diminished during a fast, which signals the organism to augment blood glucose levels by increasing appetite and HGO (23). Thus, it follows that enhanced leptin signaling may prevent a proper response to fasting, thereby resulting in diminished blood glucose concentration.

Nevertheless, we found overall liver glycogen stores were lower in both STZ-D and non-diabetic leptin-treated animals compared to vehicle-treated animals, which suggests lower glycogen stores in the fed state and therefore lower glycogen availability during fasting. Levin *et al.* and Kamohara *et al.*, likewise, showed that either chronic or acute leptin treatment significantly reduces liver glycogen levels in normal, wild-type

(WT) mice (10,34). Although enhanced glycogenolysis corresponds with increased HGO, blood glucose levels were slightly lower in leptin-treated groups of these studies. Furthermore, in the present study, blood glucose was decreased in fasted leptin-treated rats despite a greater decline in hepatic glycogen content. Although these findings appear contradictory to earlier reports of an association between leptin treatment and a markedly decreased contribution of glycogenolysis to hepatic glucose production (HGP) in normal, wild-type (WT) mice (97,98), this discrepancy may be due to procedural differences since these studies examined the acute effects of a single leptin infusion under hyperinsulinemic-euglycemic clamp.

Despite the apparent stimulation of glycogenolysis in fasted leptin-treated groups, a corresponding increase in G6Pase flux was not observed, as would be expected with greater amounts of glucose-6-phosphate derived from glycogenolysis. While the activity of G6Pase is normally lowered after feeding due to the inhibitory action of insulin on glucose production (101), enzymatic activity was significantly elevated in fed animals of all treatment groups relative to controls. This increased activity, nevertheless, was expected in insulin-deficient STZ-D rats and is consistent with reports of augmented G6Pase activity in these animals (101). However, G6Pase activity was significantly decreased in fasted CL rats relative to fed animals, despite markedly reduced fasting plasma insulin levels detected in non-diabetic rats given chronic leptin treatment (8,9). Meanwhile, enzymatic activity remained elevated during fasting in SL animals. The differences in G6Pase activity between these groups, nevertheless, do not appear to be reflective of varying glycogenolysis rates, as liver glycogen contents of both leptin-treated groups were reduced to similar levels during fasting.

In contrast, liver glycogen levels were not affected by acute fasting in vehicle-treated groups, which suggests the demand for HGO may be enhanced in fasted, leptin-treated animals. Indeed, Kamohara *et al.* reported significantly increased whole body glucose turnover and glucose uptake in central leptin-infused mice (10); thus, more efficient peripheral glucose utilization may promote lower blood glucose levels. Importantly, these effects were also observed without any elevation in plasma insulin, which was, in fact, slightly decreased after acute leptin treatment in normal mice (10). Neither body weight nor plasma glucose concentration was significantly altered in the study by Kamohara *et al.*; however, long-term leptin treatment selectively reduces fat mass (11). Since skeletal muscle relies primarily on fatty acid oxidation for energy during the postabsorbative period (102), chronic leptin-treated animals lacking adequate fat stores may depend more on glucose for fuel. In fact, Hidaka *et al.* found mRNA expression of genes related to fatty acid oxidation in skeletal muscle (i.e. carnitine palmitoyltransferase I (CPT I) and muscle-type fatty acid binding protein (M-FABP)) were down-regulated in STZ-D rats by ~ 50% with chronic leptin treatment (93), which indicates a switch to carbohydrates from fatty acids as a fuel source. Furthermore, glucose infusion following acute leptin treatment in normal WT mice increased whole-body glycolytic rates (i.e. conversion of D-3-^{3}H-glucose to ^{3}H water) by nearly 2-fold compared to vehicle treatment (10). Thus, a combination of overall lower glycogen stores and enhanced peripheral glucose uptake may contribute to the inability of leptin-treated animals to maintain euglycemia during a fast.

Moreover, as glycogenolysis does not appear to sufficiently raise blood glucose in leptin-treated rats, the contribution of gluconeogenesis to HGP may be impaired in these

animals. Nevertheless, mRNA expression levels of G6Pase and PEPCK were up-regulated during fasting in both leptin and vehicle-treated rats, which implies normal induction of gluconeogenic enzymes. Although augmented G6Pase expression during fasting seems contrary to the pattern of G6Pase activity described above, this may be a compensatory response to relatively lower enzyme activity levels observed during fasting. In addition, the expression of PGC-1 (PPARγ coactivator-1), which is a cofactor that stimulates transcription of gluconeogenic enzymes and is rapidly induced in the liver during a fast (103), was generally up-regulated in fasted animals, and leptin treatment significantly enhanced this effect. The mRNA expression of the glycolytic enzyme GCK, meanwhile, was down-regulated in STZ-D animals and during fasting, which corresponds with previous findings (11,93) and was expected given GCK is stimulated by insulin action and hepatic glucose uptake. In contrast, the STZ-D vehicle-treated group in our study did not exhibit the overall elevated PEPCK, G6Pase, and PGC-1 expression or increased G6Pase activity relative to controls reported by others (93,102,103). The reason for these discrepancies remains unclear.

Overall, leptin-mediated regulation of gluconeogenic gene expression does not appear to be significantly altered during a short-term fast compared to controls; nevertheless, these findings do not exclude the possible involvement of other regulatory mechanisms. For example, Hidaka *et al.* observed that the mRNA expression of GLUT2, a glucose transporter involved in glucose efflux from the liver, is markedly elevated in STZ-D rats due to enhanced HGO. However, these levels are reduced to ~ 75 % and 40 % of controls by chronic leptin treatment in STZ-D and normal rats, respectively (93), which is suggestive of reduced glucose synthesis and/or glucose transport out of the liver.

Although the exact mechanisms by which central leptin signaling influences blood glucose homeostasis have not been fully elucidated, our study indicates these effects are independent of insulin activity. Accordingly, adrenergic activation via the SNS has been implicated in mediating CNS signals to peripheral effects on glucose homeostasis (10,104); however, blockade of $\alpha 1$, $\beta 1$, $\beta 2$, or $\beta 3$-adrenergic receptors did not prevent the normalization of blood glucose levels, decrease in polyuria and polydypsia, or inhibit food intake of STZ-diabetic rats treated with chronic leptin infusion (105). Thus, the glucoregulatory effects of central leptin appear to be mediated by an alternative pathway in the CNS.

Indeed, activation of ATP-sensitive potassium (K_{ATP}) channels in hypothalamic neurons appears to be involved in the regulation of hepatic glucose homeostasis (66, 80). Obici *et al.* found central antagonism of these channels attenuated the inhibitory effects of insulin on hepatic glucose production (66), while Pocai *et al.* demonstrated that stimulation of these channels mediated efferent, but not afferent, signaling between the brain and liver via the hepatic vagus nerve (80). In addition, leptin was also found to influence glucose-responsive hypothalamic neuron activity by modulating K_{ATP} channels (88, 89,106). Thus, stimulation of this pathway may play an important role in central leptin's glucoregulatory effects in the liver independently of insulin action.

CHAPTER 6: CONCLUSIONS

Overall, leptin-treated rats, regardless of insulin-deficiency, display markedly reduced blood glucose levels during a short-term fast. In addition, a comparison of the effects of fasting and exogenous insulin injection demonstrates that this fasting effect, rather than an increased sensitivity to insulin, accounts for the significant decline in blood glucose in response to insulin (i.e. during ITT) previously observed in leptin-treated as compared to vehicle-treated rats. As these glucose-lowering effects are not observed when given access to food, animals may use food intake to regulate blood glucose levels. Moreover, this implies endogenous glucose production is impaired by leptin. However, glycogenolysis and the induction of gluconeogenic enzymes stimulated by fasting were not seemingly inhibited in leptin-treated rats. Nevertheless, the lack of a corresponding increase in G6Pase activity or ability to maintain normoglycemia during fasting indicates these processes are unable to adequately elevate hepatic glucose output. Thus, additional regulatory mechanisms may be involved in the mediation of central leptin's glucose-lowering effects and merit further investigation.

Table 1
Hepatic gene expression levels in leptin-treated STZ-D and non-diabetic animals during the fed and fasted states

Treatment	Condition	PEPCK	G6Pase	GCK	PGC-1
CC	Fed	1.00^{A}	$1.00^{A,B}$	1.00^{A}	1.00^{A}
	Fast	$2.36^{A,B}$	$2.56^{B,C}$	$0.27^{A,B}$	$2.42^{A,B}$
CL	Fed	0.35^{C}	$0.71^{A,B}$	1.17^{A}	$2.61^{A,B}$
	Fast	5.62^{B}	3.78^{C}	0.05^{B}	7.52^{B}
SC	Fed	$1.27^{A,B}$	$0.88^{A,B}$	$0.16^{A,B}$	$1.86^{A,B}$
	Fast	5.71^{B}	$2.52^{B,C}$	$0.16^{A,B}$	$3.33^{A,B}$
SL	Fed	$0.87^{A,C}$	0.41^{A}	$0.25^{A,B}$	5.33^{B}
	Fast	$2.50^{A,B}$	$1.35^{A,B,C}$	0.08^{B}	7.38^{B}

Data are expressed as fold-changes in gene expression relative to CC animals in the fed state. Statistical differences are based on $\Delta\Delta C_T$ values with different letters indicating statistical significance within columns, $P < 0.05$.
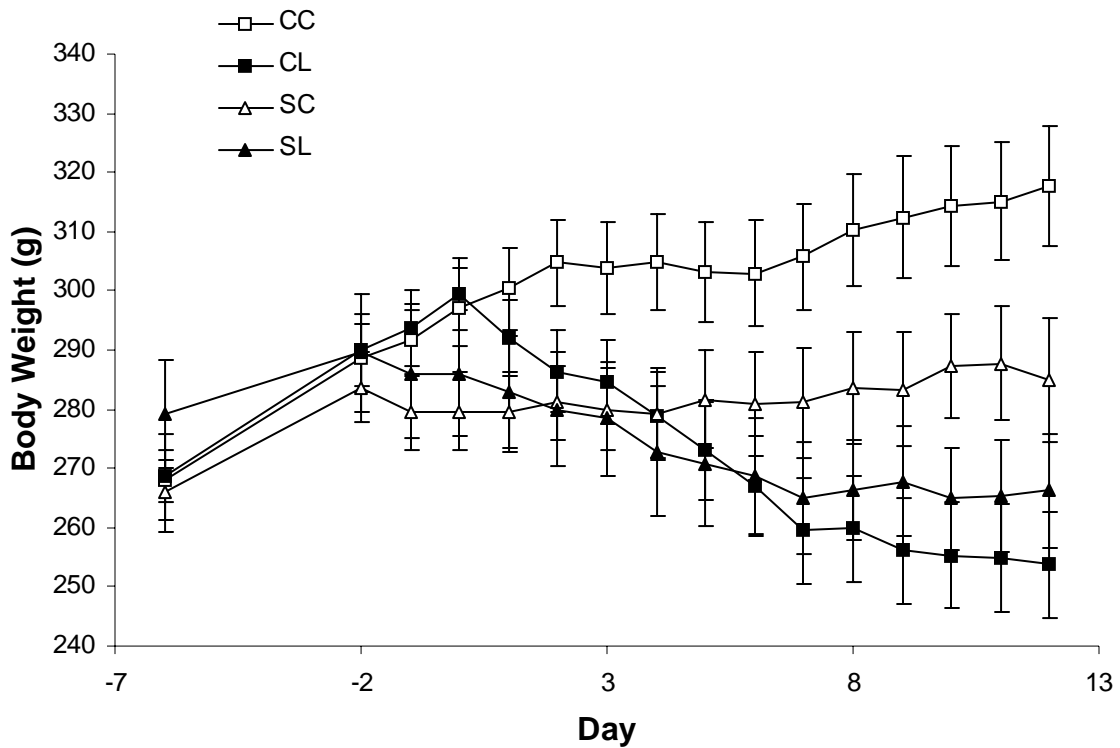
FIG. 1. Average body weight change over time in normal, vehicle-treated (CC), normal; leptin-treated (CL); STZ-diabetic, vehicle-treated (SC); and STZ-diabetic, leptin-treated (SL) rats. *Day -2* refers to induction of STZ-diabetes and *day 0*, to the initiation of icv leptin or vehicle treatment. Values are expressed as means $\pm$ SE ($n = 7$-10 per group). There was a significant overall weight-reducing effect of leptin compared to vehicle treatment ($P < 0.02$), and this effect was also significant over time ($P < 0.0001$) (MANOVA with repeated measures).
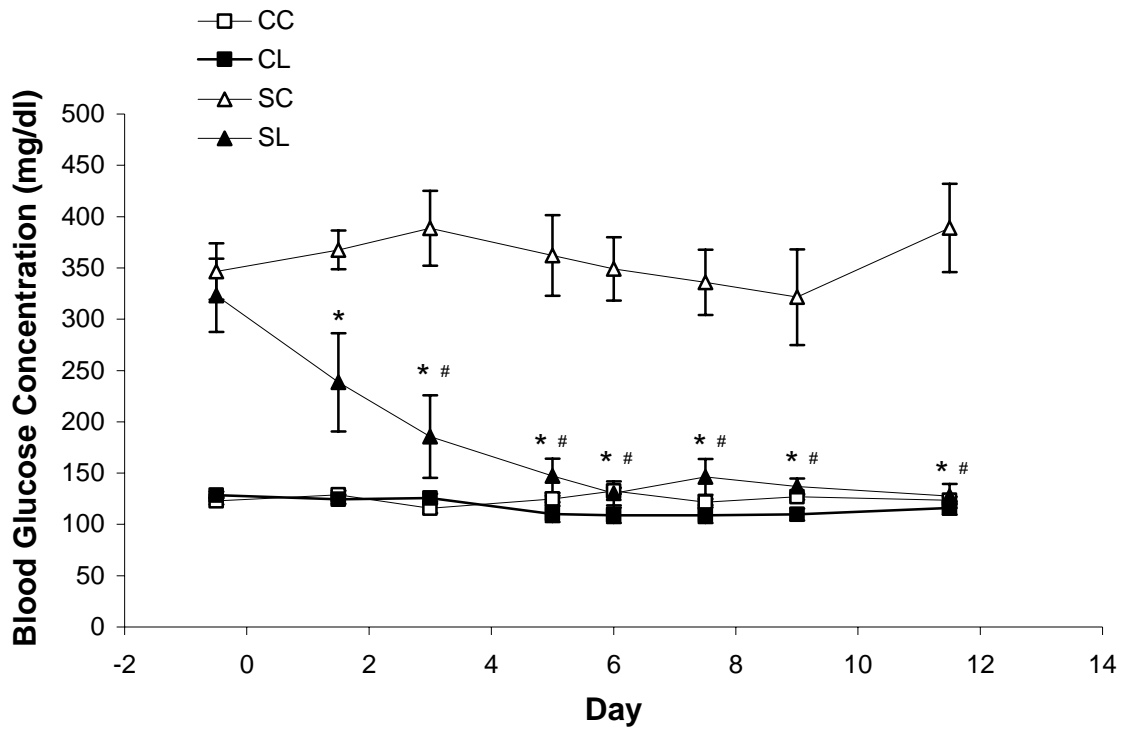
FIG. 2. Average daily blood glucose measurements (mg/dl). Blood glucose was measured approximately every other day using samples obtained from the tail-vein. *Day -0.5* refers to STZ-injection and *day 0* to initiation of icv leptin (or vehicle) treatment. Half-days (e.g. 1.5, etc…) represent the average time point determined after pooling data collected from two studies. Values are expressed as means $\pm$ SE (n = 7-10 animals per group). *P < 0.05 (SL vs. SC), [#]P = NS (SL vs. CC)
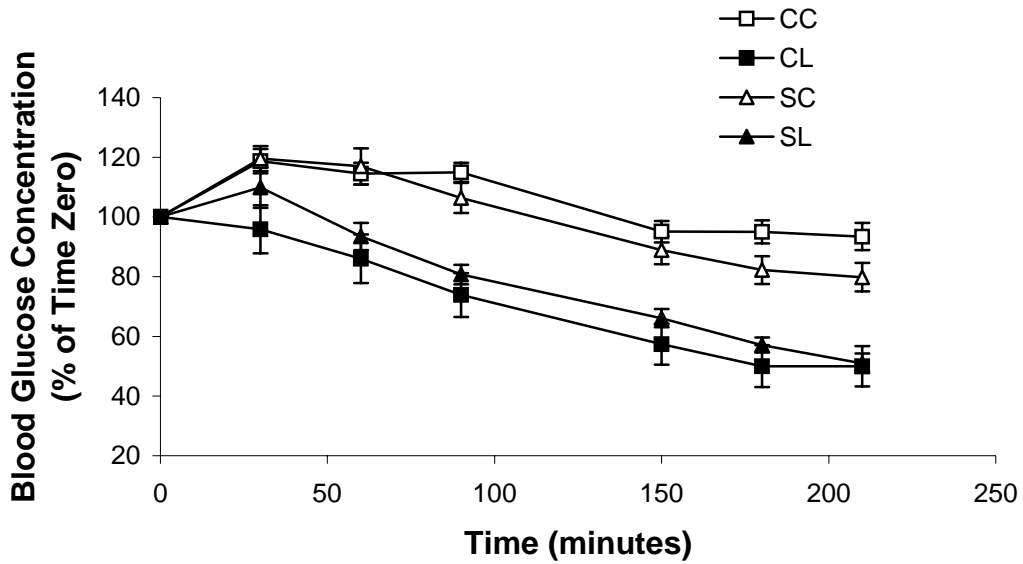
FIG 3. Blood glucose levels during a short-term (3.5 h) fast in CC, CL, SC, and SL rats. Values are expressed as means ± SE ($n = 7$-10 per group). Fasting blood glucose was significantly lowered by leptin relative to vehicle treatment ($P < 0.0001$), and this effect was significant over time ($P < 0.0001$) (MANOVA with repeated measures).
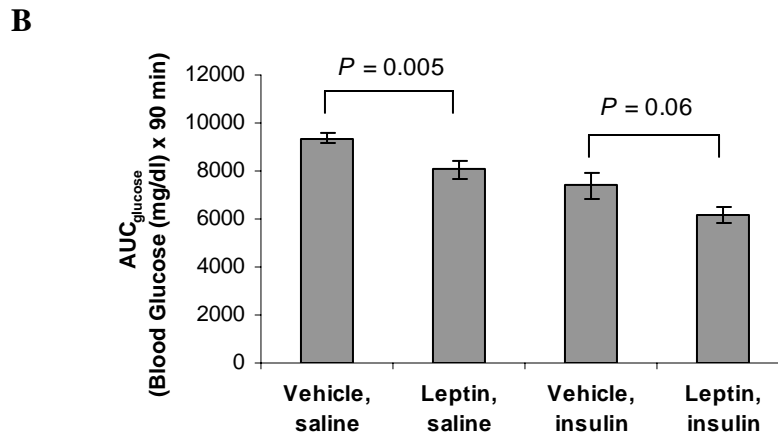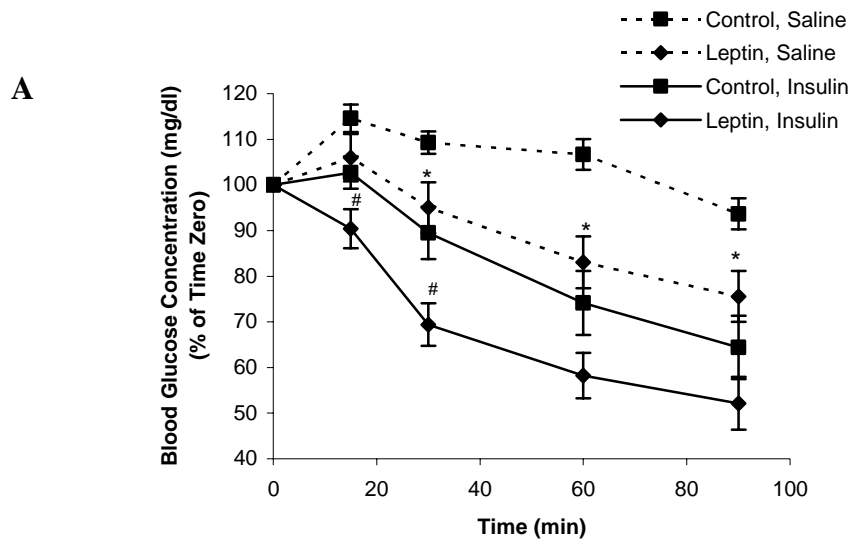
FIG. 4. A) Blood glucose response during insulin tolerance test (ITT) or following saline injection. Tests were performed over a 2-day period in a cross-over design as follows: Day 1) half the animals from each group underwent an ITT (1 U/kg), while the remaining animals received ip saline injection as a control; Day 2) tests were repeated by administering saline to animals previously given insulin and vice versa. There was no significant effect of STZ-induced diabetes on blood glucose levels during either procedure (MANOVA with repeated measures); consequently, data were pooled into two groups to examine the main effects of treatment (i.e. icv leptin or vehicle) on blood glucose in response to insulin or saline. Values represent means ± SE (n = 9-10 rats per group). *P < 0.05 (Leptin, Saline vs. Control, Saline), #P < 0.05 (Leptin, Insulin vs. Control, Insulin)
B) Area under the blood glucose response curve (AUC$_{glucose}$) during ITT. The incremental area under the blood glucose response curve (AUC$_{glucose}$) over 90 min was calculated using the trapezoidal method. Values represent means ± SE (n = 9-10 per group).
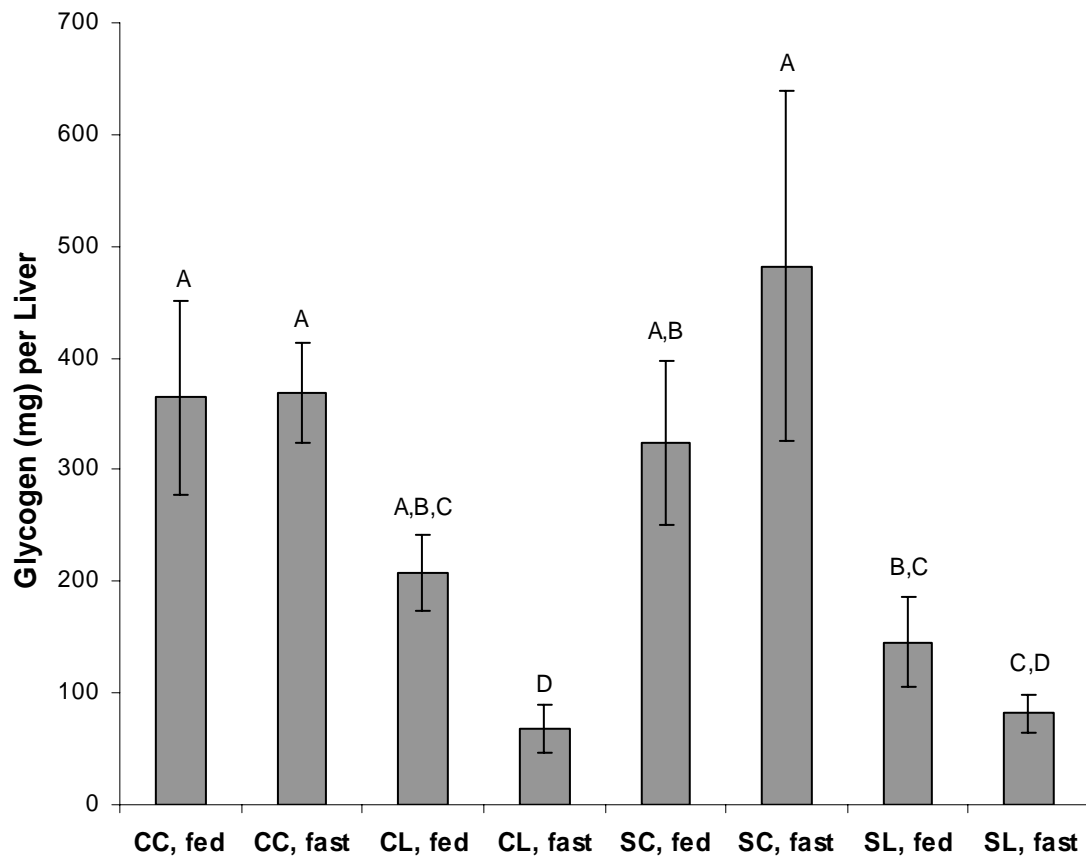
FIG. 5. Total liver glycogen measurements (mg) of CC, CL, SC, and SL animals in the fed and fasted (3 h) states (n = 3-5 per group). Values are expressed as means ± SE. Bars not sharing the same letter are significantly different, $P < 0.05$.
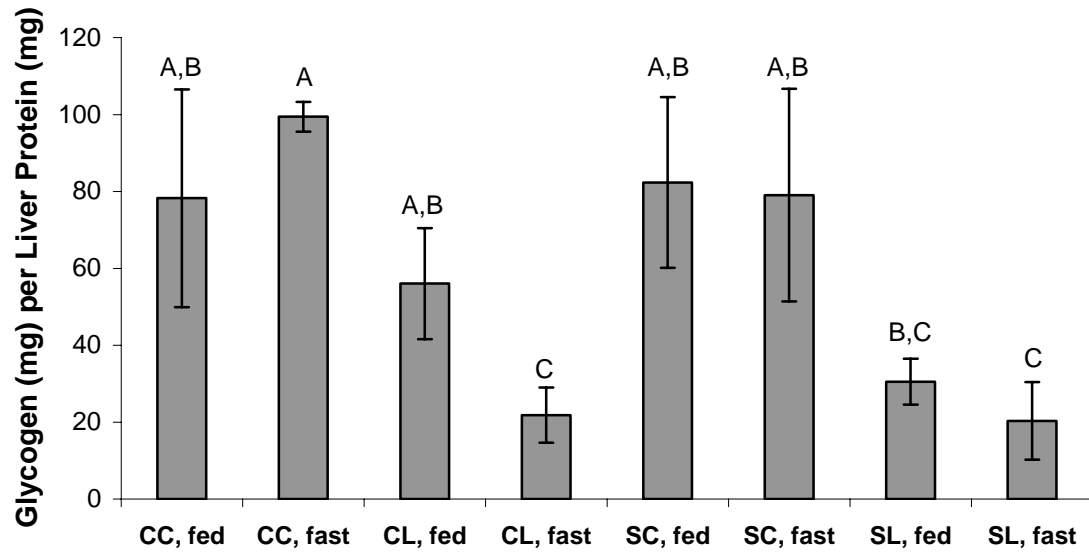
FIG. 6. Liver glycogen content expressed as glycogen (mg) per liver protein (mg) of CC, CL, SC, and SL animals in the fed and fasted (3 h) states (n = 3-5 per group). Values are expressed as means ± SE. Bars not sharing the same letter are significantly different, $P < 0.05$.

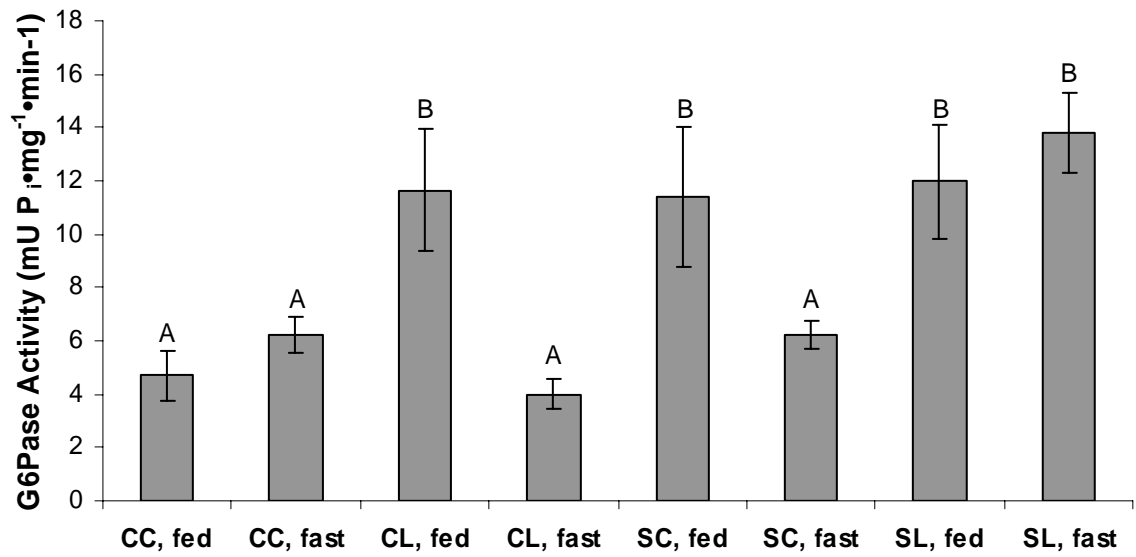**FIG 7. Hepatic microsomal G-6-Pase enzymatic activity (mU $P_i \cdot mg^{-1} \cdot min^{-1}$) of CC, CL, SC, and SL animals in the fed and fasted (3 h) states.** Values expressed as means ± SE (n = 3-5 per group). Bars not expressing the same letter are statistically different, $P < 0.05$.

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