EFFECTS OF LEPTIN AND THE SYMPATHETIC NERVOUS SYSTEM ON ADIPOSE TISSUE METABOLISM IN MALE C57BL/6 MICE AND SPRAGUE DAWLEY RATS

by

CHERIE RENEÉ ROOKS

(Under the Direction of Ruth Harris)

ABSTRACT

Leptin decreases body weight by decreasing fat stores and preserving lean tissue. Although much is known about leptin, there are mechanisms of leptin function yet to be determined. The objective of this thesis was to determine the importance of sympathetic innervation of white adipose tissue in mediating the effects of leptin on fat. Male C57Bl/6 mice and Sprague Dawley rats that were unilaterally denervated and infused with leptin showed that the sympathetic nervous system (SNS) is not necessary for leptin induced decreases on body fat in mice. In rats, however, the SNS is necessary for leptin action, of one pad but denervation also increases the sensitivity of distant fat depots to leptin. Changes in fat metabolism due to leptin were not clearly determined. Surprisingly, removal of the SNS in one pad resulted in an increased response of the contralateral pad to insulin and norepinephrine. These studies imply that the SNS may not be necessary for all leptin-induced responses of adipose tissue; however, this is not consistent in all species and still needs further investigation.

INDEX WORDS: peripheral leptin infusion, norepinephrine, lipolysis, lipogenesis, body weight, body composition

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CHAPTER 1

INTRODUCTION

Over half the adult population in the United States is termed overweight or obese [www.cdc.gov], and the incidence of obesity in children has doubled since 1980 [www.surgeongeneral.com]. Weight gain has become an accepted part of life and for every year that adult's age, they gain approximately one and one-half pounds in weight (3). Obesity increases the risk for heart disease, certain types of cancer, type II diabetes, stroke, arthritis, respiratory disorders, hypertension, and psychological disorders, such as depression [www.surgeongeneral.com]. Obesity increases the risk of mortality in individuals with cardiovascular disease and diabetes by as much as 63% (10, 14). With such alarming numbers and associated health care costs of $117 billion in 2001 (29) methods of treatment and prevention of obesity are now at the forefront of medical research (29). Questions remain as to the best methods of treatment and prevention of obesity. Because obesity is a multifactorial disease, and its mechanisms have yet to be clearly explained, physicians, dietetic professionals, and health care providers (7, 10) face a major problem in assisting their patients achieve a healthier body weight and lifestyle. Popular treatment methods such as pharmacotherapy (7), behavioral interventions, and low calorie diets all result in a relapse within as short a time as 6 months (1, 30). If any fool-proof method of treating and/or preventing obesity is to be established, then it must begin with a thorough understanding of the body's mechanism for controlling body weight and the effects that endogenous and exogenous substances have on the function of that mechanism.

It was proposed by Kennedy (19) that a circulating factor is secreted by fat to act as a negative feedback signal in the regulation of body weight. This factor has since been identified

by Zhang et al. (33) as the protein leptin. Leptin is a hormone secreted primarily by adipose tissue (33) and circulating concentrations increase in response, partially, to increases in adipose stores in rodents and humans (2, 9, 12, 16, 21). In obese humans leptin resistance accounts for high circulating levels of leptin present that are unable to decrease body fat (9, 16, 21). The mechanisms responsible for leptin resistance are uncertain, but before leptin resistance can be understood, the normal route of leptin action must be determined. Leptin decreases body weight and food intake in lean and ob/ob mice by specifically reducing body fat (15, 18, 22). Peripheral leptin infusion decreases body weight and endogenous fat stores with a transient reduction in energy intake (18, 20). Pair-feeding to leptin infused mice results in a smaller reduction in body weight and food intake implying an additional increase in energy expenditure. Increases in energy expenditure are related to increased thermogenesis and uncoupling protein -1 expression (UCP-1) in brown adipose tissue (BAT) (27). It is hypothesized that leptin increases UCP-1 mRNA expression in brown adipose tissue by activating the sympathetic nervous system (26). When leptin is administered directly to the brain it activates the sympathetic nervous system (SNS) which increases UCP-1 mRNA expression and results in fat loss (11). SNS activity is indicated by an increase in the tissue catecholamine norepinephrine (NE) content (13). Peripheral leptin injection increases NE turnover in brown adipose tissue implying an increase in sympathetic activity (8). Based on this evidence, it is apparent that leptin activates the SNS, but it is not clear whether the SNS is necessary for physiological levels of leptin to act on white fat. Fat transplants, in which fat is removed and sutured to the inner abdominal wall leaving the fat without intact sympathetic nerves intact, show that hyperleptinemia is able to decrease fat without intact sympathetic signaling (32). This does establish that a direct mechanism or activation of another hormone accounts for the ability of pharmacological levels of leptin to

decrease fat. It is uncertain, however, whether physiological levels of leptin are capable of decreasing fat in the absence of the SNS.

If leptin is acting to decrease fat either directly or through the SNS it does so by either increasing lipolysis and/or inhibiting lipogenesis. The information available on leptin and its effect on adipocyte metabolism is conflicting. In vivo studies (6, 17) show that adipocyte lipid synthesis and glucose oxidation decrease in leptin-treated animals compared to PBS-treated controls. Leptin inhibits both basal and insulin stimulated lipid metabolism (6). Lipoprotein lipase (LPL), an enzyme that transports lipid into fat cells, decreases with in vivo administration of leptin. This effect of leptin on LPL is likely indirect because insulin and triglyceride levels were also decreased (23). Consistent with this, high doses of leptin have not been shown to affect LPL activity or glucose transport in adipocyte cultures (25). In vivo studies have also shown that hyperleptinemia decreases fat stores by inducing lipolysis and by increasing fatty acid oxidation (28, 31). In older 15-week-old C57Bl mice, however, physiological doses of leptin does not induce lipolysis (4, 5).

This thesis will focus on two possible mechanisms of leptin action on body fat. In the first I will determine if the SNS is necessary for physiological concentrations of leptin to decrease fat stores. This will be achieved using chemical denervation that disrupts sympathetic terminals and degrades catecholamines (24). Secondly I will determine the effects of physiological doses of leptin on body fat by measuring the metabolic activity in white adipose tissue from leptin-infused rats. In addition to measuring the metabolic response to leptin I will also determine whether removal of sympathetic innervation changes the response to leptin.

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CHAPTER 2

LITERATURE REVIEW

LEPTIN

The lipostatic hypothesis, proposed by Kennedy in 1953 (43), states that energy stores are regulated by a factor related to body fat mass that maintains body weight by modifying energy consumption during changes in environment, excess caloric intake, and lactation. Additional evidence for regulation of body fat is provided by the compensation for weight and fat loss caused by fasting (36), or lipectomy (51) when body fat is decreased. In addition, the body fat that is gained during periods of overfeeding is lost after overfeeding stops (36).

These studies illustrate how tightly body weight is regulated. Subsequent parabiosis studies (39) provided evidence that Kennedy's factor was circulating and is involved in the regulation of body weight. It was found in parabiosis studies, in which animals share the same blood circulation, that pairing of db/db and ob/ob mice resulted in hypophagia, severe weight loss and later death in the ob/ob partner (14). Also, parabiosis of lean wild-type mice to db/db mice resulted in severe weight loss and death of the lean mice. In contrast, parabiosis of lean mice to ob/ob mice hindered the weight gain of ob/ob mice (15). From these parabiotic studies it was understood that the db/db mice produced a circulating factor but did not recognize it and that the circulating lipolytic factor is not produced in the ob/ob mice (14). It has since been determined that this factor associated with fat stores is the product of the ob gene leptin (31).

Leptin and Body Weight

Identification of the obese gene by Zhang et al (74) conveyed new insight into the mechanisms by which body weight is controlled. Mutation of the ob protein gene results in severe obesity in mice. The ob gene in mice is analogous to the human ob gene, (41), and

encodes a 16kD protein, leptin. As fat stores increase, expression of the ob gene in white adipose tissue (WAT) and secretion of leptin also increase (30, 74). Leptin (Greek meaning 'thin') has been hypothesized to maintain homeostasis in response to weight gain by decreasing food intake, increasing energy expenditure, decreasing fat stores, and acting as a satiety signal (26, 58). There is an alternative hypothesis that leptin is more important for signaling the brain during times of energy deficit rather then when energy stores are in excess (23). When leptin concentrations are low as in a state of starvation, there is an increased drive to increase food intake (23). An energy deficit (energy intake decreased by 2 MJ/d and energy expenditure increased by 0.8MJ/d) of one week results in a 54% drop in human serum leptin levels (42). Usually, during energy deficit or starvation it appears that low leptin results in inhibition of non-essential, energy expensive functions such as reproduction, thyroid and growth hormones are suppressed along with fertility to maintain energy stores for survival. Leptin administration, however, reverses these effects of starvation as if in a state of positive energy balance (2, 11). This implies leptin is naturally present as a means of maintaining energyhomeostasis during energy deficit.

Specifically, leptin-induced changes in overall body weight are due to decreases in fat stores with the maintenance of lean tissue (21). Because obese individuals are leptin-resistant (19), approaches to weight loss can be clearly specified by learning how leptin works to decrease fat stores, which are the largest energy reserves in the body (41), and why in obese individuals this pathway is inactive. In obese humans leptin mRNA expression and serum leptin concentrations are elevated, with women having higher serum leptin concentrations than men (32, 50, 57). This implies that obese humans are hyperleptinemic and unresponsive to leptin (19) but not leptin deficient. Although plasma leptin concentrations in humans are highly variable,

(32, 50) larger adipocytes do express more leptin mRNA (32). The soluble leptin receptor (SLR) Ob-Re, an isoform of the leptin receptor (Ob-R) discussed below, is found in the circulation (45). SLR is higher in lean individuals than those that are obese. Obese individuals have less leptin bound to the SLR, even though, leptin levels are higher in obese (57). This offers one possible explanation for the ineffectiveness of leptin in overweight and obese humans. There are special cases in which humans are leptin deficient, leading to diminished circulating leptin concentrations, hyperphagia, severe obesity, and decreased energy expenditure (25, 47). In these individuals the response to leptin (0.01-0.04 mg/kg for 18 months) is remarkable leading to decreases in body weight (specifically fat tissue) and food intake, decreased LDL cholesterol and triglycerides, and increased HDL cholesterol (47). In lean never obese humans that are not leptin deficient, exogenous leptin (0.3 mg/kg for 6 days) administered subcutaneously does not decrease food intake or body weight nor does it alter energy expenditure (49). In humans leptin may be present solely for detecting starvation as mentioned above, because obese humans who have plenty of leptin do not respond to their own leptin or to exogenous leptin treatment, but do respond to periods of energy deficit by decreasing serum leptin.

In rodents, Pelleymounter et al. (58) found that homozygous obese (ob/ob) mice, heterozygous obese, and normal mice given daily intraperitoneal (i.p.) injections of leptin produced changes in body weight dependent on dosage (0.1, 1.0, or 10mg/kg) and time. Genetically obese ob/ob mice that do not secrete leptin are more sensitive to leptin than lean mice (37). A larger dose response study was conducted conducted by Harris et al. (37) in which leptin (0, 1, 2, 5, 10, or 42 μg/day) was administered to five-week-old ob/ob and lean female C57BL/6 mice. The 10 μg leptin/day dosage was the lowest dose that produced circulating concentrations of leptin in the physiological range that also decreased body weight lean mice and

the 2 μg/day dose decreased weight in the ob/ob mice over the course of the study. Additionally, hyperleptinemia (8 ng/ml) decreases body weight and completely depletes fat stores in male Wistar rats (12). This decrease in fat is associated with a 30-50% decrease in food intake. Bowen et al. (5) showed that lower doses of leptin (10 μg/day) infused through a peripheral miniosmotic pump, also decrease body fat in mice. This loss of fat was not accompanied by a decrease in food intake but was influenced by gender and housing conditions. Male mice were less responsive to leptin even though they showed a reduction in fat due to leptin, and group housing prevented a loss of body fat in high-fat fed NIH Swiss mice.

In lean and ob/ob mice both central and peripheral leptin decrease body weight (9, 31, 70). ob/ob mice differ from lean mice in that they are unable to secrete leptin and are thus leptin deficient and morbidly obese (1). Peripheral leptin infusion in ob/ob mice (100 μg/ kg/day) causes a persistent reduction in weight, but peripheral leptin injection (3 μg/mouse) causes an initial drop in weight that stabilizes at the end of treatment and is accompanied by a reduction in serum insulin and glucose (70). Leptin does act as a satiety signal reducing food intake in ob/ob mice (9, 31, 37), however, the method of administration determines the effects of leptin on food intake in lean mice. Intracerebroventricular (ICV) administration of leptin (1 μg/mouse) results in a significant reduction in food intake of both ob/ob and lean mice (9). Peripheral leptin infusion (100 μg/ kg/day) significantly decreases food intake of ob/ob mice, but only leads to a transient reduction in food intake of lean mice (37, 46). db/db mice do not respond by decreasing food intake or body weight to any form of leptin administration due to a lack of the long-form leptin receptor (ObRb) (9, 31, 46).

Decreases in body weight with leptin administration that only result in a transient reduction in food intake (37, 46) imply an additional increase in energy expenditure leading to a

decrease in weight. Scarpace et al. (64) demonstrated that leptin increases energy expenditure by increasing sympathetic outflow to BAT. This increase in sympathetic outflow increases BAT UCP-1 mRNA expression and thermogenesis (18, 63). Based on the data available leptin appears to be involved in the mechanisms that the body has set in place to regulate body weight and energy stores. It is uncertain whether leptin acts directly on fat stores through its receptor discussed below, through activation of another humoral factor, or through effector systems such as the sympathetic nervous system (SNS).

Leptin and Leptin Receptors

The actions of leptin within the body are caused by leptin acting through its receptor, Ob-R. (13, 68). The leptin receptor Ob-R (66) has five isoforms (Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, Ob-Re) (45), each with matching extracellular ligand binding domains and a different intracellular carboxy terminus. Mouse Ob-R is a class I cytokine receptor that is 78% homologous to the human leptin receptor (66). Ob-R is found in various areas of the brain and in all peripheral tissues (66). Ob-Rb, the long form receptor, has a long intracellular domain that is important for intracellular signal transduction (13). The other leptin receptors (Ob-Ra, Ob-Rc, Ob-Rd) have short intracellular domains, and their physiological function is unclear. Ob-Re has no trans-membrane or intracellular domain and is present in the circulation as a binding protein (45, 48). Ob-Rb is found at high levels in the hypothalamus and lower levels in adipose tissue, testes (45), adrenal gland, and kidney (40). Ob-Ra, the short form leptin receptor, is expressed at high levels in most tissues (40). It has been suggested that because Ob-Ra is present in the choroid plexsus (66) and in microvessels, it acts as a transport protein, transporting peripheral leptin across the blood-brain-barrier (4). Its function in peripheral tissue has not been defined. Leptin is shown to act through its Ob-Rb receptor located within the hypothalamus to influence

hepatic glucose metabolism (56). Microinjection of leptin into the ventromedial hypothalamus (VMH) increases glucose uptake into brown adipose tissue, heart, skeletal muscle and spleen (53). In addition, microinjection of leptin, into the VMH, increased glucose utilization within BAT and heart but not WAT. The increase in glucose utilization occurred in the presence of leptin and insulin rather then leptin alone (33). Global sympathectomy of rats inhibits the leptin-induced decrease in glucose uptake of these tissues implying that the SNS mediates leptin action in BAT, and heart. WAT glucose utilization is not clearly demonstrated by leptin acting through the SNS, even though the SNS does play a role in WAT metabolism (3).

The Sympathetic Nervous System and White Adipose Tissue

The central nervous system (CNS) is responsible for activating the SNS that innervates adipose tissue and releases a catecholaminergic neurotransmitter, norepinephrine (NE) (29). The sympathetic innervation of white fat was confirmed through fluorescent tract tracers injected into inguinal and epididymal pads (72). Sympathetic nerves entering white fat are involved in lipid mobilization and total body fat regulation (3, 56). The method by which sympathetic nerves are disrupted inhibiting catecholamine release (60) adds insight into what function the SNS has in WAT when nerves are intact. Removal of the sympathetic signal in WAT has been demonstrated using both surgical and chemical denervation (20, 73). Surgical denervation is a permanent procedure that entails cutting both sympathetic and sensory nerves innervating white fat. The regulation of fat pad size is by the sympathetic nervous system and is demonstrated by sympathetic denervation increasing fat pad size (7, 73). Two to three month old Siberian hamsters were placed on either long "summer-like" days causing a naturally obese state, or short "winter-like" days in which body weight is reduced, and had their inguinal white adipose tissue (IWAT) surgically denervated. Norepinephrine content was decreased markedly in hamsters that

were denervated compared to their controls. The denervated fat pad also increased in weight by increasing fat cell number not size (73). Chemical denervation that specifically disrupts sympathetic terminals leaving sensory nerves intact, is not permanent with regeneration first appearing in a week. 6-Hydroxydopamine (6OHDA) and guanethidine are both chemicals used for sympathetic denervation. Injection of these chemicals disrupts sympathetic terminals by first hindering catecholamine release followed by nerve degeneration (60). Local unilateral denervation by guanethidine increases epididymal and IWAT mass in hamsters while decreasing their NE content (20).

In addition to the presence of sympathetic nerves, adrenergic receptors (AR) (α and β) are present on WAT (44). Catecholamines such as NE can be released from the adrenal medulla or sympathetic nerve terminals in vivo and stimulate lipolysis (3, 10, 56) by activating β-ARs (10). If the activation of β-AR on WAT outweigh the α-ARs then lipolysis is activated, however, if the presence of α-ARs predominates then lipolysis is inhibited (3). Activation of lipolysis causes a cascade effect within the fat cell with hormone sensitive lipase, the main lipolytic rate limiting enzyme, breaking down triglycerides into monoglycerides which are further broken down to glycerol and fatty acids that can be released from the cell (3). Insulin, however, has an inhibitory effect on lipolysis by reducing the number of adrenergic binding sites available by translocating insulin receptors from the plasma membrane to intracellular compartments. This causes a desensitization of ARs to catecholamine-stimulated lipolysis in human fat cells (24). Potentially, leptin may increase lipolysis by increasing sympathetic outflow from the CNS to WAT (16). The experiment described in chapter 4 of this thesis tested the effects of leptin and denervtion on lipolysis in white fat.

Leptin and the Sympathetic Nervous System

Leptin administration activates the sympathetic nervous system, indicated by a stimulation of thermogenesis (64) and increased norepinephrine turnover in brown fat (16). Peripheral leptin-infusion using miniosmotic pumps with doses from 10.0 μg/kg to 1,000 μg/kg dose dependently increases renal sympathetic nerve activity (SNA) (38). This leptin-induced sympathetic activation is inhibited in obese Zucker rats that have a mutation of the extracellular domain of the leptin receptor (38). In female C57Bl/6J ob/ob mice, a 40.0 μg injection of leptin did not significantly increase NE turnover in WAT but caused a significant increase in NE turnover in interscapular brown adipose tissue (IBAT) (16). On the other hand, direct injection of leptin into epididymal WAT increases renal sympathetic nerve activity (54). In similar studies, leptin injection also activates efferent nerves of the contralateral epididymal pad (55). Although leptin does not effect NE tunover of WAT (16), the presence of NE is necessary for leptin to regulate its own gene expression and UCP-1 mRNA expression in brown fat (17). The activation of the SNS by leptin (38) and the inhibition of leptin mRNA by the SNS have been shown previously (34, 62), but the role of leptin-induced sympathetic activation on lipid metabolism is uncertain. Central leptin (50ng/h) is shown to decrease body weight in globally sympathectomized rats treated with guanethidine (22). In this same study leptin-infused rats with intact sympathetic signaling lost just as much weight as sympathectomized rats however, they had a higher food intake. This implies an important role of the SNS in increasing energy expenditure (22). Weight loss is caused by an increase in energy expenditure and/or a decrease in energy intake. Removal of the SNS inhibits the ability to increase energy expenditure, thus requiring a larger reduction in energy intake to decrease body weight. There is additional evidence that leptin is able to act on fat without sympathetic innervation either by leptin

receptors located in adipose tissue (45, 69), or the activation of other hormones. In an in vivo study (69) epididymal fat pads taken from eight-week-old Wistar rats were sutured to the anterior abdominal muscle wall, disrupting the sympathetic signal into and out of the fat pad. Four weeks later adenovirus leptin was administered, and eight days after treatment the rats were sacrificed. Serum leptin levels for the control group averaged approximately 1.2 ng/ml where as those from the leptin treated group averaged over 20 times that at approximately 23 ng/ml. The disappearance of the denervated fat pad implies that leptin is able to decrease fat either by the direct action of leptin on the fat pad, through leptin affecting another substance within the body that, in turn, decreases adipose tissue, or by causing a hypermetabolic state and negative energy balance. The studies discussed here demonstrate that leptin is able to decrease body weight and fat mass. What they do not demonstrate is how leptin works normally within the body. One study delivered leptin centrally (22) when the majority of leptin is produced and secreted peripherally. In the other study the adenovirus leptin raised serum leptin levels to pharmacological levels that would not normally be seen in circulation (69). It is important to determine how more physiological levels of leptin act in the absence of the SNS.

Leptin and Adipose Tissue Lipid Metabolism

A decrease in WAT can occur through lipolysis (catabolism of triglycerides/fatty acids) and/or through inhibition of lipogenesis. Increases in lipolysis with leptin have been seen both in vivo (65) and in vitro (67, 71). The significant increase in lipolysis in cultured cells, reported by Wang et al (67), was seen with the lowest effective dose of leptin (5 ng/ml). In an in vivo study conducted by Frübeck et al. (27) lipolysis (measured as glycerol release per 100 mg lipids) was significantly increased in both lean and ob/ob mice injected with higher doses of leptin (10mg/kg of body weight). In db/db mice, however, leptin did not stimulate lipolysis, which further

implies that it requires the long form leptin receptor, Ob-Rb (13). A stimulation of lipolysis also was not observed in older (15-week), wild-type, leptin-treated mice infused with low doses of leptin (10μg/day) (6). It is not clear whether this was because a low dose of leptin was used, or because the mice were generally unresponsive to leptin. Additionally, leptin effects in vitro are inconsistent in that leptin increases lipolysis in isolated adipocytes of ob/ob and lean mice (28), but does not effect lipolysis in adipocytes of Sprague Dawley rats (52).

Inhibition of lipogenesis with physiological doses of leptin (10 μg/day) was found in lean female C57/Bl mice (35). In addition in gold thioglucose mice (GTG) a single leptin injection (25 μg/mouse) reduced lipogenesis in WAT and liver (8). GTG mice are chemically induced to be obese, and they have less hypothalamic leptin receptors then their lean counterparts. Lipogenesis was reduced in these animals implying that the leptin receptors within the brain are not necessary for leptin to inhibit lipid synthesis. In vitro, however, leptin does not reduce lipogenesis as measured by total lipid synthesis in isolated adipocytes from Sprague Dawley rats (52). In this same study and as well as one conducted in cultured rat adipocytes, leptin was unable to inhibit insulin-stimulated glucose transport (52, 61). Lipoprotein lipase (LPL), an enzyme that transports lipid into fat cells, was found to decrease in WAT, and IBAT of ob/ob mice infused with leptin (59). This effect of leptin on LPL is likely indirect because insulin and triglyceride levels were also decreased (59). Another study found conflicting evidence in that leptin did not reduce LPL activity in 3T3-L1 differentiated cells, or isolated adipocytes and adipose tissue from ob/ob mice. Because the data available are inconsistent, it is important to determine exactly how physiological doses of leptin decrease adiposity in vivo if we wish to use leptin for future obesity treatment.

Summary

Leptin, the hormone primarily secreted from adipose tissue is involved in the regulation of body weight and specifically reduces adipose stores but preserves lean tissue (58). It is unclear however, exactly how leptin is able to decrease body fat. Although the SNS is proven to innervate white fat, whether leptin acts on sympathetic nerves to induce its lipolytic effects (3) is yet to be determined. It is known that for leptin to increase UCP-1 mRNA expression in IBAT, the sympathetic neurotransmitter NE must be present (17). Fat stores are decreased either by increasing lipolysis and/or inhibiting lipogenesis, but the effect leptin has on lipid metabolism is yet to be clearly determined. If both an inhibition of lipogenesis and a stimulation of lipolysis are found then physiological doses of leptin are able to decrease the incorporation of triglycerides into adipocytes and increase their release from the cell. This thesis will address how physiological doses of leptin reduce adiposity in vivo and whether it requires intact sympathetic innervation of white fat.

Hypothesis:

It is hypothesized that leptin-induced reductions in denervated fat pads will be prevented. Leptin will reduce fat pad size by inducing lipolysis and/or inhibiting lipogenesis. In order to determine this:

- Fat pads will be chemically denervated to determine if leptin is capable of decreasing fat in the absence of the sympathetic nervous system.

AND

- Isolated adipoctytes from rats infused peripherally with physiological doses of leptin will be incubated to determine whether leptin increases lipolysis and/or inhibits lipogenesis.

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CHAPTER 3

SYMPATHETIC DENERVATION OF A SINGLE FAT PAD DOES NOT REDUCE LEPTIN SENSITIVITY IN C57BL/6 MICE BUT MODIFIES THE RESPONSE IN SPRAGUE DAWLEY RATS[1]

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ABSTRACT

Leptin administration causes weight loss due to decreases in fat rather than lean tissue. Leptin has been shown to stimulate the sympathetic nervous system (SNS), but it is not known whether sympathetic innervation of white adipose tissue is necessary for a leptin-induced decrease in fat pad size. In this study, one epididymal pad in 5-week-old male C57Bl/6 mice or 7-week-old male Sprague Dawley rats was sympathetically denervated by local injection of 6-hydroxy-dopamine or one pad was injected with phosphate buffered saline (PBS); (sham animals). Half of the denervated and half of the sham animals were intraperitoneally infused with 10 μg of leptin/day in mice and 50 μg/day in rats. Controls were infused with PBS. After 13 days of infusion, denervated mice infused with PBS were significantly heavier than all other groups of mice and leptin inhibited this increase and reduced body weight, fat pad mass, and total body fat of denervated mice. In rats, leptin inhibited weight gain in denervated animals, but denervation of one epididymal pad also decreased leptin sensitivity of inguinal and retroperitoneal fat depots, and increased mesenteric fat mass. Therefore, leptin action on white fat is not dependent on activation of the SNS in mice, but may be partially dependent on the SNS in rats. These results also suggest a direct or indirect communication between different fat pads because denervation of one epididymal pad led to changes in other fat depots.

INTRODUCTION

Leptin has been hypothesized to act as a satiety signal and to maintain homeostasis in rats by decreasing food intake, increasing energy expenditure and depleting fat stores (16, 31). Administration of the ob protein, leptin, to ob/ob (7, 18, 44) and wild type (31,18) mice dose-dependently decreases food intake while reducing body weight. Food intake, however, may not completely account for the reduction in body fat and body weight because decreases in body fat of leptin-infused mice are greater than in their pair fed counterparts (27). This implies that an increase in energy expenditure also contributes to the decrease in fat. Leptin increases energy expenditure in both ob/ob mice (31, 8) as well in lean rats (36). Increased oxygen consumption, norepinephrine (NE) turnover, and uncoupling protein - 1 (UCP-1) mRNA in brown fat reflects these increases in energy expenditure (2, 8, 31, 35, 36).

Leptin may act on fat through a number of mechanisms, three of which include: direct, neural, and/or hormonal mechanisms. The direct action is made possible through functional leptin receptors found in white adipose tissue (WAT) (26). In vitro studies show an increase in lipolysis in isolated fat tissue incubated in the presence of high concentrations of leptin (42, 17). Indirectly leptin may act through other unidentified circulating factors that, once activated, lead to responses that have been attributed to leptin. Finally, peripheral leptin increases thermogenesis by increasing brown fat UCP1 expression and NE turnover (NETO) (8,24) showing the involvement of the sympathetic nervous system (SNS) in leptin action, at least in this tissue. Centrally, a high density of leptin receptors are located within the hypothalamus (15, 14) including neurons within the paraventricular nucleus of the hypothalamus that, when activated, are part of the SNS outflow to white fat (3) that could potentially promote lipolysis.

Independent of leptin action, there is evidence that the SNS regulates the size of WAT (4). When WAT is surgically denervated, fat pad size increases due to an increase in cell number (6, 45). This enlargement of fat pad mass also occurs with denervation by the SNS neurotoxin, guanethidine (12). Chemical sympathetic denervation by 6-hydroxy-dopamine (6OHDA) or guanethidine has the advantage over surgical denervation by only removing the SNS innervation (for review see, (32)) and leaving other nerve types intact. Although there is no direct evidence that leptin promotes lipolysis in white fat via the SNS, Scarpace et al. have shown that SNS innervation is necessary for the action of leptin action on BAT (35) and leptin has been shown to increase renal SNS activity (24, 39). Collins et al. (8), however, found only a small, non-significant increase in WAT NETO of rats that received intraperitoneal injections of leptin. Therefore, it is not clear whether leptin-induced activation of SNS promotes loss of fat from WAT.

In contrast, other studies offer possibilities for leptin action without an intact SNS. Global chemical sympathectomy produced via systemic injections of guanethidine do not prevent leptin from reducing body fat (13). Specifically, complete permanent sympathectomy does not block the ability of intracerebroventricularly (ICV) administered leptin to mobilize lipid. Others also have shown that hyperleptinemia caused by a leptin secreting adenovirus reduced the size of transplanted fat that was devoid of neural connections (43). It is not known, however, whether physiological doses of peripheral leptin administered by the natural route of the peptide (peripherally) are capable of reducing body fat in the absence of the SNS in WAT. Therefore, the purpose of this study was to determine if sympathetic innervation is required for peripheral leptin to decrease body fat in mice and rats. This was achieved by infusing physiological amounts of leptin systemically following local unilateral sympathetic denervation

of one epididymal pad leaving the other pad intact as a within-animal control that was possible because this fat pad is bilaterally located and unilaterally innervated (4).

METHODS

Test of the effectiveness of 6OHDA to produce sympathetically denervated white fat

To confirm that 6OHDA decreased fat pad NE content, thirteen C57Bl/6 mice aged 5 weeks old were obtained from Harlan (Indianapolis, IN) and were acclimated to housing (73ºF with lights on 12h/day from 7:00 am) for one week with free access to food and water. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Georgia and were in accordance with the principles of the American Physiological Society (1). Animals were given free access to standard mouse chow containing (kcal%): carbohydrate (56%), fat (26%), and protein (18%) (Diet 5015; Lab diet, Purina Mills, MO) and water. The first group of six animals had one epididymal pad locally injected with 6OHDA and the contralateral pad served as the control pad. The mice were anesthetized with isofluorane and one epididymal pad was locally injected 10 times with 2 µL injections of 8 mg/ml 6OHDA (Sigma, St. Louis, MO). Injections were given using a micro-syringe fitted with a 30-gauge needle. The needle was held in place for 45 seconds after each injection to minimize backflow. Injection of 6OHDA was alternated between sides of different mice to control for possible unilateral differences between pads. Animals were decapitated one week later and both the injected and non-injected epididymal pads were snap frozen in liquid nitrogen for measurement of NE content, as described below. The surgery for the second group of 7 mice was the same as for the first group; however, animals were decapitated two weeks after surgery to determine whether sympathetic innervation returned to the injected epididymal pad within two weeks.

Experiment 1

Fifty-four male C57BL/6 mice aged 3 weeks were single housed in conditions described above. The mice were fed a diet containing (kcal%): carbohydrate (70%), fat (10%), and protein (20%) (Diet 12450B; Research Diets Inc., New Brunswick, NJ). They were single housed in cages with grid floors so as to measure food intake. One week after adaptation to these conditions baseline daily food intakes and body weights were measured for one additional week. The mice were then divided into four weight-matched groups based on an experimental design of denervation X treatment (2 X 2). Two weeks following arrival mice were anesthetized with isoflurane and each mouse received an intraperitoneal (i.p.) Alzet miniosmotic pump (Model 1002; Durect, Cupertino, CA) delivering either 0.01 M PBS or 10 μg leptin/day (recombinant murine leptin: R&D Systems, Minneapolis, MN) for 13 days. At the same time one epididymal pad was locally injected with 6OHDA or its vehicle (0.01 M phosphate buffered saline [PBS]), as described above. The PBS injections are referred to as vehicle injections from this point forward to avoid confusion with infusion of PBS from the Alzet pumps. The side of injection was alternated between animals. Daily food intakes and body weights were measured for 13 days. A small sample (~50 μl) of tail blood was collected five days after surgery to measure serum leptin concentrations (Mouse Leptin RIA: Linco Research Inc., St. Charles, MO). On Day 13, mice were food deprived for three hours before decapitation and trunk blood was collected in tubes containing 10 μl of 1% EDTA with 500 U/ml heparin. Plasma from trunk blood was taken for measurement of leptin, triglycerides (Sigma kit 337-B: Sigma Chemical Co., St. Louis, MO), and free fatty acids (FFA: NEFA C kit: WAKO Chemicals, Richmond, VA). Interscapular brown, mesenteric fat, left and right testicles, left and right retroperitoneal, and left and right epididymal pads were weighed. Interscapular brown fat (IBAT) and epididymal pads were collected and

snap frozen in liquid nitrogen for measurement of UCP1 mRNA expression in brown fat and of NE content in white and brown fat, as described below. In addition, a sample (50 mg) of each epididymal pad was fixed in osmium tetroxide for determination of fat cell size and number by Coulter Counter and Multisizer (28).

Experiment 2

Thirty-six (n = 9 per group) male Sprague-Dawley rats, 5 weeks of age (Harlan, Indianapolis, IN) were housed as described above. Rats were weight-matched and assigned to treatment groups. They were fed rat chow containing (kcal%): carbohydrate (63%), fat (11%), and protein (27%) (Diet 5012; Lab Diet, Purina Mills, MO). One week after arrival baseline daily food intakes and body weights were measured for another week. Surgeries were conducted as described above two weeks after arrival except rats were anesthetized with subcutaneous injections of ketamine (90 mg/kg) and a combination of xylazine (10 mg/kg) and glycopyrrolate (0.2 mg/ml). Each rat was fitted with an i.p. Alzet miniosmotic pump (Model 2002; Durect, Cupertino, CA) delivering either 0.01 M PBS or 50 μg leptin/day (recombinant rat leptin: R&D Systems, St. Charles, MO). At the same time, one epididymal pad was locally injected 20 times with 2 μL injections of either 9 mg/ml 6OHDA or 0.01 M PBS containing a 1% ascorbic acid solution. Daily food intakes and body weights were measured for 13 days. On day 13 of infusion rats were food deprived for 2 hours, decapitated and trunk blood was collected for measurement of serum leptin only. Tissues were weighed and collected as previously described with inguinal WAT also weighed. The remaining tissues were returned to the carcass for determination of body fat content as previously described (22).

Determination of Adipocyte Size and Number

A 50-mg sample of each pad was fixed in osmium tetroxide for determination of fat cell size and number by Coulter Counter. Tissue samples were rinsed in saline and immediately placed in collidine-HCl buffered osmium tetroxide (5 g osmium tetroxide in 100 ml of 50 mM collidine-HCl buffer). Isolation and measurement of adipocyte size and number were conducted as previously described (21) using a Coulter Counter (model S/ST II, Beckman/Coulter Electronics, Hialeah, FL) and Multisizer (model Multisizer II E).

Measurement of Catecholamines

To confirm WAT denervation, NE was measured by reverse – phase HPLC following our modifications of the method of Mefford (29). Briefly, frozen WAT (100 – 250 mg) was homogenized in perchloric acid buffer (0.2 M perchloric acid with 0.3 mg/ml ascorbic acid) with 1000 ng/ml of dihydroxybenzylamine (internal standard). Samples were centrifuged and catecholamines absorbed from the supernatant using alumina and 0.5M Trizma base (Sigma). Catecholamines were eluted into perchloric acid buffer. Catecholamines were assayed using an ESA, Inc (Chelmsford Bedford, MA) HPLC system with electrochemical detection (Culochem II). The mobile phase was Cat-A-Phase II and the column was a C-18 reverse phase column (ESA, Inc., Chelmsford, MA).

Northern Blot Analysis

Total tissue RNA was isolated from brown fat using Trizol reagent (Invitrogen Life Technologies; NY) according to manufacturer's directions. Brown fat UCP1 mRNA expression was measured according to the methods of Haltiner (19). UCP1 probe was biotin labeled (Brightstar Psoralen - Biotin labeling Kit Ambion, TX) and membranes were hybridized at 65° C for 3 hours. Detection of mRNA on the Nylon membranes (Hybond N^+; Amersham, Piscataway,

NJ) was detected with chemical luminescence by following the manufacturer's instructions (Ambion Detection Kit; Ambion, TX) and imaged on x-ray film. Following detection of UCP1 mRNA the membranes were stripped and rehybridized with a probe for 28S rRNA (34). The density of hybridized bands was measured (Un-Scan-It gel, Urem, UT) and expressed as a ratio of UCP1 mRNA to 28S rRNA.

Statistical Analysis

Body weight, food intake, and adipocyte size and number were compared between groups using a Repeated Measure ANOVA (Statistica Software 99' Edition, Stat Soft, Tulsa OK). Body weight and food intake measured immediately before surgery were used as covariates to determine changes between groups. Pad size, tissue NE content, serum leptin, free fatty acids, triglycerides, and glycerol were compared using a two-way ANOVA. Differences between specific groups were identified by post-hoc Duncan's Multiple Range test. The size of the denervated versus intact epididymal fat pads within an animal were compared by paired t-tests. In addition retroperitoneal and inguinal fat pads were compared using paired t-tests in rats. Differences were considered significant if $P < 0.05$.

RESULTS

Test of the effectiveness of 6OHDA to produce sympathetically denervated white fat

Injection of 6OHDA into one epididymal pad significantly reduced NE content of the pad at both 1 and 2 weeks after injection by 47% and 43%; respectively (Figure 1: 6OHDA: $P < 0.013$, Duration: NS, 6OHDA X Duration: NS). One-tailed paired t-test at 1 and 2 weeks after injection showed a significant reduction in NE content of the injected pad.

Experiment 1

The combination of epididymal denervation and leptin infusion had a significant effect on daily body weight of the mice (Figure 2A: 6OHDA: NS, Leptin: $P< 0.0001$, Day: $P < 0.0001$, 6OHDA X Leptin: $P < 0.04$, Leptin X Day $p < 0.006$). The major effect, after the initial loss of weight due to surgery, was that PBS infused mice with one denervated fat pad were significantly heavier after surgery than any other group, even though all the groups were weight matched before surgery. In addition, denervated mice infused with leptin weighed significantly less than both the denervated and non-denervated PBS controls. There was an interaction between leptin and denervation for total weight gained between surgery and the end of the experiment (Figure 2B: 6OHDA: NS, Leptin: NS, 6OHDA X Leptin: $P<0.02$) with the PBS-infused, 6OHDA-treated mice gaining significantly more weight than all other groups. This difference in weight occurred without any change in food intake (data not shown). At the end of the study we found differences between the injected and non-injected epididymal pads of both 6OHDA and vehicle injected mice (Figure 3A: Treatment: $P<0.0001$, Injection: NS). Sympathetic denervation alone did not change the weight of the injected pad of PBS-infused animals, but leptin infusion reduced the weight of 6OHDA-injected pads ($P < 0.03$). As expected leptin infusion also caused a significant reduction in weight of the non-injected pad of leptin-infused, vehicle ($P<0.03$) and 6OHDA treated mice ($P<0.03$). Leptin did not change the weight of epididymal pads, retroperitoneal, or mesenteric fat depots in vehicle injected mice (Figure 3A and Table 1). Analysis of the weights of the total dissectible fat from the different groups nearly reached statistical significance ($P<0.057$). Post-hoc analysis indicated a significant increase in the weight of white fat from PBS-infused, 6OHDA-injected animals compared with all other groups (Figure 3B).

There was no effect of denervation or leptin on adipocyte size or number in the injected epididymal pads (Figure 4A: 6OHDA: NS, Leptin: NS, Size: $P<0.001$). In contrast, in the non-injected epididymal pads, 6OHDA increased the number of small cells (20-30 μm) in PBS infused mice and leptin inhibited this increase (Figure 4B: 6OHDA: NS, Leptin: $P<0.046$, Size: $P<0.000$, Leptin X Size: $P<0.006$). The leptin-infused, vehicle-treated animals had fewer cells than PBS-infused, 6OHDA-treated mice ($P<0.05$). There was no difference in total fat cell number between treatment groups of either the injected or the non-injected epididymal pad (Figure 4C). At the end of the study there was no difference in NE content of either the injected or the non-injected epididymal fat pad (Figure 5: 6OHDA: NS, Leptin: NS, 6OHDA X Leptin: NS). In addition, there was no difference in IBAT UCP1 mRNA expression or NE content (data not shown).

Five days after pump placement, denervation and leptin infusion each affected serum leptin independently without any interaction (Figure 6: 6OHDA: $P<0.00006$, Leptin: $P<0.013$, 6OHDA X Leptin: NS). Leptin-infused, 6OHDA-treated mice had significantly higher serum leptin concentrations than all other groups. Denervation alone also increased serum leptin in PBS-infused mice ($P<0.03$). There were no differences in serum leptin, triglycerides, glycerol, or free fatty acids at the end of the experiment (Table 1).

Experiment 2

Neither leptin nor denervation of one epididymal fat pad had a significant effect on food intake of the rats during the experimental period when it was considered on either a daily basis or cumulatively over the 13 days of infusion (data not shown). There was a significant interaction between denervation and leptin on daily body weights of the rats (Figure 7: 6OHDA: NS, Leptin: $P<0.02$, Day: $P<0.0001$, Leptin x Day: $P<0.0001$). All of the rats lost weight immediately after

surgery and then regained weight during the experimental period. The denervated, leptin-infused rats, however weighed less than all the other rats from day 3 to 7 of infusion. There was no significant difference between any of the groups when total weight gained from immediately before surgery to the end of the experimental period was compared (data not shown).

At the end of the experimental period there were site-specific effects of denervation and/or leptin on fat depot weight. When the weights of individual epididymal fat pads were compared, leptin was the only factor that caused a significant difference in weight (Figure 8A: Injections: NS, 6OHDA: NS, Leptin: $P<0.01$, Interactions: NS). Post-hoc unpaired t-tests showed that leptin reduced the size of epididymal fat in all treatment conditions, but the only pad in which this reached statistical significance was the non-injected fat pad of vehicle-treated animals. Leptin infusion increased the NE content of the non-injected pad of vehicle-treated animals (Figure 8B: $P<0.02$ by t-test), but there was no indication of an increase in NE content of the non-injected pad of the leptin-infused, 6OHDA-treated animals. Leptin infusion did not increase NE content of injected pads from either vehicle- or 6OHDA-treated animals. Injecting epididymal fat with either vehicle or 6OHDA decreased the total number of cells present in the range of 20 to 170 um (Figure 9A: $P<0.001$), but there was no significant effect of 6OHDA or of leptin. 6OHDA treatment increased the number of cells in the 60-70 um size range in the vehicle-injected pad of PBS-infused, rats compared with all other groups (Figure 9B: 6OHDA: NS, Leptin: NS, Size: $P<0.0001$, 6OHDA x Leptin x Size: $P<0.004$). The difference in cell number was significant for cells in the range of 60 to 170 um for the non-injected pads (Figure 9C).

Denervation of one epididymal fat depot also had a significant effect on the leptin response of other fat depots within the same animal. Because the responses of retroperitoneal

and inguinal fat were not restricted to the ipsilateral side of the 6OHDA-injected epididymal pad, these depots are represented as the total weight of left plus right pad. Leptin reduced the size of inguinal (Figure 10A: 6OHDA:NS, Leptin: $P<0.01$, Interaction: NS) and retroperitoneal fat depots (Figure 10B: 6OHDA: $P<0.55$, Leptin: $P<0.004$, Interaction: NS). The reduction in fat pad size was significant for vehicle-injected rats, but not for 6OHDA-injected rats. In contrast, leptin had no significant effect on the size of the mesenteric fat depot, but denervation of one epididymal pad caused a significant increase in the amount of mesenteric fat present in PBS-infused rats (Figure 10C: 6OHDA: $P<0.006$, Leptin: $P<0.05$, Interaction: NS). There was no significant effect of either denervation or of leptin infusion on either the weight of IBAT or on the NE content of the IBAT pads (data not shown). Despite the fat depot specific effects of epididymal pad denervation, there was no significant effect of denervation on total dissected body fat content of the rats although leptin caused a significant decrease in carcass fat content of both vehicle- and 6OHDA-injected animals (Figure 10D: 6OHDA: NS, Leptin: $P<0.0006$, Interaction: NS). There were no differences in serum leptin concentrations at the end of the study (data not shown).

DISCUSSION

The activation of the SNS in some tissues by leptin (24) and the inhibition of adipose leptin mRNA expression by the SNS (20, 33) strongly suggest that these two factors interact to affect body fat. The objective of this study was to determine whether physiological doses of leptin would reduce body fat in the absence of an intact sympathetic neural signal to white fat. Epididymal fat was selected because our previous experiments have shown this pad to be responsive to peripheral infusion of leptin (5, 37). In mice, leptin decreased the mass of denervated epididymal depots to the same degree as depots with intact sympathetic nerves. In

rats, however, leptin action in animals with a denervated fat pad was decreased in all fat pads measured, even though only one fat pad was denervated. These results indicate that, in rats, the effect of leptin on white adipose tissue mass are mediated in part by the SNS and that there also is an integrated response between pads that is mediated through the effects of leptin on other humoral factors and/or via neural factors. In both species leptin reduced adipocyte size to the same degree in both the denervated and vehicle-injected pads. Therefore, even though there were few significant changes in the weights of epididymal pads in rats, leptin did cause some reduction in adipocyte lipid content suggesting a leptin-triggered increase in lipid mobilization. We did not find any substantial effect of denervation on cell size in the injected pads in this study, but there was a significant increase in the number of cells present in the larger size range of the non-injected pad of PBS-infused denervated rats and mice. This suggests that removal of SNS in one depot triggered lipid accumulation in a distant fat depot that could potentially be achieved if SNS in the distant pad was reduced thereby inhibiting lipolysis. Unlike previous reports (6, 11, 45), we did not find any significant increase in fat cell number, suggestive of proliferation, due to denervation in this study possibly because the adrenal glands were left intact, or because the rats and mice in this study were young and already were growing rapidly resulting in a ceiling effect on preadipocyte proliferation.

In the experiments described herein, we gave the rats and mice peripheral infusions of physiological doses of leptin. Previous studies have shown that these doses effectively reduce body fat even though they are lower than those used by other investigators (23). Evidence is available for both central leptin and for higher peripheral doses of leptin to increase sympathetic output (10, 24, 35, 38), but this has only been studied once in WAT and only in the retroperitoneal fat depot (8). Here we found that low doses of peripherally administered leptin

increased NE content in the non-injected pad of leptin-infused, vehicle-injected mice and rats. Preliminary data showed that 6OHDA decreased NE content in fat pads of mice for up to two weeks after the injections, but in both experiments reported here, there was no effect of 6OHDA injection on white fat NE content by the end of the study. There were, however, obvious effects on pad weight and body weight that strongly suggest that the sympathetic innervation was compromised in the early stages of the study that triggered the significant changes in body weight and fat at the end of the study. If the 6OHDA injection did not have any effect, then there should have been no differences between denervated and non-denervated animals on these measures. Therefore, these data suggests that 6OHDA denervation was effective early, but that the effects may have begun to decline or reverse towards the end of the study reminiscent of the recovery seen after systemic 6OHDA injections in brown adipose tissue 10 but not 4 days post-injection (40).

In vehicle-injected animals leptin infusion increased the NE content of the non-injected epididymal pad and denervation prevented this increase, suggesting that SNS denervation was still partially effective in 6OHDA-injected tissue further supporting the above contention that 6OHDA was effective, at least early on, in diminishing the SNS innervation of white fat. The increase in NE content was small, but this is consistent with the results of Collins et al. (8) who reported that peripheral injections of leptin caused a small but non-significant increase in the NE turnover in white adipose tissue of ob/ob mice. The failure of leptin to increase NE content in the vehicle injected fat depots suggests that the surgery caused some damage to the pads. Although we attempted to minimize physical trauma to the pads by using 30 gauge needles for injections, there may have been some ancillary damage that made them less responsive to leptin thus potentially accounting for the significant difference in size of the injected and non-injected

epididymal pad of PBS-infused, vehicle-injected mice. Even though leptin appeared to increase white fat NE content, there was no evidence for increased NE content in brown adipose tissue or UCP1 mRNA expression in any leptin-treated rats or mice. Others have shown that central and peripheral administration of leptin activates brown fat (10, 11); therefore, it is possible that the physiological doses of leptin used in this study were too low to activate thermogenesis.

The leptin infusions used in this study caused relatively small increases in serum leptin concentration, reinforcing our intent of creating physiological levels of the hormone. Denervation alone, however, did increase serum leptin and this was exaggerated in leptin-infused, 6OHDA-injected mice. This is consistent with the observation that activation of the SNS inhibits white adipose tissue leptin mRNA expression (9) and with observations that spinal cord injury patients lacking a functional sympathetic chain show increased circulating serum leptin concentrations (25). Although denervating one pad might not be expected to change circulating leptin levels, if denervation of one pad inhibits SNS activity in all white fat, then there could be a significant increase in circulating leptin. There is evidence of interconnections between white fat pads in that leptin injected directly into one epididymal pad triggered increased sympathetic activity in the contralateral pad (30) suggesting that the notion of neural interplay among fat pads may exist.

Although the objective of this study was to determine the role of the SNS on leptin activity, some unexpected responses in the non-injected pads of 6OHDA treated animals were observed. Denervation of one epididymal pad caused an enlargement of the mesenteric depot in rats and hindered leptin induced decreases in fat pad weight of inguinal and retroperitoneal fat pads. Because local injections of 6OHDA would not cause global denervation (32), it would appear that there is some communication between pads resulting in changes in SNS drive which

has been suggested previously with leptin injection (30), as noted above. The effect of denervation was not large during these 2-week studies, but did result in a significant 20% increase in total dissected fat and total weight gain of mice. This effect may have been greater if the duration of the study had been extended, but because the focus of this study was the effect of leptin on fat pad size the experiment only lasted for 13 days. In other studies, however, denervation of WAT results in similar decreases in NE content for 8 to 12 weeks resulting in substantial increases in WAT fat pad mass and fat cell number (45). Furthermore, although it was expected that denervation would decrease the responsiveness to leptin, the interaction of denervation plus leptin did not result in an increase but instead triggered a decrease in body weight in mice. In rats, animals that had one denervated fat pad and that were infused with leptin had body weights that were significantly lower than those of denervated rats infused with PBS, even though the response was not as pronounced as in mice. This argues against the SNS being essential for leptin action (41) on body weight and body fat, but also suggests that denervation of a single fat depot may influence the whole animal response to leptin.

In summary, the results from these experiments suggest that SNS outflow into white fat is not essential for leptin to reduce fat mass in mice, but that it may be involved in the leptin response of rats. Because we used low concentrations of peripherally administered leptin it is possible that opposite effects or more marked effects may be seen if leptin is administered centrally or if larger peripheral doses are used. Surprisingly, denervation of one fat depot caused significant changes in neurally intact distal fat pads such as mesenteric WAT in rats and in total dissected fat in mice. Therefore, it is likely that in both species there is some communication between the denervated pad and the non-denervated pads leading to changes in these depots. Whether this signal is a circulating factor or a neural signal from pad to pad is unknown. Further

studies are needed to determine exactly which metabolic pathways are modified by leptin to reduce body fat mass and whether these changes are induced indirectly or by leptin acting directly on white fat. In addition, studies should be conducted to identify the nature of the signal between pads that facilitates an orchestrated response to sympathetic denervation of a single fat depot. Finally, a more thorough test of the ability of physiological amounts of peripherally administered leptin to affect NETO across various WAT pads is needed.

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Table 1: Fat pad weights per body weight and serum measures in mice at the end of Experiment 1

Fat Depots (mg)/ Bodyweight (g)	***6OHDA/ Leptin***	***6OHDA/ PBS***	***Vehicle/ Leptin***	***Vehicle/ PBS***
Mesenteric	6.1 ± 0.6	6.2 ± 0.5	7.0 ± 0.7	6.2 ± 0.3
Retroperitoneal	2.7 ± 0.3	3.3 ± 0.3	2.6 ± 0.2	2.6 ± 0.2
Brown Fat	2.6 ± 0.4	2.1 ± 0.3	2.8 ± 0.3	2.5 ± 0.2
Serum				
Triglyceride (mg/dl)	95 ± 14	96 ± 27	104 ± 14	80 ± 12
Glycerol (mg/dl)	7.1 ± 1.0	7.2 ± 2.0	7.9 ± 1.1	6.1 ± 0.9
FFA (mEq/L)	1.3 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.4 ± 0.1
Leptin (ng/ml)	6.6 ± 1.1	8.5 ± 1.3	9.5 ± 2.2	7.9 ± 1.5

Values are means ± sem for 12 to 14 mice in Experiment 1. There were no differences between groups for any fat depot or serum value.

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Figure Legends

Figure 1: Preliminary data for epididymal pad NE. Data are means ± sem for groups of 5 to 6 mice. Asterisks indicate a significant difference in NE content of control and 6OHDA-injected pads.

Figure 2: Daily body weight (Panel A) and total weight gain (Panel B) of C57Bl/6 mice in Experiment 1. Data are means ± sem for groups of 12 to 14 mice. In panel A, * indicates a significant difference ($P<0.05$) between PBS-infused, 6OHDA-treated mice from both groups of leptin-infused mice. A ¥ represents a significant difference between the leptin-infused, 6OHDA treated mice from both groups of vehicle-treated mice. # Indicates a difference between leptin-infused 6OHDA-treated mice from both PBS-infused groups and the PBS-infused, 6OHDA-treated mice from all groups. + Indicates a difference between PBS-infused, 6OHDA-treated mice from all other groups. Panel B, shows that 6OHDA-treatment increased total weight gain of PBS-infused mice compared with all other groups. Values with different superscrscripts are significantly different ($P<0.05$).

Figure 3: Injected vs. non-injected epididymal pad weights (Panel A) and total weight of dissected fat pads (Panel B) of mice in Experiment 1. Data are means ± sem for groups of 12 to 14 mice. In panel A and B values with different superscripts are significantly different ($P<0.05$).

Figure 4: Fat cell size distribution of the injected (Panel A) vs. non-injected (Panel B) epididymal pad depots and total cell number of each pad (Panel C) in mice in Experiment 1. Data are means ± sem for 12 to 14 mice. In Panel B, * represents significant difference between PBS-infused, 6OHDA-treated mice, and leptin-infused, vehicle-injected mice, and # indicates a

difference between PBS-infused, 6OHDA treated mice from both leptin-infused groups ($P<0.05$).

Figure 5: Injected vs. Non-injected epididymal pad NE content for mice in Experiment 1. Data are means ± sem for 12 to 14 mice. There was no significant difference between treatment groups.

Figure 6: Serum leptin in mice in Experiment 1 5 days after treatment. Data are means ± sem for 12 to 14 mice. Values that do not share the same superscript are significantly different ($P<0.05$).

Figure 7: Daily body weights of rats in Experiment 2. Data are means ± sem for groups of 7 to 9 rats. # indicates a significant difference between vehicle-treated, leptin infused rats and all other groups. An asterisk indicates a significant difference between 6OHDA-treated, leptin infused rats and all other groups.

Figure 8: Weights of epididymal fat pads (Panel A) and of epididymal fat pad NE content (Panel B) for rats in Experiment 2. Data are means ± sem for groups of 7 to 9 rats. The asterisk indicates a significant difference in the weight (Panel A) and NE content (Panel B) of the non-injected pads from leptin-infused and PBS-infused, vehicle treated rats.

Figure 9: Fat cell number (Panel A) and cell size distribution for the injected (Panel B) and non-injected (Panel C) epididymal fat pads of rats in Experiment 2. There were no differences in the

total number of measurable cells in the different fat pads. Asterisks in both Panel A and Panel B indicate a significant difference in the number of cells present within a specific size range for the 6OHDA-treated, PBS-infused rats compared with other groups.

Figure 10: Weights of different fat depots and carcass fat content of rats in Experiment 2. Values are means for 7 to 9 rats and, within a specific axis, those that do not share a common superscript are significantly different at $P<0.05$. Leptin decreased the size of inguinal (Panel A) and retroperitoneal (Panel B) fat depots but denervation of one epididymal fat depot ameliorated this effect. Denervating an epididymal pad increased the size of mesenteric fat in PBS-infused rats but this effect was partially reversed by leptin (Panel C). Leptin-infusion significantly reduced carcass fat in both vehicle-treated and 6OHDA-treated rats (Panel D).

Figure 1

Preliminary Data: Epididymal Pad Norepinephrine

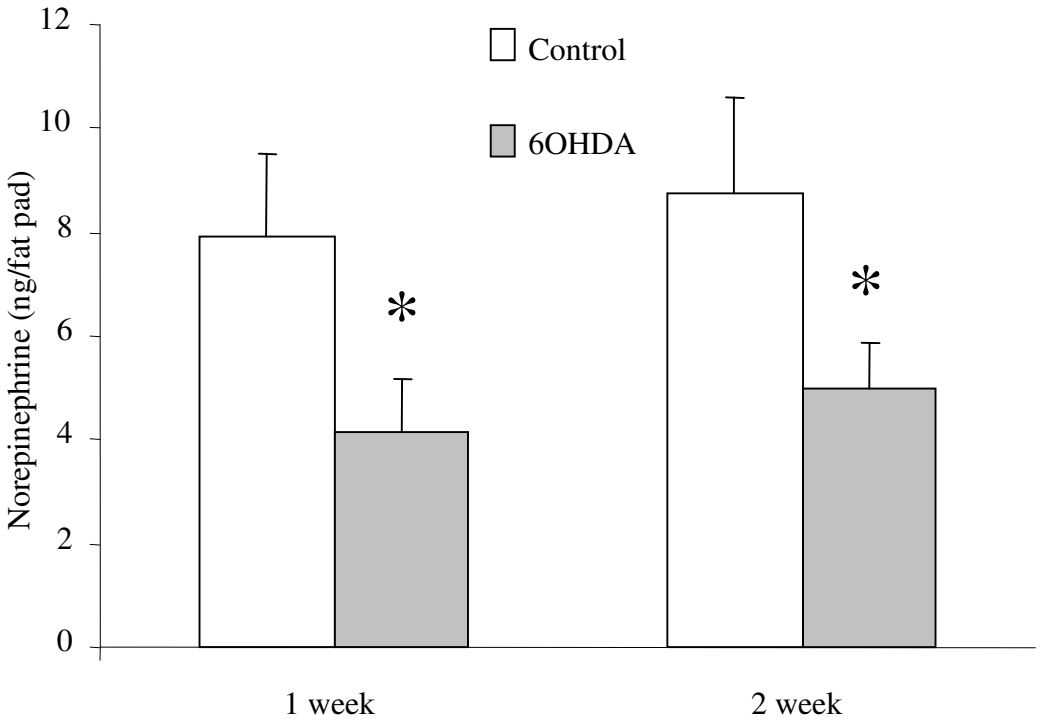
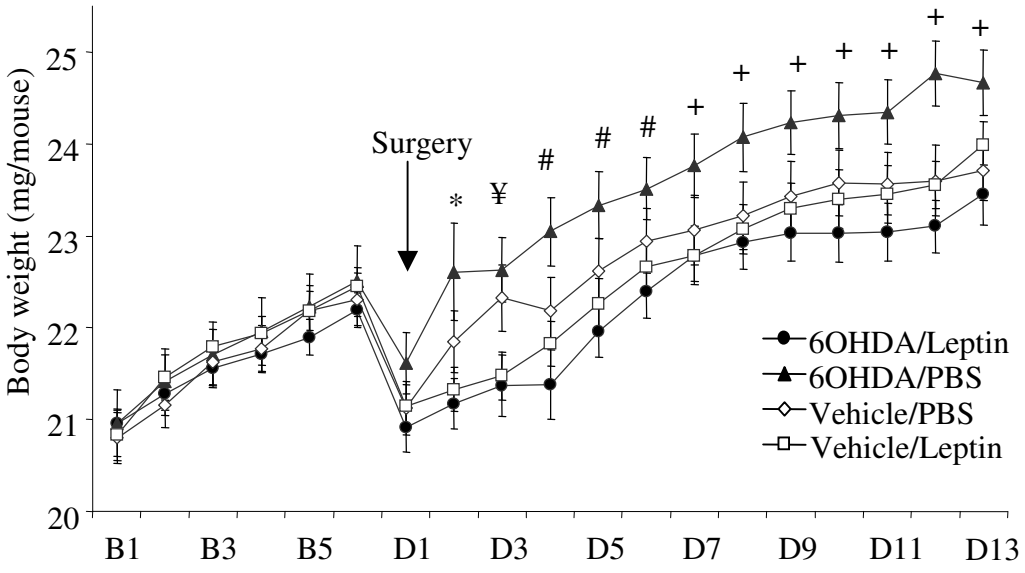
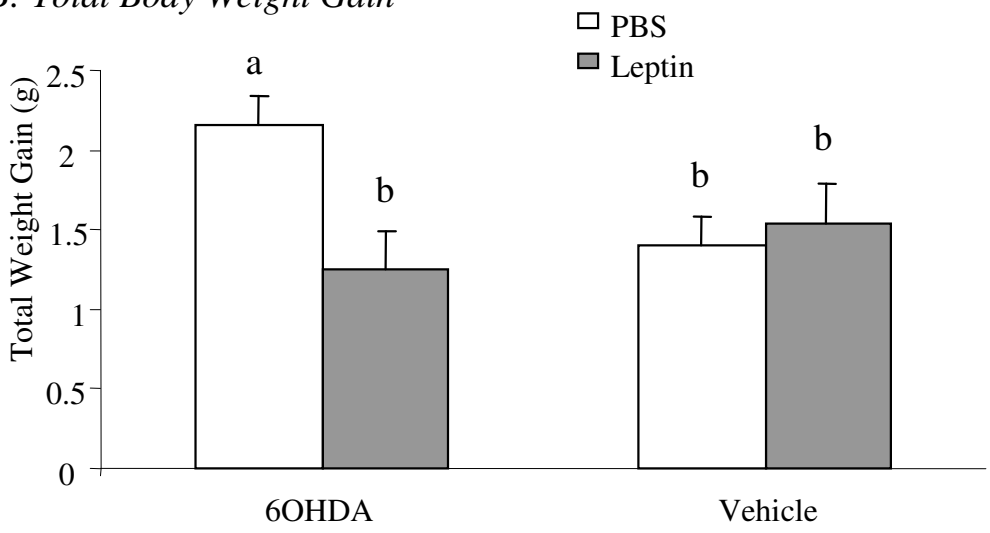


Figure 2

A: Daily Body Weight

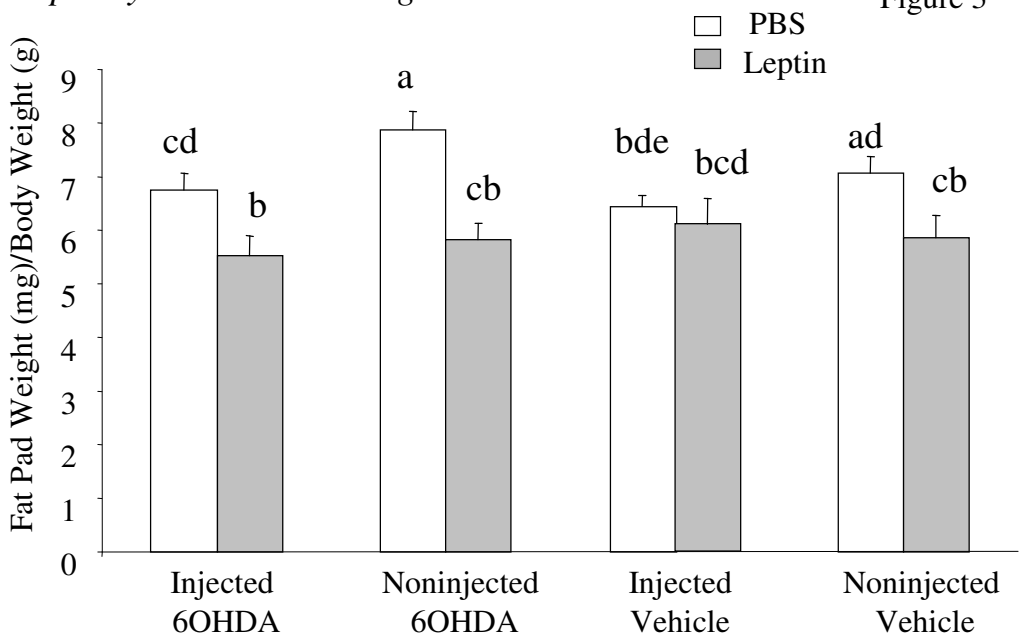


B: Total Body Weight Gain



A: Epididymal Fat Pad Weight

Figure 3



B: Total Dissected Fat

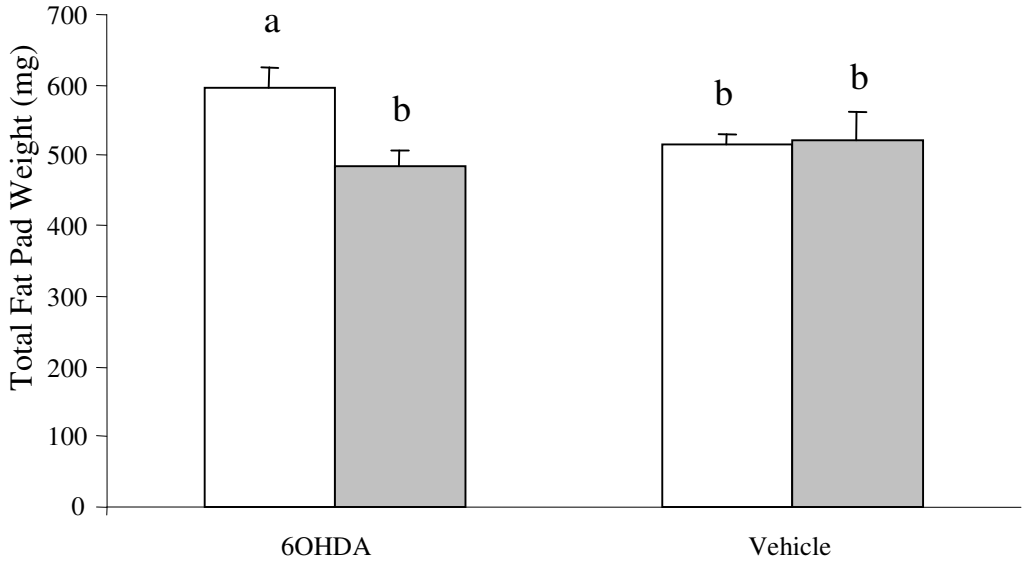
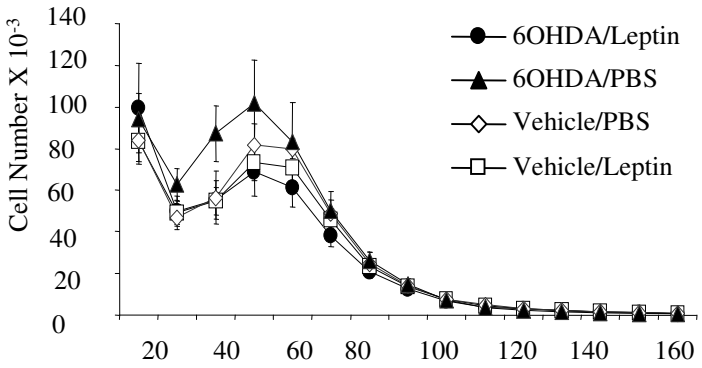
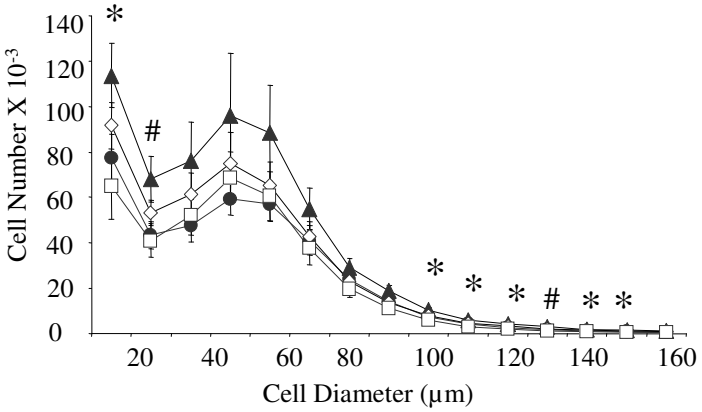


Figure 4

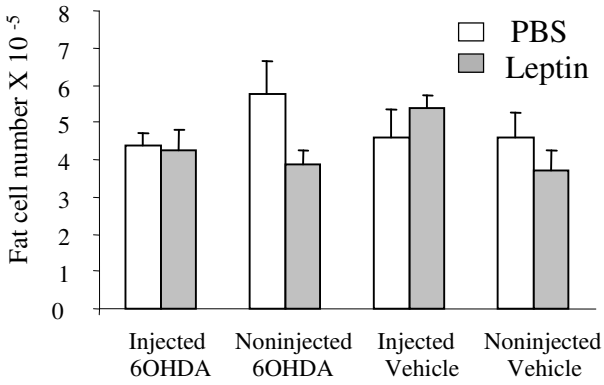
A: Injected Epididymal Pad



B: Non-Injected Epididymal Pad

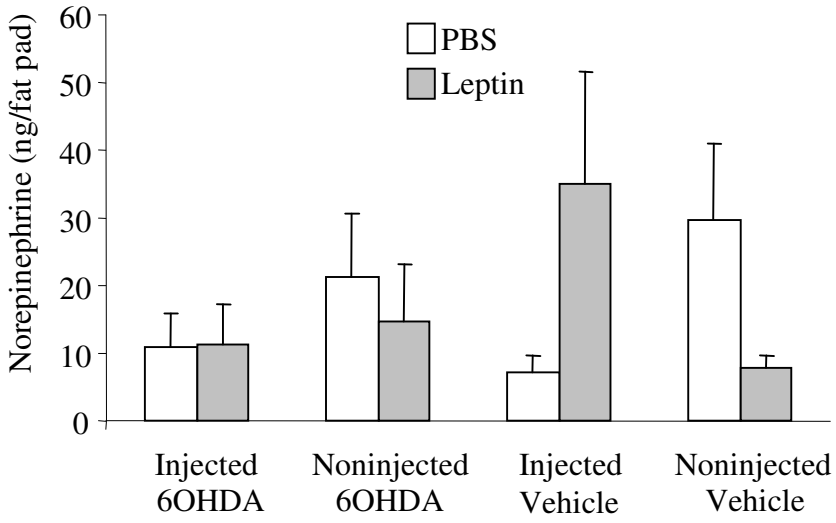


C: Cell Number



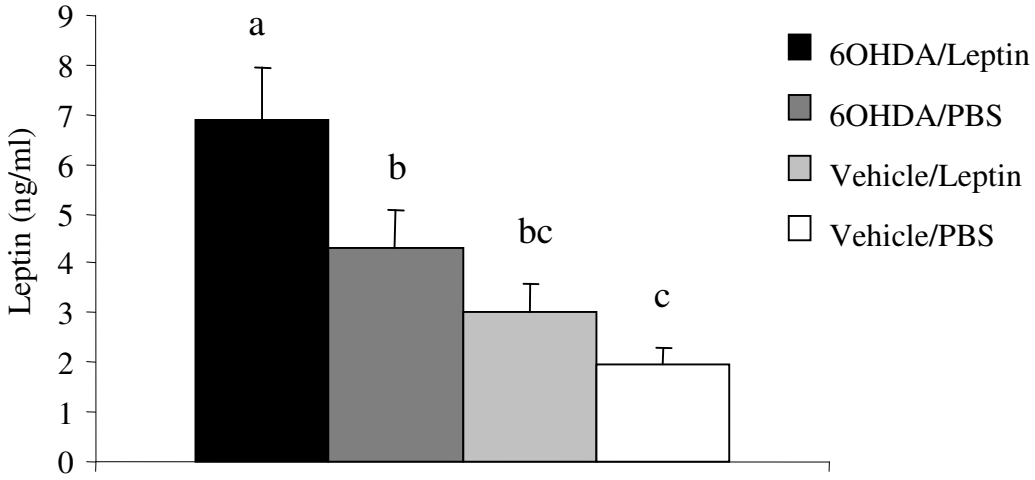
Epididymal Pad Norepinephrine

Figure 5



Serum Leptin: Day 5

Figure 6



Daily Body Weight

Figure 7

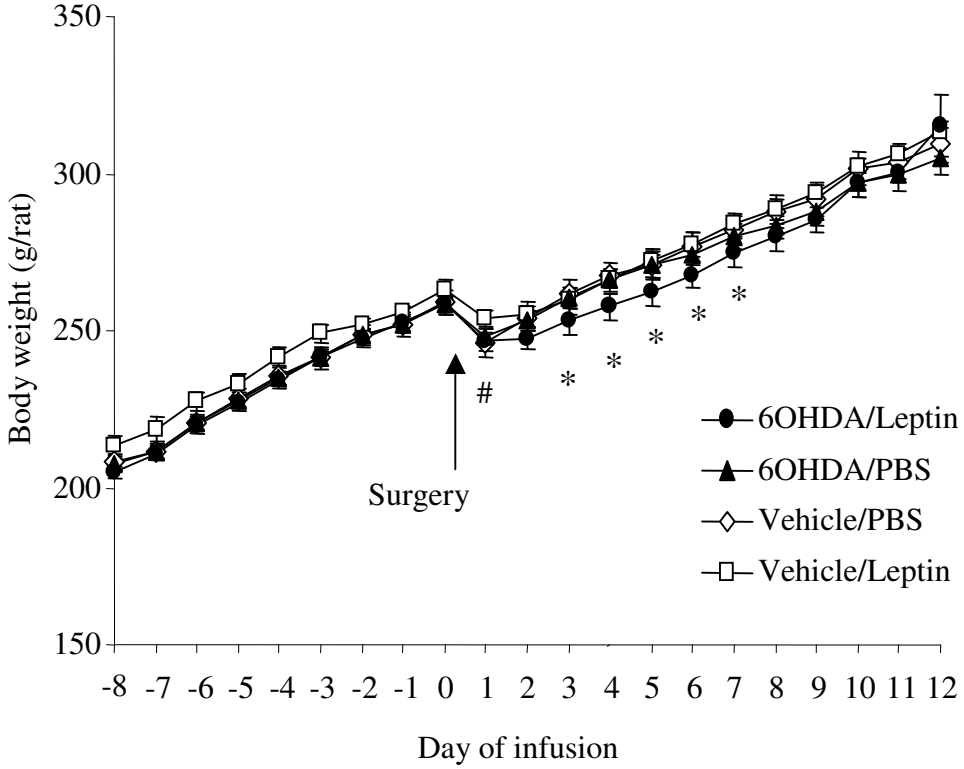


Figure 8

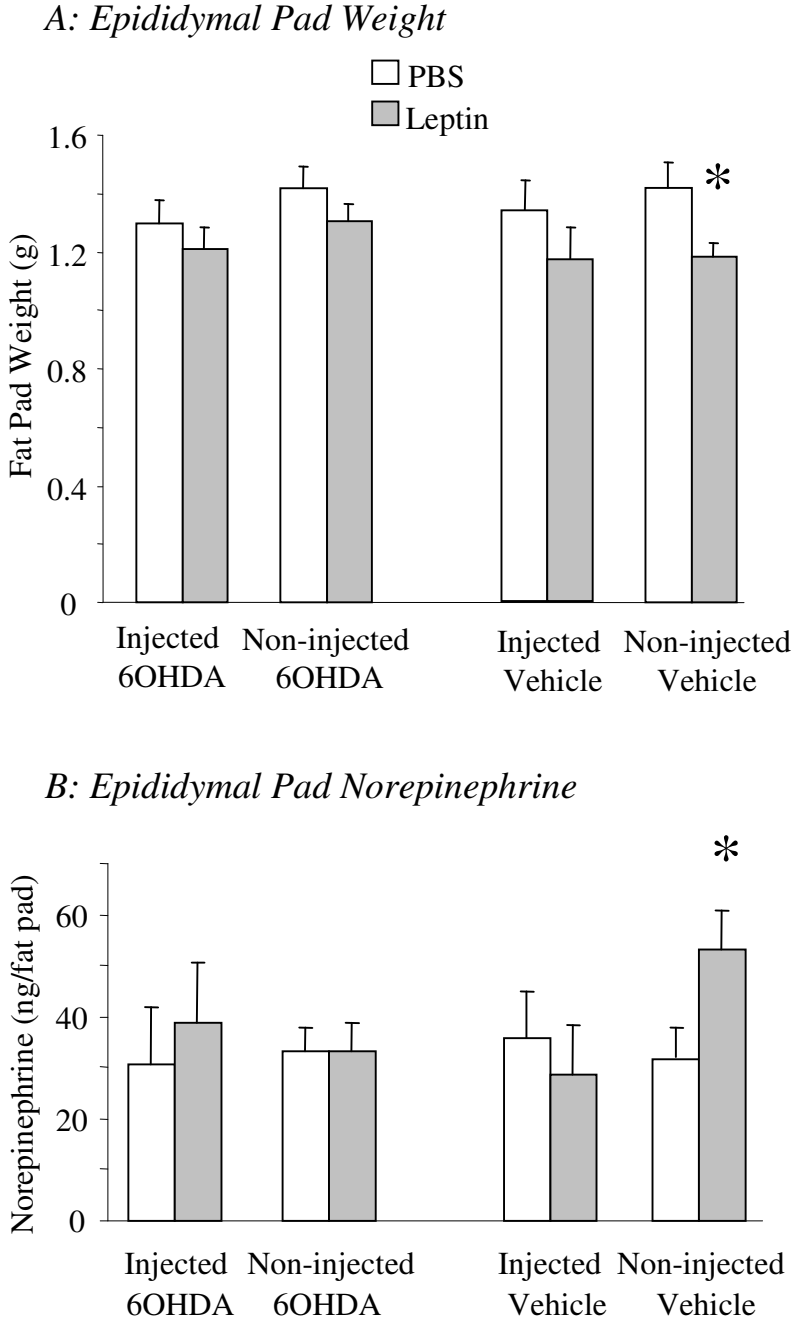

A: Epididymal Pad Weight
PBS
Leptin
Fat Pad Weight (g)
1.6
1.2
0.8
0.4
0
*
Injected 6OHDA
Non-injected 6OHDA
Injected Vehicle
Non-injected Vehicle
B: Epididymal Pad Norepinephrine
Norepinephrine (ng/fat pad)
60
40
20
0
*
Injected 6OHDA
Non-injected 6OHDA
Injected Vehicle
Non-injected Vehicle

Figure 9

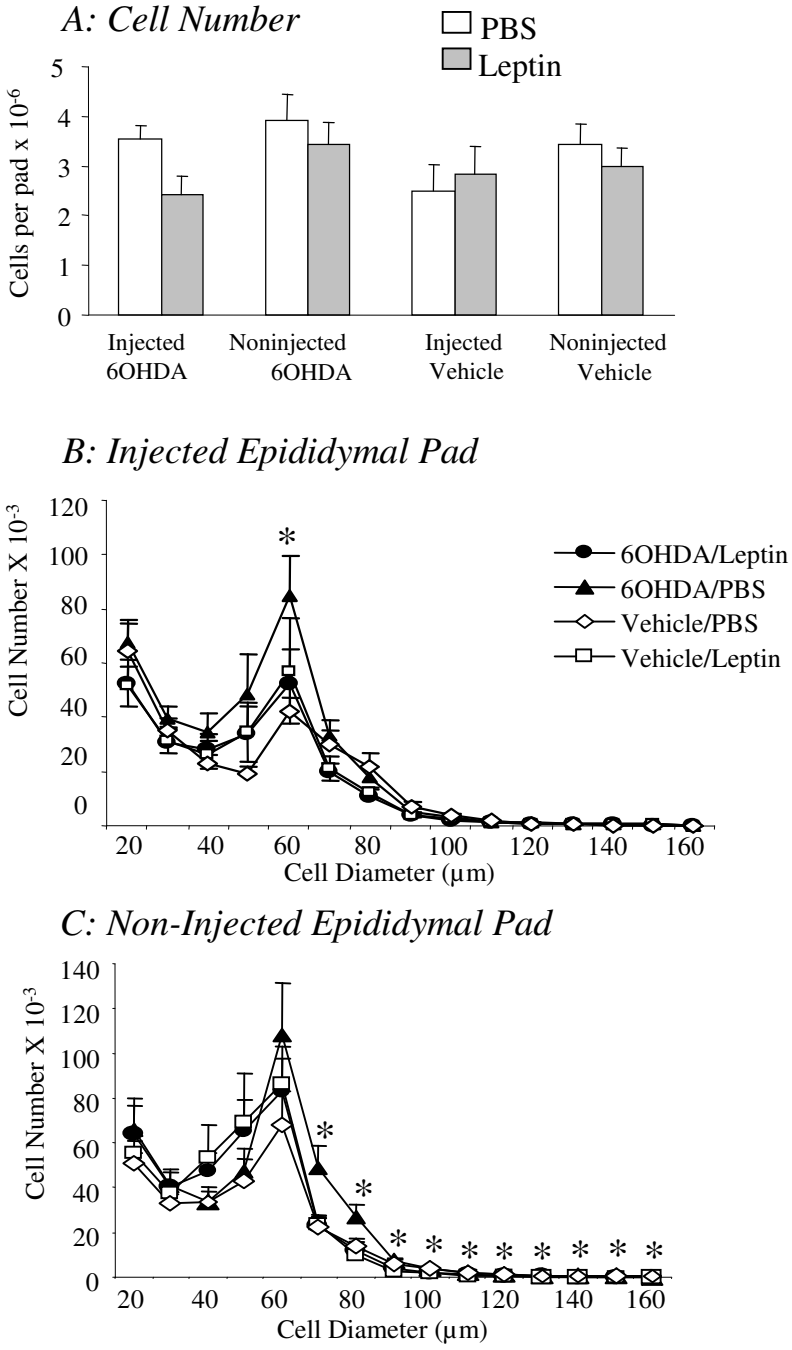
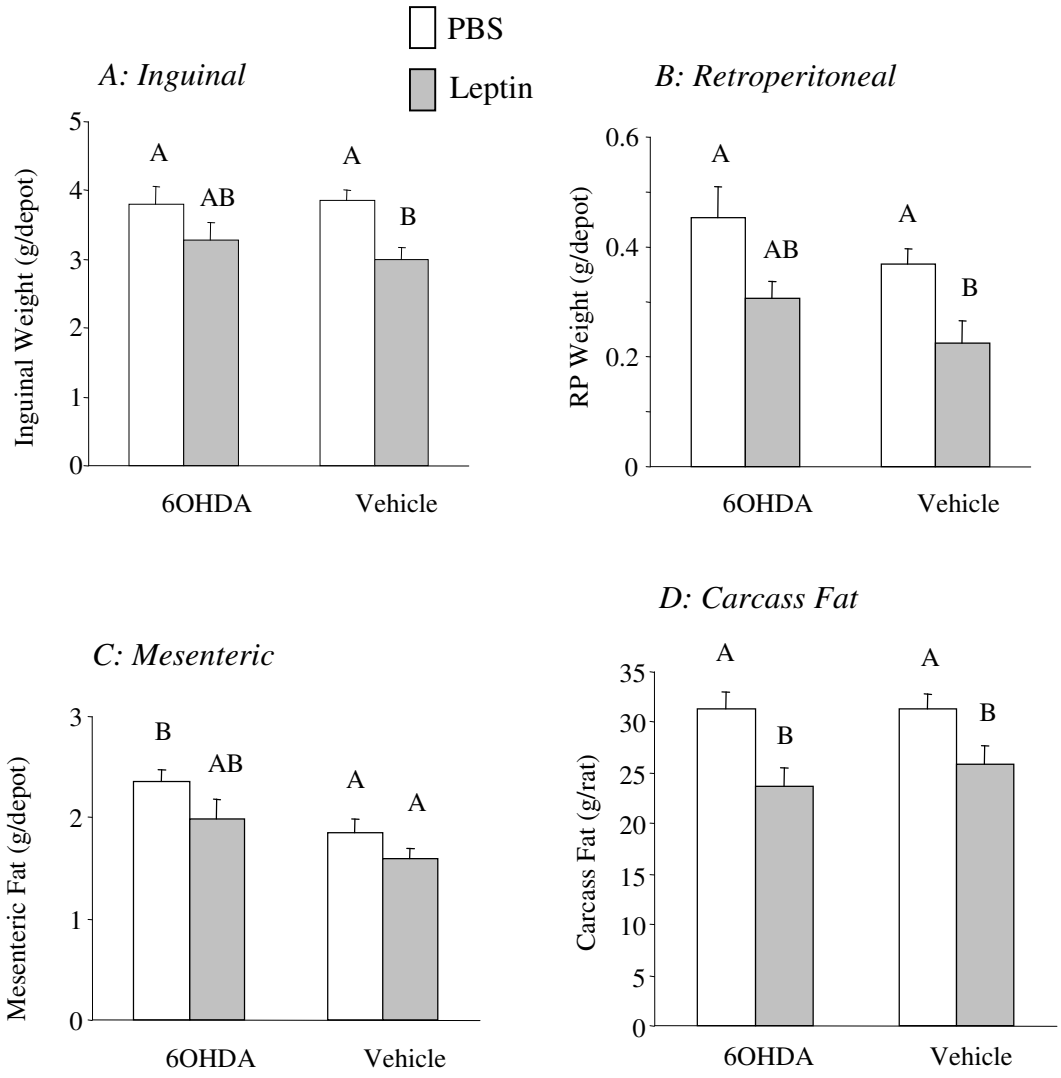

A: Cell Number
PBS
Leptin
Cells per pad x 10-6
0
1
2
3
4
5
Injected 6OHDA
Noninjected 6OHDA
Injected Vehicle
Noninjected Vehicle
B: Injected Epididymal Pad
Cell Number X 10-3
0
20
40
60
80
100
120
20
40
60
80
100
120
140
160
Cell Diameter (µm)
6OHDA/Leptin
6OHDA/PBS
Vehicle/PBS
Vehicle/Leptin
C: Non-Injected Epididymal Pad
Cell Number X 10-3
0
20
40
60
80
100
120
140
20
40
60
80
100
120
140
160
Cell Diameter (µm)

Figure 10


PBS
Leptin
A: Inguinal
Inguinal Weight (g/depot)
A
AB
A
B
6OHDA
Vehicle
B: Retroperitoneal
RP Weight (g/depot)
A
AB
A
B
6OHDA
Vehicle
C: Mesenteric
Mesenteric Fat (g/depot)
B
AB
A
A
6OHDA
Vehicle
D: Carcass Fat
Carcass Fat (g/rat)
A
B
A
B
6OHDA
Vehicle

CHAPTER 4

LEPTIN INHIBITS BODY WEIGHT GAIN IN YOUNG RATS WITH UNILATERAL SYMPATHETIC DENERVATION BUT DOES NOT INDUCE METABOLIC CHANGES IN EPIDIDYMAL WHITE ADIPOSE TISSUE[2]

[2] Rooks, C.R., Mitchell, T.D., Kelso, E.W., Harris, R.B.S. To be submitted to the *American Journal of Physiology: Regulatory, Integrative, and Comparative Physiology.*

ABSTRACT

Leptin is involved in the overall regulation of body weight by reducing fat stores, and food intake, and increasing energy expenditure. The role of the SNS in these responses is unclear. Decreases in white adipose tissue (WAT) by leptin are presumed to occur through increasing lipolysis and/ or inhibiting lipogenesis, but the data available is inconsistent. In this study, one epididymal pad in 7-week old male Sprague Dawley rats was denervated by locally injecting 6-hydroxydopamine (6OHDA). Sham animals were injected with vehicle. At the same time as denervation a miniosmotic pump was placed in the intraperitoneal cavity to infuse leptin (50μg/day) or PBS (sham), for 13 days. After 13 days rats were decapitated and both the injected and non-injected epididymal pad of each rat was removed, and cells were isolated for measurement of lipolysis and fatty acid esterification. Leptin inhibited body weight gain in both denervated and non-denervated rats. There was no effect of denervation or leptin on epididymal pad weight; however, denervation plus leptin did increase lipolysis in the presence of norepinephrine but not in basal conditions. The exact role that leptin plays in regulating metabolism within WAT is still unclear but it does appear that the SNS together with leptin interact to determine sensitivity to metabolic hormones.

INTRODUCTION

The lipostatic theory proposes a regulation of body weight by maintaining a relatively constant level of energy stores as a result of balancing energy intake and expenditure (27). The adipose-derived circulating factor, leptin, identified forty years later (51) has led to a more detailed look into how body weight is controlled. As energy stores increase leptin secretion from fat cells increases (51) signaling to the CNS a need to decrease adipose tissue, and energy consumption, and to increase energy expenditure (17, 33). Circulating leptin increases in proportion to the size of fat stores, with larger cells expressing more leptin mRNA than smaller cells (12, 22). Leptin dose dependently decreases body weight by specifically reducing adipose stores but not lean tissue in both normal and genetically obese mice (33). Similarly, hyperleptinemia causes an extreme loss in body fat of male Wistar rats, but retains lean tissue (8).

Decreases in white adipose tissue occur by increasing lipolysis (triglyceride breakdown) and/or inhibiting lipogenesis (triglyceride synthesis). Changes in lipolysis and lipogenesis have both been demonstrated with leptin administration (43, 47, 49). Single intraperitoneal (i.p.) injections of leptin increased adipocyte glycerol release in both ob/ob and lean mice but had no effect in their db/db counterparts lacking the long-form leptin receptor (18, 28). In transgenic mice over-expressing leptin, basal free fatty acids (FFA) and glycerol levels are not elevated in the circulation (26). In these mice a significant reduction in fat stores leaves these animals with small fat cells and presumably little triglyceride available for lipolysis. The presence of long and short-form leptin receptors on adipocytes allows for the possible direct stimulation of lipolysis by leptin both in vivo and in vitro (28). Leptin increases glycerol release from adipocytes isolated from female C57Bl/6 mice (19), and male lean Zucker rats (46). Although many

lipolysis studies are conducted with pharmacological concentrations of leptin (20, 29, 36, 38, 46), Siegrest-Kaiser et al. (43) show an increase in lipolysis in adipocytes incubated with physiological levels of leptin (1.8 ng/ml). The effect of leptin (16.0 ng/ml) on increasing lipolysis does not seem to be changed by age or anatomical location of epididymal, perirenal, and subcutaneous fat taken from 6 and 20 week old male Sprague Dawley rats (38). There is, however, conflicting evidence in which a 30 min. exposure to leptin (200ng/ml) did not increase lipolysis in isolated adipocytes from male Sprague Dawley rats (29). In addition, in human infant and adult adipose tissue leptin does not increase lipolysis whereas isoprenaline caused a significant increase from basal lipolysis (16).

An inhibition in white adipose tissue (WAT) lipogenesis was seen in gold thioglucose mice, a chemically induced obese model containing less leptin receptors in the hypothalamus then its lean equivalent (6). The inhibition in lipogenesis was seen with a single peripheral leptin (25 μg/mouse) injection reinforcing the autocrine or paracrine role of peripheral leptin (19). Lipoprotein lipase (LPL), the enzyme that regulates the rate of fatty acid uptake in WAT, is stimulated by insulin and glucocorticoids. ob/ob, compared with lean mice, have increased levels of LPL activity in WAT and interscapular brown adipose tissue (IBAT) that is inhibited by leptin infusion (100μg/kg body weight) (35). In isolated adipocytes from Sprague Dawley rats, however, neither total lipid synthesis (29) nor LPL (37) are reduced with acute leptin exposure (200 ng/ml) (29). In the same study insulin (10^{-8} mol/L) was also ineffective at increasing total lipids and glucose transport (29).

Leptin activates the SNS as indicated by an increase in uncoupling protein-1 (UCP-1) expression (11) and norepinephrine (NE) turnover (9) in IBAT. This mechanism is dependent on the SNS (41) and without NE, UCP-1 and leptin mRNA expression are not effected by

exogenous leptin (10). The SNS, which is found to innervate WAT (2) increases NE release in plasma from the adrenal medulla when activated (44). Removal of the SNS in WAT has been effectively shown through both surgical and chemical denervation (14, 50). In the absence of the SNS WAT mass increases in some species (13, 14, 50) and NE levels decrease. Lipolysis can be stimulated via the SNS by activating the release of catecholamines, that bind to β-adrenergic receptors, and inhibit LPL activity (2). Renal (45) and adrenal (34) sympathetic nerve activity (SNA) is increased with leptin administration, but renal SNA is inhibited in obese Zucker rats with a mutated gene for the leptin receptor (34). These results imply that leptin could potentially activate SNA in WAT. There is evidence that leptin can reduce body fat without the SNS. Central leptin is able to decrease body weight with global sympathectomy (15), and peripheral hyperleptinemia decreases transplanted WAT (48). This study will aim to determine whether leptin is able to decrease leptin action in unilaterally denervated WAT. Secondly, the effect of peripheral leptin on lipid metabolism will be determined and whether the SNS innervation must be present for these effects to occur.

METHODS

Thirty-five, five week old male Sprague Dawley rats were purchased from Harlan Sprague Dawley (Indianapolis, IN). Rats were acclimated to housing (73°F with lights on 12h/day from 7:00 am) for one week with free access to rat chow (Diet 5012; Lab Diet, Purina Mills, MO) and water. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Georgia and were in accordance with the guidance of the American Physiological Society (1). Rats were single housed in hanging wire cages to allow measurement of food intake. After one week of measuring baseline daily food intake and body weight animals were matched into four groups of 12 animals based on an experimental design of

denervation X leptin (2 X 2). Rats were anesthetized with ketamine (90 mg/kg) and a combination of xylazine (10 mg/kg) and glycopyrrolate (0.2 kg/ml). They were fitted with intraperitoneal (i.p) Alzet miniosmotic pumps (Model 2002; Durect Cupertino, CA). The pumps delivered 50 μg leptin/day (recombinant rat leptin: R&D Systems, St. Charles, MO) or 0.01 M phosphate buffered saline (PBS). At the same time one epididymal pad was injected 20 times with either 6-hydroxydopamine (6OHDA) (9mg/ml) or vehicle (0.01 M PBS containing 1% ascorbic acid). The side of surgery was alternated between animals. Daily food intake and body weight were measured for the next 13 days. Five days after surgery rats were food deprived for 2 hours and tail blood (~50 μl) was collected to measure serum leptin concentration (Rat Leptin RIA: Linco Research Inc., St. Charles, MO). On day 13 rats were food deprived for 2 hours followed by decapitation to collect trunk blood. Serum from trunk blood was collected for measurement of free fatty acids (NEFA C kit: WAKO Chemical Co., Richmond, VA) and triglycerides (L-Type triglyceride kit: WAKO Chemical Co.). IBAT, mesenteric, and both epididymal and retroperitoneal pads were weighed. IBAT and samples of epididymal WAT were snap frozen for determination of UCP-1 mRNA expression in IBAT and NE content of both tissue samples by reverse phase HPLC. The remaining fat from the left and right epididymal fat pads was digested separately and cells isolated in order to determine lipolysis and fatty acid esterification. Cells from both right and left epididymal pads were isolated by collagenase digestion. First, fat was minced with scissors and incubated with shaking in Krebs buffer containing 5.0 mM sodium bicarbonate, 5.0 mM glucose, 2.0% BSA (Bovine Serum Albumin), 10 mM HEPES, 0.5 mM palmitate, containing 2mg/ml collagenase pH 7.45 at 37°C for 30 minutes. The cells were then washed three times in collagenase-free buffer and resuspended in 10 ml Krebs buffer.

Measurement of Lipolysis

Glycerol release was used as an indirect measure of lipolysis. Aliquots of isolated cells were incubated in a final 1ml volume of Krebs buffer containing 5.0 mM $NaHCO_3$, 5.0 mM glucose, 2.0% BSA, 10mM HEPES, 0.5 mM palmitate, 0.1mM ascorbic acid and 0.8 U/ml adenosine deaminase. Triplicate samples were treated with acid at the start of the incubation to account for glycerol already present in the media. Glycerol release was determined in basal conditions and in the presence of 10 μM norepinephrine, established by Bowen et al (5) to be the most effective concentration to induce a maximal rate of lipolysis in similar conditions. Incubation flasks containing each sample were gassed with 95% O_2/5% CO_2 and incubations took place at 37°C, 90 oscillations/min. for 1 hour. The reaction was stopped by injecting 0.5mL 1.0N H_2SO_4 into the media. Samples were stored at -80° C until glycerol determination.

Glycerol Determination

Incubation media was centrifuged at 4° C and supernatant was removed. A 200 μl volume of sample was added to 200 μl of chilled 0.65 N perchloric acid. Samples were vortexed and neutralized with an adequate volume of Imidazole-KOH-KCL solution (0.52M Imidazole and 0.52 M KCl in 2.6 M KOH) to bring pH to 7.00. The neutralized solution was vortexed and centrifuged at 4° C. Supernatant was removed and triplicate samples were used in a colorimetric enzymatic assay to determine free glycerol content of the media using free glycerol reagent (Sigma: F6428). Triplicates of a glycerol standard (100 μM) and blank containing Krebs was measured with each plate assayed. Final values were determined by using absorbance readings from the blank (1X krebs) and standard (100 μM) to calculate free glycerol present per aliquot of cells. Data is expressed as free glycerol released nmol/ hour/10^6 cells.

Measurement of Fatty Acid esterification

Fatty acid esterification was measured by incubating 0.5 mL aliquots of isolated cells in a 2 ml final volume of Krebs bicarbonate buffer containing 5.0 mM $NaHCO_3$, 5.0 mM glucose, 2.0% BSA, 10mM HEPES, 0.5 mM palmitate, and 1.33 μCi/ml U-^{14}C-palmitate for 2 hours at 37°C. An additional aliquot of cells was fixed in osmium tetroxide for determination of cell number by coulter counter as described below. Triplicate aliquots of cells were incubated in basal conditions or with 100 μU/ml insulin (Illetin R Eli Lilly Corp., Indianapolis, IN). Reactions were stopped by addition of 0.5 ml 1.0 N H_2SO_4. Cell suspensions were transferred to tubes and total lipids extracted as described previously (24). U-^{14}C palmitate incorporation was determined by scintillation counting and fatty acid esterification expressed as nmol incorporated palmitate/10^6 cells/2hr.

Determination of Adipocyte Size and Number

An aliquot of cells from each digested pad was fixed in osmium tetroxide for determination of fat cell number and cell size distribution. At the initiation of incubation a 0.5 ml aliquot of isolated cells were placed in 1.0 ml collidine-HCl buffered osmium tetroxide (5 g osmium tetroxide in 100 ml of 50 mM collidine-HCl buffer). Cells were rinsed with 0.159 M saline and adipocyte size and number were measured using a Coulter Counter (model S/ST II, Beckman/Coulter Electronics, Hialeah, FL) and Multisizer (model Multisizer II E)as described previously (23).

Measurement of Catecholamines

NE content of WAT and IBAT was measured by reverse – phase HPLC. Tissue was sonicated on ice (3X at 30 sec) in 800 μl of 0.2 M perchloric Acid containing 3μg/ml ascorbic acid and 25 ng/ml dihydroxybenzylamine hydromide (internal standard). The sonicated solution

was centrifuged for 15 min. at 4°C at 9000rpm. The infranate was filtered through a 0.2µm, Nylon, 22mm syringe filter (Fisherbrand, Pittsburgh, PA). A 50µl sample of filtrate was injected into the HPLC system for measurement of catcholamines. Catecholamines were assayed using an ESA (Bedford, MA) HPLC system consisting of a Model 582 Solvent Delivery Module, a Model 542 auto sampler maintained at 6°C, and a Model 5600A CoulArray detector at 350mV. The column was a Phenomenex (150 x 4.6mm) SYNERGI, 4u, Max RP-80A. The mobile phase consisted of 0.1 M sodium phosphate monobasic, 0.1mM disodium EDTA, 0.3mM 1-octanesulfonic acid and 4% acetonitrile in ultra pure water. The pH of the mobile phase was adjusted to 3.1 with phosphoric acid. Chromatograms were analyzed with CoulArray for Windows, v.1.04, and NE content calculated from a standard curve. Data was expressed as ng NE/fat depot.

Northern Blot Analysis of UCP-1 mRNA

Tissue total RNA was isolated, from brown fat using Trizol reagent (Invitrogen Life Technologies; NY) according to manufacturer directions. Brown fat UCP1 mRNA expression was measured by Northern Blot according to the methods of Haltiner (21). UCP-1 probes were biotin labeled (Brightstar Psoralen - Biotin labeling Kit Ambion, TX) and membranes hybridized at 65° C for 3 hours. Hybridized probe on the nylon membranes (Hybond N^+; Amersham, Piscataway, NJ) was detected with chemical luminescence by following the manufacturer's instructions (Ambion Detection Kit; Ambion, TX) and imaged on x-ray film. Following detection of UCP1 mRNA the membranes were stripped and rehybridized with a probe for 28S rRNA (40). The density of hybridized bands was measured (Un-Scan-It gel, Urem, UT) and expressed as a ratio of UCP1 mRNA to 28S rRNA.

Statistical Analysis

Body weight, food intake, and adipocyte size, free glycerol, and fatty acid esterification were compared between groups using a Repeated Measure ANOVA (Statistica Software 99' Edition, Stat Soft, Tulsa OK). Body weights and food intake values taken the morning before surgery were used as covariates to determine changes in body weight and food intake that occurred after initial treatment to determine differences between groups. Pad size, serum leptin, free fatty acids, triglycerides, and glycerol were compared using a two-way ANOVA. A Duncan's multiple range test was used to determine differences between specific groups. The size of the denervated versus intact epididymal fat pads and tissue NE content within an animal were compared by paired t-tests. In addition mesenteric, retroperitoneal and inguinal fat pads were compared using a two-way ANOVA in rats. Differences were considered significant at $P < 0.05$.

RESULTS

In this study male Sprague Dawley rats were unilaterally denervated and infused with leptin. Leptin had an overall effect of decreasing daily body weight (Figure 1: 6OHDA: NS, Leptin: $P < 0.0065$, Leptin X Day: $P < 0.033$). The 6OHDA/PBS rats were significantly heavier than both groups of leptin-infused rats on day 5 of treatment. On days 6 through 12 leptin infused animals were significantly smaller than both PBS infused groups. There was no significant difference in total weight gain between groups over the 13 day experimental period (data not shown). There were no associations between leptin-induced body weight reduction and changes in food intake or in energy expenditure (data not shown) as measured by UCP-1 mRNA expression in IBAT.

Contrasting the previous study conducted in rats (39), neither 6OHDA nor leptin had an effect on either the injected or non-injected epididymal pad weight (Figure 2A). To confirm the effectiveness of denervation within an animal, a two-tailed t-test was conducted on NE content of epididymal pads within an animal. Leptin increased NE content of the non-injected pad of 6OHDA/Leptin rats. Denervation inhibited this increase (Figure: 2B: $P < 0.042$). Leptin did not, however, increase NE content of Vehicle/Leptin rats over their Vehicle/PBS counterparts. Neither leptin nor 6OHDA affected IBAT NE consistent with previous data (data not shown). The lack of difference of epididymal pad weights was reflected in cell size and number distribution in that there were no treatment differences between groups (Figure 3A and 3B). In contrast, both denervation and NE significantly influenced glycerol release from isolated epididymal fat cells (Figure 4A: 6OHDA: $P < 0.029$, Leptin: NS, NE: $P < 0.0001$, Leptin X 6OHDA: NS). There were no effects of either denervation or leptin infusion on basal glycerol release. In the presence of NE however, there was a denervation effect (Figure 4B: 6OHDA: $P < 0.018$, Leptin: NS, 6OHDA X Leptin: NS). Specifically, the non-injected pad of 6OHDA/PBS and 6OHDA/Leptin rats had higher rates of NE-stimulated glycerol release then the Vehicle/Leptin rats. In addition, adipocytes from the injected epididymal pad of 6OHDA/Leptin rats had higher rates of NE-stimulated glycerol release than Vehicle/Leptin rats. The largest difference within animals was the non-injected pad of 6OHDA/PBS rats which had higher glycerol release then the injected pad of that group. There was no difference in glycerol release between 6OHDA/PBS or 6OHDA/Leptin groups.

There was no overall effect of either denervation or leptin alone on ^{14}C fatty acid esterification. There was, however, an interaction between insulin and denervation (Figure 4B: 6OHDA: NS, Leptin: NS, Insulin: $P < 0.060$, 6OHDA X Insulin: $P < 0.03$). In basal conditions,

the non-injected pad of 6OHDA/PBS rats had lower rates of esterification compared to the injected pad of the Vehicle/PBS and the non-injected Vehicle/Leptin rats. No significant differences were seen between groups incubated in the presence of insulin, but the non-injected pad of 6OHDA/PBS rats showed a trend of being the most responsive to insulin.

Although no difference in epididymal pad weights were seen, there was an effect of leptin on other fat depots. Leptin significantly reduced inguinal WAT (Table 1: $P < 0.01$) and retroperitoneal WAT (Table 1: $P < 0.03$) but not mesenteric (Table 1) consistent with results from the previous study with rats (39). There were no differences found between left or right inguinal or retroperitoneal fat depots when weights for individual pads were compared. The Vehicle/Leptin animals had significantly smaller inguinal pads than either of the PBS-infused groups. Overall differences were not seen in the retroperitoneal pad. There were no differences between total dissected fat pad weights or percent carcass fat (data not shown). Leptin had an overall effect on grams of protein per rat body weight (Table 1: 6OHDA: NS, Leptin: $P < 0.03$). The 6OHDA/Leptin rats had significantly lower protein levels then their 6OHDA/PBS counterparts.

On day 5 of treatment leptin-infused rats had significantly higher serum leptin levels than either of the PBS-infused groups. An insignificant trend ($P< 0.19$) showed that the serum leptin levels of the 6OHDA/Leptin group were lower than the Vehicle/Leptin group (Table 1: 6OHDA: NS, Leptin: $P < 0.014$, 6OHDA X Leptin: NS). This change in serum leptin was not accompanied with changes in serum triglycerides, glycerol, or free fatty acids (Table 1).

DISCUSSION

In this study unilateral sympathetic denervation did not inhibit leptin from decreasing body weight consistent with the previous study done in rats (39). In contrast, local denervation

did not result in any additional increase in body weight reported previously (14). Therefore any changes occurring due to denervation are small enough not to effect overall body weight, but result in changes within the injected pad. The epididymal pad has been found to be responsive to both leptin (4, 42) and unilateral denervation (14); however, no changes in epididymal pad weight, cell number, or cell size by leptin or 6OHDA were demonstrated in this experiment. The rats were young and all animals were growing rapidly. Leptin only inhibited weight gain by reducing retroperitoneal and inguinal fat and carcass protein and not epididymal fat. Food intakes and energy expenditure were not different between groups possibly because small changes may not have been distinguishable by the measurements taken. In addition measurement of IBAT UCP-1 mRNA suggests that thermogenesis was not dramatically increased. In this study and the previous study (39) epididymal pad weight did not increase with 6OHDA injection. Changes may have been seen in both studies if the experiment was extended for a larger period of time, however; the point of this study was to determine metabolic changes occurring due to leptin with and without the SNS. In the previous study we speculated injecting 6OHDA into white adipose damaged the fat depot response. This study argues against that since identical injections were conducted and there were no differences between the weights of injected and non-injected pads within an animal or in cell size distribution between the pads.

The effects of physiological doses of leptin given peripherally on WAT lipolysis and lipogenesis are still unclear after this study. No effect of leptin was seen on lipolysis in basal or in NE stimulated conditions for animals with intact sympathetic signals. This failure was not due to difficulties with the assay, since NE did increase glycerol release in the isolated adipocytes Although there is in vivo evidence that leptin alone increases lipolysis (18); this was seen in lean mice given a single injection of leptin after an overnight fast and sacrificed within an hour after

injection. In addition, it was only with the most concentrated dose (10.0 mg/kg) that any lipolytic effect of leptin was seen in lean mice. In this study, after the initial administration of leptin, animals had free access to food and were not decapitated for another two weeks. This gave the rats time and energy to grow and reverse any acute leptin-induced changes on lipolysis. Chemical denervation did not affect lipolysis measured in basal conditions which is similar to previous studies completedwith surgical denervation (30). It is presumed that the sympathetic nervous system activates lipid mobilization (2). Consequently, sympathetic denervation should inhibit lipid mobilization. There was however, no decrease in glycerol release in adipocytes from 6OHDA-treated fat pads. These results are consistent with the report that surgical denervation, which does cause an increase in fat mass implying a reduced level of lipid mobilization, does not decrease fatty acid release, glycerol production or hormone sensitive lipase (HSL) activity either (3,13). This comes as a surprise since surgical denervation of retroperitoneal WAT does not inhibit adrenergic stimulation in vitro (7, 13). In this study, however, chemical denervation inhibited glycerol release in the presence of NE compared to the control pad. Cousin et al. (13) found that denervation does not effect adrenergic receptor expression, therefore any rate of lipolysis should be consistent in both pads. Since this apparently does not hold true with chemical denervation the control pad must be able to undergo lipolysis through a mechanism not active in the denervated pad. It is unknown what this pathway may be, but in future studies it will important to measure changes in HSL activity to see if chemical denervation effects the cAMP cascade that activates HSL and increases lipolysis or if adrenerigic receptor number is increased (32). Communication between pads also offers a reasonable explanation for the change lipolysis in the control pad of PBS-infused, 6OHDA treated rats when no change was reported within the injected pad. Denervation of one pad may be signaling either

neurally or humorally to the non-injected pad to respond more to NE as a compensatory action. If the control pad was acting independently from the denervated pad, then its response to NE should have resembled that of PBS-infused pads with intact sympathetic signaling.

It is likely that the same mechanism of NE-stimulated glycerol release in adipocytes of denervated PBS-infused rats is similar to that of both bilateral epididymal pads of denervated leptin-infused rats because the response to NE of cells from 6OHDA/Leptin rats was substantially increased. Others have also reported that adipocyte lipolysis stimulated by isoprenaline is more sensitive to the inhibitory action of insulin when fat pads are denervated with 6OHDA (25). This increased sensitivity may not be selective to insulin, but may include leptin. This may explain why leptin was able to increase lipolysis in denervated rats, but not in intact rats. In addition, the presence of leptin in vivo may cause a metabolic change in 6OHDA-injected pads that facilitate an increased response to NE, which is not present in 6OHDA/PBS rats. Since leptin plus denervation did not raise lipolysis any further than denervation alone, leptin is not having an additive effect on the response to denervation, but more likely is acting through the same pathway. By administering leptin the inhibitory effect of direct injection of 6OHDA on lipolysis is curtailed allowing denervated leptin-infused cells to respond to NE.

In animals with intact sympathetic signaling leptin did not directly effect lipogenesis in basal conditions. This is consistent with previous data in which lipogenesis is unaffected with leptin exposure (37). It is only after prolonged (16 hrs.) exposure to leptin that any change in lipogenesis is observed (30). Although in this study insulin, that acts to increase lipogenesis did not raise fatty acid incorporation in non-denervated rats, and leptin did not inhibit insulin action on adipocytes (29, 37). The data presented does show that sympathetic innervation is not necessary for control of lipogenesis since the injected pad of denervated rats did not differ from

injected pads of non-denervated rats. The main lipogenic effect demonstrated within this study is the reduced level of ^{14}C labeled palmitate incorporation in the non-injected pad of denervated rats compared to adipocytes from epididymal pads of non-denervated rats. The non-injected pad of the denervated rats was also the most responsive to NE-stimulated lipolysis. Surprisingly, the non-injected pad in denervated rats was the most responsive to insulin. There is limited data to support what may have been occurring within the non-injected pad of denervated rats. Chemical sympathectomy using 6OHDA is shown to increase insulin sensitivity and the number of high-affinity insulin receptors in white fat (25). In contrast, other studies suggest that leptin may cause insulin resistance in adipocytes (30). If resistance to insulin is occurring with leptin administration in vivo then leptin may be acting through the SNS to cause this resistance. Therefore, removal of the SNS in this study would explain the exaggerated response of denervated adipocytes to insulin. The only discrepancy is why the exaggerated response occurred within the non-injected pad rather then the injected. The differences between the injected and non-injected pad cannot be attributed to surgery damaging the integrity of the pad since no difference was seen in cell size distribution or fat pad weight. One explanation for leptin not effecting lipogenesis may be the young age of rats. As age increases leptin mRNA expression in WAT increases in male Wistar rats aged 2 and 20 months (31). This is associated with a reduced level of fatty acid synthesis, and reduced activity of acetyl CoA carboxylase, fatty acid synthase, ATP-citrate lyase, malic enzyme, and glucose 6-phosphate dehydrogenase in older versus younger rats. Therefore younger, growing rats with less WAT leptin mRNA expression naturally are predisposed to be in a lipogenic state more than their older counterparts. This may explain why any differences due to leptin were inhibited in the rats in this study.

Based on the data, leptin administration and sympathetic denervation were both functional since serum leptin levels increased in leptin-infused non-denervated rats and slightly increased in denervated rats. 6OHDA treatment also inhibited leptin from increasing NE content in the injected epididymal pad of 6OHDA/Leptin rats. This effect of 6OHDA was only seen in leptin-infused rats and not the 6OHDA/PBS rats because leptin increased NE content enough to cause an increase in NE content however, with no stimulation in the PBS-infused groups no difference between groups were seen. In addition, leptin has not been determined to definitively increase sympathetic activity (9). In the previous study (39) in rats epididymal NE content increased with leptin, but not consistently in both pads measured with intact sympathetic signaling.

In conclusion, denervation did not inhibit leptin from decreasing body weight. There were no differences between the injected and non-injected epididymal pad weights due to denervation, but this could be a species effect with rats epididymal pads being less effected by 6OHDA. It is important to mention that denervation and leptin were effective, based on serum leptin levels and NE content. Results from lipolysis and lipogenesis did not clearly explain the role leptin has on lipid metabolism with or without the SNS. Since age does affect metabolism this study should be conducted in older rats when growth begins to slow down and the rats have larger fat stores. Surprisingly we found that denervation of one fat pad increased the sensitivity of its non-injected controlateral pad to both lipolytic and lipogenic hormones. Also, that denervation and leptin combined also increased the lipolytic response to norepinephrine.

Table 1: Total Carcass weights (g/rat), Fat pad weights (g/depot), serum measurements at the end of the Experiment, and serum leptin measurements on Day 5 of treatment.

Carcass Composition (g)	***6OHDA/ Leptin***	***6OHDA/ PBS***	***Vehicle/ Leptin***	***Vehicle/ PBS***
Ash	13 ± 1.3	11 ± 0.6	12 ± 0.8	12 ± 0.9
Fat	0.17 ± 0.01	0.19 ± 0.01	0.17 ± 0.01	0.19 ± 0.16
Protein	81 ± 2.2^{b}	89 ± 4.4^{a}	81 ± 1.3^{ab}	85 ± 1.9^{ab}
Water	198 ± 2.1	200 ± 3.5	198 ± 3.0	204 ± 4.9
Fat Depots (g)				
Inguinal*	3.5 ± 0.2^{ab}	3.8 ± 0.2^{b}	3.2 ± 0.1^{a}	3.9 ± 0.2^{b}
Mesenteric	2.1 ± 0.1	1.7 ± 0.2	2.1 ± 0.2	1.8 ± 0.2
Retroperitoneal*	0.7 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	0.8 ± 0.1
Brown Fat	0.3 ± 0.02	0.3 ± 0.01	0.3 ± 0.01	0.3 ± 0.01
Serum				
Triglyceride (mg/dl)	67 ± 5	54 ± 3	64 ± 5	63 ± 5
Glycerol (mg/dl)	74 ± 9	51 ± 6	72 ± 9	77 ± 12
FFA (mEq/L)	0.3 ± 0.04	0.3 ± 0.02	0.3 ± 0.02	0.3 ± 0.03
Leptin (ng/ml)	1.7 ± 0.15^{ab}	1.2 ± 0.19^{b}	2.2 ± 0.35^{a}	1.4 ± 0.18^{b}

Values are ± sem for 8 to 11 rats. * Indicates an overall leptin effect in the retroperitoneal (RP) and inguinal fat depot with no differences between individual groups for the RP. Values with unlike superscripts are significantly different.

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FIGURE LEGENDS

Figure 1: Daily body weights ± sem for 8 to 11 rats. # indicates 6OHDA/Leptin rats were smaller than both PBS-infused groups, and the 6OHDA/PBS rats were larger than both leptin-infused groups. * indicates the leptin-infused, 6OHDA-treated rats were smaller than both PBS-infused groups, the 6OHDA/PBS treated rats were heavier than both leptin-infused groups, and the Vehicle/PBS rats were heavier than both leptin-infused groups. Differences were significant at $P < 0.05$.

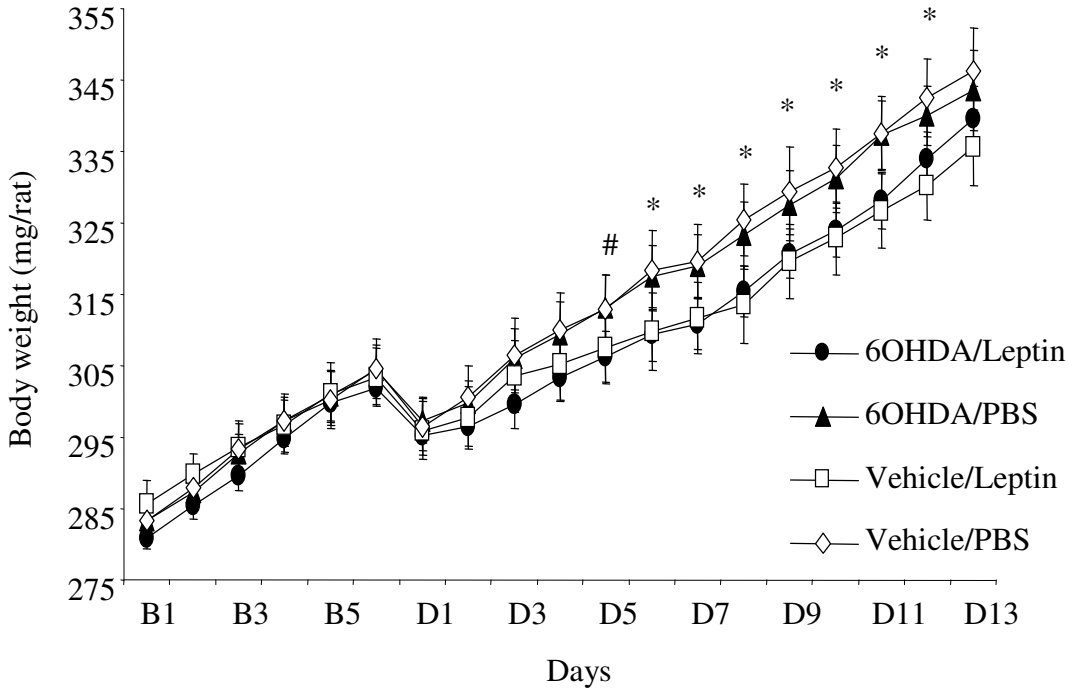
Figure 2: Epididymal pad weight (Panel A) and NE content (Panel B) ± sem for 8 to 11 rats. There was no difference in epididymal pad weight between groups in Panel A. In Panel B an asterisk represents a significant difference ($P < 0.05$) in NE content of injected and non-injected pads of 6OHDA/Leptin rats.

Figure 3: Cell size and number distribution ± sem of isolated adipocytes taken from the injected (Panel A) and non-injected (Panel B) epididymal pads of 8 to 11 rats. No differences between groups were determined in either epididymal pad.

Figure 4: Glycerol release (Panel A) and fatty acid esterification (Panel B) ± sem of isolated adipocytes from both epididymal pads of 8 to 11 rats. In Panel A ** indicates a difference ($P < 0.05$) between the injected and non-injected pad of 6OHDA/PBS rats. The injected pad of Vehicle/Leptin was different from the non-injected pad of 6OHDA/PBS rats represented by an asterisk. The injected (#) and non-injected (§) pad of 6OHDA/Leptin rats are also both different from the injected pad of Vehicle/Leptin rats. All these differences occurred in the presence of

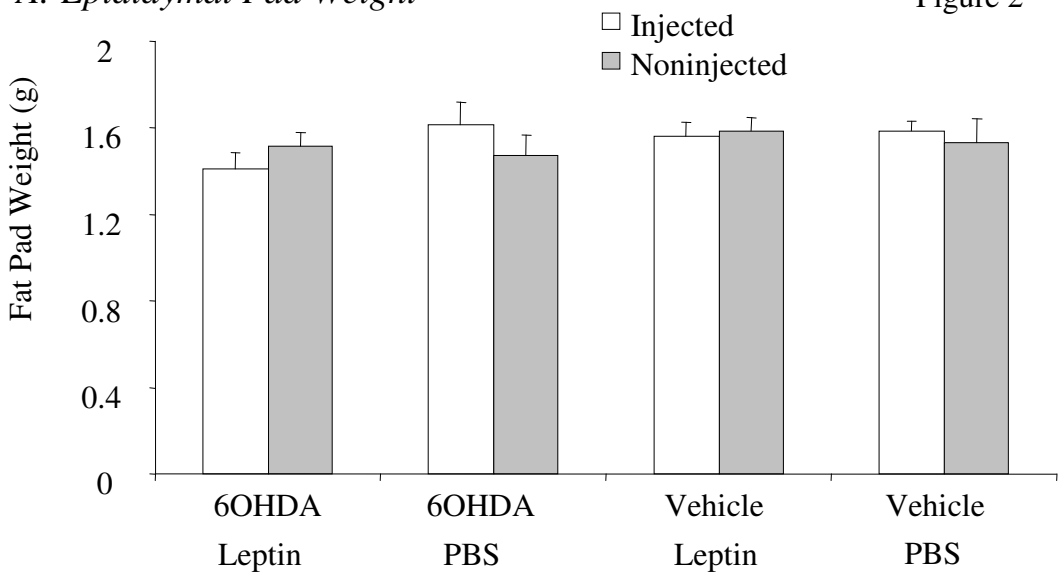
NE. In panel B the non-injected pad of the 6OHDA/PBS rats was different from both the non-injected pad of the Vehicle/Leptin rats (¥) and the injected pad of Vehicle/PBS rats in basal conditions.

Daily Body Weight Figure 1



A: Epididymal Pad Weight

Figure 2



B: Epididymal Pad Norepinephrine

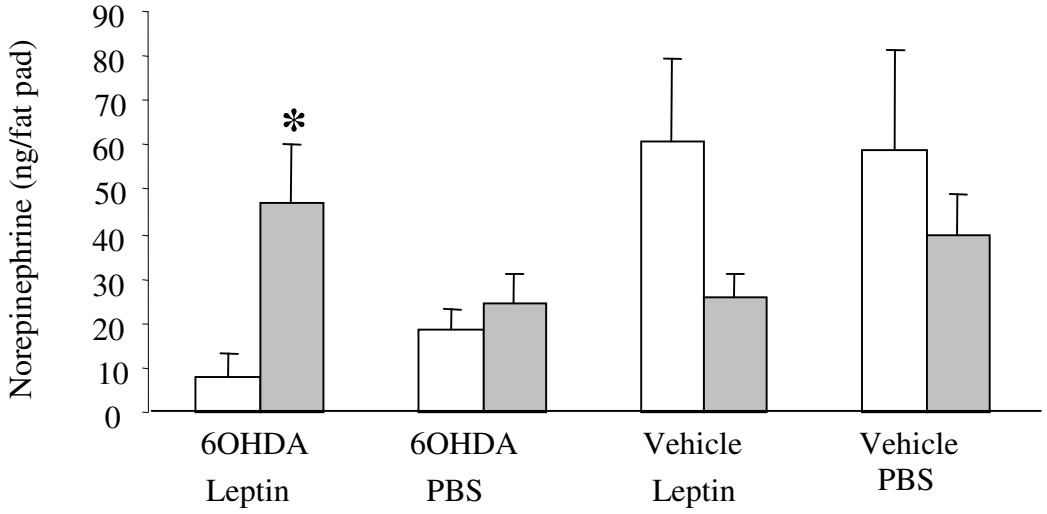
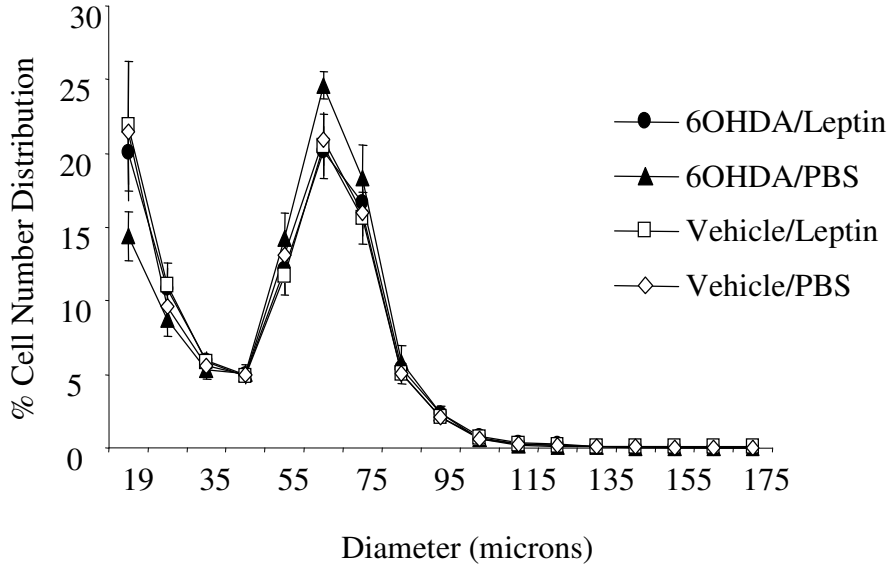
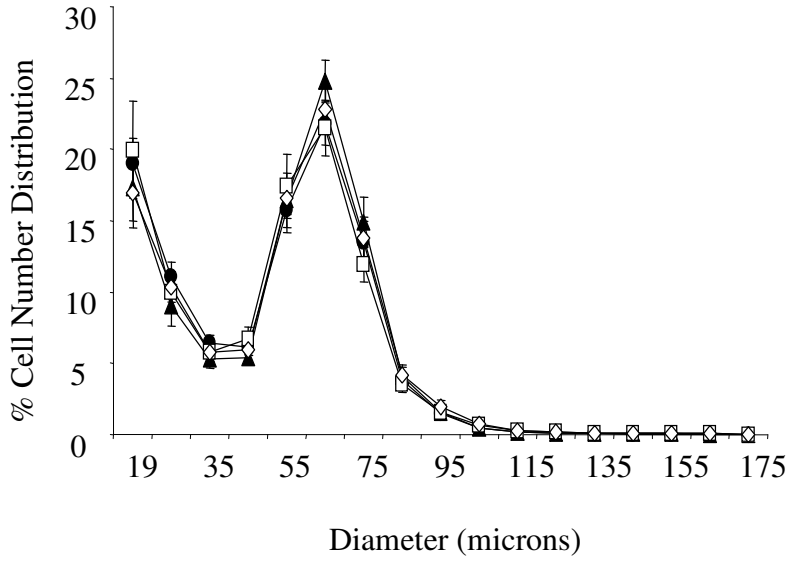


Figure 3

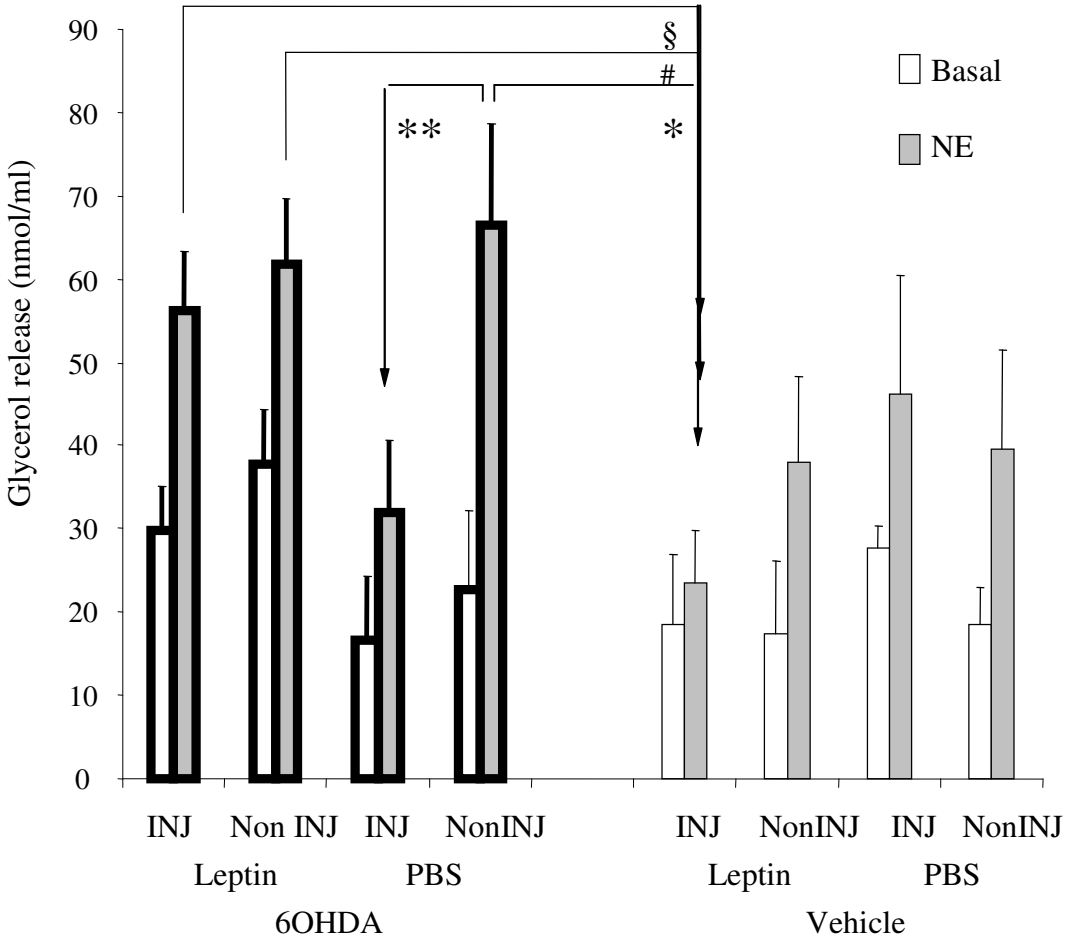
A: Injected Epididymal Pad



B: Non-Injected Epididymal Pad

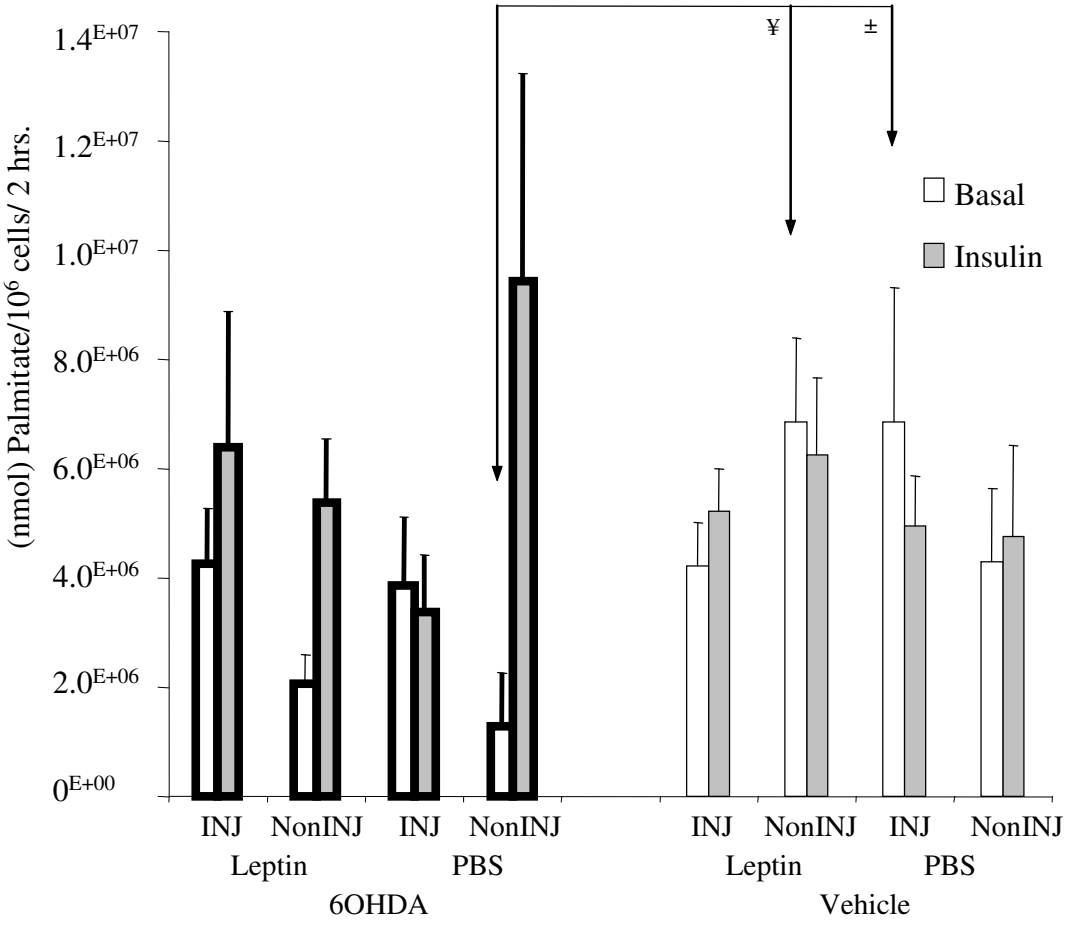


A: Epididymal Glycerol Release Figure 4



B: Fatty Acid Esterification

Figure 4



CHAPTER 5

CONCLUSION

In the study described in Chapter 3 both C57Bl/6 mice and Sprague Dawley rats were unilaterally denervated while infused peripherally with physiological doses of leptin. Leptin decreased the size of fat pads in mice regardless of sympathetic innervation. Leptin decreased body weight and total dissected fat in both intact and, denervated mice. In rats however, the SNS was necessary for leptin to decrease white fat. Fat pads of leptin infused denervated rats were larger than rats infused with leptin with intact sympathetic signals. Interestingly, denervation of one fat pad caused changes in distant fat pads that were not denervated. To determine metabolically how these changes were occurring in fat and whether the SNS played a role, the study was repeated, but after sacrifice adipocytes from both the denervated and non-denervated fat pads of rats were isolated and for measurement of fatty acid esterification and lipolysis in basal and stimulated conditions. Leptin did not effect lipolysis or lipogenesis in rats with intact sympathetic signals therefore it is still unclear what role leptin plays in metabolism. The most important finding for both lipolysis and lipogenesis was that leptin increased the sensitivity of the non-injected pad of denervated rats to external stimuli, insulin and norepinephrine.

The purpose of these studies was to determine the mechanism of leptin action on white fat in vivo. Obese humans have elevated serum leptin levels that are not signaling an excess in energy stores leading to a decrease in weight (2, 5). The reason for leptin resistance in humans has not yet been determined but before we can treat leptin resistance we must first determine exactly how leptin works. Chemical denervation using 6OHDA was somewhat effective at

determining if leptin can act without the sympathetic nervous system. Unfortunately, 6OHDA did not consistently decrease NE content at the end of each experiment, making it difficult to determine whether pads were in fact denervated. It is possible that 6OHDA was effective early during treatment and its effects dissipated toward the end of each study. This explains why changes due to denervation were apparent at the end of the experiment even though NE levels were not decreased.

Whether unilateral chemical denervation inhibits leptin action appeared to be a simple question and we expected a straightforward outcome. Other studies report that hyperleptinemia (6) and central leptin (4) are able to decrease white fat and body weight; respectively without intact sympathetic signaling. Similar results were reported in this thesis in mice, however in rats it appears the SNS is necessary. What complicated matters was the effect that leptin plus denervation had not only on the denervated pad but also on the bilateral and distal fat pads. In addition, the increased sensitivity of the contralateral pad to insulin and NE was unexpected because others have reported that surgical denervation has had no effect on lipid metabolism (1, 3). The results of these studies imply that other factors that regulate fat metabolism are induced by leptin administration and/or inhibited with the presence of the SNS. Future studies should determine exactly the role of leptin in lipid metabolism since the results of experiments described here did not provide a clear answer. In addition it is important to determine how unilateral denervation causes changes in distal pads, and increases sensitivity of the contralateral pad to external stimuli.

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