EFFECTS OF HIGH-FAT FEEDING ON LEPTIN SENSITIVITY IN WEANLING AND ADULT MICE

by

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(Under the direction of Ruth Harris)

ABSTRACT

Genetically obese mice exhibit mutations in leptin or its receptor and are inappropriate for studying human obesity, a hyperleptinemic condition with an intact leptin system. High-fat diets reportedly induce resistance to peripherally administered leptin and may better represent obese humans. 35-day-old, C57BL/6J high-fat (45% kcal fat) fed mice housed individually did not decrease food intake or body weight in response to central or peripheral leptin injections. Peripheral infusions of leptin reduced body fat of 35-day-old mice and 15-week-old mice fed high-fat diet but had no effect on adipocyte glycerol or free fatty acid release in adult mice suggesting that leptin reduces body fat by mechanisms other than lipolysis. Group-housing young mice on bedding prevented fat loss in high-fat fed female C57BL/6J mice suggesting that the lipopenic effect of leptin is in part due to increased thermogenesis. These studies suggest the development of leptin resistance is complex and involves additional factors.

INDEX WORDS: Leptin, Leptin Receptors, Leptin Resistance, Body Weight, Food Intake, Body Composition, Lipolysis, Mice

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INTRODUCTION

In the United States, the increasing incidence of obesity is described as an epidemic with one of five adults classified as obese and more than half of the adult population being overweight (13). Obesity is associated with an increased risk for diseases such as hypertension, Type II diabetes, and coronary heart disease (15). Obesity is a disease of increased adipose tissue mass, which is not only a site of long-term energy storage but also is an active participant in the regulation of energy balance. One way in which adipose tissue participates in the regulation of energy balance is through the production and secretion of leptin (18). The maintenance of body weight during various conditions including food restriction and overfeeding suggests that body fat mass is tightly regulated, however, the precise regulatory mechanism has not been defined. The existence of internal regulators of body fat mass was suggested by early parabiosis studies, which established a role for a circulating adiposity signal(s) (3). Discovery of leptin in 1994 reinforced the idea that there are biological determinants of body fat mass and leptin was proposed to be the signal produced by the fat tissue that indicates to the hypothalamus the size of the fat stores (18). This notion was supported by evidence that leptin could enter the central nervous system by a saturable transport system (1), which allows the protein to interact with hypothalamic leptin receptors, Ob- Rs. The expression of Ob-Rs is not limited to the hypothalamus and ubiquitous expression of the receptors in peripheral tissues suggests that leptin is involved in processes in the periphery.

Rodent models either lacking leptin, the ob/ob mouse, or its receptor, the db/db mouse, have helped us to better understand leptin's role in energy balance. Studies administering leptin to ob/ob mice and lean littermates have shown that leptin reduces food intake and body weight; the weight loss is a specific loss of adipose tissue (7, 8, 14). Failure to observe such effects in db/db mice indicates that the receptor is critical for leptin's function (2, 7, 9). There is a continuum of leptin sensitivity: db/db mice are unresponsive or 'resistant' due to lack of the leptin receptor, ob/ob mice are extremely sensitive to the effects of leptin, and lean mice fall somewhere in between the db/db and ob/ob mouse in terms of responsiveness.

Mutations such as those that occur in ob/ob and db/db mice are rare in humans (5, 12). In contrast, the majority of obese humans are hyperleptinemic but fail to reduce food intake and maintain body weight in response to elevated levels of endogenous leptin (4). Similar observations have been made in mice where increased leptin concentrations associated with increased body mass fail to limit intake of energy and expansion of adipose tissue (6). This observation has lead to the idea that 'leptin resistance' is a potential cause of obesity (11). Mice fed a high-fat diet also have been described as resistant to leptin's effects on food intake and body weight (10, 16) and, therefore, may be a more appropriate model for studying human obesity than the genetically obese rodents which are not representative of the majority of obese humans. Studies describing 'leptin resistance' have focused on leptin's effects on energy intake and body weight and have not determined the effects of high-fat feeding on leptin's peripheral effects, although emerging evidence suggests that leptin exerts direct effects in the periphery to

reduce body fat mass (17). Additionally, the mechanisms underlying the described 'resistance' are still undefined.

The objectives of this thesis are (1) to further characterize the development of high-fat diet induced 'leptin resistance' by examining the effects of gender, strain, housing conditions, and method of leptin administration on the development of diet induced 'leptin resistance'; (2) to determine the effects of high-fat feeding on leptin-induced lipolysis; (3) to determine if 'leptin resistance' is associated with reduced leptin receptor protein in peripheral tissues.

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CHAPTER 2

LITERATURE REVIEW

LEPTIN

Leptin, the protein encoded by the ob gene, was identified using positional cloning and proposed to be the hypothesized adipose-derived signal important for regulation of fat stores (108). Adipose tissue is the primary source of leptin (108) but ob mRNA is expressed at lower levels in other tissues including the stomach (6, 74, 89) and skeletal muscle (44, 50, 98). Leptin circulates in the blood as a 16 kD protein in concentrations proportional to the percent body fat (23). Both tissue expression (83) and serum leptin levels (3) display a diurnal rhythm linked with feeding patterns and may in part be regulated by insulin (83). In mice, serum leptin levels increase around midnight with feeding and reach a maximum in early morning before onset of the light cycle (3). Leptin circulates in serum in both bound and free forms with different distributions observed in lean and obese individuals: lean subjects have more bound leptin whereas obese subjects have primarily free leptin (46, 88). The soluble leptin receptor (Ob-Re), described in more detail below, binds leptin with high affinity (25, 62) and is proposed to be the primary binding protein in the human circulation (55). The role of bound leptin in the serum is not completely understood but may result in a prolonged half-life of leptin and/or delayed leptin clearance (47, 75). In addition, bound leptin has been suggested to be biologically inactive (47). It remains to be determined if bound leptin is protected from degradation during transport or if binding renders the protein inactive.

LEPTIN RECEPTORS

The leptin receptor, a single-membrane spanning receptor and a member of the class I cytokine receptor family, was cloned from the mouse choroid plexus by Tartaglia and colleagues (93). Six splice variants have been identified: Ob-Ra (56), Ob-Rb (56), Ob-Rc (56), Ob-Rd (56), Ob-Re (56), and r-Ob-Rf (100). Ob-Rb, the long-form receptor contains a long intracellular domain important for intracellular signal transduction (19, 93). Ob-Ra, Ob-Rc, and Ob-Rd are short-form receptors in that they have an identical extracellular and transmembrane domain to Ob-Rb but lack a complete intracellular domain (56). Ob-Re is a soluble receptor lacking both transmembrane and intracellular domains (56). r-Ob-Rf has been identified as a short isoform in the rat (100). Ob-Rb is expressed at high levels in the hypothalamus (56, 63, 100) but also has been detected in most peripheral tissues including the lung (63, 95, 100), adipose tissue (12, 56, 63), testes (56, 63), and stomach (63, 74, 89, 100) at a low level of expression. The short-form receptors are ubiquitously expressed with high levels in a number of tissues including the testes (45, 56), liver (100), kidney (45, 100), adipose tissue (45, 56), and lung (45, 100). The function of these receptors is not fully understood, however, the observed variation in tissue expression suggests differential effects in the tissues. Short-form receptors expressed in the choroid plexus (56, 78, 93) and the brain microvessels (10) may be involved in transport of leptin into the central nervous system. This is supported by studies showing that rats lacking the short-form receptor have reduced leptin transport across the blood brain barrier; however, the transport is not eliminated in these rats suggesting the presence of an additional transporter (53). Ob-Re, the soluble receptor, is expressed in adipose tissue, hypothalamus, heart, and testes (56) and, as described above,

is capable of binding leptin in the circulation (25, 62) where it likely functions in leptin transport. The specific functions of the leptin receptor isoforms remain to be fully elucidated.

LEPTIN SIGNALING

Leptin signaling occurs through activation of the JAK-STAT pathway. Leptin binding to the Ob-Rb receptor results in dimerization of the leptin receptor (105) and phosphorylation of tyrosine residues of Jak2, a member of the janus kinase family (36). The autophosphorylation of Jak2 results in its activation and phosphorylation of the tail of the receptor, which binds the Src homology 2 (SH2) domain of the signal transducers and activators of transcription (STATS) (24, 49). STAT bound to the leptin receptor is phosphorylated on tyrosine and forms either homo- or heterodimers that translocate to the nucleus where the STATS regulate gene transcription (24). In contrast to *in vitro* studies indicating leptin receptor signaling activates STAT 1 (8), STAT 3 (8, 37), STAT 5 (8, 37), and STAT 6 (37), studies *in vivo* have found that leptin binding to the receptor activates only STAT 3 (73, 96). The short-form of the receptor appears to be incapable of activating the JAK-STAT signaling pathway (8, 36, 37, 96) but may have some signaling capabilities through other pathways (76).

EFFECTS OF LEPTIN

Leptin has demonstrated roles in energy balance (1, 42), glucose metabolism (35, 42), reproduction (42, 84), angiogenesis (87), and immune function (27, 42). The studies described here focus on leptin's role in the regulation of energy balance and its specific effects on adipose tissue although leptin has important roles in liver glucose turnover (52) and muscle fatty acid oxidation (90).

The critical role of leptin in energy balance is best demonstrated by two spontaneous single point mutations in the mouse: ob/ob and db/db mice. The ob/ob mouse has a mutation that is a premature stop codon in the ob gene, which results in production of an inactive protein (108). ob/ob mice are characterized by extreme obesity, hyperphagia, hypothermia, and infertility (22). Genetically obese and diabetic db/db mice exhibit a syndrome similar to the ob/ob mouse described above with obesity, hyperphagia, and severe Type II diabetes (22, 48). Unlike the ob/ob mouse, the db/db mouse produces leptin but has a mutation in the db gene encoding the leptin receptor, which results in an absence of the Ob-Rb receptor and replacement by the short-form receptor (19) that has little or no signaling capabilities (8, 36, 37, 76, 96).

As expected, central and peripheral leptin administration reverses many abnormalities of the ob/ob mouse including reduced food intake and body weight (15). Leptin's effects on body weight are associated with specific reductions in fat while maintaining lean body mass (40, 43, 80). In addition to reducing food intake and body weight, ob/ob mice also respond to peripheral leptin by increasing activity (80) and body temperature (43, 80) while normalizing oxygen consumption (80), serum cholesterol (57), serum glucose and insulin concentrations (43, 57, 80, 91), reducing corticosterone (91), and increasing uncoupling protein (UCP) expression (43). Due to lack of the leptin receptor, db/db mice do not reduce food intake or lose weight in response to adminstration of exogenous leptin (15, 40, 57).

Leptin also results in small transient reductions in food intake and decreased body weight when administered centrally (15) or peripherally (39, 40, 43, 57, 80) to normal, lean mice. In all cases, however, ob/ob mice respond to a much greater degree

than lean mice indicating they are especially sensitive to the effects of leptin (43, 80). In both lean and ob/ob mice, small doses (0.6-40 μg/kg) of leptin administered centrally reduce food intake and body weight and the same effects can only be seen with larger peripheral doses (400-4000 μg/kg) supporting the role of the central nervous system in mediating leptin's effects (91). The hypothalamus has been the proposed target of leptin, however, there is new evidence to suggest that other areas of the brain, including the caudal brain stem (38), are involved in mediating leptin's inhibitory effects on food intake and body weight.

Leptin's effects on body weight with exclusive loss of fat are not simply secondary effects of reduced food intake (43) as evidenced by pair-feeding studies (57). Leptin administered by adenoviral gene transfer results in complete elimination of body fat at circulating leptin levels of 8 ng/ml (18) and at extremely high concentrations (>20 ng/ml) leptin can eliminate adipose tissue through mechanisms that do not require neural input to the adipose tissue (104), supporting the notion that leptin may have direct actions on adipocytes (103).

The mechanisms by which leptin specifically reduces body fat mass are poorly understood. Leptin could potentially limit adipocyte development by decreasing adipocyte differentiation and/or proliferation, however, studies in this area have shown conflicting results. Supraphysiologic concentrations of leptin have little or no effect on the differentiation of human preadipocytes (5) but increase both proliferation and differentiation of rat subcutaneous preadipocytes (67). In a human marrow stromal cell line, hMS2-12, leptin's effects on adipocyte differentiation markers were confusing and conflicting but the overall effect appeared to be reduced adipocyte differentiation (94).

The number of adipocytes available for lipid storage is also determined by the rate of apoptosis or programmed cell death. Few studies have investigated leptin's role in induction of apoptosis, although, recent evidence suggests that central (81) and peripheral leptin treatment (21) may induce apoptosis in adipocytes.

Additionally leptin may reduce body fat mass by inhibiting lipogenesis or stimulating lipolysis. Studies investigating leptin's effects on lipogenesis are thus far inconclusive. In human adipocyte cultures, leptin concentrations at 10 ng/ml and 100 ng/ml decreased insulin-stimulated glucose uptake by adipocytes, therefore, limiting substrate availability for lipogenesis (107). *In vivo* leptin injections reduced the lipogenic rate in white adipose tissue of lean and gold thioglucose treated mice, a model of obesity due to hypothalamic infarction (13). In contrast, *in vivo* administration of leptin in rats did not alter lipogenic rate in white adipose tissue (65). Harris (41) reports that adipocyte lipid synthesis and glucose utilization is reduced with *in vivo* peripheral leptin infusions (10 µg/ day) but found no effect of *in vitro* leptin (6.3 nM) exposure on adipocyte lipid synthesis and glucose utilization. Studies investigating leptin's ability to stimulate lipolysis have also produced mixed results. *In vitro* studies suggest a direct lipolytic effect of leptin, which may be mediated through adipocyte Ob-Rb (31, 33, 82, 86). The *in vitro* effect of leptin on lipolysis is dose-dependent (34) and depot specific with subcutaneous adipocytes having higher rates of lipolysis than omental adipocytes (32). In contrast, *in vitro* ovine adipocyte metabolism was unaffected by physiological levels of leptin (77). Likewise, lipolytic rates of human adipocytes expressing Ob-Rb were unaffected by large doses of leptin (500 ng/ml) (5). One study suggests that lipolysis induced by leptin is of a different nature than lipolysis induced by norepinephrine (99).

Norepinephrine-stimulated lipolysis resulted in release of both glycerol and free fatty acid from rat adipocytes (99). In contrast, leptin-induced lipolysis was associated with glycerol but not free fatty acid release suggesting an increase in free fatty acid oxidation (99). Other studies investigating leptin-stimulated lipolysis found that 0.1 nM leptin increased fatty acid release by 177% in mouse epididymal adipose tissue in comparison with controls (54). *In vivo* studies using high doses of leptin have shown lipolytic effects. Leptin (10 mg/kg) administered as a single intraperitoneal injection increased glycerol release (~50%) in adipocytes from lean C57BL/6 mice (30). Similarly, leptin transfer into ob/ob mice through the muscle increased *in vivo* lipolysis (71). Currently there are no *in vivo* studies investigating the effects of physiological concentrations of leptin on lipolysis. The studies described in this thesis will determine if physiological doses of leptin are capable of inducing lipolysis *in vivo.*

LEPTIN RESISTANCE

The spontaneous mutations in ob/ob and db/db mice have greatly enhanced our understanding of leptin and its functions, but similar mutations in humans are rare (28, 70). A majority of the obesity in humans is characterized by high levels of leptin and increased expression of ob mRNA, however, these levels are incapable of regulating or limiting body weight gain (23, 64). Similar observations have been made in mice where the high leptin concentrations associated with increased body mass fail to limit intake of energy or expansion of adipose tissue (29). This observation has lead to the idea that 'leptin resistance' is a potential cause of obesity (69). 'Leptin resistance' may be defined as a reduced response or a lack of response to leptin's ability to reduce food intake and

maintain body weight. In addition to obesity, consumption of high-fat diets (60, 97) and aging (102) have been found to induce 'leptin resistance'.

LEPTIN RESISTANCE INDUCED BY A HIGH-FAT DIET

Rodents fed a high-fat diet reportedly develop peripheral leptin resistance. C57BL/6 mice fed a high-fat diet are insensitive to peripheral leptin injections (60, 97) and a continuous leptin infusion (0.4 mg/kg/day) is incapable of preventing the obesity and diabetes caused by consumption of a high-fat diet (92). The effects of high-fat feeding on central leptin responsiveness are less clear. Centrally administered leptin produces dose-dependent reductions in food intake and body weight of peripherally resistant high-fat fed mice (97). Similarly, high-fat fed rats decrease food intake in a dose-dependent manner in response to intracerebroventricular leptin (61). In contrast, C57BL/6J mice fed a high-fat diet for prolonged periods (19 weeks) become insensitive to central leptin (60). High-fat fed rats also reportedly have a reduced sensitivity to centrally injected leptin (106). These studies suggest that the progressive development of 'leptin resistance' that begins with early peripheral resistance followed by later development of central resistance is dependent on time of exposure to a high-fat diet. The mechanisms by which high-fat feeding inhibits the normal feeding response to exogenous leptin treatment have yet to be determined. It appears that there is an initial attempt by the body to maintain body weight and energy homeostasis when challenged with a high-fat diet. Acute exposure to a high-fat diet decreases tissue expression of peptides such as neuropeptide Y and Agouti related peptide (109), both of which normally increase food intake, and stimulate leptin secretion (109). These changes would be expected to decrease food intake and maintain body weight, however, despite these

changes body weight increases in the high-fat fed animals (109). The specific aspect of the system that eventually gives way to dysregulation of body weight is unknown.

It is not clear if 'leptin resistance' develops in response to dietary components or the increasing adiposity that accompanies high-fat feeding. There is evidence to support an association between diet composition and 'leptin resistance'. Development of peripheral leptin resistance in high-fat fed rats is not immediate but does occur within 5 days of exposure to a high-fat diet and leptin responsiveness is restored if low-fat diet is provided immediately after leptin treatment, suggesting it is not due to adiposity (58). Most studies, however, have not separated diet from adiposity.

Various mechanisms for 'leptin resistance' have been suggested including abnormal leptin synthesis or secretion (1), impaired transport into the brain (16, 85), dysfunctional leptin receptors (72), and altered post-receptor signaling (1). Observations that high-fat diets lower endogenous leptin secretion (4) and interrupt the normal diurnal variation in serum leptin (17) support the possibility that altered synthesis or secretion of leptin contributes to 'leptin resistance'. Studies examining the role of leptin transport in the development of 'leptin resistance' have failed to produce conclusive results. High-fat diets have been shown to increase the expression of Ob-Ra at the blood brain barrier (11) an effect that would be expected to increase leptin entry to the brain given the potential role for Ob-Ra in regulation of leptin transport into the central nervous system. In contrast, a separate study reported reduced leptin transport at the blood brain barrier in animals with high-fat diet-induced obesity (14). Also, observations that leptin transport into the brain is saturated at circulating leptin levels found in lean rats further suggests that increased levels of leptin, as found in obese animals, may have limited access to

receptors in the central nervous system (7). Further studies are needed to clarify the effect of feeding a high-fat diet on leptin transport into the brain.

Studies examining the effects of high-fat feeding on leptin receptor mRNA expression have also produced conflicting results. Several studies have shown no effect of high-fat feeding on Ob-Ra or Ob-Rb mRNA expression in the brain (26, 68). Similarly, male Wistar rats fed a high-fat diet for 15 weeks did not have changes in either Ob-Rb or Ob-Ra mRNA expression in the hypothalamus, white adipose tissue, brown adipose tissue or liver (79). In contrast, in situ hybridization showed an increase in leptin receptor mRNA expression in the mouse choroid plexus and arcuate nucleus after 8 weeks of high-fat feeding followed by a decrease in expression after 19 weeks of high-fat feeding (59). Although the effects of leptin on mRNA expression of receptors remain unclear, there is evidence that high-fat diets down-regulate Ob-Rb and Ob-Ra protein levels suggesting translational regulation of the receptors (68).

Some studies suggest that 'leptin resistance' may be due to post-receptor signaling defects. El-Haschimi (26) showed that peripheral leptin (100 μg) was incapable of inducing STAT 3 DNA binding in the hypothalamus of C57BL/6J mice fed high-fat diet and that central leptin (0.5 μg) produced a smaller response (75% lower) in mice fed a high-fat diet than in mice fed a low-fat diet (26). Because leptin receptor protein was not measured, the loss of STAT 3 DNA binding could be secondary to a reduced number of receptors. Additional studies have investigated the role of inhibitory proteins such as suppressor of cytokine signaling-3 (SOCS-3), which has been shown to interfere with leptin signal transduction (9), and protein inhibitor of activated STAT (PIAS-3), which inhibits DNA-binding activity of STAT 3 (20) in the development of leptin resistance.

One study has found increased SOCS-3 mRNA in epididymal fat pads from high-fat fed rats (101). In contrast, other studies using high-fat fed rodents have failed to show changes in mRNA expression of either SOCS-3 or PIAS-3 (26, 79).

Few studies have examined how high-fat feeding alters leptin's action in the periphery. Steinberg and Dyck (90) demonstrated an inhibition of leptin's stimulatory effect on lipid oxidation and inhibitory effect on triglyceride synthesis in the soleus muscle of rats with high-fat feeding. High-fat feeding is also associated with a decrease in lumbar sympathetic nerve activity and mean arterial pressure in leptin-treated Wistar rats while there is an increase in both measures in low-fat fed, leptin-treated animals (66). This is a relevant finding considering leptin's effects on metabolism is in part mediated by the sympathetic nervous system increasing thermogenesis and energy expenditure (2). Johnson et al. (51) found that adipose tissue taken from high-fat fed, centrally resistant Sprague-Dawley rats had a similar *in vitro* lipolytic response to leptin as chow-fed, leptin responsive rats. The high-fat fed rats, however, were less responsive to isoproterenol-stimulated lipolysis than their chow-fed counterparts. The studies described in this thesis will determine if leptin-induced lipolysis is altered in mice made leptin resistant by high-fat feeding.

SUMMARY

Leptin in the presence of its receptor(s) plays a very important role in energy balance that is best demonstrated by the phenotypes of the ob/ob and db/db mouse models. The mechanisms whereby leptin regulates food intake and reduces body fat mass are still unclear. Furthermore, it is puzzling why a condition such as obesity occurs in the presence of high levels of leptin. One explanation is that obese persons are

resistant to the effects of leptin. Mice fed high-fat diet are resistant to leptin's effects on food intake and body weight (97) but the point in the leptin pathway that is altered by high-fat feeding, giving way to body weight dysregulation, remains undefined. The purposes of this thesis are to characterize high-fat diet induced leptin resistance in mice and to investigate whether inhibition of leptin-induced lipolysis or downregulation of peripheral leptin receptor(s) is involved in the development of leptin resistance.

HYPOTHESIS TO BE TESTED

- Mice weaned onto a high-fat diet will be resistant to peripherally but not centrally administered leptin at 6 weeks of age.
- Adult mice fed a high-fat diet will be resistant to the fat-reducing effects of leptin administered as a constant peripheral infusion.
- Leptin resistance in adult mice fed a high-fat diet will be associated with an inhibition of leptin-induced lipolysis and a downregulation of leptin receptors in adipose tissue.

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CHAPTER 3

METHOD OF LEPTIN DOSING, STRAIN, AND GROUP HOUSING INFLUENCE LEPTIN SENSITIVITY IN HIGH-FAT-FED WEANLING MICE[1]

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ABSTRACT

High-fat diets are reported to induce resistance to peripherally administered leptin. In an attempt to develop a model of juvenile diet-induced obesity mice were weaned onto high-fat diet. Male and female, 35 day-old, C57BL/6J high-fat (45% kcal fat) fed mice housed individually on grid floors did not decrease food intake or body weight in response to intraperitoneal (30 ug), lateral ventricle (5ug) or 3rd ventricle (0.5 ug) injections of leptin. Body weight and fat were significantly reduced by 13-day intraperitoneal infusions of 10 ug leptin/day, which doubled circulating leptin. Leptin infusion also reduced body fat in weanling, high-fat fed NIH Swiss mice. Group-housing mice on bedding prevented loss of fat in high-fat fed male and female NIH Swiss and female C57BL/6J mice. These results indicate that peripherally infused leptin reduces fat, in part, by increasing thermogenesis and that inhibition of food intake in high-fat fed mice requires either chronic activation of central leptin receptors or is independent of receptors that inhibit feeding in response to an acute central injection of leptin.

INTRODUCTION

Leptin, a 16 kD protein secreted by the adipose tissue, has been hypothesized to act as a signal from the periphery to the central nervous system (CNS) indicating the size of energy stores (39). Leptin enters the brain by a saturable transport system (3), where it activates hypothalamic long-form leptin receptors, Ob-Rb. Although five leptin receptor isoforms exist in mice, only Ob-Rb is abundantly expressed in the hypothalamus and appears to possess full signaling capabilities (36). These receptors are responsible for the inhibitory effect of leptin on food intake and other physiological functions including reproduction. Initially it was assumed that leptin would act as a lipostatic signal, inhibiting food intake during periods of positive energy balance and enlargement of body fat mass (39). It has been reported that in young lean rats there is a direct negative correlation between circulating concentrations of leptin and body fat mass when leptin remains within the narrow range normally found in these animals (6). Leptin administration also induces weight loss and a transient inhibition of food intake in lean, wild-type, mice (17, 27). Low doses of leptin administered directly into the brain produce effects comparable to those seen with larger doses administered in the periphery, supporting the concept that leptin-induced changes in energy balance are mediated by receptors in the CNS (34). In obese animals (16) and humans (5) circulating concentrations of leptin increase in proportion with body fat mass and the relationship between leptin and adiposity is lost. It is now hypothesized that, rather than increased levels of leptin signaling energy excess, a reduction in circulating concentrations of leptin conserves energy during periods of energy deficit by inhibiting activity of some energy expensive processes (1).

A majority of human obesity is characterized by high circulating levels of leptin and increased adipose tissue expression of leptin mRNA. These high levels of leptin do not down regulate body fat mass, which has led to the concept of leptin-resistance (5), defined as a defect in leptin signaling that allows a dysregulation of energy balance and a failure to decrease body weight and food intake in response to increasing leptin concentrations (7, 9). In addition, the leptin levels in obese humans appear unresponsive to short-term changes in nutritional status, as large changes in body mass (>7%) are required before circulating concentrations of leptin change significantly (19). Several authors have reported that male mice and rats exposed to a high-fat diet also are unresponsive to peripheral injections of leptin (23, 37), and that continued consumption of a high-fat diet eventually results in reduced central leptin sensitivity (24), suggesting that this model accurately reflects the human obese condition. The development of peripheral leptin resistance appears to be rapid, developing within 16 days in 5-week old mice on a 45% kcal fat diet (37) and within 5 days in rats fed a 56% kcal fat diet (23).

In a previous experiment, we found that female C57BL/6J mice fed a high-fat (45% kcal fat) diet for 15 weeks remained responsive to peripheral administration of leptin both as a constant peripheral infusion and as a single bolus injection (14). The obvious explanation for the conflicting results with high-fat fed mice in our study and those of others (23, 37) was that the gender of the mice influenced sensitivity to leptin, as we have previously reported significant effects of gender on the response to both central and peripheral administration of leptin in mice that over-express agouti protein (15). The initial objective of this study was to identify factors that contribute to the development of leptin resistance in young mice weaned onto a high-fat diet. Due to the failure to induce

leptin resistance in older female mice (14) we included both males and females in this study to determine the importance of gender in the development of leptin resistance in high-fat fed mice. Mice were offered the high-fat diet from 10 days of age, while they were still suckling, so that they were never exposed to a low-fat diet. Based on the observation that 5-week old mice were leptin resistant after 16 days on a 45% kcal fat diet (37), we anticipated that the high-fat fed mice would be leptin resistant, providing an appropriate model for the study of juvenile, diet-induced obesity. The initial experiments demonstrated that high-fat fed male and female mice were, at least partially, resistant to central injections of leptin but were fully responsive to peripheral infusions of leptin, therefore, additional experiments compared the effects of strain, gender, and housing conditions of the mice on the sensitivity to peripherally infused leptin in 5-week old mice weaned onto either low- or high-fat diet.

METHODS

Animals and Diet

Male and female C57BL/6J and NIH Swiss mice were obtained from breeding colonies maintained at the University of Georgia. Mice were housed at 73°F with lights on 12 hours/day from 7.00 a.m. They had free access to food and water except where specified. Dams and their litters were fed either low-fat diet containing 10% kcal as fat (Diet 12450B; Research Diets, NJ) or high-fat diet containing 45% kcal as fat (Diet 12451; Research Diets, NJ) starting 10 days postpartum. Pups were weaned at 28 days of age and, at 30 days of age, single-housed mice were housed individually in cages with grid floors to allow for measures of food intake. Group-housed mice were housed 3-4 mice per cage on bedding. Only two male and two female pups were taken from each

litter for incorporation into studies to minimize litter-specific responses. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Georgia and were conducted in conformity with the APS Guiding Principles in the Care and Use of Animals (2).

Experiment 1: The effects of intracerebroventricular leptin injection on single-housed C57BL/6J mice

This study tested whether young, high-fat fed C57BL/6J mice responded to central leptin injections by reducing food intake or weight gain. Male and female single-housed C57BL/6J mice, aged 34 days, were fitted with bilateral cannulas of the lateral ventricle using the procedure of Guild and Dunn (12). Briefly, a 1 cm saggital incision was made along the skin covering the skull followed by placement of the cannulae at 0.6 mm posterior and +/- 1.6 mm lateral of the bregma. One-week later mice within each dietary treatment were divided into 2 groups of 7-9 males or 8-13 females and were injected unilaterally with 5 µg leptin (recombinant murine leptin; R&D Systems, MN) in 1.5 ul of PBS or an equal volume of PBS. This dose was in the middle of the range of doses used by Van Heek et al. (37) to demonstrate development of peripheral but not central leptin resistance in mice fed a 45% kcal fat diet for 56 days. The mice were housed in shoe-box cages with an elevated grid floor to allow measurement of food intake. Food was placed on the grid floor and intake, measured to 0.01 g, was corrected for spillage that collected below the grid. Food intakes and body weights were recorded at 24 hour intervals for 3 days before injections. On test days, mice were food deprived from 7.00 a.m. and were injected at 4.00 p.m. Food was returned to the cages one hour after injection and food intake was recorded 4, 14, 24, 38, and 48 hours post-injection.

Body weights were recorded 14 and 38 hours post-injection. One week later, the procedure was repeated with treatments switched. At the end of the study, methylene blue dye was injected into the cannulae and placement was verified by visually examining staining of the ventricles.

Because the results of this experiment indicated that the high-fat fed mice were resistant to leptin, but subsequent experiments showed that the mice responded to peripheral infusions of leptin, we repeated the study using mice with 3rd ventricle cannulas. A 30 gauge guide cannula (Plastics One, VA) was placed using the stereotaxic co-ordinates of 0.8 mm posterior, 0.2 mm lateral and -2.5 mm dorsal to the bregma. The mice were allowed to recover from surgery for one week and then baseline daily food intakes and body weights were recorded at 5.00 p.m. each day for 3 days. On the test day the mice were food deprived from 7.00 a.m. until 5.00 p.m. when they received a 3rd ventricle injection of 0.5 ug leptin in 0.5 ul PBS or an equal volume of PBS. Food was returned to the cages and intakes were measured 4, 14, 24, 48 and 72 hours after injection. Body weights were recorded at 24, 48 and 72 hours after injection. One week after the first injection the procedure was repeated except that the treatment groups were switched. There were 5-6 mice per treatment group. At the end of the experiment the mice were injected with 4 ug NPY at 8.00 a.m. and those that did not eat at least 0.5 g of food within 2 hours were excluded from the experiment.

Experiment 2: The effects of peripheral leptin infusion on single-housed C57BL/6J mice

The results of the previous experiment indicated that the young mice weaned onto high-fat diet were resistant to central injections of leptin. The objective of this experiment was to test whether the high-fat fed mice were also resistant to the effects of

peripheral leptin infusion on food intake, body composition and insulin status. C57BL/6J mice fed low- or high-fat diet were single-housed and daily food intakes and body weights were recorded for 3 days. Male and female mice from each dietary treatment were further divided into 2 weight-matched groups of 5-6 male mice or 7-9 female mice. At 34 days of age the mice were fitted with an intraperitoneal (i.p.) Alzet miniosmotic pumps (Model 1002; Durect Corp., CA). One group was infused with PBS and the other with 10 µg leptin /day for 13 days. Food intakes and body weights were measured daily. On Day 10 of infusion, mice were deprived of food from 7.00 a.m. to 12.00 p.m. and small blood samples were collected from the tail for measurement of fasting insulin (Mouse Insulin RIA; Linco Research Inc., MO) and glucose (Accumet glucometer; Boehringer Mannheim, Gmg., Germany). On day 13, mice were decapitated and trunk blood was collected for measurement of serum leptin (Mouse Leptin RIA; Linco Research Inc.). Gonads, gonadal, mesenteric, and retroperitoneal fat were weighed and the gut was cleaned. Tissues were returned to the carcass for determination of body composition as described previously (18).

Efficiency of energy gain for the different groups of mice was calculated. The proportion of carcass weight that was protein or fat at the end of the experiment and weight gain during the period of infusion were used to calculate gain in carcass energy, assuming that the energy cost of gaining either fat or protein is 12.6 kcal/g (29). This value was divided by total energy consumed during the period of infusion to give an estimate of the efficiency of gain.

Experiment 3: *The effects of peripheral leptin infusion on single-housed NIH Swiss mice.*

In Experiment 2, we found that C57BL/6J mice fed high-fat diet responded to peripheral leptin infusions by reducing body fat content. Therefore, in this experiment we tested whether the retention of leptin-responsiveness was strain-specific and determined whether NIH Swiss mice became leptin-resistant when they were fed a high-fat diet. These mice showed a 96% preference for the high-fat over the low-fat diet when offered a choice between the two diets and older male NIH Swiss mice significantly increased their body fat mass when offered the high-fat diet used in this study. The experimental design was exactly the same as in Experiment 2 except that groups of 5 male and 4-5 female single-housed NIH Swiss mice were used in this study and carcass composition was not determined although fat depot weights were recorded.

Experiment 4: The effects of peripheral leptin infusion on group-housed C57BL/6J mice

In Experiments 2 and 3 we found that single-housed mice fed high-fat diet responded to peripheral infusions of leptin, independent of strain. Leptin reduced body fat content without causing any substantial inhibition of food intake, implying that there was a leptin-induced stimulation of thermogenesis. Therefore, we conducted this study with the mice housed in conditions that minimized the need for heat production and tested whether group-housed C57BL/6J mice fed high-fat diet responded to peripheral infusions of leptin. The experimental design was the same as for Experiment 2 except that mice were group-housed, as described above, so that food intakes could not be recorded. There were 8-9 male mice and 7-8 female mice per treatment group.

Experiment 5: The effects of peripheral leptin infusion on group-housed NIH Swiss mice

Group-housed C57BL/6J mice in Experiment 4 responded to leptin by reducing their body fat content, therefore, we tested whether responsiveness was dependent upon the strain of the mouse. The experimental design was the same as for Experiment 4 except that there were 7-9 male and 7-8 female NIH Swiss mice per treatment group.

Experiment 6: The effects of peripheral leptin injection on single-housed C57BL/6J mic

All of the high-fat fed mice in Experiments 2-5 responded to peripheral infusions of leptin by reducing body fat content. Others have reported leptin-resistance in high-fat fed mice given daily peripheral injections of leptin (24, 37). Therefore, in this study we tested whether single-housed C57BL/6J mice were resistant to i.p. injections of leptin. Single-housed male and female mice from each dietary treatment were divided into 2 groups and daily food intakes and body weights were recorded for 3 days before initiation of injections. Mice were 35 days of age on the first day of injection. On three consecutive test days each mouse was injected i.p. with either PBS or 30 µg leptin (~1.5 mg/Kg). Food intakes and body weights were recorded for 5 days after the first injection. One hour after the last injection, small blood samples were obtained from the tail vein for measurement of serum leptin concentration. The procedure was repeated one week later with treatments switched.

Statistics

Body weight, weight change, and energy intake measures were analyzed by repeated measure ANOVA with day or time as the repeated measure. Baseline measures of body weight or energy intake were used as covariates in body weight and energy intake analysis and in experiments where mice served as their own control, mouse was

considered a covariate. Organ weights, body composition, and serum measurements were analyzed by ANOVA. Each analysis was initially conducted with data for both males and females (Statistica, StatSoft, OK). The analysis was then repeated independently for each sex. In some instances, the mice were further separated by diet to detect differences between control and leptin treated mice. Differences between individual groups on a specific day was determined by post-hoc Duncan's multiple range test. Differences were considered significant at $p<0.05$.

RESULTS

Experiment 1: The effects of intracerebroventricular leptin injection on single-housed C57BL/6J mice

This experiment tested the effects of centrally injected leptin on male and female C57BL/6J mice fed low-fat or high-fat diet. Weight gain was influenced by both gender and leptin (Gender: $p<0.01$, Leptin: $p<0.001$, Leptin x Time: $p<0.05$: Figure 3.1). Leptin had no effect on weight gain of any of the male mice but reduced weight gain 38 hours after the injection in female mice fed the low-fat diet, but not those fed high-fat diet (Figure 3.1B). The changes in weight gain even of low-fat fed mice were small but were in the same range (3-5% of body weight) reported by others testing the response of C57BL/6J mice to central injections of leptin (24). Male mice fed the high-fat diet had higher cumulative energy intakes than those fed the low-fat diet at 4, 24, 38, and 48 hours post-injection ($p<0.05$: Figure 3.2A) but leptin did not have any significant effect on intake of male mice in either dietary treatment at any time point. Female mice fed high-fat diet had higher energy intakes than those fed the low-fat diet 24 hours after injection ($p<0.01$). Leptin reduced energy intake of low-fat fed female mice 38 ($p<0.01$) and 48

hours ($p<0.04$) post-injection but did not have any significant effect on mice fed high-fat diet (Figure 3.2B). The reduction in energy intake of low-fat fed mice was small (15.7% for females) but similar to that reported by others (24) for mice receiving lateral ventricle injections of leptin.

Leptin injection into the 3rd ventricle had no significant effect on energy intakes of any of the mice (data not shown). Leptin did inhibit weight gain in low-fat fed male mice at 72 hours post-injection and low-fat fed female mice 48 hours post-injection, but did not significantly inhibit weight gain in high-fat fed mice (Gender: NS, Leptin: NS, Diet x Gender: $p<0.03$, Leptin x Diet: $p< 0.05$, Gender x Time: $p<0.02$: Figure 3.3).

Experiment 2: The effects of peripheral leptin infusion on single-housed C57BL/6J mice

This experiment tested the effects of peripheral leptin infusion on single-housed male and female C57BL/6J mice. Serum leptin concentrations were higher in female than male mice and increased with leptin infusion (Table 3.1). Fasting glucose and insulin concentrations on Day 10 of infusion were higher in males than females and the high-fat diet further increased fasting insulin in male mice. Leptin reduced fasting glucose levels in female mice fed low-fat diet and fasting insulin levels in male mice fed high-fat diet (Table 3.1).

Male mice were heavier than females but diet had no effect on their body weight. In contrast, high-fat fed female mice were heavier than low-fat fed females (Figures 3.4C and D). Leptin significantly reduced the body weight of all animals (Gender: $p<0.05$, Diet: NS, Leptin: $p<0.0001$, Gender x Day: $p<0.0001$, Leptin x Day: $p<0.0001$, Gender x Leptin x Day: $p<0.01$: Figure 3.4). Low-fat fed male mice infused with leptin weighed less than their controls from Day 2 of infusion, the difference in body weights was

significant from Day 4 for high-fat fed male mice (Figures 3.4A and B). Similar responses were found in female mice (Figures 3.4C and D).

Male mice had significantly higher energy intakes than females and mice fed the high-fat diet consumed more energy than those fed low-fat diet ($p<0.05$: Figure 3.5), but leptin significantly reduced energy intake of all animals (Gender: $p<0.02$, Diet: $p<0.0005$, Leptin: $p<0.00001$, Day: $p<0.00001$, Gender x Diet: $p<0.05$, Gender x Day: $p<0.0005$, Diet x Day: $p<0.01$: Figure 3.5).

At the end of the experiment, male mice had significantly heavier gonadal and retroperitoneal adipose depots than females (Table 3.2) and high-fat diet increased the size of both retroperitoneal and epididymal fat pads, but leptin reduced the size of these pads in all male mice. In contrast, there was no effect of either diet or leptin treatment on the weight of the mesenteric depot or testes weight. In females, high-fat feeding significantly increased, whereas leptin decreased, the weights of all fat depots measured except the mesenteric depot in high-fat fed mice. There was no effect of either diet or leptin infusion on uterine weight. Due to increased body size, male carcasses were heavier than females with more fat, water, and protein (Table 3.2). Male mice fed high-fat diet had more carcass fat than those fed low-fat diet but leptin reduced carcass fat content in both dietary groups. There were no effects of diet or leptin treatment on carcass weight, water, protein, or ash for male mice. Female mice fed a high-fat diet had heavier carcasses, more fat and more protein than those fed the low-fat diet. In females, leptin infusion reduced carcass weight, fat, and protein in both dietary groups but the effect on protein did not reach significance in mice fed low-fat diet ($p=0.06$). There was no effect of diet or leptin on carcass ash or water in female mice. There was a substantial

(50 - 90%) reduction in efficiency of energy utilization for gain of carcass tissue (Table 3.3) in all leptin infused mice.

Experiment 3: *The effects of peripheral leptin infusion on single-housed NIH Swiss mice.*

Results for the leptin-infused single-housed NIH Swiss mice were similar to those found in C57BL/6J mice and are summarized in Table 3.4. There was no effect of gender on serum leptin concentrations but leptin infusion significantly increased serum leptin levels in all treatment groups. Fasting glucose concentrations were higher in males than females and leptin reduced fasting glucose concentrations in female mice fed low-fat diet ($p=0.05$). There were no differences in fasting insulin levels between any of the groups.

Male mice gained more body weight than females but leptin inhibited body weight gain in both high-fat and low-fat fed male and female mice. In contrast to the C57BL/6J weanlings, there were no gender or diet effects on energy intake. Leptin significantly reduced the total amount of energy consumed by male high-fat fed, but not low-fat fed, mice during the 13-day infusion period. There were no differences in total energy intakes of leptin infused female mice fed either diet. The effects of diet, gender and leptin on organ weights were also similar to those observed in C57BL/6J mice. Male mice had significantly heavier gonadal and retroperitoneal fat pads than females. In male mice, high-fat feeding increased the size of the mesenteric, retroperitoneal, and epididymal adipose pads while leptin infusion decreased the weight of the epididymal and retroperitoneal depots but not the mesenteric depot. No effects were observed on testes weight. In female mice, mesenteric and retroperitoneal fat pad weights were increased with high-fat feeding and leptin reduced the size of the mesenteric,

retroperitoneal, and parametrial adipose depots. There were no effects of diet or leptin on uterine weight.

Experiment 4: The effects of peripheral leptin infusion on group-housed C57BL/6J mice

This experiment tested the effects of peripheral leptin infusion on group-housed male and female mice. Serum leptin concentrations were higher in male than female mice and were approximately doubled with leptin infusion (data not shown). Fasting serum glucose concentrations were higher in male than female mice and neither diet nor leptin influenced fasting glucose or insulin concentrations (data not shown). There was a significant effect of leptin on body weight of all of the mice (Gender NS, Diet NS, Leptin $p<0.0001$, Day $p<0.0001$, Gender x Diet $p<0.05$, Gender x Day $p<0.0001$, Leptin x Day $p<0.0001$, Gender x Diet x Day $p<0.005$: Figure 3.6).

At the end of infusion male mice fed high-fat diet had heavier fat pads than those fed low-fat diet (Table 3.5) but there was no effect of diet on fat pad weights in females. Leptin tended to reduce the weights of all fat pads in all mice but the difference was only significant for the epididymal fat in low-fat fed males, the retroperitoneal fat pad in high-fat fed males and the mesenteric depot in high-fat fed females. There was no effect of diet or leptin on the weights of the testes or uterus of male or female mice, respectively. All aspects of carcass composition except fat were greater for male than female mice (Table 3.5) and leptin reduced carcass weight ($p<0.05$). Carcass fat content was increased in high-fat fed male, but not female, mice compared with low-fat fed animals and leptin tended to reduce carcass fat content in all groups but the difference was only significant for males fed high-fat diet and females fed low-fat diet. The lack of a statistically significant response in low-fat fed males may be due to the minimal amount

of body fat present (<1 g) in these animals because leptin reduced carcass fat content by 30%.

Experiment 5: The effects of peripheral leptin infusion on group-housed NIH Swiss mice

This experiment was conducted to determine whether failure to consistently induce leptin resistance in group-housed mice fed high-fat diet was due to the strain of the mice. Male and female NIH Swiss mice were group-housed and infused with leptin for 13 days and the data are summarized in Table 3.6. Serum leptin levels were higher in males than females and were increased by leptin infusion. The difference did not reach statistical significance in female mice fed high-fat diet. Serum glucose concentrations were higher in male than female mice and increased in female mice fed a high-fat diet. Leptin reduced fasting glucose concentrations of female mice fed the low-fat diet ($p<0.05$). There were no gender, diet, or leptin effects on fasting insulin concentrations.

Leptin significantly inhibited weight gain in female but not male NIH Swiss mice. Gonadal and retroperitoneal fat was significantly heavier in male than female mice and high-fat diet increased the size of these depots in male but not female mice, when all treatment groups within gender were considered. In low-fat fed male mice, leptin reduced the size of the mesenteric, retroperitoneal, and epididymal fat. In low-fat fed females, leptin reduced the weight of the retroperitoneal and parametrial fat. Similar to the results for group-housed C57BL/6J mice, all aspects of carcass composition were greater for male than female mice. Leptin had no effect on protein, water, or ash in any group but reduced body fat content of male and female mice fed the low-fat diet. There was no effect of leptin on body fat content of male or female mice fed high-fat diet.

Experiment 6: The effects of peripheral leptin injection on single-housed C57BL/6J mice

This experiment was conducted to determine if peripheral leptin resistance in high-fat fed mice was dependent upon the method of leptin administration. A single i.p. injection of 30 µg leptin produced an approximate 100-fold increase in circulating leptin concentrations in all mice, measured 1 hour after the last injection (data not shown). There were no effects of diet or leptin on weight gain in male mice, however, leptin inhibited weight gain on the second and third day of injection in low-fat fed female mice (Gender NS, Diet NS, Leptin NS, Day $p<0.0001$, Gender x Leptin $p<0.05$, Gender x Leptin x Day $p<0.05$: Figure 3.7A). There was no significant effect of diet or leptin on the energy intakes of male mice. In female mice, leptin inhibited intake of low-fat fed females on the days of leptin injection (Figure 3.7B)

DISCUSSION

The initial objective of these studies was to develop a mouse model of juvenile diet-induced leptin resistance and we chose to wean mice onto a high-fat diet because it was anticipated that this would accelerate the development of leptin resistance. Other investigators have reported that exposure of 5 week-old mice to a high-fat diet for 16 days induces peripheral, but not central, leptin resistance (37). Therefore, it was surprising to find that 6 - 7 week-old mice that had been weaned onto a high-fat diet showed an attenuated response to the central effects of leptin on food intake and weight gain but remained fully responsive to the effects of peripheral infusions of leptin on body weight and body fat mass. These results are consistent with those of Halaas et al (13) who found that female mice fed a 45% kcal fat diet for 10 weeks lost weight in response to daily i.p. injections of leptin, but contrast with those of Van Heek et al (37) who found

that male mice became resistant to the effects of i.p. injections of leptin on food intake and body weight when they were fed a 45% kcal fat diet for only 16 days. In Experiment 6 we tested whether the method of peripheral leptin administration influenced our interpretation of whether, or not, the mice were leptin resistant. We found that single daily injections of 30 ug leptin, which induced a 100-fold increase in circulating concentrations of leptin, had no significant effect on either food intake or body weight of low-fat or high-fat fed male mice. Thus it is possible that previous studies that found mice resistant to peripheral injections of leptin would find them responsive to peripheral infusions of leptin.

Van Heek et al (37) reported that high-fat fed C57BL/6 mice became resistant to peripheral injections of leptin faster than high-fat fed AKR mice, implying that background strain influences the development of leptin resistance and that a factor, other than dietary fat content, such as body mass or adiposity, is responsible for the leptin resistance (37). In the experiments described here we tested both C57BL/6J and NIH Swiss mice on the high-fat diet and found that both strains of mice were fully responsive to the peripheral infusions of leptin, even though the fat pads of some of the high-fat fed mice were double the size of those in low-fat fed mice. The role of body fat mass in determining leptin-responsiveness also is challenged by a study that shows that rats become resistant to peripheral leptin within 5 days of being offered a high-fat diet, but high-fat fed rats responded to leptin within 1 day of being returned to the low-fat diet (23). These changes in sensitivity to peripheral leptin are too rapid to be determined by the size of body fat stores.

Mistry et al. (25) examined the energetic responses to centrally administered leptin in lean and *ob/ob* pups and found that lateral ventricle injection of leptin did not inhibit food intake until the pups were 28 days old, whereas energy expenditure was stimulated in 17-day old pups. Therefore, although the centrally-mediated stimulation of thermogenesis by leptin develops earlier than centrally-mediated inhibition of food intake in mice, this cannot account for the development of central, but not peripheral, leptin resistance in mice in our experiments because they were 5 to 7 weeks old at the time that leptin was administered. Older mice fed a high-fat diet for 8 weeks become resistant to peripheral injections of leptin but remain responsive to centrally administered leptin. With continued exposure to the high-fat diet the response to centrally injected leptin is attenuated after 16 weeks on the diet (24). In this experiment, we found that consumption of a high-fat diet from weaning attenuated the response to a lateral ventricle and a 3rd ventricle injection of leptin in 6 to 7-week old mice. These results indicate that, in mice that have never consumed a low-fat diet, sensitivity to centrally administered leptin is compromised more quickly than when older animals are switched from a low-fat (chow) to a high-fat diet.

A previous study in lean C57BL/6J mice reported changes in food intake of neonatal mice as early as 30 minutes after central injections of leptin (25). In contrast, we did not observe significant changes in food intake or body weight until 38 hours after lateral or 3rd ventricle injections. The mice in the previous study were only food deprived for 4 hours before the leptin injections, whereas our mice were food deprived for 9 hours. The longer period of food deprivation, and increased hunger of the mice, may have resulted in immediate eating by both leptin-treated and control groups of mice.

The observed delay in the response to leptin may also be explained by observations that lateral ventricle injections of leptin do not immediately block feeding but decrease the size of meals and the rate of feeding in rats (21). A study in which male Wistar rats received 3rd ventricle injections of leptin showed that meal size was only reduced with the second meal after injection (8), which may explain why we did not find any differences in food intake 4 hours after injection in Experiment 1.

Few studies have examined the effects of peripheral leptin infusions in mice fed high-fat diet. One long-term study investigated whether leptin infusions could prevent the development of obesity and diabetes in mice fed a high-fat diet (35). Although subcutaneous infusion of leptin (0.4 mg/kg/day, 8-10 ug/mouse/day) reduced body weight and food intake during the first 5 weeks of infusion, there were no differences in body weights, food intakes, or fat pad weights of leptin-infused and control mice at the end of the 12 week study. The mice in this study (35) were group housed and the results from experiments described here suggest that the housing conditions and the gender of the mice may influence whether leptin infusions change body fat mass.

The differences between single-housed and group-housed mice may be attributable to differences in thermogenic capacity because group-housed animals huddle and reduce the requirement for heat production from each animal. The degree of huddling varies according to ambient temperature and gender, with females more likely to huddle than males (4). Because of the reduced requirement for heat production, brown adipose tissue thermogenesis (20, 22) and food intake (28) are inversely related to number of animals per cage. One of the mechanisms by which leptin induces weight loss is by stimulating heat production (25,27) and leptin has been shown to increase brown

adipose tissue mRNA expression of uncoupling proteins (UCP2 and 3) (31). These observations suggest that loss of body fat in single-housed, leptin treated mice may be, at least partially, dependent on leptin-induced thermogenesis, which is consistent with our observations of decreased efficiency of energy utilization in Experiment 2. In group-housed animals the need for thermogenesis is reduced and the effect of leptin on body composition is limited. This is consistent with observations by Stehling et al. (32) that the reduced body fat mass of juvenile lean Zucker rats injected subcutaneously with leptin was entirely due to an increase in energy expenditure, but that leptin did not stimulate energy expenditure when the rats were reared in thermoneutral conditions (33). Because group-housing in our experiment inhibited leptin activity in high-fat fed, but not low-fat fed mice, diet composition must directly influence the mechanisms by which leptin induces loss of body fat in mice.

In the experiments described here peripheral infusions of leptin reduced the body fat mass of single-housed, high-fat fed mice that had a reduced sensitivity to central injections of leptin. These results suggest either that the central receptors in high-fat fed, but not low-fat fed, mice need to be chronically stimulated by leptin for there to be an effect on food intake or that the body fat-reducing effects of peripheral leptin are not mediated by the same receptors that are responsible for the central inhibition of food intake by leptin, and that some of the response may result from a direct action of leptin on peripheral tissues. *In vitro* (11, 30) and *in vivo* (10) studies have shown that leptin directly stimulates lipolysis in adipocytes and it has been reported that dennervated fat depots are reduced in hyperleptinemic rats (38), implying that the metabolic changes responsible for loss of fat in leptin- treated animals are independent of the activation of

central leptin receptors. The mechanisms by which fat is specifically decreased by peripheral infusions of leptin in high-fat fed weanling mice need to be clarified, but our results suggest that leptin may act directly in the periphery and that part of the response is due to an increase in thermogenesis.

As shown in the results, peripheral infusions of leptin produced variable increases in serum leptin concentrations ranging from no change in concentration (low-fat fed group-housed C57BL/6J females) to a 4-fold increase (single-housed C57BL/6J females). Although all mice received the same dose of leptin and it was not adjusted for body weight, these differences are unlikely to be due to the size of the animals because the group-housed females were smaller than most of the other animals in this study. In addition, the measured serum leptin concentrations did not necessarily correlate with leptin response because body fat content was significantly lower in low-fat fed, leptin-treated group-housed female C57BL/6J mice compared with their controls. One possible explanation for this discrepancy is that the RIA kit we used to measure leptin has been reported to measure leptin binding protein (Ob-Re) in addition to leptin (26), thus no distinction is made between free (bioactive) leptin, bound leptin and leptin binding protein. The effect of leptin infusions on serum fasting glucose and insulin concentrations also varied between treatment groups but the reason for the inconsistent changes in this study are unknown and were not investigated.

In summary, the experiments described here show that mice weaned onto a high-fat diet develop an insensitivity towards peripheral and central injections of leptin at 5-7 weeks of age, however, these mice respond to peripheral infusions of leptin by specifically reducing body fat mass. The results from the group-housing experiments

suggest that leptin partially exerts its effects on body fat in high-fat fed mice through increased thermogenesis and, in situations where thermogenic capacity is reduced, leptin has limited effects on body weight regulation. Because human obesity is associated with 'leptin resistance', characterized by maintenance of an enlarged body fat mass in the presence of increased concentrations of endogenous leptin (5), it is important that we find an appropriate animal model to study this condition. Mice fed a high-fat diet have been reported to be resistant to peripheral injections of leptin (24, 37) but the studies described here demonstrate that, in weanling mice fed a high-fat diet, the response to peripherally administered leptin is determined by gender, strain, housing conditions and method of leptin administration. Therefore, we need to develop a better understanding of the factors that influence the development of leptin resistance before we can evaluate its role in facilitating the development and maintenance of an obese state.

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Table 3.1: Serum hormone concentrations for single-housed C57BL/6J mice in Experiment 2.

	Low-Fat		**High-Fat**		Statistical Summary
	PBS	*Leptin*	*PBS*	*Leptin*	
Serum Leptin (ng/ml)					
Males	1.8 ± 0.7	3.7 ± 1.4	1.8 ± 0.4	3.8 ± 0.6	G: $p<0.005$, L: $p<0.001$,
Females	1.5 ± 0.2^{A}	8.0 ± 0.9^{B}	3.1 ± 1.3^{A}	11.0 ± 2.6^{B}	G x L: $p<0.01$
Fasting Glucose (mg/dl)					
Males	157 ± 10	137 ± 6	139 ± 9	131 ± 7	G: $p<0.01$, L: $p<0.003$
Females	134 ± 11^{A}	117 ± 6^{AB}	141 ± 4^{A}	99 ± 11^{B}	
Fasting Insulin (ng/ml)					
Males	0.38 ± 0.6^{A}	0.22 ± 0.07^{A}	2.46 ± 0.46^{B}	0.50 ± 0.14^{A}	G: $p<0.001$, D: $p<0.001$, L: $p<0.001$,
Females	0.37 ± 0.13	0.18 ± 0.04	0.58 ± 0.22	0.37 ± 0.11	G x D: $p<0.001$, G x L: $p<0.01$,
					D x L: $p<0.005$, G x D x L: $p<0.005$

Data are means ± sem for groups of 5-6 male mice or 7-9 female mice. Fasting glucose and insulin was measured on Day 10 of an i.p. infusion of either PBS or 10 ug leptin/day. Serum leptin levels were measured at the end of the 13 day infusion. Values for a specific parameter that do not share a common superscript are significantly different at $p<0.05$, determined by a two-way ANOVA and post-hoc Duncan's Multiple Range Test. G: Gender, D: Diet, L: Leptin.

Table 3.2: Organ weights and body composition of single-housed male and female C57BL/6J mice infused with leptin or PBS.

	Low-fat		**High-fat**		Statistical Summary
	PBS	*Leptin*	*PBS*	*Leptin*	
Male					
Organ Weights (mg)					
Mesenteric Fat	151 ± 20	119 ± 19	112 ± 15	123 ± 8	G x D: $p<0.02$
Retroperitoneal Fat	70 ± 7^{A}	29 ± 8^{B}	100 ± 13^{C}	61 ± 8^{A}	G: $p<0.001$, D: $p<0.005$, L: $p<0.001$
Epididymal Fat	265 ± 29^{AC}	128 ± 32^{B}	365 ± 29^{C}	193 ± 42^{AB}	G: $p<0.001$, D: $p<0.03$, L: $p<0.001$
Testes	184 ± 6	182 ± 5	185 ± 6	169 ± 23	
Carcass Composition (g)					
Carcass Weight	20.4 ± 0.6	19.1 ± 0.9	22.4 ± 2.1	19.4 ± 0.6	G: $p<0.001$
Fat	1.9 ± 0.1^{A}	1.1 ± 0.2^{B}	2.5 ± 0.3^{C}	1.6 ± 0.2^{AB}	G: $p<0.001$ D: $p<0.008$, L: $p<0.001$
Water	13.3 ± 0.5	13.1 ± 0.5	14.1 ± 1.3	12.7 ± 0.4	G: $p<0.001$

Protein	4.5 ± 0.1	4.2 ± 0.2	5.0 ± 0.5	4.3 ± 0.1	G: $p<0.001$
Ash	0.80 ± 0.02	0.77 ± 0.03	0.86 ± 0.10	0.79 ± 0.04	
Female					
Organ Weights (mg)					
Mesenteric Fat	124 ± 17^{AB}	78 ± 13^{A}	188 ± 18^{C}	142 ± 18^{BC}	G x D: $p<0.02$, D: $p<0.001$, L: $p<0.008$
Retroperitoneal Fat	33 ± 5^{A}	8 ± 2^{B}	75 ± 7^{C}	20 ± 5^{AB}	G: $p<0.001$, D: $p<0.001$, L: $p<0.001$, D x L: $p<0.006$
Parametrial Fat	170 ± 19^{A}	77 ± 8^{B}	261 ± 29^{C}	127 ± 14^{AB}	G: $p<0.001$, D: $p<0.001$, L: $p<0.001$
Uterus	119 ± 13	118 ± 12	97 ± 14	118 ± 18	
Carcass Composition (g)					
Carcass Weight	17.2 ± 0.6^{A}	15.8 ± 0.3^{B}	18.5 ± 0.4^{C}	16.6 ± 0.4^{AB}	G: $p<0.001$, D: $p<0.02$, L: $p<0.001$
Fat	1.3 ± 0.2^{A}	0.6 ± 0.1^{B}	2.0 ± 0.2^{C}	0.9 ± 0.2^{B}	G: $p<0.001$, D: $p<0.004$, L: $p<0.001$

Water	11.5 ± 0.4	11.0 ± 0.2	11.6 ± 0.3	11.3 ± 0.4	G: $p<0.001$
Protein	3.6 ± 0.1^{AB}	3.4 ± 0.04^{A}	4.1 ± 0.2^{C}	3.7 ± 0.1^{B}	G: $p<0.001$, D: $p<0.001$, L: $p<0.003$
Ash	0.75 ± 0.02	0.74 ± 0.01	0.78 ± 0.02	0.75 ± 0.02	

Data are means ± sem for groups of 5-6 male mice or 7-9 female mice. Body composition was determined after 13 days of infusion of either PBS or 10 μg leptin/day. Values for a specific parameter that do not share a common superscript are significantly different at $p<0.05$, determined by a two-way ANOVA and post-hoc Duncan's Multiple Range Test. G: Gender, L: Leptin, D: Diet.

Table 3.3: Efficiency of Energy Utilization in male and female C57BL/6J mice infused with leptin or PBS.

	Low-fat		**High-fat**		Statistical Summary
	PBS	*Leptin*	*PBS*	*Leptin*	
Male					
Gain in Carcass Energy (kcal/12 days)	9.9 ± 1.6^{A}	4.9 ± 1.3^{B}	11.9 ± 1.1^{A}	4.1 ± 1.0^{B}	D: NS, L: p<0.0001, Int: NS
Energy Intake (kcal/12 days)	188 ± 3^{A}	171 ± 5^{B}	216 ± 6^{C}	197 ± 7^{A}	D: P<0.0001, L: p<0.004, Int: NS
Efficiency of gain (%)	5.3 ± 0.8^{A}	2.8 ± 0.8^{B}	5.5 ± 0.6^{A}	2.0 ± 0.5^{B}	D: NS, L: p<0.0002, Int: NS
Female					
Gain in Carcass Energy (kcal/12 days)	7.7 ± 2.6^{A}	0.8 ± 0.9^{B}	8.3 ± 1.4^{A}	1.4 ± 0.7^{B}	D: NS, L: p<0.0001, Int: NS
Energy Intake (kcal/12 days)	177 ± 3^{A}	157 ± 4^{B}	184 ± 6^{C}	169 ± 3^{A}	D: p<0.0001, L: p<0.0001, Int: NS
Efficiency of gain (%)	4.2 ± 1.5^{A}	0.4 ± 0.6^{B}	4.4 ± 0.7^{A}	0.8 ± 0.4^{B}	D: NS, L: p<0.0001, Int: NS

Data are means $\pm$ sem for groups of 5-6 male mice or 7-9 female mice. Gain in carcass energy was calculated from weight gain during infusion and proportional body composition at the end of infusions. It was assumed that the energy cost of gaining 1 g of either fat or protein was 12.6 kcal (29). Energy intake was the total energy consumed during the leptin infusion and efficiency was calculated as energy gain divided by energy intake. Values for a specific parameter that do not share a common superscript are significantly different at $P<0.05$.

Table 3.4: Body weight, energy intake, organ weights, and serum hormones of single-housed NIH Swiss mice infused with leptin or PBS in Experiment 3.

	Low-fat		**High-fat**		Statistical Summary
	PBS	*Leptin*	*PBS*	*Leptin*	
Male					
Preinfusion Weight (g)	25.0 ± 1.3^{A}	25.4 ± 0.9^{A}	29.6 ± 0.7^{B}	29.2 ± 0.8^{B}	D: $p<0.001$
Weight Change (g/13 d)	3.4 ± 0.7^{AC}	0.8 ± 0.7^{B}	4.0 ± 0.4^{A}	1.9 ± 0.5^{BC}	G: $p<0.001$, L: $p<0.002$
Total Energy Intake (kcal/13 d)	227 ± 13^{A}	213 ± 9^{A}	265 ± 6^{B}	233 ± 12^{A}	L: $p<0.005$
Organ Weights (mg)					
Mesenteric Fat	150 ± 21^{AB}	92 ± 16^{A}	200 ± 30^{B}	190 ± 21^{B}	D: $p<0.005$
Retroperitoneal Fat	93 ± 20^{AB}	13 ± 5^{A}	208 ± 35^{C}	103 ± 34^{B}	G: $p<0.001$, D: $p<0.005$, L: $p<0.005$, G x D: $p<.05$

Epididymal Fat	363 ± 72^{A}	90 ± 24^{B}	726 ± 90^{C}	389 ± 78^{A}	G: p<0.001, D: p<0.001, L: p<0.001, G x D: p<0.05
Testes	171 ± 10	174 ± 18	191 ± 9	176 ± 7	
Serum Hormones					
Leptin (ng/ml)	2.2 ± 0.4^{A}	4.9 ± 0.8^{B}	2.2 ± 0.2^{A}	4.1 ± 0.7^{B}	L: p<0.001
Fasting Glucose (mg/dl)	117 ± 24	77 ± 11	121 ± 22	133 ± 9	G: p<0.05
Fasting Insulin (ng/ml)	0.15 ± 0.05	0.50 ± 0.37	0.31 ± 0.17	0.60 ± 0.32	
Female					
Preinfusion Weight (g)	20.9 ± 1.2	21.3 ± 0.6	23.4 ± 0.6	22.8 ± 0.7	D: p<0.05
Weight Change (g/13 d)	1.9 ± 0.8^{AC}	-0.7 ± 0.5^{B}	2.2 ± 0.17^{A}	-0.1 ± 0.6^{B}	G: p<0.0005, L: p<0.001
Total Energy Intake (kcal/13 d)	217 ± 7	212 ± 9	227 ± 7	$210 \pm +6$	
Organ Weights (mg)					
Mesenteric Fat	144 ± 19^{A}	59 ± 7^{B}	203 ± 17^{C}	139 ± 16^{A}	D: p<0.001, L: p<0.001
Retroperitoneal Fat	49 ± 9^{A}	11 ± 7^{B}	91 ± 12^{C}	27 ± 15^{AB}	G: p<0.001, D: p<0.05, L: p<0.001, G x

					D: $p<0.05$
Parametrial Fat	170 ± 26^{AB}	50 ± 27^{B}	314 ± 56^{A}	150 ± 96^{AB}	G: $p<0.001$, L: $p<0.05$, G x D: $p<0.05$
Uterus	164 ± 19	191 ± 36	182 ± 18	153 ± 11	
Serum Hormones					
Leptin (ng/ml)	1.4 ± 0.4^{A}	3.6 ± 0.3^{BC}	1.7 ± 0.5^{AB}	4.8 ± 1.2^{C}	L: $p<0.002$
Fasting Glucose (mg/dl)	103 ± 12	54 ± 20	99 ± 11	88 ± 14	G: $p<0.05$
Fasting Insulin (ng/ml)	0.25 ± 0.12	0.25 ± 0.20	0.13 ± 0.10	0.84 ± 0.49	

Data are means ± sem for groups of 5 male mice or 4-5 female mice. Mice were infused with PBS or 10 µg leptin/day for 13 days from 34 days of age. Weight change is the difference between body weights on Day 13 and preinfusion weight. Fasting glucose and insulin were measured on Day 10 of infusion and serum leptin was measured at the end of the 13-day infusion. Values for a specific parameter that do not share a common superscript are significantly different at $p<0.05$, determined by a two-way ANOVA and post-hoc Duncan's Multiple Range Test. G: Gender, D: Diet, L: Leptin.

Table 3.5: Organ weights and body composition of group-housed C57BL/6J mice infused with leptin or PBS in Experiment 4.

	Low-fat		**High-fat**		Statistical Summary
	PBS	*Leptin*	*PBS*	*Leptin*	
Male					
Organ Weights (mg)					
Mesenteric Fat	102 ± 10	91 ± 12	154 ± 33	131 ± 23	D: p<0.05
Retroperitoneal Fat	45 ± 5^{AB}	17 ± 3^{A}	112 ± 18^{C}	71 ± 21^{B}	D: p<0.005, L p<0.05
Epididymal Fat	221 ± 6^{A}	130 ± 21^{B}	382 ± 54^{C}	285 ± 56^{AC}	D: p<0.001, L: p<0.05, G x D: p<0.05
Testes	189 ± 5	187 ± 4	189 ± 4	190 ± 3	
Carcass Composition (g)					
Carcass Weight	20.7 ± 0.5	19.6 ± 0.7	20.6 ± 0.7	20.0 ± 0.7	G: p<0.001
Fat	1.01 ± 0.18^{AB}	0.74 ± 0.10^{A}	2.24 ± 0.25^{C}	1.59 ± 0.26^{B}	D: p<0.001, L: p<0.05, G x D: p<0.05

Water	14.0 ± 0.3	13.6 ± 0.5	13.0 ± 0.4	13.1 ± 0.3	G: $p<0.001$
Protein	4.9 ± 0.1	4.4 ± 0.2	4.6 ± 0.2	4.5 ± 0.1	G: $p<0.001$
Ash	0.86 ± 0.02^{A}	0.90 ± 0.03^{A}	0.79 ± 0.01^{B}	0.79 ± 0.02^{B}	G: $p<0.005$, D: $p<0.005$
Female					
Organ Weights (mg)					
Mesenteric Fat	138 ± 14^{AB}	126 ± 14^{A}	165 ± 13^{B}	103 ± 9^{A}	L: $p<0.01$
Retroperitoneal Fat	44 ± 8	20 ± 7	62 ± 12	48 ± 18	
Parametrial Fat	249 ± 43	130 ± 24	258 ± 42	195 ± 52	L: $p<0.05$, G x D: $p<0.05$
Uterus	114 ± 16	122 ± 20	112 ± 17	93 ± 9	
Carcass Composition (g)					
Carcass Weight	16.1 ± 0.3	15.1 ± 0.5	16.8 ± 0.7	15.8 ± 0.6	G: $p<0.001$
Fat	2.1 ± 0.3^{A}	1.1 ± 0.2^{B}	2.0 ± 0.3^{AB}	1.7 ± 0.4^{AB}	L: $p<0.05$, G x D: $p<0.05$
Water	10.1 ± 0.3	10.2 ± 0.3	10.7 ± 0.3	10.1 ± 0.2	G: $p<0.001$

Protein	3.3 ± 0.1	3.1 ± 0.1	3.5 ± 0.1	3.8 ± 0.5	G: $p<0.001$
Ash	0.75 ± 0.09	0.71 ± 0.03	0.77 ± 0.04	0.70 ± 0.08	G: $p<0.005$

Data are means ± sem for groups of 8-9 male mice or 7-8 female mice. Mice were housed 3 or 4 per cage and were infused with PBS or 10 µg leptin/day for 13 days from 34 days of age. Values for a specific parameter that do not share a common superscript are significantly different at $p<0.05$, determined by a two-way ANOVA and post-hoc Duncan's Multiple Range Test. G: Gender, D: Diet, L: Leptin.

Table 3.6: Body weight, organ weights, body composition and serum hormones of group-housed NIH Swiss mice in Experiment 5.

	Low-fat		**High-fat**		Statistical Summary
	PBS	*Leptin*	*PBS*	*Leptin*	
Male					
Preinfusion Weight (g)	28.8 ± 1.2	28.6 ± 1.0	31.6 ± 1.1	31.2 ± 0.9	D: $p<0.05$
Weight Change	1.7 ± 0.5	1.0 ± 0.7	0.9 ± 0.7	1.3 ± 0.1	
Organ Weights (mg)					
Mesenteric Fat	225 ± 29^{A}	143 ± 24^{B}	244 ± 24^{A}	203 ± 29^{A}	D: $p<0.05$
Retroperitoneal Fat	167 ± 31^{A}	54 ± 24^{B}	196 ± 30^{A}	146 ± 28^{A}	G: $p<0.001$, D: $p<0.05$, L: $p<0.01$
Epididymal Fat	460 ± 90^{A}	225 ± 73^{B}	627 ± 66^{A}	562 ± 92^{A}	G $p<0.001$, D $p<0.005$, G x D $p<0.05$
Testes	169 ± 6^{A}	179 ± 8^{A}	185 ± 8^{AB}	211 ± 17^{B}	D $p<0.05$
Body Composition (g)					
Carcasss Weight	27.0 ± 1.1	26.4 ± 1.3	28.9 ± 1.5	28.8 ± 0.9	G $p<0.001$

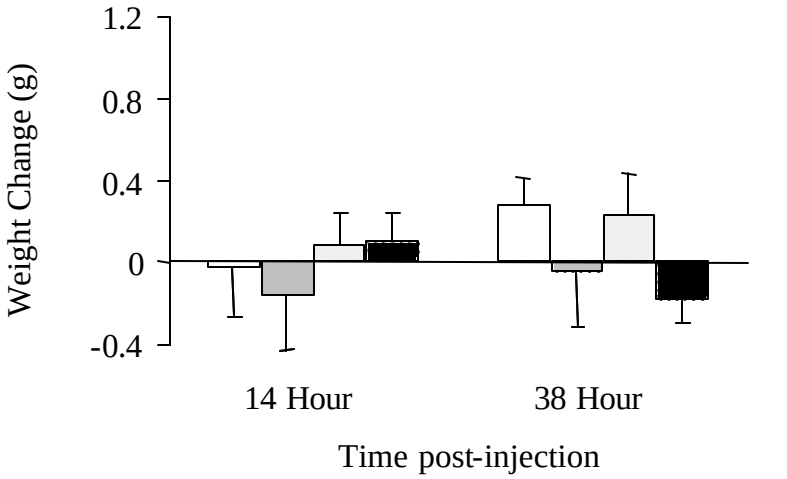
Fat	2.8 ± 0.5^{A}	1.5 ± 0.3^{B}	2.6 ± 0.3^{A}	2.9 ± 0.3^{A}	G $p<0.001$, L $p<0.05$
Water	17.1 ± 0.5	17.9 ± 0.8	18.8 ± 1.0	18.4 ± 0.9	G $p<0.001$
Protein	6.0 ± 0.3	5.8 ± 0.3	6.2 ± 0.3	6.3 ± 0.2	G: $p<0.001$
Ash	1.10 ± 0.02	1.19 ± 0.05	1.26 ± 0.09	1.18 ± 0.06	G: $p<0.001$
Serum Hormones					
Leptin (ng/ml)	1.4 ± 0.3^{A}	3.8 ± 0.5^{B}	1.6 ± 0.3^{A}	3.1 ± 0.5^{B}	G: $p<0.05$, L: $p<0.001$
Fasting Glucose (mg/dl)	117 ± 7	96 ± 7	112 ± 9	115 ± 13	G: $p<0.005$
Fasting Insulin (ng/ml)	0.68 ± 0.20	0.29 ± 0.04	0.72 ± 0.28	0.56 ± 0.19	
Female					
Preinfusion Weight (g)	21.5 ± 0.5	21.7 ± 0.7	22.0 ± 0.9	22.1 ± 1.0	
Weight Change (g/13 d)	2.6 ± 0.2^{A}	1.2 ± 0.2^{B}	2.3 ± 0.3^{A}	1.0 ± 0.4^{B}	L $p<0.001$
Organ Weights (mg)					
Mesenteric Fat	209 ± 15	148 ± 17	161 ± 12	173 ± 36	
Retroperitoneal Fat	60 ± 14^{A}	23 ± 4^{B}	63 ± 7^{A}	45 ± 16^{AB}	G $p<0.001$, L $p<0.05$

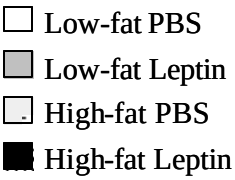
Parametrial Fat	252 ± 51	122 ± 17	223 ± 29	218 ± 64	G $p<0.001$, G x D $p<0.05$
Uterus	190 ± 12	156 ± 14	149 ± 17	167 ± 13	
Body Composition (g)					
Carcasss Weight	21.3 ± 0.4	20.1 ± 0.6	21.1 ± 0.7	20.4 ± 0.8	G $p<0.001$
Fat	2.0 ± 0.2^{A}	1.2 ± 0.1^{B}	1.9 ± 0.1^{A}	1.7 ± 0.2^{AB}	G $p<0.001$, L $p<0.01$
Water	14.2 ± 0.2	13.8 ± 0.4	13.5 ± 1.0	14.6 ± 0.6	G $p<0.001$
Protein	4.1 ± 0.1	4.2 ± 0.2	4.2 ± 0.2	3.4 ± 0.5	G $p<0.001$
Ash	0.94 ± 0.02	1.10 ± 0.11	1.06 ± 0.10	0.96 ± 0.04	G $p<0.001$
Serum Hormones					
Leptin (ng/ml)	1.2 ± 0.1^{A}	2.8 ± 0.3^{B}	1.6 ± 0.4^{A}	2.1 ± 0.4^{AB}	G $p<0.05$, L $p<0.005$
Fasting Glucose (mg/dl)	90 ± 8^{A}	66 ± 9^{B}	109 ± 7^{A}	93 ± 8^{A}	G $p<0.005$, D $p<0.01$, L $p<0.05$
Fasting Insulin (ng/ml)	0.75 ± 0.46	0.24 ± 0.07	0.39 ± 0.11	0.21 ± 0.04	

Data are means ± sem for groups of 7-9 male mice and 7-8 female mice. Mice were group housed and received 13-day i.p. infusions of PBS or 10 ug leptin/day starting at 34 days of age. Fasting glucose and insulin were measured on Day 10 of infusion and serum leptin was measured at the end of the 13-day infusion. Values for a specific parameter that do not share a common superscript are significantly different at $p<0.05$, determined by a two-way ANOVA and post-hoc Duncan's Multiple Range Test. G: Gender, D: Diet, L: Leptin.

Figure 3.1: Change in body weight of male (A) and female (B) C57BL/6J single-housed mice fed low- or high-fat diet and given a lateral ventricle injection of 5 ug leptin or PBS in Experiment 1. Data are means ± sem for groups of 7-9 males or 8-13 female mice. Values for weight change in female mice at 38 hours that do not share a common superscript are significantly different at $P<0.05$, determined by two-way ANOVA and post-hoc Duncan's multiple range test.

A: Male Mice





B: Female Mice

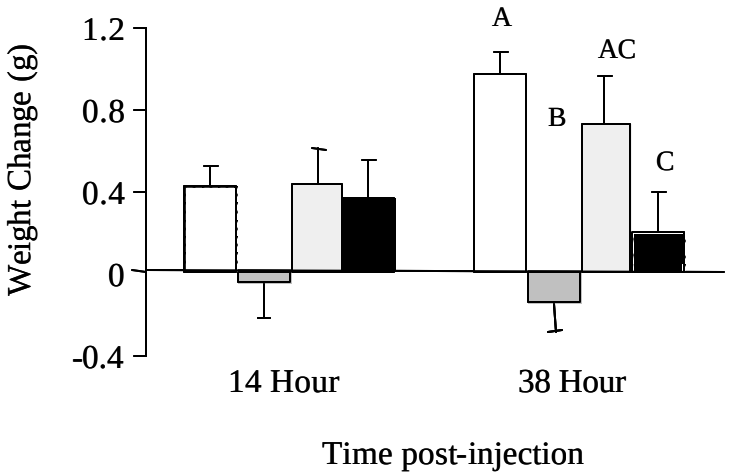
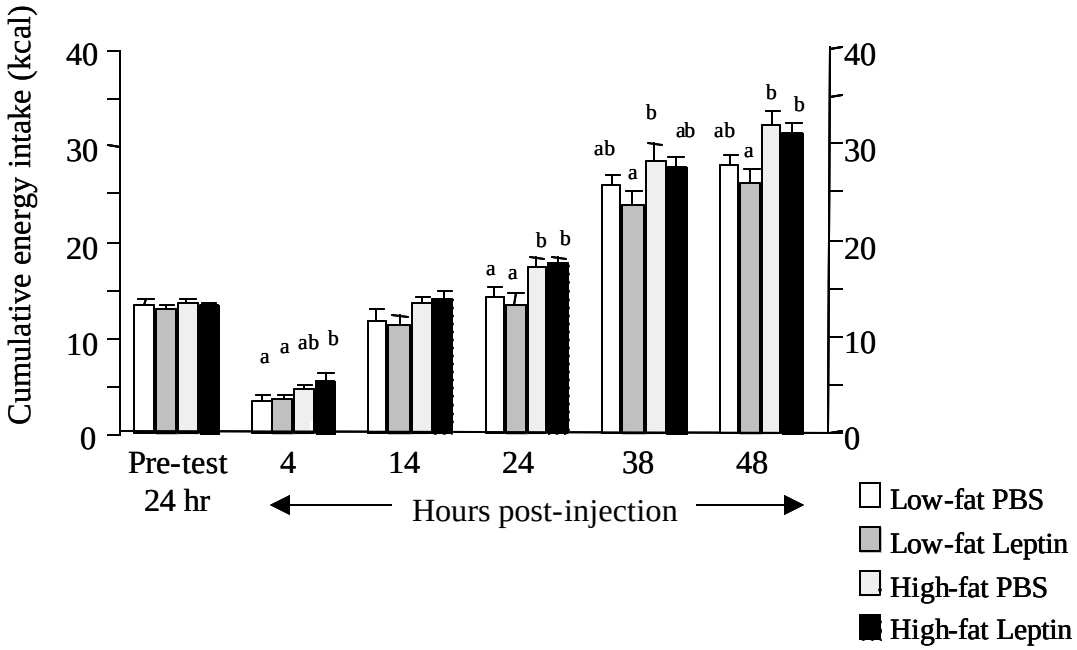


Figure 3.2: Cumulative food intake of male (A) and female (B) C57BL/6J single-housed mice fed low- or high-fat diet and given a lateral ventricle injection of 5 ug leptin or PBS in Experiment 1. Data are means + sem for groups of 7-9 males or 8-13 female mice. Values for intake at specific time points that do not share a common superscript are significantly different at $P<0.05$, determined by two-way ANOVA and post-hoc Duncan's multiple range test. The absence of superscripts indicates no difference between groups at that time point.

A: Male Mice



B: Female Mice

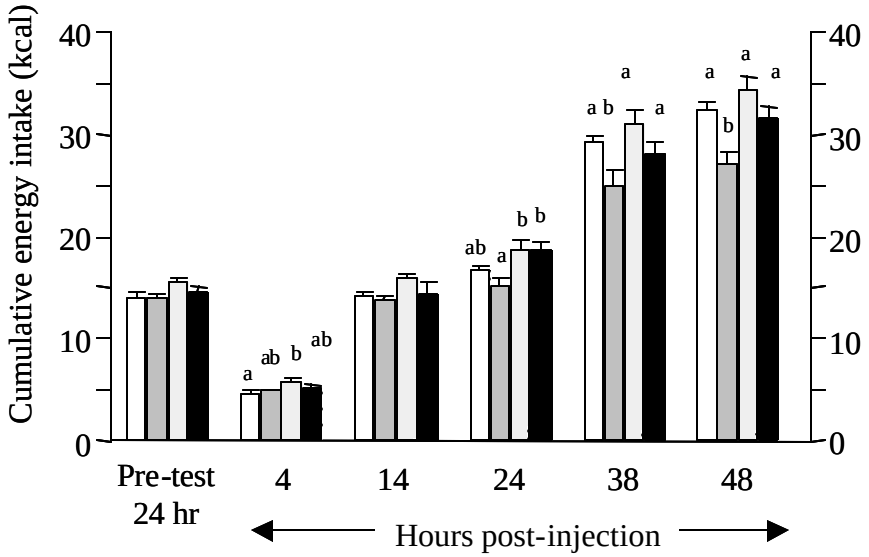


Figure 3.3: Weight change of male (A) and female (B) single-housed C57BL/6J mice that received a 3rd ventricle injection of 0.5 ug of leptin or PBS in Experiment 1. Data are means ± sem for groups of 5 or 6 mice. Values for weight change of females 48 hours after the injection that do not share a common superscript are significantly different at $P<0.05$, determined by two-way ANOVA and post-hoc Duncan's multiple range test.

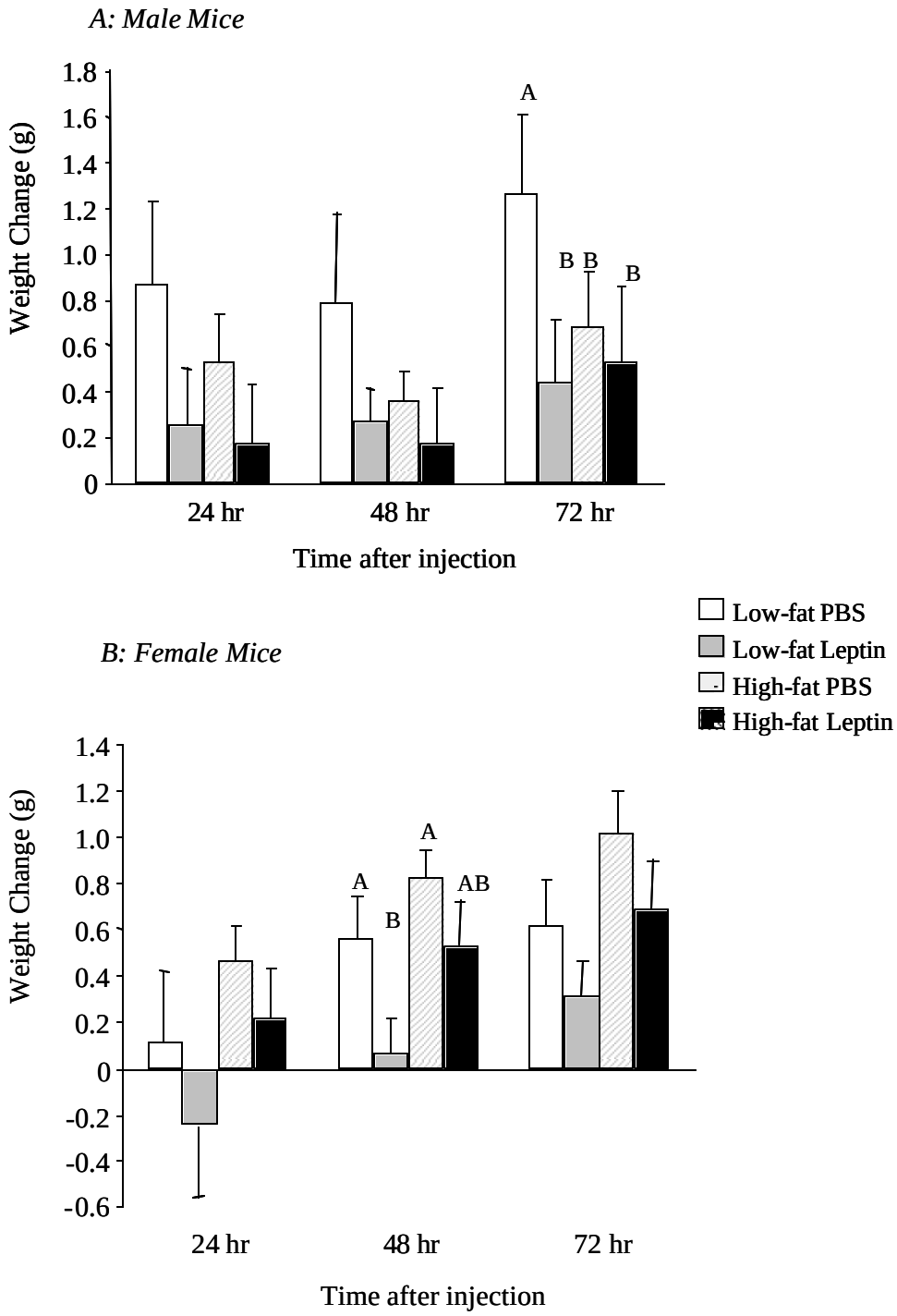
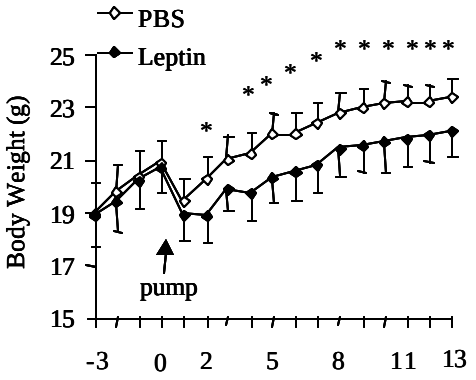
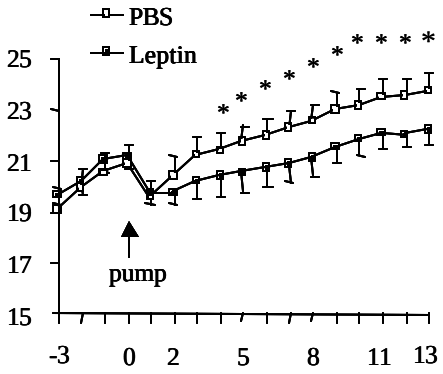

A: Male Mice
Weight Change (g)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
A
B B
B
24 hr
48 hr
72 hr
Time after injection
Low-fat PBS
Low-fat Leptin
High-fat PBS
High-fat Leptin
B: Female Mice
Weight Change (g)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
A
B
A
AB
24 hr
48 hr
72 hr
Time after injection

Figure 3.4: Daily body weights of male (A and B) and female (C and D) single-housed C57BL/6J mice that were infused with 10 ug leptin/day from age 34 days in Experiment 2. Data are means ± sem for groups of 5-6 male mice or 7-9 female mice. Asterisks indicate a significant ($P<0.05$) difference between leptin-treated and control mice, determined by repeated measures analysis of variance and post-hoc t-tests.

A: Single Housed Low-fat Male

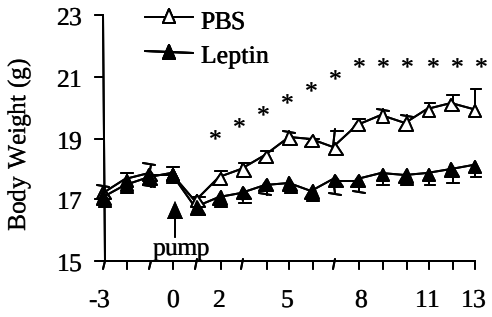


B: Single Housed High-fat Male

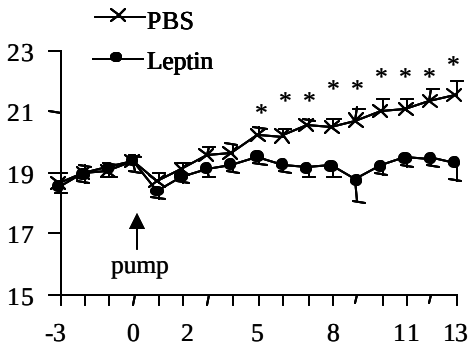


Day of Experiment

C: Single Housed Low-fat Female



D: Single Housed High-fat Female



Day of Experiment

Figure 3.5: Daily food intakes of male (A and B) and female (C and D) single-housed C57BL/6J mice that were infused with 10 ug leptin/day from age 34 days in Experiment 2. Data are means ± sem for groups of 5-6 male mice or 7-9 female mice. Asterisks indicate a significant ($P<0.05$) difference between leptin-treated and control mice, determined by repeated measures analysis of variance and post-hoc t-tests.

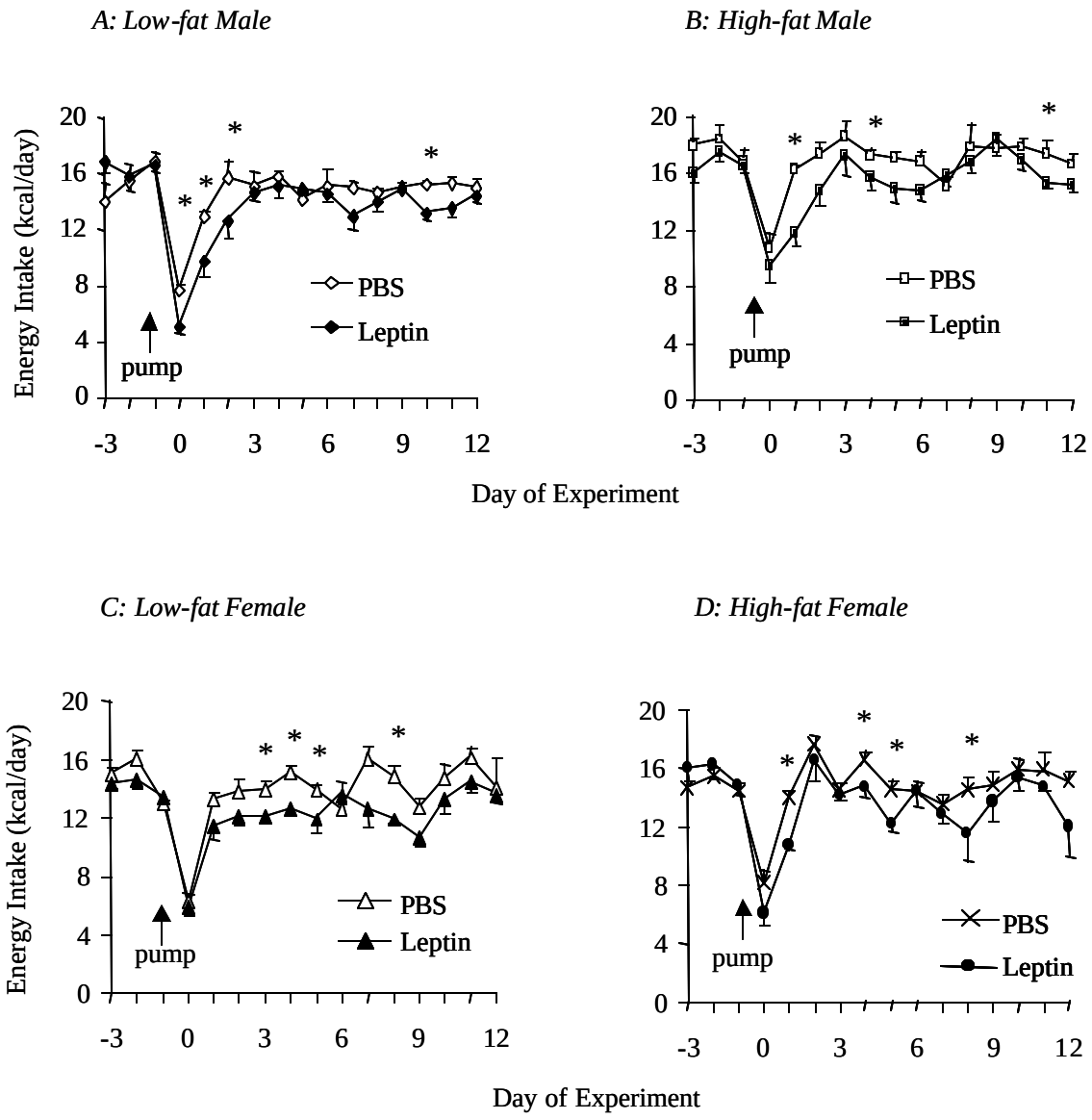
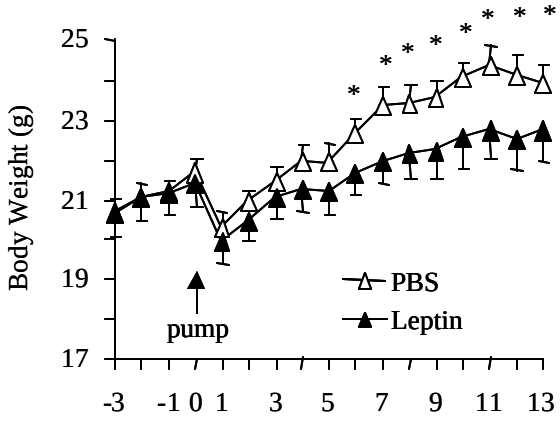
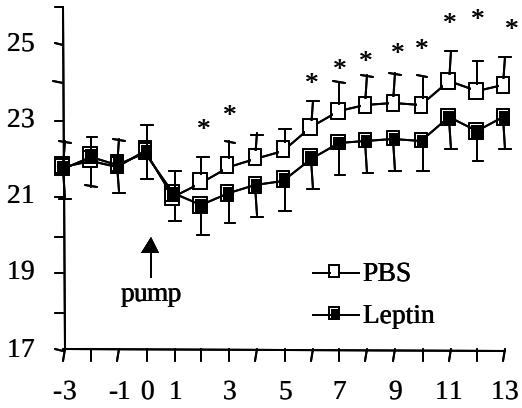

A: Low-fat Male
B: High-fat Male
C: Low-fat Female
D: High-fat Female
Energy Intake (kcal/day)
Day of Experiment
PBS
Leptin
pump
0
4
8
12
16
20
-3
0
3
6
9
12

Figure 3.6: Daily body weights of male (A and B) and female (C and D) group housed C57BL/6J mice that were infused with 10 ug leptin/day from age 34 days in Experiment 4. Data are means ± sem for groups of 8-9 male mice or 7-8 female mice. Asterisks indicate a significant ($P<0.05$) difference between leptin-treated and control mice, determined by repeated measures analysis of variance and post-hoc t-tests.

A: Group Housed Low-fat Male

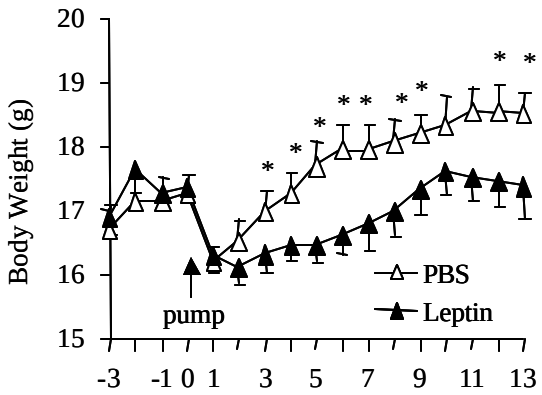


B: Group Housed High-fat Male

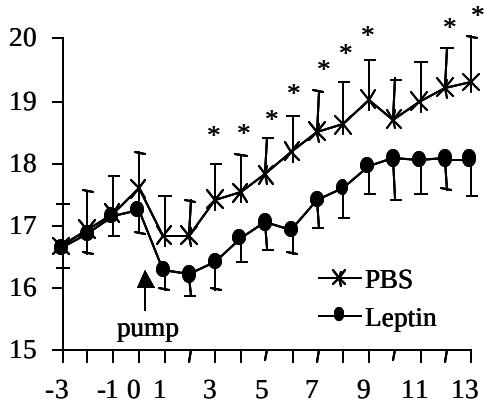


Day of Experiment

C: Group Housed Low-fat Female



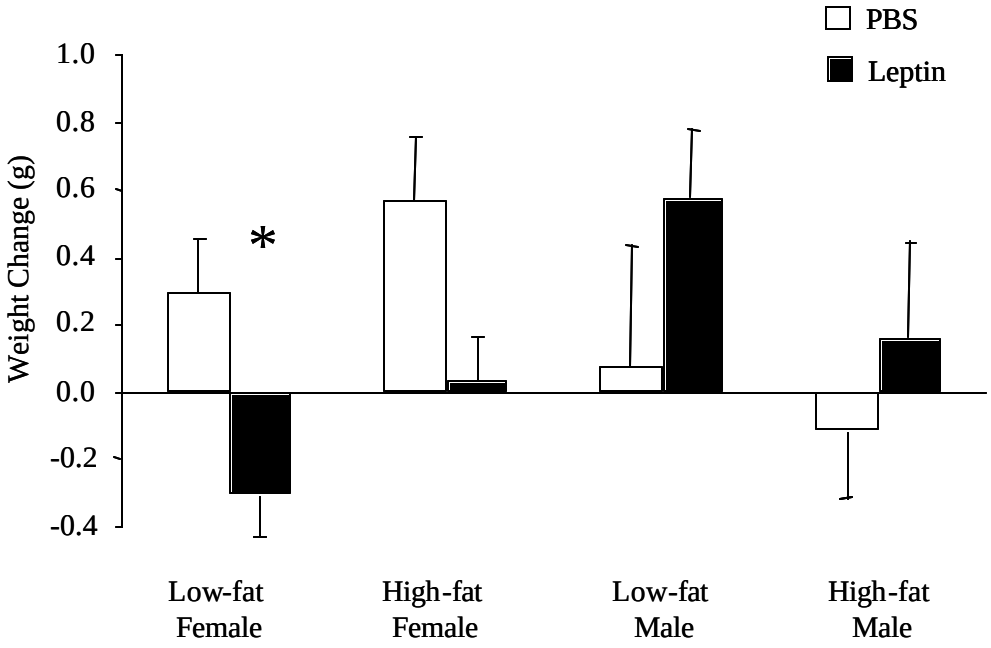
D: Group Housed High-fat Female



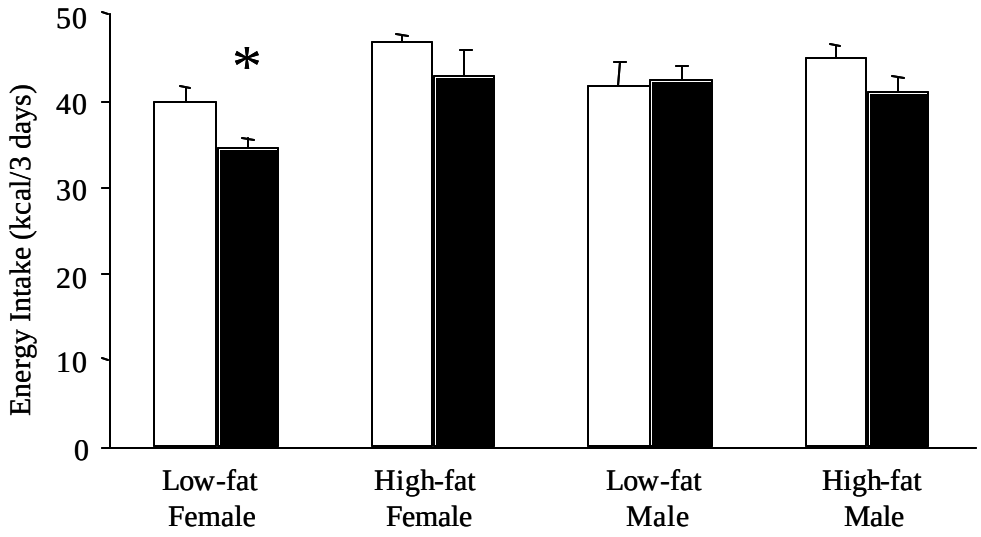
Day of Experiment

Figure 3.7: Weight change (A) and food intake (B) of single-housed C57BL/6J mice that were injected i.p. with 30 ug of leptin in each of three days in Experiment 6. Data are means + sem for groups of 5 or 6 mice. Asterisks indicate significant differences ($P<0.05$) between control and leptin-treated low-fat fed female mice, determined by repeated measures analysis of variance and post-hoc t-test.

A: Weight Change on Day 3



B: Energy Intake



CHAPTER 4

PERIPHERAL LEPTIN INFUSIONS FAIL TO STIMULATE LIPOLYSIS IN ADIPOCYTES FROM MICE FED EITHER LOW-FAT OR HIGH-FAT DIET[2]

[2] Bowen, H.M. and R.B.S. Harris. To be submitted to American Journal of Physiology

ABSTRACT

The aim of this study was to determine whether mice fed high-fat diet were resistant to peripheral leptin infusions and if the resistance was associated with reduced leptin-induced lipolysis. It was further determined if there were changes in leptin receptor protein levels in serum or peripheral tissues. Peripheral leptin infusions (10 μg/day) for 13 days had no effect on the body weight or food intake of 15-week-old male C57BL/6J mice fed low-fat (10% kcal fat) or high-fat (45% kcal fat) diet for 5 weeks. Leptin-infused mice fed a high-fat diet had smaller epididymal and mesenteric fat depots and reduced body fat content compared with their controls. Five-day infusions of leptin in 15-week-old male mice fed low-fat or high-fat diet for 5 weeks had no effect on adipocyte glycerol or free fatty acid release. There were no changes in leptin receptor protein levels in the serum, liver, lung, or white adipose tissue in response to diet or leptin treatment. These results suggest that leptin resistance is determined by factors beyond dietary fat content and the mechanisms involved remain to be determined.

INTRODUCTION

Leptin, a16 kD circulating protein, is secreted primarily by adipose tissue and is hypothesized to be the adipose-derived signal important for regulation of fat stores (42). Peripheral (13, 15, 20, 30, 12) and central (3) leptin administration has been shown to reduce food intake and body weight in lean mice and the changes in food intake and body weight are thought to be mediated through hypothalamic long-form leptin receptors, Ob-Rb (35). Expression of Ob-Rb is not limited to the hypothalamus but has been detected in most peripheral tissues including the lung (24, 36, 39) and adipose tissue (2, 19, 24). In mice, there are three short-isoforms of the leptin receptor, Ob-Ra, Ob-Rc, Ob-Rd, that are ubiquitously expressed in both central and peripheral tissues (16, 19, 39). In addition, there is a soluble leptin receptor, Ob-Re, capable of binding leptin in the serum (6, 23). It is unknown whether this binding renders the protein inactive or protects the protein from degradation during transport. The roles of the short- and soluble-isoforms of the leptin receptor in mediating leptin's actions remain to be determined.

Leptin causes weight loss that is primarily loss of body fat but the magnitude of fat loss exceeds that expected from the reduced energy intake (15, 20), which implies that energy expenditure must be increased by leptin treatment. The mechanisms by which leptin specifically reduces body fat are poorly understood. It has been proposed that, at high concentrations, leptin may have direct effects on adipocyte metabolism (41). Leptin, at supraphysiologic concentrations, has recently been shown to stimulate lipolysis both *in vivo* (10) *and in vitro* (9, 11, 31, 34). Currently, there are no *in vivo* studies on the effects of physiological concentrations of leptin on lipolysis.

In obese persons serum leptin levels are high, however, these high levels do not inhibit food intake or body weight (5). From this observation, it has been proposed that obesity is a condition associated with 'leptin resistance' (26), which may be defined as a reduced response, or a lack of response, to leptin's ability to reduce food intake and maintain a constant body weight. Studies in mice have shown a resistance to leptin's effects on food intake and body weight in animals fed a high-fat diet (22, 37). Similarly, we have found that 15-week-old Swiss mice fed a high-fat diet for 5 weeks do not lose body fat in response to peripheral infusions of leptin (carcass fat content: low-fat controls: 2.5 ± 0.3 g, low-fat leptin: 1.2 ± 0.3 g, high fat control: 2.6 ± 0.2 g, high-fat leptin: 2.6 ± 0.4 g). Various mechanisms for 'leptin resistance' have been suggested including abnormal leptin synthesis or secretion (1), impaired transport of leptin into the brain (4, 33), dysfunctional leptin receptors (27), and altered post-receptor signaling (1). Studies suggest that high-fat diets do not affect Ob-Ra or Ob-Rb mRNA expression in the brain (8, 25), liver, brown adipose tissue, or white adipose tissue (29) but down-regulate hypothalamic Ob-Rb and Ob-Ra protein levels (25).

Using C57BL/6J mice, we sought to confirm the high-fat diet induced leptin resistance observed in 15-week old Swiss mice and to examine the effects of high-fat feeding on leptin's peripheral actions. Therefore, we examined the effects of high-feeding on leptin receptor protein in serum and peripheral tissues including the liver, white adipose tissue, and lung. We expected high-fat diets to induce leptin resistance by inhibiting leptin-induced lipolysis and down-regulating leptin receptor protein levels in white adipose tissue.

METHODS

Animals and Diet

Nine-week-old male C57BL/6J mice were purchased from Harlan Laboratories (Indianapolis, IN). Mice were housed on bedding at 76°F with lights on 12 hours/day from 7.00 a.m. with free access to food and water. From 10 weeks of age, mice were fed a low-fat diet containing 10% kcal as fat (Diet 12450B; Research Diets, NJ) or a high-fat diet containing 45% kcal as fat (Diet 12451; Research Diets, NJ). At 14 weeks of age, the mice were placed in cages with grid floors to allow for measures of food intake.

Experiment 1: Leptin Sensitivity

This experiment was conducted to determine if adult C57BL/6J mice fed high-fat diet develop leptin resistance. After 3 days of adaptation to the grid floors, daily food intakes and body weights were measured for 3 days and mice in each diet treatment were divided into 2 weight-matched groups of 8-9 mice. At 15 weeks of age, mice were anaesthetized by isoflourane inhalation and were fitted with intraperitoneal (i.p.) Alzet miniosmotic pumps (Model 1002; Durect Corp., CA). One group was infused with PBS and the other with 10 µg leptin /day (recombinant murine leptin: R&D Systems, MN). Five days after pump placement, mice were deprived of food from 8.00 a.m. to 11.00 a.m. and blood samples were obtained by tail-bleeding for measurement of triglycerides (Sigma kit 337-B: Sigma Chemical Co., MO) and free fatty acids (FFA: NEFA C kit: WAKO Chemicals, VA). Blood glucose was measured using a glucometer (Glucometer Elite; Bayer Corp., NY). After 13 days of infusion, mice were decapitated and trunk blood was collected. Inguinal, mesenteric, retroperitoneal, and epididymal fat depots and

testes were dissected and weighed and the gut was cleaned. All tissues were returned to the carcass for determination of body composition, as described previously (14).

Experiment 2: Effects of high-fat diet on leptin-induced lipolysis and leptin receptor protein

This experiment was designed to test the effects of high-fat feeding and *in vivo* peripheral leptin infusions on lipolysis and leptin receptor protein levels. After 3 days of adaptation to the grid floors, daily food intakes and body weights were measured for 3 days and mice in each diet treatment were divided into 2 weight-matched groups of 10-11 mice. At 15 weeks of age, mice were anaesthetized by isoflourane inhalation and were fitted with intraperitoneal (i.p.) Alzet miniosmotic pumps (Model 1007D; Durect Corp., CA). One group was infused with PBS and the other with 10 µg leptin /day (recombinant murine leptin: R&D Systems, MN). On Day 5 of infusion, mice were food deprived from 7.00 a.m. to 10.00 a.m. At 10.00 a.m., mice were decapitated and trunk blood collected for measurement of serum leptin (Mouse Leptin RIA: Linco Research Inc., MO), triglycerides (Sigma kit 337-B: Sigma Chemical Co., MO), and free fatty acids (FFA: NEFA C kit: WAKO Chemicals, VA). Liver, lung, inguinal, retroperitoneal, and epididymal adipose tissues were weighed. Epididymal and retroperitoneal depots were combined for measurement of glycerol and free fatty acid release from isolated adipocytes. Samples of liver, lung, and inguinal fat were snap-frozen for measurement of short- and long-form leptin receptor protein by Western blot.

Epididymal and retroperitoneal fat pads were pooled and adipocytes were isolated from the tissue by collagenase digestion [1 mg collagenase/ ml in Krebs Ringer bicarbonate buffer (KRBC), 5.0 mM glucose, 4% BSA, pH 7.5] for approximately 30

minutes at 37°C. The cells were filtered through a 250 µm nylon screen and washed 3 times in collagenase-free KRBC. Following a thirty-minute rest period, 0.2 ml of isolated cells (45,000-65,000 cells) were incubated in shaking water baths with 0.4 mL KRBC, 5 mM glucose, 2% BSA, 0.1 mM ascorbic acid and 0.8 U/ml adenosine deaminase for 2 hours at 37°C. Glycerol release into media was measured in triplicate for basal conditions and in duplicate for norepinephrine-stimulated conditions (100 nM and 10 µM norepinephrine). All reactions were stopped by injecting 0.5 ml 1.0 N H_2SO_4 into each flask. Incubation media was aspirated from beneath the cells and frozen at –80°C for measurement of glycerol and free fatty acid concentrations.

Glycerol content was determined in deproteinized media samples by an enzymatic/fluorometic method described by Edens et al. (7). Samples of incubation media were deproteinized with 0.65 N perchloric acid and neutralized with imidazole-KOH buffer. The reaction mixture consisting of glycine buffer [0.21 M glycine, 0.1% 2 M magnesium chloride, 0.002 M ATP, pH 9.1], 67.5 U glycero-3-phosphate dehydrogenase, 15.3 U glycerokinase, 115 ul hydrazine hydrate was added to neutralized media samples in a 96-well plate. After the addition of NAD^+, glycerol release was determined by flourescence of NADH. Free fatty acid content of incubation media samples were determined by enzymatic colorimetric assay (FFA: NEFA C kit: WAKO Chemicals, VA). Glycerol and fatty acid release were expressed per 10^6 cells per 2 hours. Cell number and size distribution were determined by fixing 0.2 ml aliquots of isolated cells in osmium tetroxide (3%) (Electron Microscopy Sciences, PA). Fixed adipocytes were rinsed with 0.9% NaCl and filtered through a 240 µm nylon screen and collected on

a 20 μm nylon screen. Samples were suspended in 4% NaCl and analyzed on a Coulter electronic particle counter (Coulter Multisizer, Coulter Electronics, FL).

The liver, lung, and inguinal pads from each mouse fed either a low- or high-fat diet were homogenized in Krebs bicarbonate buffer, pH 7.5, containing protease inhibitors (10 μM leupeptin, 2 U/ml aprotinin, and 1 μM phenylmethylsulfonylflouride). A crude membrane fraction was prepared by centrifuging the homogenate for 10 min at 6,000 x g and then recentrifuging the supernatant at 6,000 x g for 10 min. Samples (50 μg) of the resulting supernatant from each tissue and 3 μl samples of serum were separated by SDS-PAGE in a 9% acrylamide gel in a Tris glycine buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS, pH 8.3). The gel was cut so that proteins larger than 66 kD were contained on the upper half of the gel and proteins smaller than 66 kD were on the bottom half. Proteins from multiple gels were transferred to a single polyvinylidene difluoride membrane (Boehringer Mannheim, Mannheim, Germany) in 25 mM Tris, 192 mM glycine, and 20% methanol buffer. Leptin receptor was detected by Western blot using rabbit antimouse OB-R antibody (Affinity Bioreagents Golden, CO) at a 1:500 dilution and an HRP-linked antirabbit IgG POD secondary antibody (Boehringer Mannheim) at a dilution of 1:10,000. Actin was detected using a monoclonal antibody (Monoclonal AC-40: Sigma Chemical Co., MO) at a 1:1,000 dilution and a peroxidase conjugated antimouse IgG secondary antibody at a dilution of 1:4,000. The blot was developed using a chemiluminescence system (BM Chemiluminescence Blotting Substrate, Boehringer Mannheim) according to the manufacturer's directions. The X-ray films were scanned and band density for the short-form and long-form leptin receptors

and actin were quantified using UnScan-It software (Silk Scientific Inc., Orem, UT). The amount of Ob-R protein was expressed as a ratio to actin.

Statistics

Body weight, weight change, and energy intake measures were analyzed by repeated measure ANOVA with day/time as the repeated measure. Baseline measures of body weight or energy intake were used as covariates in body weight and energy intake analysis. Organ weights, body composition, serum measurements, lipolysis, and cell size distribution were analyzed by ANOVA. Differences between individual groups were determined by post-hoc Duncan's multiple range test. Differences were considered significant at $p<0.05$.

RESULTS

Experiment 1: Leptin Sensitivity

This experiment was conducted to determine if 15-week old male C57BL/6J mice fed a high-fat diet for 5 weeks were resistant to peripheral infusions of leptin. Neither diet nor leptin significantly reduced body weight (Figure 4.1) or energy intake (Figure 4.2), however, there was an effect of day ($p<0.001$) for both body weight and energy intake and an interaction of diet and day ($p<0.01$) for body weight only. On Day 7, mice fed high-fat diet had higher energy intakes than mice fed low-fat diet and there was a significant reduction of energy intake in high-fat fed leptin treated mice ($p<0.05$) compared to their controls.

High-fat diet increased the weight of all fat pads examined (Table 4.1). Leptin reduced the size of the epididymal, mesenteric, and retroperitoneal depots in mice fed

high-fat diet but not mice fed low-fat diet. There were no effects of diet or leptin on testes weight.

Mice fed the high-fat diet had heavier carcasses than mice fed low-fat diet with more fat and protein (Table 4.1). Mice fed the low-fat diet had higher ash content than mice fed the high-fat diet, indicating greater skeletal growth in mice fed low-fat diet. High-fat diet increased body fat content. Leptin reduced body fat content of mice fed high-fat but not low-fat diet. When fat was expressed as a percent of carcass weight, the effect in high-fat fed leptin infused mice was significantly different compared to their controls ($p<0.05$; low-fat control 11.4%; low-fat leptin 10.3%; high-fat control 16.3%; high-fat leptin 13.3%).

Mice fed the high-fat diet were hyperglycemic compared to mice fed the low-fat diet (Table 4.2). Serum triglycerides, glycerol, and free fatty acids were not affected by diet or leptin (Table 4.2).

Experiment 2: Effects of high-fat diet on leptin-induced lipolysis and leptin receptor protein

In this study, the effects of peripheral infusions of leptin on lipolysis were determined in C57BL/6J mice fed low-fat or high-fat diet for 5 weeks. Leptin inhibited body weight gain, however, the effect did not reach significance in mice fed low-fat diet compared to their controls (Table 4.3). Leptin inhibited total energy intake during the infusion period. Leptin had no effect on fat pad weights at the end of the 5-day infusion, however, high-fat feeding increased the size of all depots examined.

Isolated adipocytes from mice fed high-fat diet had higher rates of glycerol and free fatty acid release per 10^6 cells than those from mice fed low-fat diet (Figure 4.3).

Addition of both 100 nM and 10 µM norepinephrine to the incubation media stimulated glycerol and free fatty acid release. There were no effects of leptin on basal or norepinephrine stimulated glycerol or free fatty acid release. When norepinephrine-stimulated glycerol or free fatty acid release was expressed as percent of basal lipolysis there were no differences between any of the groups (data not shown).

Cell number and size distribution was determined for aliquots of adipocytes isolated from the combined epididymal and retroperitoneal adipose pads. For all groups, more than 90% of the total cell volume was composed of cells less than 150 µm in size (Figure 4.4). Control mice fed a low-fat diet had a higher percentage of cells in the size range of 51-75 µm compared to control mice fed high-fat diet.

Leptin receptors were measured by Western blot in the serum, inguinal adipose tissue, liver, and lung. The short-form receptor(s) were detected in all tissues examined and the long-form receptor was detected in the lung (Figure 4.5). There were no differences in leptin receptor protein in any tissue tested for any of the groups (data not shown).

At the end of the 5-day infusion, serum leptin concentrations increased in all animals infused with leptin, however, the effect did not reach significance in high-fat fed mice (Table 4.3). Serum triglycerides were decreased in leptin infused mice fed high-fat diet compared to the other groups. There were no effects of leptin or diet on serum glycerol concentrations. Mice fed low-fat diet had higher serum free fatty acids compared to mice fed high-fat diet.

DISCUSSION

This study was conducted to determine the effects of feeding a high-fat diet on leptin-induced lipolysis and leptin receptor protein levels in peripheral tissues. Others have reported that mice fed a high-fat diet develop a resistance to leptin's effects on food intake and body weight (37). Preliminary studies in our laboratory demonstrated that 15-week-old male Swiss mice consuming a high-fat diet for 5 weeks were resistant to the lipopenic effects of peripheral leptin infusions whereas leptin significantly reduced body fat content of low-fat fed mice. Therefore, it is surprising that in this study, high-fat fed mice remained metabolically sensitive to infused leptin. The results described here suggest that 'leptin resistance' is not determined by dietary fat content and the differences between this study and our preliminary data suggests that there may be an effect of background strain on leptin responsiveness. The influential effects of strain are supported by Van Heek et al. (37) who found that C57BL/6J mice fed high-fat diet became resistant to the effects of peripheral leptin injections faster than AKR mice fed the same high-fat diet. Furthermore, AKR mice fed both low-fat or high-fat diet for 56 days failed to decrease food intake and body weight in response to peripheral leptin injections, indicating that a factor other than dietary fat content is responsible development of resistance (37). In addition to diet, it has been shown that aging is associated with leptin resistance. Wang et al. recently demonstrated that 18-month old Zucker rats have reduced responses to adenovirus-induced hyperleptinemia in comparison with 2-month old rats (40). Similarly, 12-month-old C57BL/6J mice peripherally infused with leptin (0.15-1.5 μg/hour) have delayed decreases in food intake, body weight, and body fat loss (18). Scarpace et al. (32) found that aged rats (30 months

old) were less sensitive than young rats (6 months old) to 7-day infusions of pharmacological doses (1 mg leptin/day) with smaller changes in food intake and no change in oxygen consumption.

Both high-fat feeding and increasing age are associated with concurrent increases in adiposity, therefore, it is unclear from the studies described above if fat mass, dietary components, or age of the animals determines the development of leptin resistance. One study found that acute changes in dietary composition determine leptin sensitivity in rats (21). For example, leptin sensitivity was reduced after only 5 days of high-fat diet exposure and returned as early as 1 day after reintroducing a low-fat diet (21). These changes occured too quickly to be due to adiposity. In contrast, a study by Vaselli et al. (38) suggests that resistance to inhibitory effects of leptin on feeding and body weight are determined by adiposity and not diet composition. The complexity of leptin resistance requires additional studies to help us clearly define and understand those factors contributing to its development.

In a separate experiment, we examined how leptin-induced lipolysis and peripheral leptin receptors were affected by dietary fat content. Although this experiment was conducted in a separate group of mice it is reasonable to assume that these mice were reflective of those in the first experiment as they were the same age, sex, and strain. In addition, we measured serum triglycerides and free fatty acids in both groups and compared the measurements. Serum samples from each experiment were collected after a similar period of fasting at the same time during the infusion, however, the samples obtained in Experiment 1 were from tail-bleeds whereas those in Experiment 2 were collected from the trunk at sacrifice. There were no differences in free fatty acid

concentrations between the two groups of mice and there was no effect of leptin on serum glycerol concentrations although glycerol levels were twice as high in Experiment 1 compared with those in Experiment 2.

In Experiment 2, we found that adipocytes isolated from 16-week old mice infused with physiological doses of leptin did not have increased rates of glycerol release. This is in contrast to another study that found increasing lipolytic rates in adipocytes obtained from ob/ob and lean mice after a single intraperitoneal injection leptin one hour prior to sacrifice (10). One milligram per kilogram doses of leptin were sufficient to increase glycerol release in adipocytes from ob/ob mice, however, glycerol release was only increased in lean mice after 10 mg/kg (~300 µg); doses that are probably not reflective of physiological effects of leptin. The lack of response in adipocytes isolated from high-fat fed mice which had a significant reduction in body fat in Experiment 1 suggests, that at least in early stages of leptin infusion, there are no measurable increases in lipolytic activity to account for fat loss. Although this study focused on lipolysis the fat loss observed in these mice may be due to decreased lipogenesis or secondary to reduced cell number.

In the present study, we found no differences in protein levels of Ob-Re in the serum or the short- and long-isoform(s) of the leptin receptor in peripheral tissues, suggesting that high-fat diets do not change leptin receptor protein levels in the serum, liver, lung, and inguinal adipose tissue. The leptin receptor in the serum represents the soluble leptin receptor, Ob-Re, which binds leptin in the circulation (6, 23). The role of the leptin bound to Ob-Re in the circulation is not completely understood but the binding may prolong the half-life of leptin and/or delay leptin clearance (17, 28). In addition,

bound leptin may be biologically inactive (17). The role of the short-isoforms of the receptors are not clear, however, expression of the long-form receptor in the lung has been suggested to mediate leptin's effects on cell proliferation in this tissue (36). Although several studies have reported Ob-Rb mRNA expression in the lung (24, 36), this is the first study to report protein expression in the lung.

In conclusion, we show that development of leptin resistance occurs in response to factors other than dietary fat content. These factors, which may include adiposity or aging, should be further studied so that appropriate intervention therapies can be developed. Additionally we show that physiological concentrations of leptin administered *in vivo* are incapable of stimulating glycerol or free fatty acid release. Feeding a high fat diet fails to change leptin receptor protein levels in the serum, lung, liver, and inguinal adipose tissue.

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Table 4.1: Organ weights and body composition of C57BL/6J mice infused with leptin or PBS.

	Low-fat		**High-fat**		
	PBS	Leptin	PBS	Leptin	Statistical Summary
Organ Weights (mg)					
Inguinal Fat	383 ± 43^{A}	333 ± 37^{A}	689 ± 56^{B}	550 ± 78^{B}	D $p<0.0001$
Epididymal Fat	469 ± 58^{A}	416 ± 29^{A}	770 ± 52^{B}	579 ± 92^{A}	D $p<0.001$
Retroperitoneal Fat	146 ± 23^{AB}	93 ± 11^{B}	250 ± 22^{C}	181 ± 28^{A}	D $p<0.0005$, L $p<0.05$
Mesenteric Fat	175 ± 19^{A}	184 ± 12^{A}	254 ± 22^{B}	185 ± 18^{A}	D $p<0.05$, D x L $p<0.05$
Testes	177 ± 4	187 ± 4	188 ± 5	178 ± 7	
Carcass Composition (g)					
Carcass Weight	25.4 ± 0.7^{AB}	24.5 ± 0.4^{A}	27.0 ± 0.3^{B}	26.3 ± 0.6^{B}	D <0.01
Fat	2.9 ± 0.3^{AB}	2.5 ± 0.2^{B}	4.4 ± 0.2^{C}	3.6 ± 0.4^{A}	D $p<0.001$, L $p<0.05$

Water	15.7 ± 0.4	15.5 ± 0.4	15.4 ± 0.3	15.7 ± 0.1	
Protein	5.8 ± 0.2^{AB}	5.5 ± 0.1^{A}	6.2 ± 0.1^{B}	6.1 ± 0.2^{B}	D $p<0.01$
Ash	0.98 ± 0.02	0.99 ± 0.02	0.95 ± 0.01	0.95 ± 0.01	D $p<0.05$

Data are means ± sem for groups of 7-9 mice fed either a low-fat (10% kcal fat) or high-fat (45% kcal fat) diet from 10 weeks of age. Intraperitoneal pumps were placed at 15 weeks of age and PBS (control) or 10 µg leptin/day infused for 13 days. Values for a specific parameter that do not share a common superscript are significantly different at $p<0.05$, determined by a two-way ANOVA and post-hoc Duncan's Multiple Range Test. D: Diet, L: Leptin.

Table 4.2: Serum hormones of C57BL/6J mice infused with leptin or PBS.

	Low-fat		**High-fat**		
	PBS	Leptin	PBS	Leptin	Statistical Summary
Glucose (mg/dl)	125 ± 3^{A}	117 ± 4^{A}	146 ± 7^{B}	138 ± 4^{B}	D $p<0.001$
Triglycerides (mg/dl)	87 ± 21	96 ± 10	59 ±10	67 ± 11	
Glycerol (mg/dl)	90 ± 30	84 ± 24	74 ± 20	69 ± 16	
Free Fatty Acids (mEq/l)	1.11 ± 0.15	1.21 ± 0.13	0.98 ± 0.11	0.96 ± 0.11	

Data are means ± sem for groups of 7-9 mice fed either a low-fat (10% kcal fat) or high-fat (45% kcal fat) diet from 10 weeks of age. Intraperitoneal pumps were placed at 15 weeks of age and PBS (control) or 10 µg leptin/day infused for 13 days. Serum glucose, triglycerides, glycerol, and free fatty acids were determined from fasting serum samples collected on Day 5 of infusion. Values for a specific parameter that do not share a common superscript are significantly different at $p<0.05$, determined by a two-way ANOVA and post-hoc Duncan's Multiple Range Test. D: Diet, L: Leptin.

Table 4.3: Serum hormones, organ weights and body composition of C57BL/6J mice infused with leptin or PBS for five days.

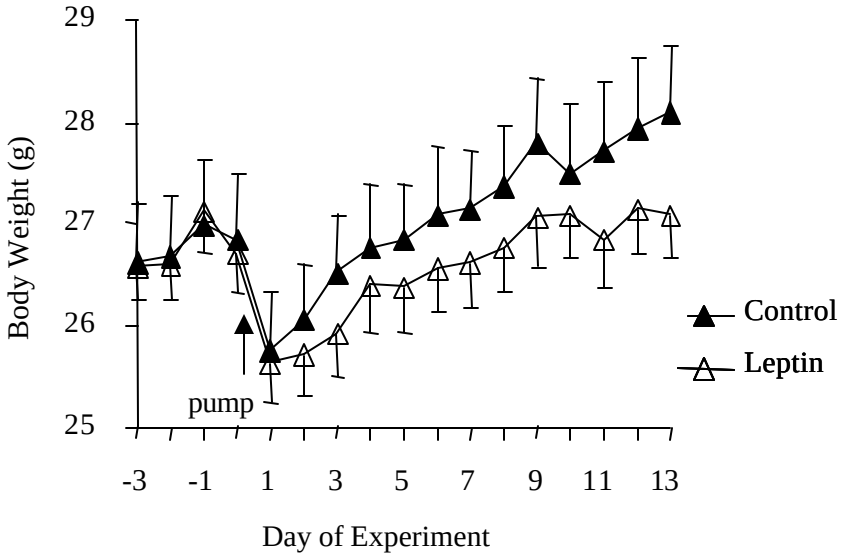
	Low-fat		**High-fat**		
	PBS	Leptin	PBS	Leptin	Statistical Summary
Serum Hormones					
Leptin (ng/ml)	2.4 ± 0.3^{A}	6.7 ± 1.3^{B}	4.0 ± 1.0^{AB}	6.4 ± 1.7^{B}	L $p<0.01$
Triglycerides (mg/dl)	51 ± 6^{A}	51 ± 4^{A}	55 ± 8^{A}	39 ± 4^{B}	D $p<0.05$
Glycerol (mg/dl)	48 ± 2	45 ± 4	43 ± 5	36 ± 2	
Free Fatty Acids (mEq/l)	1.30 ± 0.04^{A}	1.17 ± 0.07^{A}	1.10 ± 0.10^{AB}	0.93 ± 0.05^{B}	D $p<0.005$, L $p<0.05$
Body Weight					
Body weight change (g)	-0.4 ± 0.3^{A}	-1.1 ± 0.3^{A}	-0.4 ± 0.3^{A}	-2.2 ± 0.4^{B}	L $p<0.001$
Energy Intake					
Total Intake (kcal)	63 ± 2^{A}	55 ± 2^{B}	66 ± 2^{A}	53 ± 4^{B}	L $p<0.001$

Organ Weights (mg)					
Inguinal Fat	433 ± 55^{A}	407 ± 68^{A}	672 ± 99^{B}	533 ± 85^{AB}	D $p<0.05$
Epididymal Fat	574 ± 98^{AB}	533 ± 75^{A}	838 ± 109^{B}	722 ± 107^{AB}	D $p<0.05$
Retroperitoneal Fat	168 ± 30^{A}	145 ± 26^{A}	272 ± 38^{B}	222 ± 40^{AB}	D $p<0.01$

Data are means ± sem for groups of 10-11 mice fed either a low-fat (10% kcal fat) or high-fat (45% kcal fat) diet from 10 weeks of age. Intraperitoneal pumps were placed at 15 weeks of age and PBS (control) or 10 µg leptin/day infused for 5 days. Serum leptin, triglycerides, glycerol and free fatty acids were measured from fasting serum samples collected on Day 5 of infusion. Values for a specific parameter that do not share a common superscript are significantly different at $p<0.05$, determined by a two-way ANOVA and post-hoc Duncan's Multiple Range Test. D: Diet, L: Leptin.

Figure 4.1: Daily body weights of male C57BL/6J mice infused i.p. with leptin (10 μg/day) or PBS (Control) for 13 days. Mice had been fed either low-fat (10% kcal fat) or high-fat (45% kcal fat) from 10 weeks of age and pumps were surgically inserted at 15 weeks of age. Data are means ± sem for groups of 7-9 mice. An asterisk indicates a significant difference ($p<0.05$) between control (PBS-infused) and leptin infused mice.

A: Low-Fat



B: High-Fat

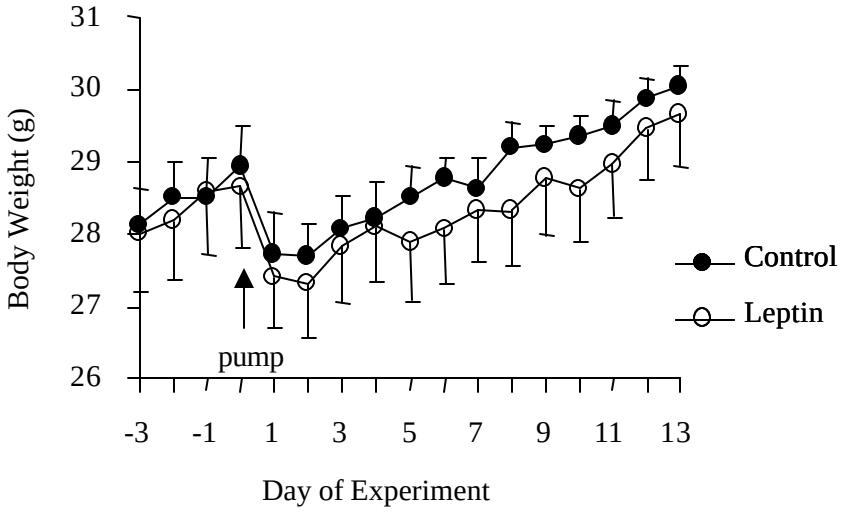
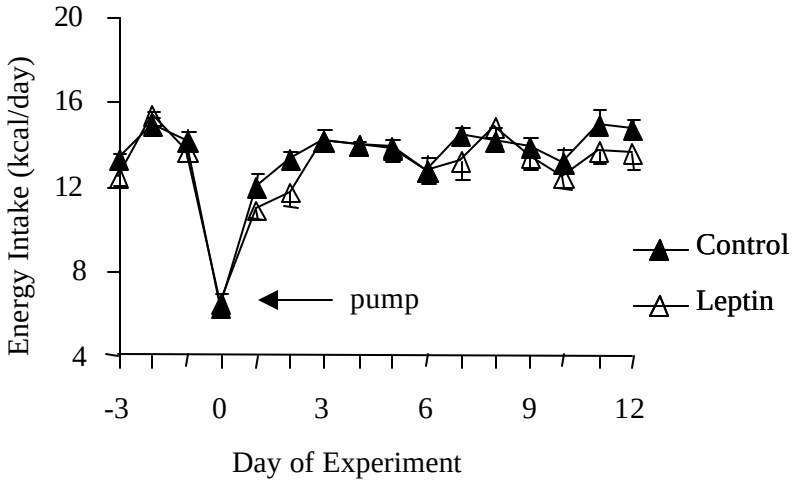


Figure 4.2: Daily energy intakes of male C57BL/6J mice infused i.p. with leptin (10 μg/day) or PBS (Control) for 13 days. Mice had been fed either low-fat (10% kcal fat) or high-fat (45% kcal fat) from 10 weeks of age and pumps were surgically inserted at 15 weeks of age. Data are means ± sem for groups of 7-9 mice. An asterisk indicates a significant difference ($p<0.05$) between control (PBS infused) and leptin infused mice.

A: Low-Fat



B: High-Fat

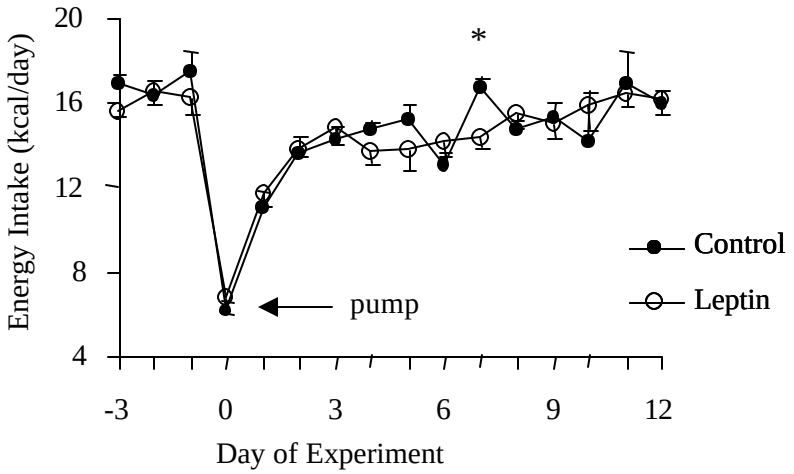
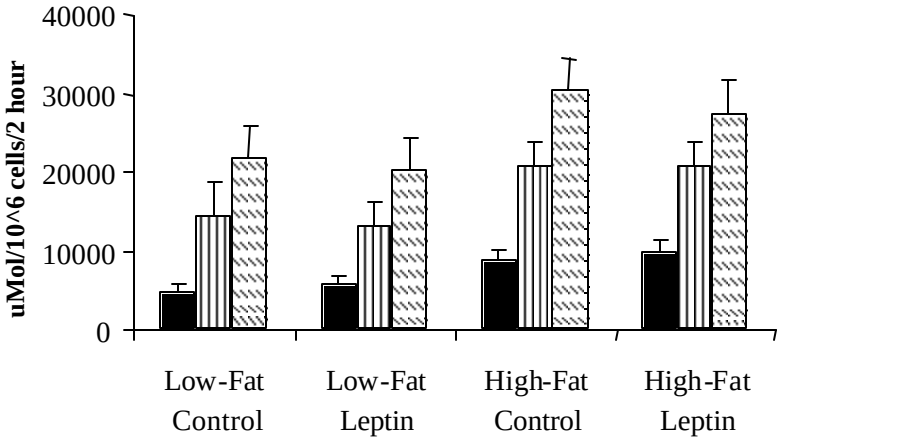
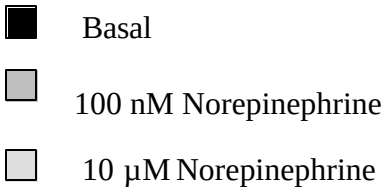


Figure 4.3: Adipocyte lipolysis in mice fed either low-fat (10% kcal fat) or high-fat (45% kcal fat) from 10 weeks of age and infused i.p. with 10 µg/leptin/day or PBS for 5 days. Data are means ± sem for groups of 10-11 mice.

A: Glycerol Release





B: Free Fatty Acid Release

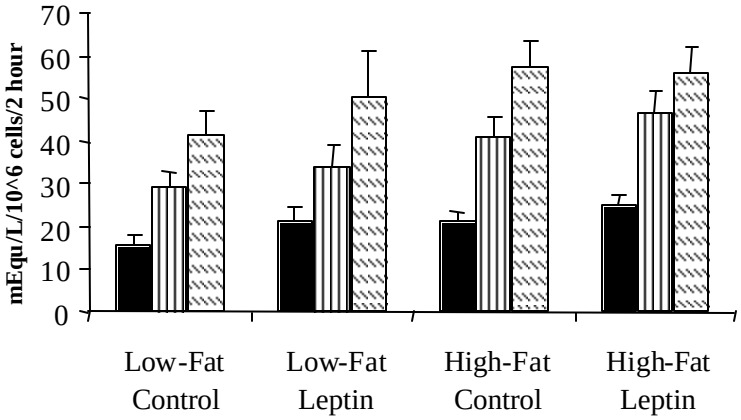


Figure 4.4: Cell size distribution for adipocytes from the combined epididymal and retroperitoneal fat depots of mice fed either low-fat (10% kcal fat) or high-fat (45% kcal fat) from 10 weeks of age and infused i.p. with 10 µg/leptin/day or PBS for 5 days. Data are means ± sem for groups of 10-11 mice. Within a given size range, groups that do not share a common letter are significantly different at $p<0.05$.

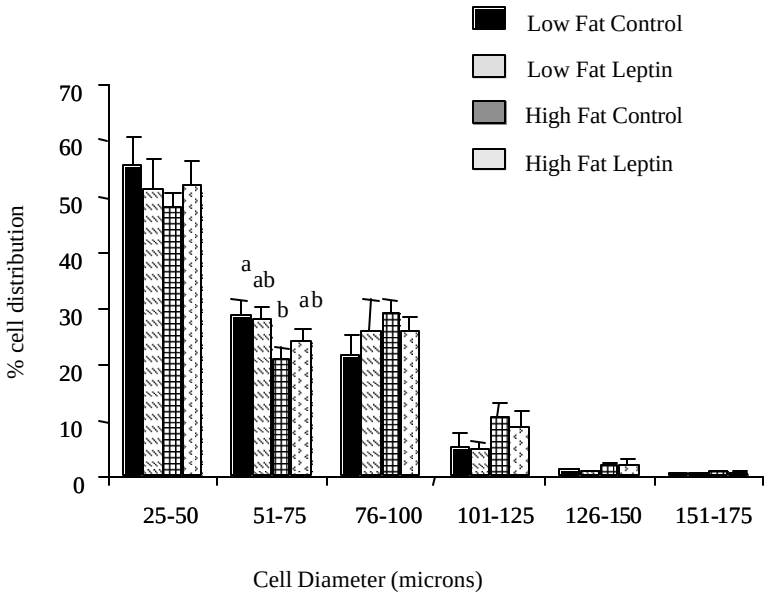
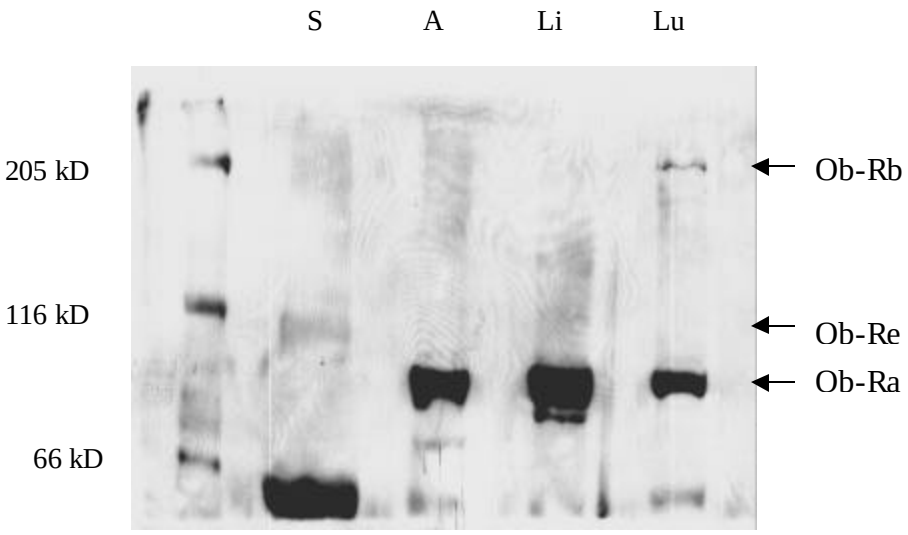

Low Fat Control
Low Fat Leptin
High Fat Control
High Fat Leptin
% cell distribution
70
60
50
40
30
20
10
0
a
ab
b
ab
25-50
51-75
76-100
101-125
126-150
151-175
Cell Diameter (microns)

Figure 4.5: Leptin receptor detected by Western blot using a polyclonal primary antibody raised to the extracellular portion of the receptor. The blot is a representative sample from a low-fat control mouse. S: Serum, A: Adipose, Li: Liver, Lu: Lung.


S
A
Li
Lu
205 kD
116 kD
66 kD
Ob-Rb
Ob-Re
Ob-Ra

CHAPTER 5

CONCLUSIONS

The studies described in Chapter 3 show that young mice weaned onto a high-fat diet have a reduced sensitivity to central and peripheral injections of leptin but that these mice are still responsive to peripheral leptin infusions evidenced by a reduction in body fat mass. It is suggested by the studies on group-housed weanling mice that the response to peripheral leptin infusions is partially mediated through an increase in thermogenesis. The results from experiments described in Chapter 4 show that adult mice fed low-fat but not high-fat diet do not reduce body fat when peripherally infused with leptin. Independent of the effect of leptin on body fat mass, there was no effect of leptin on adipocyte lipolysis measured as glycerol and free fatty acid release. There also were no changes in leptin receptor protein levels in the serum or peripheral tissues that were associated with leptin infusion or dietary fat content.

These studies were conducted to identify an appropriate model for studying human obesity. Human obesity is a condition associated with increased serum leptin and, due to a failure to regulate body weight in the presence of increased endogenous leptin, has been described as a state of 'leptin resistance' (1). Genetically obese mice do not serve as a good model of human obesity because of the mutation in leptin or its receptor. The majority of obese humans have all aspects of the leptin system but fail to respond to high endogenous levels of leptin. Other investigators have reported that mice fed a high-fat diet fail to decrease food intake and body weight in response to peripheral injections

of leptin and, therefore, may serve as a good model to study human obesity (2, 3). The results summarized above suggests that dietary fat content does not determine the development of leptin resistance but the response to peripherally administered leptin is influenced by age, gender, strain, housing conditions, and method of leptin administration. It is necessary to determine how each of these factors contributes to the development of leptin resistance in order to determine its role in the development of obesity. If leptin resistance is due to adiposity then the resistance is secondary to obesity rather than a cause of obesity. Furthermore, mechanisms involved in the development of leptin resistance must be identified to develop appropriate therapies that target the portion of the leptin pathway that is altered and eventually gives way to increased body fatness. The studies described here suggest that the development of leptin resistance is more complicated than failure to transport leptin across the blood brain barrier or a failure to inhibit food intake.

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