Leptin Treatment on Mouse Diabetic Models - ob/ob Mice and db/db Mice: Relevance to Leptin Treatment on Human Diabetic Subjects

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Abstract

Leptin is the first fat-cell derived peptide hormone to be discovered and regulates glucose homeostasis and other effects by binding on receptors in the lateral hypothalamus to inhibit the effects of neuropeptide Y (NPY), which is an appetite promoter secreted by endocrine cells in the gut and the hypothalamus. Animal models of human type 2 diabetes, ob/ob mouse and db/db mouse, have been extensively studied on the effects of leptin on glucose homeostasis and body weight. Ob/ob mice present with hyperphagia, extreme obesity and hyperglycemia due to mutated, biologically inactive leptin and revert to normoglycemia and normal body weight by exogenous leptin injection and leptin gene-treatment. Db/db mice present similar symptoms as in ob/ob mice with relatively severer hyperglycemia and less obesity due to the mutated inactive leptin-receptor in Ob-Rb and do not respond to leptin treatment or leptin gene treatment. Human obese individuals have ample circulating leptin in the blood due to mutated leptin-receptor and do not respond to leptin treatment due to the mutated leptin receptor similar in db/db mice. Serum leptin levels display diurnal rhythm in both humans and rodents. This circadian rhythm in mammals is generated by a central clock located in the lateral hypothalamus that constantly synchronizes to external solar light cues and controls subordinate clocks active in all peripheral tissues via circadian output pathways. There have been leptin experimental treatments going on in human type 1 and type 2 diabetic individuals. Nonobese type 1 individuals have shown some beneficial effects on glucose homeostasis with a recombinant human leptin (metreleptin, Myalept®) but no such beneficial data have been reported in obese, type 2 diabetic individuals. Metreleptin effectively improves glucose and lipid homeostasis in the lipodystrophy individuals, including type 1 and type 2 diabetic subjects with severe lipodystrophy, who have less or no leptin in the blood. More studies with metreleptin have been going on with both type 1 and type 2 diabetic individuals.

Keywords: Db/db mouse, Diabetes, Fat Cell, Gene treatment, Glucose, Glucose tolerance test, Hypothalamus, Hyperglycemia, Insulin, Insulin resistance, Leptin, Leptin deficient, Leptin resistant, Metreleptin, Nod mouse, NPY, Ob/ob mouse, Obesity, Pancreas

Abbreviations: hr: hour, ip: intraperitoneal, min: minute, mo: month, NOD: nonobese diabetic, NPY: Neuropeptide Y, sc: subcutaneous

Unit of measurement: Blood and plasma glucose concentration: 100 mg/dl= 0.56 mmol/l, 200 mg/dl= 1.11 mmol/l, 300 mg/dl=1.67 mmol/l

Introduction

Leptin was the first fat cell-derived hormone to be discovered [1]. Leptin is a peptide hormone, adipokine, produced by adipose tissue that regulates number of physiological processes and behaviors including appetite, body weight, neuroendocrine functions and glucose homeostasis [2,3]. Leptin has the structure of a long chain helical cytokine and is expressed in adipose tissue in proportion to adipocyte size [2,3]. Leptin acts on receptors in the lateral hypothalamus to inhibit hunger by counteracting...
the effects of NPY, a potent hunger promoter secreted by cells in the gut and in the hypothalamus and acts also on the medial hypothalamus to stimulate satiety [1,4,5].

Leptin and insulin inform arcuate nucleus of lateral hypothalamus about energy status of the body. When LepR-b (leptin receptor-b) and Ins-R (insulin receptor) are activated by leptin and insulin, they promote changes in lateral hypothalamic NPY, which causes alterations in peripheral function to restore energy balance and glucose and lipid metabolism, namely in the liver and skeletal muscle (Figure-1) [6]. Absence of leptin in ob/ob mouse are indistinguishable from the lean littermates at birth but become heavier within 1 month after birth [5,6]. Insulin resistance progresses with aging through ever increasing obesity in ob/ob mice, as seen in the aging humans with type 2 diabetes [3,4]. Obese human individuals generally exhibit more leptin in the blood than lean individuals due to more leptin secreting fat cells and exogenous leptin injection does not usually reduce obesity in humans in resistance to leptin [2]. In contrast, ob/ob mice will revert obesity to normal weight by exogenous leptin injection or leptin gene treatment [2,8,9,10].

**Figure 1:** Hypothalamic leptin and insulin signals regulate several peripheral functions. Leptin and insulin secreted by white adipose tissue (WAT) and the pancreas, respectively, inform the hypothalamus about the energy status of the organism. When LepR-b and InsR are activated by these hormones, they promote changes in hypothalamic neuropeptide expression, which cause alterations in peripheral functions to restore energy balance and glucose metabolism. Arc, arcuate nucleus; InsR, insulin receptor; LepR-b, leptin receptor b; POMC, proopiomelanocortin; VMH, ventromedial hypothalamus.

**Ob/ob (C57BL/6J) mice-Leptin deficient mouse model:**

Ob/ob mice present hyperphagia, marked obesity, hyperglycemia, glucose intolerance, severe hyperinsulinemia and low energy due to leptin deficiency [9]. Ob/ob mice are indistinguishable from the lean littermates at birth but already has sign of chubby appearance with 1/3 heavier body weight within 1 mo of age when plasma glucose levels were already twice as much as that of lean mice (Figure-2) [9-12].

Body weight progressively increases up to 6 months reaching 70 g as twice as heavy as lean mice where plasma glucose peaked at 3 months and subsequently decreased to 6 months twice as much as that of lean mice (Figure-2) [11]. Glucose tolerance test, 2 mg glucose per g of body weight intraperitoneal, ip, revealed a marked difference of plasma glucose and insulin levels between ob/ob mice and lean littermates: lean mice showed consistently high plasma glucose levels 12-13 mmol/l at 30 min from 1 to 6 months of age whereas 1- mo old ob/ob mice revealed the highest glucose levels of 30 mmol/l at 30 or 60 min after glucose injection (Figure-3) [12]. Plasma insulin levels of 1- mo old ob/ob mice were about twice as much as that of lean mice and subsequently increased to fivefold that of lean mice at 6 mo of age (Figure-4) [12]. Thus, glucose tolerance test showed much higher plasma glucose and insulin levels in ob/ob mice than in lean mice (Figure-3,4) [12].
plasma glucose levels up to 120 min. In ob/ob mice, plasma glucose levels reached their highest levels at 60 min, with an increment of 15-24 mmol/L higher than that of lean mice. The highest level glucose was recorded in the 1-month-old ob/ob mice after glucose infusion. O, p < 0.05, ●, p < 0.01. Number of animals is shown in parentheses: lean, 1 month (7), 3 months (6), 6 months (4), ob/ob, 1 month (4), 3 months (6), 6 months (4).

**Figure 2:** Comparison of body weight and serum glucose concentration in lean and ob/ob mice as a function of age. Data plotted are mean ± SEM and show the relevant numbers of mice studied. O-O lean controls ●-● ob/ob. a Body weight as a function of age; b Serum glucose concentrations in the same animals at various ages.

**Figure 3:** Glucose tolerance test; plasma glucose level (millimoles per liter). In lean mice, plasma glucose reached its maximal level of 13 mmol/L or above at 30 min after glucose infusion, with an increment of 6-8 mmol/L from the zero time levels, while maintaining moderately elevated plasma glucose levels up to 120 min. In ob/ob mice, plasma glucose levels reached their highest levels at 60 min, with an increment of 15-24 mmol/L higher than that of lean mice. The highest level glucose was recorded in the 1-month-old ob/ob mice after glucose infusion. O, p < 0.05, ●, p < 0.01. Number of animals is shown in parentheses: lean, 1 month (7), 3 months (6), 6 months (4), ob/ob, 1 month (4), 3 months (6), 6 months (4).

**Figure 4:** Glucose tolerance test: plasma insulin levels (micro-moles per liter). In lean mice, plasma insulin levels were lowest at 1 month of age and increased from 3 to 6 months of age. In ob/ob mice, little increase of plasma insulin was noted from 1 to 3 months of age, whereas 6-months-old mice showed an incremental plasma insulin of 23 μmol/L, the highest of all groups studied. O, p < 0.05; ●, p < 0.01. Number of animals is shown in parentheses; lean, 1 month (7), 3 months (6), 6 months (4); ob/ob, 1 month (4), 3 months (6), 6 months (4).

Insulin tolerance, porcine insulin 150 mU/ml, 1 mU/g of body weight, ip, revealed that lean mice responded by the lowest plasma glucose to 3 to 4 mmol/l from 30 to 120 min after insulin injection whereas 1- mo old ob/ob mice revealed the lowest plasma glucose to 7 mmol/l in 1- mo of age, 9 mmol/l in 3 mo and 10 mmol/l in 6 mo [12]. Plasma insulin levels were about the same in both ob/ob mice and lean mice of 1-mo old [12]. However, plasma insulin levels of ob/ob mice reached the highest levels at 3 mo, peaked at 45 μmol/l of three- fold higher than that of lean mice, and slightly decreased until 6 mo at 40 μmol/l 30 min after insulin injection [12]. Plasma insulin levels stayed 3- to 4- fold higher in ob/ob mice than in lean mice [12]. After 7-day treatment of leptin (100 μg/day, sc), isolated islets from ob/ob mice showed improved insulin secretion in vitro at 5.5 mmol at 15% and 16.7 mmol glucose solution by 85% and leptin-treatment improves
glucose-induced insulin secretion in isolated islets in vitro [13].

**Db/db (C57BL/Ks) mouse-Leptin resistant mouse model:**

In contrast to ob/ob mice, a small increased plasma insulin is detected by 10-12 days of age when they already have extra fat deposits with overt obesity observed. Serum insulin continues slowly until weaning, after which a rapid rise to 6-10 times greater than lean littermates by 2-3 mo of age [5]. Db/db mice are severely hyperglycemic throughout life but hyperinsulinemia is transient: after 3 mo, plasma insulin falls rapidly, pancreatic islets atrophy and mice lose weight before death between 6 and 8 mo of age [5,14,15]. Leptin excerts its effects through leptin receptor (Ob-R), which has at least five splice variants (Ob-Ra, -Rb, -Rc, -Rd and –Re) [16].

Mutation of the Ob-Rb gene occurs in db/db mice [17]. Ob-Rb is highly expressed in the hypothalamus and its receptor essential for the inhibition of food intake by leptin and its activity depends on the activation of phosphoinositol-3-kinase [18].

**Leptin treatment on ob/ob mice and db/db mice:**

Systemic injection of leptin normalized food intake, body weight and blood glucose levels in ob/ob mice [8]. An action of leptin in the brain is to mediate NPY neurons in the hypothalamus arcuate nucleus where NPY release lowers energy expenditure and stimulates food intake [9,10,13,19]. Since hypothalamic NPY gene expression is elevated in both ob/ob and db/db mice, increased NPY gene synthesis and release may contribute to the development of obesity and metabolic abnormalities in the mouse models of diabetes [19]. The male ob/ob mice C57BL/6J receiving leptin reduced daily food intake with successive day of treatment. By the 5th day, food intake was 54% below that of saline-injected ob/ob mice, resulting in a 4% reduction of body weight as compared to 1.2% increase of body weight of the lean littermates. In contrast, leptin treatment had no effect on food intake and body weight of db/db mice (Figure-5) [19]. Daily ip leptin injection reduced NPY mRNA hybridization in the arcuate nucleus of ob/ob mice by 43% as compared with saline-

![Figure 5: Food intake and body weight in ob/ob mice (n=8 per group) (A) or db/db mice (n=6 per group) (B) receiving daily intraperitoneal injections of 150 μg of either recombinant murine (□) or saline (injection volume = 1 ml). Saline-treated mice were either fed ad libitum (O) or pair-fed to the food intake of leptin-treated mice (●). The duration of the injection protocol is illustrated by the horizontal bar. *P < 0.03 **P < 0.002 by two-tailed Students t test for comparison of the ad libitum-fed group treated with leptin to the ad libitum-fed group that received saline.](image-url)
treated ob/ob mice. Leptin injection markedly reduced serum glucose in ob/ob mice (8.3 ± 1.2 vs 24.5 ± 3.8 mmol/l) and insulin levels (7,263 ± 1,309 vs 3,150 ± 780 pmol/l) compared with saline injected ob/ob mice, but there was no effect on plasma glucose and insulin in db/db mice between leptin injected mice and saline-injected mice (Figure-6) [19].

Thus, in ob/ob mice leptin injection inhibits NPY gene overexpression through a specific action in the arcuate nucleus and exerts a hypoglycemic action, which is partly independent of its weight-reducing effects. Defective leptin signaling due to either leptin deficiency in ob/ob mice or leptin resistance in db/db mice leads directly to hyperglycemia and the overexpression of hypothalamic NPY that is implicated in the obesity syndrome [19].

Serum glucose levels were sharply reduced in ob/ob mice treated with leptin (Figure-6A) while this reduction was also seen in pair-fed ob/ob controls, which was significantly lesser than leptin-treated ob/ob mice (Figure-6A). This hypoglycemic action of leptin was a parallel decline in serum insulin levels in leptin-treated ob/ob mice but not in the pair-fed groups (Figure-6B).

In contrast, db/db mice receiving ip saline were characterized by marked hyperglycemia with higher serum glucose and lower insulin levels than in comparably treated ob/ob mice and leptin administration was not effective in lowering serum glucose or insulin levels in db/db mice (Figure-6). Because of the mutated leptin receptor (Leprdb/db) in db/db mice, leptin treatment and leptin gene therapy have no effect for hyperphagia, obesity and hyperglycemia [5,19,20].

There are different strains of db/db mice, which present variable levels of hyperphagia, hyperglycemia, obesity and metabolic conditions [21,22].

**Leptin gene therapy on ob/ob mice:**

A replication-deficient recombinant adenoviral vector was constructed that contains the mouse leptin cDNA under the transcriptional control of aRSV long terminal repeat promoter [10]. Administration of Ad.RSV-mLeptin to 18 to 22-week-old ob/ob mice resulted in a dramatic decrease in food intake and body weight (Figure-7) [10]. Food intake of the leptin gene-treated mice had declined by 93 ± 4% below the pretreatment level at 1 week after viral injection. Leptin -gene -treated mice lost 56 ± 3% of their body weight after 3 weeks, which matched with that of age-matched C57BL/6J mice [10].
mice [10]. By glucose tolerance test, saline injected ob/ob mice showed much greater glucose levels, whereas leptin-gene-treated ob/ob mice showed normalization of glucose tolerance test (Figure-8) [10].

Obese and hyperglycemic phenotype in ob/ob mice were corrected by leptin gene therapy.

Leptin therapy on nonobese (NOD) mice-nonobese model of type 1 diabetes:

The NOD mice were developed at Shionogi Research Laboratory in Japan and is a mouse model of type 1 diabetes for humans [5,23,24].

Eight-week-old NOD mice were injected with alloxan (80 mg/kg at 7-day interval) to produce type 1 diabetes, and food intake, body weight and blood glucose were measured. Hyperglycemia of the 15 diabetic mice ranged from 220 to 572 mg/dl and were implanted with an Alzet Osmic pump to deliver 20 μg of leptin per hour for 12 days. The other mice were treated with insulin at 0.01 or 0.01 U sc twice daily. Mean plasma leptin levels ranged between 20 and 50 ng/ml [24].

Plasma glucose levels declined in all 15 leptin-treated mice and averaged 88 ± 28 mg/dl after 12 days compared with 160 ± 32 mg/dl on the insulin pellet [24]. Plasma free FA were also dramatically lowered by leptin to 0.25 ± 0.04 mmol after 12 days of treatment compared with 0.54 ± 0.1 mmol in the insulin group and 1.9 ± 0.4 mmol in saline-treated controls [24].

Leptin treatment profoundly reduced food intake from 10 ± 1.5 g/day in untreated hyperphagic diabetic mice to 2.8 ± 0.8 g/day. The leptin-treated mice lost 2.5 g of body weight and 76% of body fat during the 12 days, whereas the saline treated mice lost 2.0 ± 1.7 g of body weight in 12 days, 57% of which was body fat [24].

Thus, leptin therapy normalizes blood glucose and body weight as well as multiple chemical classes. Leptin lowers both lipogenic and cholesterol genic factors and reduces plasma and tissue lipid. Leptin treatment may have multiple short- and long-term advantages over insulin alone therapy for type 1 diabetes.

Circadian rhythm in mammals:

Serum leptin levels show diurnal rhythm in both humans and rodents. Restriction of food access to the resting phase reverses serum leptin circadian rhythm, while fasting abolishes leptin diurnal rhythm and decreases plasma leptin below the basal level [25].

External food cues have been thought to be the main factor controlling diurnal rhythm of leptin signaling. Circadian rhythm in mammals generated by a central clock is located in the lateral hypothalamus that constantly synchronizes to external solar light cues and controls subordinate clocks active in all peripheral tissues via circadian output pathways. At the molecular level, both central and peripheral clock is operated by feedback loops of circadian genes driven by heterodimers of the bHL-PAS transcription factors CLOCK/BMAL1 or NPAS2/ BMAL1, which induce the expression of their repressors via E box sequences in gene promoters. The rhythmic interaction of the core circadian genes over a 24 hr period defines intrinsic circadian rhythmicity of the molecular clock [26,27].

Plasma leptin concentrations in the humans reach an acrophase (peak) after midnight between midnight and 2:30 am and the lowest levels (nadir) occur between noon and early afternoon [25]. The phasing of this rhythm is similar in circadian rhythm [25]. The 24 hr mean leptin level was statistically greater in obese type 2 diabetic men than in healthy men (24.07 ± 1.71 ng/ml vs 9.47 ± 0.66 ng/ml) [28].

Leptin treatment on humans with type 1 and type 2 diabetes:

The form of leptin available for human therapy is recombinant methionyl human leptin, metreleptin (Myalept®, Amylin Pharmaceuticals, recently acquired by AstraZeneca plc), initially available as Leptin A-100. Metreleptin is composed of the 146 amino acids of mature human leptin with additional methionyl residue at the N-terminal end of the recombinant protein [29]. Myalept® has been approved by the FDA for treating congenital or acquired generalized lipodystrophy with leptin deficiency [30, 31].

Figure 8: Glucose tolerance test in ob/ob mice after leptin gene treatment. The ob/ob mice were injected with Ad.RSV-mLeptin(●) or Ad.RSV-hAAT (Δ) 3 x 10⁸ pfu/mouse or PBS only (O). Five weeks (A) and 17 weeks (B) after injection, glucose tolerance test were performed on treated mice. Blood glucose were measured prior to and at indicated times after intraperitoneal glucose injection. Glucose tolerance test was also performed with age-matched C57BL/6J mice for comparison. Each value represents mean ± SEM of six to eight mice.
The recommended starting dose is 0.13 mg/kg if body <40 kg and 10 mg/kg if body weight > 40 kg. Metreleptin is administered once daily at the same time every day, sc [30]. Leptin therapy is effective improving blood glucose levels in mouse type 2 diabetic model like ob/ob mice with mutated leptin gene [30,31].

There is a rationale to use metreleptin as an adjuvant to insulin for type 1 diabetes, particularly patients with relatively leptin-deficient condition [31].

During normal routine activities, serum leptin levels were significantly higher in obese type 2 diabetic subjects (30.8 ± 6.7), obese non-diabetic subjects (41.7 ± 9 ng/ml) than lean control subjects (12.0 ± 4.4 ng/ml) [29]. Serum leptin levels were highest between midnight and early morning and lowest around noon to midafternoon. The average amplitude between acrophase and nadir was 60.7% in obese type 2 diabetic subjects, 51.7% in obese non-diabetic subjects and 75.6% in lean control subjects [32].

The jury is still out until clinical trials are to be completed in near the future (Clinical-Trial. Gov identifier: NCT0128644). Eighteen sedentary obese subjects with newly diagnosed type 2 diabetes had been treated with low-dose (30mg /day) and high-dose (80mg/day) metreleptin injected for 14 days [30]. Neither low-dose and high-dose therapy had a beneficial effect on insulin-mediated suppression of glucose, glycerol or palmitate rates of appearance in plasma compared with saline injected control subjects [30]. Metreleptin treatment did not increase insulin-mediated stimulation of glucose disposal, thus metreleptin does not have effects on glucose homeostasis or weight loss [33,34]. In newly diagnosed type 2 diabetic subjects, leptin therapy for 16 days has not shown significantly affect glucose homeostasis, body weight, body composition, insulin sensitivity and HbA1c after 16 weeks of treatment [34]. Non-obese diabetic patients with normal or low leptin levels may benefit from metreleptin treatment, especially those with hypoleptinemia, insulin resistance and metabolic syndrome since they frequently have low adipose tissue mass and are more leptin-sensitive [35].

Leptin replacement treatment has shown beneficial effects on both type 1 and type 2 diabetic subjects with lipodystrophy [36]. Nine females subjects (six type 1, two type 2 diabetic and one non-diabetic subjects) with lipodystrophy and serum leptin levels < 4ng/ml received metreleptin, sc, twice a day for 4 mo. During the treatment, the serum leptin levels increased from a mean 1.3 ± 0.3 ng/ml to 11.1 ± 2.5 ng/ml. Four months of therapy average triglyceride levels decreased by 60% and liver volume by 28% and the absolute decrease in HbA1c was 1.9% [36]. Thus, leptin replacement therapy improved glycemic control and decreased triglyceride levels in subjects with lipodystrophy and leptin deficiency. Leptin deficiency contributes to the insulin resistance and other metabolic abnormalities associated with severe lipodystrophy [36].

**Discussion**

Leptin is fat-cell derived peptide hormone and exerts its effects by binding to the receptor at the hypothalamus, which secretes NPY and leptin counteracts the action of satiety factor of NPY. The two type 2 diabetic mouse models, ob/ob mice and db/db mice have been extensively studied for efficacy of leptin and leptin gene-treatment in leptin-deficient ob/ob mice and leptin-resistant db/db mice. Ob/ob mice responded to reduce hyperglycemia and body weight by leptin and leptin gene treatment, however db/db mice with mutated, inactive leptin receptor showed no response to leptin treatment. The NOD mice made type 1 diabetic with alloxan injection responded to a better glucose and fatty acid homeostasis to leptin injection [37]. Because of these diabetic animal experimental results, there has been a high expectation for relevance to leptin treatment for human type 1 and type 2 diabetic individuals [38].

So far type 1 diabetic individuals have shown favorable results of glucose and fatty acid homeostasis by human leptin injection, metreleptin, as an adjuvant to insulin treatment, and type 2 diabetic individuals with severe lipodystrophy and hypolipidemia have shown efficacy in both glucose and lipid metabolism by leptin treatment [36,38]. More studies are now underway, and thus more results will become available in near future.

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