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Leptin increases osteoblast-specific osteocalcin release through a hypothalamic relay ¹

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Abstract

Enhanced long-term expression of leptin by gene therapy selectively in the hypothalamus, without leakage to the systemic circulation, abrogated skeletal abnormalities and reinstated weight and insulin-glucose homeostasis in leptin deficient *ob/ob* mice. Whether increases in osteocalcin, a hormone produced by osteoblasts and known to play a role in bone growth and recently in glucose-insulin homeostasis, may link these benefits of central leptin was assessed. The effects of a single intraventricular injection of non-immunogenic, non-pathogenic recombinant adeno-associated virus vector encoding leptin gene (rAAV-lep) or green fluorescent protein gene (rAAV-GFP, control) were studied in three genotypes, wildtype (wt), obese diabetic, hyperinsulinemic *ob/ob* and non-obese, diabetic insulinopenic Akita mice. Selective hypothalamic leptin expression with central rAAV-lep treatment decreased weight, fat mass, food intake, suppressed insulin levels in *ob/ob* and wt mice, and conferred euglycemia by suppressing blood glucose in all three genotypes. Contemporaneously, rAAV-lep treatment also augmented blood osteocalcin levels. In wt mice, osteocalcin rose by 51% and, whereas, basal osteocalcin levels in *ob/ob* and Akita mice were significantly lower as compared to those in wt mice (26% and 55%, respectively), gene therapy reinstated levels to the control range in *ob/ob* mice, and raised 40% above the wt range even in the absence of insulin in Akita mice. These findings demonstrate that the central beneficial effects of leptin on bone growth involve increased hypothalamic relay of signals that augment osteocalcin efflux from osteoblasts into the general circulation, a response that, in turn, may also modulate glucose-insulin and weight homeostasis.

Keywords

Osteocalcin; Osteoblast; Leptin; Hypothalamus; Bone remodeling; Glucose-insulin homeostasis

1. Introduction

Bones are constantly remodeled through a normally balanced process of bone resorption by osteoclasts and bone formation by osteoblasts [32,35,36,51,54,58]. Osteocalcin, secreted

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selectively by osteoblasts, is the most abundant non-collagenous protein in bone and dentin and comprises about 2% of total protein in the human body. Although increased osteocalcin secretion by osteoblastic cells is observed during the bone formation process, its precise role in bone remodeling remains to be ascertained [24,32,36,46,51,54]. Relative to wild type (wt) mice, osteocalcin null mice exhibited increased bone mass [18,24,32,46,51,64]. Intriguingly, these osteocalcin deficient mice became obese with age and concomitantly displayed decreased circulating insulin levels with hyperglycemia [28,32,46,64]. Administration of osteocalcin improved insulin-glucose homeostasis and stimulated insulin secretion in vitro from pancreatic β -cells of these mice. Although these observations taken together suggested a role for osteocalcin in energy homeostasis and bone remodeling, whether the pathways mediating this coupling reside in the periphery or brain is not known.

Leptin, produced primarily by adipocytes, is a pleiotropic hormone [1,2,8,10,14,19,21,29,37]. In the periphery, leptin modulates immune system, reproduction, angiogenesis and lipolysis, among others [8,16,19,37,47,56]. Centrally, leptin is an essential component of the feedback circuitry that integrates energy homeostasis, primarily by modulating the hypothalamic peptidergic network involved in energy intake and expenditure [14,20,29,37–39,41,65]. In the absence of leptin the mutant *ob/ob* mice display a spectrum of metabolic disorders, including unremitting hyperphagia culminating in morbid obesity, hyperglycemia and hyperinsulinemia, and leptin replacement either peripherally or centrally readily corrects these metabolic abnormalities [10,12,14,20,21,29,37,40,41,45,59,60].

In addition, it became apparent recently that leptin may play a role in bone remodeling by mobilizing leptin receptors expressed by cells in bones and/or neurons in the hypothalamus [23–26,28,31,35,36,51,54,58,63]. Leptin deficient *ob/ob* mice exhibit a phenotype with varied skeletal abnormalities characterized by reduced bone length, but increased cancellous bone mass as compared to wt mice [23,24,28,31,35,36]. Leptin replacement by either peripheral or central routes corrected this skeletal phenotype [18,23,24,26,31,35,36,46,58,64]. Given the experimental evidence that leptin administered centrally is readily transported to the peripheral circulation [7,42,43,49,53], the identity of central versus peripheral leptin receptors involved in reinstating the skeletal phenotype remains to be ascertained [35,36].

We have recently employed leptin gene transfer technology to increase leptin availability in the hypothalamus [1,2,10,11,20,21,40,59,60]. A single intracerebroventricular (icv) administration of a non-replicative, non-immunogenic and non-pathogenic adeno-associated virus vector encoding leptin gene (rAAV-lep) augmented leptin availability selectively in the hypothalamus for the lifetime of the rodents. We observed that, in addition to reversing obesity and metabolic disorders, this selective reinstatement of leptin action in the hypothalamus abrogated abnormalities in bone architecture, and restored the skeletal phenotype in *ob/ob* mice to values that did not differ from wt mice [35,36]. Leptin was undetectable in the rAAV-lep treated *ob/ob* mice, thereby reinforcing the view that activation solely of leptin-responsive neural pathways in the hypothalamus resulted in normalization of skeletal phenotype and that leptin may normally be required for optimal peak bone growth and quality [36,37].

In view of this emerging evidence linking osteoblast specific osteocalcin and adipocyte leptin as key players in integration of energy homeostasis and bone volume and architecture, we undertook to investigate the relationship between leptin and osteocalcin, if any, in manifestation of central effects of leptin on energy balance and skeletal phenotype [24,28,46,64]. In the current study, we took advantage of single icv administration of rAAV-lep paradigm to selectively raise leptin levels in the hypothalamus of three genotypes; the leptin-deficient hyperinsulinemic and hyperglycemic *ob/ob* [10,12,35–37,40,59,60], the insulinopenic non-obese Akita [59,60,62,66], and wt mice in attempts to assess the effects of leptin on circulating

osteocalcin levels in relation with its impact on body weight, adiposity, energy intake, and metabolic indicators, blood insulin and glucose levels.

2. Methods

2.1 Experimental design

The effects of icv rAAV-lep on body weight (BW), food intake (FI) and blood levels of osteocalcin, insulin and glucose in three genotypes, *ob/ob*, Akita and wt mice were examined as follows.

Mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed individually in a temperature (21° C) and light controlled room (lights-on 06:00–18:00h) under specific pathogen-free conditions. Regular chow diet (11 Kcal% fat; caloric density 3.4 Kcal/g; LM-485 Teklad, Madison, WI) and water were available *ad libitum*. Mice were adapted to the animal rooms for one week before experimental procedures were begun. BW and FI were measured on a weekly basis throughout the experiment to monitor the condition of the animals and confirm the effectiveness of the treatments. The BW data in *ob/ob* mice have been published elsewhere [22]. Only the BW recorded at the time of sacrifice and food consumed the week prior to the day of sacrifice in these three animal models are presented here. The Institutional Animal Care and Use Committee of the University of Florida approved the animal protocols.

In three separate experiments, 4 weeks old male *ob/ob* mice (27–31 g) and age-matched control C57 male mice (16–18 g) in the first experiment, 4–10 weeks old Akita mice, as available (15–20 g), along with their wt male control littermates (18–22 g) in the second experiment, and normal adult male C57 wt mice of varying ages (21–28g) in the final experiment, were employed.

2.2 Surgical procedure

Mice were anesthetized by intraperitoneal injection of sodium pentobarbital (90 mg/kg for wt and *ob/ob* mice and 80mg/kg for the lean Akita mice). They were placed in a stereotaxic instrument for intracerebral injection of either a non-immunogenic, non-pathogenic rAAV encoding the green fluorescent protein gene (rAAV-GFP, 1.8×10^{10} particles in 1.5 μ l) or rAAV encoding the rat leptin gene (rAAV-lep, 2.4×10^{10} particles in 1.5 μ l) as described earlier [10,12,59,60]. The vectors used were packaged, purified, concentrated and titered as previously described [20,21,40]. The stereotaxic coordinates for third ventricle injections were on the midline, 0.3mm posterior of bregma, and 4.2mm below the dura [10,12,59,60]. The rAAV-GFP or rAAV-lep solution was slowly infused over a 2 minute period and the injector was left in place for an additional 5 minutes to allow diffusion of the injected solution. The age-matched wt mice in the *ob/ob* and Akita mouse experiments were left untreated to serve as additional controls.

The *ob/ob* mice were sacrificed 13 weeks, Akita mice 9–10 weeks and wt mice 3 weeks post-icv injection. On the day of sacrifice mice were anesthetized with sodium pentobarbital for withdrawal of blood by orbital sinus puncture as described [10,59,60], and then killed by decapitation between 10:30 and 15:00 h. Abdominal WAT was dissected out and weighed. Blood glucose was measured at sacrifice with a drop of blood from the orbital sinus using a glucose meter (Glucometer Elite XL; Bayer, Elkhart, IN). Plasma was stored at –20° C for analysis of osteocalcin by IRMA kit (ALPCO Diagnostics, Salem, NH) and insulin by RIA kit (Sensitive Rat Insulin RIA kit, Linco Research, Inc., St. Charles, MO), as described earlier [10,59,60].

2.3 Statistical analyses

The results from the *ob/ob* and Akita mice were analyzed using one-way ANOVA and Newman-Keuls multiple comparison post-hoc tests. The results from the wt mice experiment were analyzed using Student's *t*-test. The significance was set at $p < 0.05$ for all analyses.

3. Results

3.1 Effects of rAAV-lep on BW, Visceral WAT mass and FI

As evident in Fig. 1A, a single rAAV-lep icv injection suppressed BW in obese *ob/ob* and wt mice as compared to their respective rAAV-GFP controls ($p < 0.05$). In fact, BW in rAAV-lep treated *ob/ob* mice was rapidly normalized to that found in the age-matched wt mice [22]. However, as observed earlier [60], BW in Akita mice after rAAV-lep injection was not affected (Fig. 1A, $p > 0.05$).

In conjunction with suppression of BW, visceral WAT was also significantly reduced in *ob/ob* and wt mice after rAAV-lep treatment (Fig. 1B, $p < 0.05$). The WAT mass of rAAV-GFP treated *ob/ob* mice was more than 12 times that of age-matched untreated wt control mice ($p < 0.01$), which was reduced to the control range after rAAV-lep treatment. In contrast, WAT was indiscernible for measurement in both rAAV-GFP control and rAAV-lep treated Akita mice.

In association with rAAV-lep induced diminution in BW and WAT mass, hyperphagia seen in rAAV-GFP *ob/ob* mice was suppressed and the cumulative FI during the week preceding the termination of the experiment was normalized by rAAV-lep injection (Fig. 1C, $p < 0.05$). Similarly, hyperphagia in Akita mice receiving rAAV-GFP, as indicated by higher cumulative FI (49%) compared to that of their wt littermates, was suppressed to the control range after rAAV-lep injection (Fig. 1C, $p < 0.05$). In wt mice, cumulative FI was also significantly reduced by rAAV-lep injection (Fig. 1C, $p < 0.05$).

3.2 Effects of rAAV-lep on circulating insulin and glucose levels

In hyperinsulinemic *ob/ob* mice, rAAV-lep suppressed blood insulin levels to the range significantly below that observed in wt untreated control mice (Fig. 2A, inset). Plasma insulin in Akita mice was undetectable (not shown). As reported earlier [10,59,60], plasma insulin levels were significantly reduced in wt mice after rAAV-lep injection (Fig. 2C, inset $p < 0.01$).

Hyperglycemia was also abrogated and euglycemia prevailed after rAAV-lep injection in *ob/ob* and Akita mice (Fig. 2A and B, $p < 0.05$). Also, blood glucose levels in *ob/ob* rAAV-lep injected mice fell below those seen in control untreated mice (Fig. 2A, $p < 0.05$). As expected [10,59,60], rAAV-lep suppressed plasma glucose in control wt mice (Fig. 2C). It should be noted that reduction in blood glucose response was contemporaneous with suppressed plasma insulin levels in both *ob/ob* and wt mice after rAAV-lep injection.

3.3 Effects of rAAV-lep on plasma osteocalcin levels

Unlike suppression of blood glucose and insulin responses, rAAV-lep injection elicited an opposite osteocalcin response in all three genotypes. In rAAV-GFP treated *ob/ob* mice, basal osteocalcin levels were significantly lower (26%) than in wt untreated control mice (Fig. 3A, $p < 0.05$) and rAAV-lep treatment reinstated osteocalcin levels to the range seen in controls ($p < 0.01$). Similarly, osteocalcin levels in insulin-deficient rAAV-GFP injected Akita mice were 55% below those in wt untreated controls (Fig. 3B, $p < 0.001$), and rAAV-lep injection stimulated them above those observed in rAAV-GFP treated Akita mice ($p < 0.001$). In fact, unlike the reinstatement response seen in *ob/ob* mice, rAAV-lep in Akita mice raised osteocalcin levels to a 40% higher range than in untreated wt littermates ($p < 0.01$). Also, rAAV-

lep injection in the wt mice was effective in eliciting a significant increase (51%) in circulating osteocalcin levels over that found in controls (Fig. 3C, $p < 0.05$).

4. Discussion

In accord with previous reports [10–12,21,50,59,60], increased leptin transgene expression to augment the supply of biologically active leptin within the hypothalamus by icv rAAV-lep injection, suppressed BW, adiposity and FI, and blood insulin levels concomitant with sustenance of euglycemia in leptin-deficient hyperinsulinemic *ob/ob* mice and wt mice. In leptinopenic and hyperglycemic Akita mice, central leptin gene therapy similarly attenuated BW and hyperphagia and engendered euglycemia even in the absence of detectable insulin [60]. The possibility that these long-term benefits on energy homeostasis may be attributed to diminished energy consumption and increased energy expenditure, in conjunction with markedly suppressed fat depot due to decreased availability of adipogenic insulin and increased energy metabolism, has been previously addressed [1,2,17,20,21,33,37–40,55,59,60].

We now report that by similarly enhancing leptin signaling in the hypothalamus one can increase blood levels of osteocalcin secreted from osteoblasts of all three genotypes. Leptin infusion centrally in rats attenuated the calorie-restriction induced decrease in blood osteocalcin levels, an observation suggestive of the stimulatory effects of leptin on osteocalcin levels [30]. Furthermore, we report for the first time that either in the complete absence, as in *ob/ob* mice, or markedly diminished circulating leptin levels, as in Akita mice, secretion of osteocalcin is significantly less than that in wt mice. Additionally, whereas leptin expression centrally reinstated osteocalcin levels in *ob/ob* mice to the normal control range, a similar enhanced leptin signaling in Akita mice elevated osteocalcin levels significantly above the normal circulating range in wt mice. Seemingly, leptin can act centrally to augment osteocalcin secretion by osteoblasts, and this centrally mediated stimulatory response may not require insulin participation, as illustrated by Akita mice. Interestingly, parathyroid hormone and simvastatin are the other therapies that have been found to increase osteoblast activity [15, 27,52,63]. The possibility that increased central leptin action may participate in stimulation of osteocalcin by these therapies is under investigation.

Convergence of evidence in recent years is in line with the view that central leptin feedback is an important pathway in linking energy homeostasis and bone remodeling [26,31,35,36,46, 63,64]. Indeed, skeletal abnormalities, the hallmark of leptin mutant *ob/ob* mice, can be alleviated by leptin replacement peripherally, presumably, in this case, by the active leptin transfer across the BBB to hypothalamic targets [7,42,43]. We reported that icv rAAV-lep-induced leptin gene expression selectively in the hypothalamus rescued the *ob/ob* skeletal phenotype by promoting growth in femoral length and total bone volume, along with decreasing the femoral and vertebral cancellous bone volume to that observed in wt mice [35,36]. A similar reduction in cancellous bone volume in vertebrae was also seen after icv infusion of leptin in a short-term experiment [23,31]. The current observation of high osteocalcin levels in circulation in association with normalization of cortical as well as cancellous bone volume [35,36], strongly imply a permissive role of central leptin in bone remodeling, in part, conferred by increased osteocalcin secretion from osteoblasts in *ob/ob* mice. Whether high circulating osteocalcin levels in Akita mice and wt mice after icv rAAV-lep administration exert similar positive effects remains to be determined.

There are few studies examining the causal interaction among various metabolic markers in the periphery conferred by leptin. Lee and Karsenty [46] recently reported that mice lacking osteocalcin display hyperglycemia in association with markedly reduced blood insulin levels, decreased pancreatic islet cell size and insulin immunoreactivity in pancreatic β -cells (Fig. 4A). That osteocalcin deficiency underlies the disruptions in glucose-insulin homeostasis was

underscored by the finding that osteocalcin administration increased insulin secretion from β -cells and normalized blood glucose levels. In contrast, we have consistently observed that enhanced expression of leptin in the hypothalamus suppressed pancreatic insulin secretion and blood glucose levels concomitantly with fat depletion [1,2,10,22,37,40,45]. Our current finding that elevated osteocalcin levels are associated with suppressed blood insulin levels is not consistent with the proposed stimulatory role of osteocalcin on pancreatic insulin secretion [34,46]. It is plausible that increased leptin signaling from hypothalamus to pancreatic β -cells restrains insulin secretion to an extent that it is not overcome by high circulating levels of osteocalcin or the osteocalcin stimulatory and insulin inhibitory responses are independently propagated by central leptin feedback (Fig. 4B).

Despite suppression of circulating insulin, however, rAAV-lep treatment was also shown to increase insulin sensitivity in all three genotypes [10,59,60]. Osteocalcin administration was also shown to enhance insulin sensitivity in osteocalcin-mutant mice [34,46]. Thus, it is possible that the rAAV-lep evoked increased insulin sensitivity is, in part, conferred by elevated osteocalcin levels. In that case, how rises in circulating osteocalcin impart increased insulin sensitivity independent of insulin, as in Akita mice [59], remains to be understood.

In this context, the possibility that stimulation by osteocalcin of adiponectin secretion from adipocytes contributed to enhanced insulin sensitivity was also advanced [28,46]. However, we found that central leptin gene therapy enhanced insulin sensitivity contemporaneously with suppressed blood adiponectin levels [59]. Seemingly, the mechanism(s) responsible for increasing insulin sensitivity in these paradigms are complex and varied and likely to include those that exclude increased adiponectin secretion.

How abrogation of hyperglycemia in *ob/ob* and Akita mice and suppression of blood glucose in wt mice contemporaneous with high circulating osteocalcin levels after icv rAAV-lep treatment is causally related, is not clear. Increased leptin signaling by icv injection of either leptin or rAAV-lep readily augments energy expenditure and glucose metabolism, even in the absence of increased energy intake [1,2,17,20,21,32,59,60] and these centrally generated effects participate substantially in diminishing blood glucose levels [20,21,59,60]. Therefore, the question of whether increases in osteocalcin, in turn, modulates glucose homeostasis either independently or in concert with increased energy expenditure imposed by stimulation of hypothalamic leptin receptors is under investigation.

Finally, It is likely that hypothalamic leptin target sites involved in energy homeostasis are a part of the substrate network that also modulates osteocalcin secretion from osteoblasts [37–41,65]. Central leptin gene therapy reliably repressed orexigenic NPYergic signaling by decreasing hypothalamic NPY gene expression and release [39–41]. The NPY receptor system in the hypothalamus has also been implicated in bone remodeling [3–5,48]. Indeed, deletion of hypothalamic Y2 and Y4 NPY receptors impacted bone and adipose tissue in varied ways [3–5,48]. Therefore, we propose that the effects of central leptin therapy on bone remodeling are mediated by hypothalamic NPYergic signaling involving Y2 and Y4 receptors.

The descending efferent pathways from hypothalamus that relay leptin-induced signals to promote osteocalcin secretion from osteoblasts, remain to be deciphered (Fig. 4B). It is possible that sympathetic innervations from hypothalamus transmit these signals to bone cells in the periphery [9,13,34,57]. Based on the fact that track-tracing investigations have mapped distinct hypothalamic neural links to those organs in the periphery, such as WAT [44], brown adipose tissue [6,9], pancreas [13,46] and liver [44,61], that participate in the integration of energy balance by leptin, one can speculate that a similar discrete descending neural pathway between leptin-responsive hypothalamic neurons and bone cells is involved in the osteoblast activation (Fig. 4B). Furthermore, since leptin also modulates various neuroendocrine functions [37–

41], the additional possibility that these may secondarily assist in stimulation of osteocalcin deserves consideration.

In summary, the present study clearly demonstrates that leptin can stimulate osteoblast-specific osteocalcin secretion by involving receptors exclusively resident in the hypothalamus and this response may, in part, underlie the well-documented benefits of leptin on skeletal architecture and energy homeostasis.

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Figure 1.

The effects of icv rAAV-lep injection in *ob/ob*, Akita and wt mice on body weight (A), visceral WAT (B) and food intake (C). Body weight and visceral WAT data represent the values at the time of sacrifice and the food intake data represents cumulative food consumption during the week immediately preceding sacrifice. For details see text. Numbers in parentheses indicate number of animals and bars with similar letters are not significantly different from each other ($p > 0.05$) in this and subsequent figures.

Figure 2.

The effects of icv rAAV-lep on blood glucose in *ob/ob* (A), Akita (B) and wt (C) mice and plasma insulin levels (inset) in *ob/ob* (A) and wt mice (C) at the time of sacrifice. Insulin was undetectable in Akita mice.

Figure 3.
The effects of icv rAAV-lep on plasma osteocalcin levels in *ob/ob* (A), *Akita* (B) and wt (C) mice at the time of sacrifice.

Figure 4.

Regulation of circulating levels of osteocalcin and insulin by leptin. **A.** Lee and Karsenty [46] proposed that a deficit in leptin signaling in the hypothalamus stimulates osteocalcin release from skeletal osteoblasts that, in turn, augments insulin secretion from pancreas. **B.** The results of the current investigation advocate that the observed inverse relationship between osteocalcin and insulin levels due to a deficit in leptin signaling is engendered by independent neural relays from hypothalamus to the two targets in the periphery.