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Central leptin gene therapy corrects skeletal abnormalities in leptin-deficient *ob/ob* mice

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ABSTRACT

Skeletal growth is tightly coupled to energy balance via complex and incompletely understood mechanisms. Leptin-deficient *ob/ob* mice are obese and develop multiple pathologies associated with the metabolic syndrome. Additionally, *ob/ob* mice have skeletal abnormalities. The objective of this study was to evaluate the effects of leptin deficiency and long duration selective central leptin repletion via recombinant adeno-associated virus-leptin (rAAV-lep) gene therapy on bone in growing *ob/ob* mice. The *ob/ob* mice were injected in the hypothalamus with either rAAV-lep or rAAV-GFP (control vector). Treated *ob/ob* and untreated wild-type (WT) mice were then maintained on a normal diet for 15 weeks. In a second experiment, similarly treated mice along with a group of pair-fed mice were maintained for 30 weeks. Leptin was not detected in blood of either rAAV-lep- or rAAV-GFP-treated mice although rAAV-lep-treated mice displayed leptin transgene expression in the hypothalamus. As expected, rAAV-lep normalized body weight and food intake. Compared to WT mice, rAAV-GFP-treated *ob/ob* mice had decreased femoral length (by 1.6 mm or 10%, $P < 0.001$), decreased total femur bone volume (by 3.3 mm^3 or 19%, $P < 0.001$), but increased cancellous bone volume in the distal femur (by 0.04 mm^3 or 60%, $P < 0.09$) and lumbar vertebrae (by 0.26 mm^3 or 118%, $P < 0.001$). Treatment with rAAV-lep rescued the *ob/ob* skeletal phenotype by increasing femoral length and total bone volume, and decreasing femoral and vertebral cancellous bone volume, so that at 15 weeks post-rAAV-lep injection the *ob/ob* mice no longer differed from WT mice. No further skeletal changes in either the femur or lumbar vertebra were observed at 30 weeks post-rAAV-lep administration. The results suggest that hypothalamic leptin functions as an essential permissive factor for normal bone growth.

1. Introduction

Osteoporosis, with no cure on the horizon, contributes to over 1,500,000 fractures annually in the United States alone [52]. Because a low peak bone mass predisposes individuals to osteoporotic fractures, an understanding of the factors that determine peak bone mass is essential for the prevention of this disease. The acquisition of peak bone mass occurs during childhood and the decade following puberty, and is tightly coupled to energy metabolism. At the extremes, obesity and anorexia each result in skeletal adaptations. Leptin, the protein product of the *ob* (*Lep*) gene [55], is a pleiotropic

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hormone that acts on multiple organs, including bone. Leptin is produced predominantly by fat cells, functions as a sentinel of energy balance, and has been recently identified as an important bone regulatory factor [26]. The obese *ob/ob* mouse, which cannot produce leptin due to an inactivating mutation in the leptin gene has skeletal abnormalities; compared to wild-type (WT) mice, *ob/ob* mice have reduced bone length and reduced overall bone mass [27,32,33,36,44]. These findings suggest leptin is required for optimal peak bone growth and quality. However, the mechanisms of action of leptin on the skeleton are not fully understood. In part, this is because the hormone has the potential to affect bone through multiple pathways; an indirect pathway involving a central/hypothalamic relay [3,26,45] and a direct pathway involving the binding of leptin to its receptors directly on cartilage and bone cells [17,20,32,39,46–48].

Some of the indirect effects of leptin on bone may be mediated through the sympathetic arm of the autonomic nervous system; the binding of leptin to its receptors in the hypothalamus stimulates the peripheral cells of the sympathetic nervous system to release noradrenalin which, in turn, could influence bone formation via adrenergic receptors expressed on osteoblasts and other cells [26,42,45]. Additional indirect effects may be mediated through changes in systemic metabolic bone regulatory factors such as IGF-I [10,15,34]. The direct action of leptin on bone and cartilage cells has been amply demonstrated in cell culture [20,36,39,48]. The systemic administration of leptin, via intraperitoneal or subcutaneous injection, has also been shown to increase total body bone mass and bone length in *ob/ob* mice and increase bone length in calorie-deprived mice, suggesting that bone growth is regulated by the circulating levels of leptin [30,32,44]. Increasing systemic leptin within the physiological range has consistently been shown to increase hypothalamic leptin levels, but the effects of central infusion on circulating leptin levels are less consistent [7,26,29,38,43,46,54]. As a consequence, neither systemic nor central applications of the hormone can unambiguously differentiate between the contributions of systemic versus central leptin in the putative regulation of skeletal metabolism. In addition, all experiments to date investigating the actions of peripheral and central leptin on bone metabolism in rodents have been of short duration ($\leq$1 month). Finally, no studies have investigated the effects of leptin on peak bone mass.

We have recently employed central leptin gene transfer technology by non-replicative, non-immunogenic and non-pathogenic virus vector (rAAV) to augment leptin availability selectively in the hypothalamus [21,22,56]. These studies demonstrated that microinjection of rAAV encoding leptin gene (rAAV-lep) into either the third cerebroventricular or other selective hypothalamic sites enhanced leptin transgene expression locally in the hypothalamus and not at distant non-hypothalamic sites for the duration of the experiments [1,2,8,9,11–14,21–24,34,37,50,51]. The leptin protein transduced in neurons was expressed in amounts sufficient to exert leptin-specific biological effects but insufficient for detection in the hypothalamic tissue surrounding the injection site, cerebroventricular fluid or peripheral circulation [1,2,11, 14,21,34,50,51]. Therefore, the objective of this study was to evaluate the effects of leptin deficiency and selective hypothalamic leptin expression of long duration by a single intracerebroventricular (icv) injection of rAAV-lep on bone size and cancellous bone architecture in growing *ob/ob* mice.

2. Materials and methods

2.1. Experimental animals

Male, 8–10-week-old leptin-replete WT (C57BL/6) and leptin-deficient *ob/ob* (weighing 40–50 g) mice, obtained from Jackson Laboratories (Bar Harbor, Maine), were used in the experiments. The mice were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Florida (Gainesville, FL). The mice were housed individually in a temperature (21–23 °C)- and light-controlled room (lights on 6 a.m. to 6 p.m.) under specific pathogen-free conditions. Food and water were available *ad libitum*.

2.2. Construction and packaging of rAAV Vectors

The rAAV-lep and rAAV-green fluorescent protein (rAAV-GFP) vectors were constructed and packaged as described elsewhere [9]. In brief, the vector pTR-CBA-Ob *Eco*RI fragment of pCR-rOb (a gift from Dr. Roger H. Unger, University of Texas Southwestern Medical Center, Dallas, TX) containing rat leptin cDNA was subcloned into rAAV vector plasmid pAAVβGEnh after deleting the *Eco*RI fragment carrying the β-glucoronidase cDNA sequence [21–23,25,56]. The control vector, rAAV-GFP, was similarly constructed to encode the *GFP* gene [21,23,25,56].

2.3. Experiment 1

Male WT and *ob/ob* mice were used in the experiment. The WT mice (n = 3) were untreated. The *ob/ob* mice were anesthetized with sodium pentobarbital (60 mg/kg, ip) and placed on a Kopf stereotaxic apparatus with mouse adapter for intracerebroventricular (icv) injection. The coordinates employed for microinjector placement were 0.3 mm posterior to bregma, 0.0 lateral to midline, and 4.2 mm below the dura [11,50,51]. Various procedures to verify icv injection are detailed elsewhere [1,2,8,9,11–14,21–24]. Mice were injected icv with either rAAV-GFP (control vector, 9×10^7 particles; n = 8) or rAAV-lep (9×10^7 particles; n = 9). All mice were then maintained on standard mouse chow fed *ad lib* for 15 weeks. Food intake and body weight response are detailed elsewhere [11,14]. Also detailed elsewhere are hormone levels (e.g., insulin and leptin) and hypothalamic leptin mRNA expression in response to rAAV-lep therapy in mice [11,14,50,51].

2.4. Experiment 2

Male *ob/ob* mice received an icv injection of either rAAV-lep (n = 9) or rAAV-GFP (n = 9) as described above. Additional controls included untreated (n = 7) and pair-fed (mice allowed to consume amounts of food equivalent to that of the rAAV-lep-treated mice, n = 7) *ob/ob* mice. The mice were maintained on standard mouse chow for 30 weeks post-vector adminis-