LEPTIN'S ROLES IN BONE GROWTH AND ADIPOSE TISSUE IN *ob/ob* MICE AND THEIR RELATIONSHIP TO OSTEOPOROSIS

by

SHOSHANA MAL-LEE BARTELL

(Under the Direction of Clifton A. Baile)

ABSTRACT

Disorders of body composition, specifically obesity and osteoporosis, have emerged in the last decade as major health concerns. The research was conducted to evaluate leptin's role in bone growth, throughout the skeleton, and adipose tissue in *ob/ob* mice and their relationship to osteoporosis. The studies utilized a model of an aged (15-wk) obese *ob/ob* mice, who cannot produce leptin due to an inactivating mutation in the leptin gene. These mice have the skeletal abnormalities observed in an individual suffering from osteoporosis. Leptin treatment increased the expression levels of genes associated with osteogenesis (eg., Runx2), while those associated with osteoclastogenesis (eg., RANK) were decreased. This is consistent with the observed leptin-stimulated bone growth as demonstrated by the increased BMD, BMC, and mineral apposition rates (MAR) throughout the skeleton and the inclination of cells to differentiate into osteoblasts. Both central and systemic injections of leptin decreased adipogenesis in the bone marrow and adipose tissue, demonstrating an enhanced sensitivity to leptin-stimulated adipocyte apoptosis, which resulted in fat loss but not to the detriment of bone mass. These results indicate that increasing concentrations of leptin promoted expression of pro-osteogenic factors in the bone marrow and enhanced bone formation in *ob/ob* mice. Leptin induced adipocyte apoptosis

in the bone marrow and adipose tissue, reduced cell differentiation into osteoclasts and adipocytes, and promoted osteoblast differentiation as demonstrated by changes in gene expression, bone histology, serum marker concentrations, and body composition. Both central and peripheral leptin treatment increased serum IGF-1 concentrations that most likely enhanced muscle and bone growth via muscle-derived mechanical stimuli. Thus, leptin exhibited characteristics important for the prevention of osteoporosis by preserving muscle mass, bone mass, and bone integrity as exhibited by the changes in gene expression, bone histology, serum marker concentrations, and body composition. Moreover, leptin administration reversed the primary osteoporosis-causing mechanisms, which future therapeutic treatments need to mimic to be effective agents and demonstrate that the *ob/ob* mouse is a useful model for studying osteoporosis.

INDEX WORDS: leptin, *ob/ob*, mice, osteoporosis, bone, apoptosis, adipocyte, osteoblast, adipose tissue, body composition, osteoclast, gene expression, bone marrow

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DEDICATION

To my family, thank you for all your love, prayers, and encouragement, always being there to support me with open arms, and listening to all of my frustrations. I could never have made it as far as I have without your love and support to drive me to the next level. Without you, my life would be incomplete.

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TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

Recently, disorders of body composition, such as obesity and osteoporosis, have increased in prevalence. Regulation in the hypothalamus and bone marrow of homeostatic mechanisms controlling body mass highlight major bone – fat interactions. Bone and fat are centrally modulated by the hypothalamus via the sympathetic nervous system by regulating appetite, insulin sensitivity, energy utilization, and bone remodeling. In the bone marrow, osteoblasts (bone forming cells) and adipocytes (fat cells) arise from the same precursor, the mesenchymal stem cell. To further complicate the paradox between adipose tissue and the skeleton is the balance of the positive effects of fat as "insulation" on cortical bone and the detrimental effects on trabecular bone by the inherent capacity of adipose tissue to function as an endocrine organ secreting inflammatory cytokines, which illicit bone resorption.

The hypothalamus regulates bone turnover and fat distribution. Obesity and osteoporosis can coexist and excess visceral fat might contribute to bone loss. On the other hand, some evidence has shown that obesity is correlated with increased bone mass, suggesting a protective effect of fat mass on bone and a reduction in body weight may actually induce bone loss. Thus, body composition, such as fat mass, percent body fat, and lean mass, more than body weight is highly correlated with bone mass and density.

Bone and energy are functionally related through a complex neuroendocrine circuit that features leptin (which is derived from fat), the hypothalamus, and the sympathetic nervous system. Leptin regulates food intake, bone metabolism, and energy metabolism by crossing the blood-brain barrier and binding to its receptor in the arcuate nucleus of the hypothalamus. The binding of leptin triggers a cascade of events resulting in the activation of the sympathetic

nervous system. Resistance to or deficiencies of leptin cause obesity, changes in food intake, and impaired bone metabolism.

Osteoporosis is characterized by the reduction of muscle mass (sarcopenia) and strength and loss of bone mass and strength that leads to fragility fractures. Skeletal fragility can result from a skeleton with suboptimal development of mass and strength during growth, excessive bone resorption by osteoclasts (bone resorbing cells) resulting in decreased bone mass and microarchitectural deterioration of the skeleton, or inadequate bone formation response by osteoblasts in response to increased resorption during bone remodeling. Osteoporosis has been considered the obesity of bone because as one ages, the propensity of bone marrow cells to differentiate into adipocytes instead of osteoblasts increases, resulting in skeletal fragility and enhanced bone resorption. With osteoporosis exploding in prevalence, there is a need for more research in the understanding of the regulation of bone metabolism, the connection between fat and bone, and possible biotargets of effective treatment methods. Any target for the treatment of osteoporosis needs to inhibit excessive bone marrow cell differentiation into osteoclasts, drive cell differentiation into osteoblasts rather than adipocytes, and/or induce adipocyte apoptosis (cell death). Ideally, all three activities in bone would be driven by the same agent. The purpose of this research was to evaluate leptin's roles in bone growth and adipose tissue in *ob/ob* mice and their relationship to osteoporosis.

CHAPTER 2

LITERATURE REVIEW

Emerging health concerns from the past decade are body composition disorders, such as obesity and osteoporosis. Homeostatic mechanisms regulated in the hypothalamus and bone marrow illuminate bone-fat connections. Many facts give support to this idea. First, bone and fat are centrally modulated by the hypothalamus via the sympathetic nervous system by regulating food intake, insulin sensitivity, energy utilization, and bone remodeling. In fact, processing in the hypothalamus from efferent fat depots regulates bone turnover. Second, in the bone marrow, both osteoblasts (bone forming cells) and adipocytes (fat cells) arise from mesenchymal stem cells. Third, changes in glucose and fat metabolism may adversely affect the skeleton as in diabetes mellitus. Similarly, bone-specific proteins secreted from osteoblasts have been shown to regulate glucose metabolism. Finally, body weight or body composition has been demonstrated to relate to bone mineral density and bone mass. Adipose tissue may provide protection against bone damage during falls and contribute to bone growth by its biomechanical effects or it can cause detrimental effects by acting as an endocrine organ secreting inflammatory cytokines that promote bone resorption. There is a sensitive balance between adipose tissue and the skeleton and leptin has emerged as a potential candidate as the link between fat and bone mass.

Regulation of Adipose Tissue Mass

Adipose Tissue

Adipose tissue is the main organ providing fuel reserves for the organism. Without the fat stores in adipose tissue, an individual could not live for more than a few days without eating. Adipose tissue belongs to loose connective tissue, and is composed of adipocytes (about 90% of mass) filled mainly with triglycerides, blood cells, immune cells, and fibroblasts (1). Fat tissue is highly vascularized and innervated, indicating its high metabolic activity, which is under tight neural and hormonal control (2). This tissue may develop almost anywhere, but is mainly located subcutaneously and viscerally. Although there is no limit for adipocytes to store lipid, animals maintain their body weight fairly constantly. An adiposity signal in the hypothalamus reflects total body fat content by either direct or indirect adipose tissue regulation.

The understanding of the control of energy balance and lipid metabolism in rodents and humans has been furthered by the successful cloning of the ob gene from *ob/ob* mice (3) and the db gene from *db/db* mice (4), as well as the subsequent characterization of leptin and its receptor. It is now widely accepted that leptin acts as an adiposity signal from body stores to the hypothalamus, binding to receptors to regulate energy balance. Leptin inhibits feeding behavior and stimulates energy expenditure by modulating sympathetic activity, thermogenesis, oxygen consumption, and locomotor activity. Furthermore, down-regulation of leptin expression with food restriction triggers a series of physiological responses to conserve energy.

Leptin and Adipose Tissue

Leptin, the product of the ob gene, acts primarily on the brain to regulate energy balance and food intake in a negative-feedback loop regulating the mass of adipose tissue (3). It is produced almost exclusively from adipose tissue, and is released into the bloodstream in

proportion to the amount of adipose tissue as a signal of adiposity (3). Although leptin is primarily secreted from adipose tissue, it is also present in the stomach (5), skeletal muscle (6), mammary gland and placenta (7). Ob-Rb, one isoform of the leptin receptor (8), is predominantly localized in the hypothalamus, and is known to be responsible for transmitting and activating the intracellular signal of leptin (9). Within the hypothalamus, different neuronal populations mediate leptin's downstream effects on body weight and energy balance.

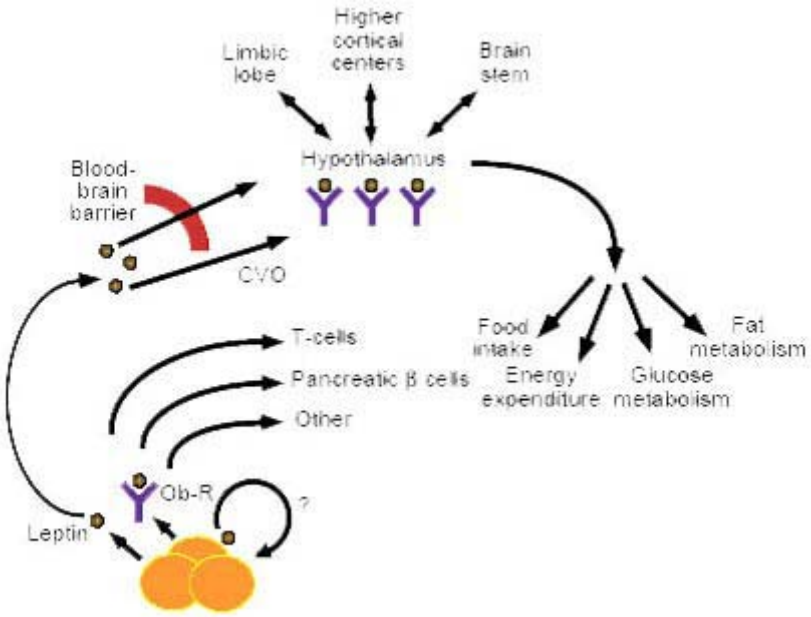

Figure 2.1 Leptin and the regulation of adipose tissue mass (10)

Leptin decreases expression of orexigenic peptides such as neuropeptide Y (NPY) (11) and agouti-related peptide (AGRP) (12), and increases expression of anorexigenic peptides such as proopiomelanocortin (POMC) (11) and cocaine and amphetamine regulated transcript (CART) (13), leading to an overall decrease in appetite and body weight (14).

Interest in leptin stems from its special weight-reducing effects in rodents. Central (ICV) and peripheral (SQ) administration of leptin results in a dose-dependent decrease in body weight for both *ob/ob* and wild-type mice (15-16), and leptin-induced weight loss is specifically

restricted to adipose tissue while lean body mass is spared (2,17). Furthermore, leptin-treated *ob/ob* mice exhibited a greater decrease in body and fat weights than pair-fed *ob/ob* mice (18). Following reduced food intake, *ob/ob* mice treated with leptin maintained high energy expenditures, while food-restricted mice demonstrated a reduction in energy expenditure (16). In addition to regulating food intake and thermogenesis, leptin also protects skeletal muscle (19), pancreatic islets (20), and myocardium (21) from lipotoxicity and lipoapoptosis (19). Although leptin can regulate adiposity indirectly by modulating food intake and energy expenditure (3), there is emerging evidence (22-23) indicating that leptin directly acts on adipocytes in regulating cellular lipid balance and promoting β-oxidation.

Leptin and Lipid Metabolism

The amount of adipose tissue is defined by the size and number of constituent adipocytes. In adult animals, changes in triglycerol content determine changes of fat mass. Numerous studies have shown that leptin has a direct action on the rates of synthesis and degradation of lipids as an autocrine and/or paracrine signal. Fruhbeck and colleagues demonstrated that in vitro leptin induced lipolysis in a dose-dependent manner in mouse adipocytes from *ob/ob* mice (24). It was also demonstrated that lipolytic rates were increased in adipocytes from *ob/ob* (no functional leptin), while no changes were observed in *db/db* mice (no functional leptin receptor) after intraperitoneal (i.p.) injection of leptin (24). In addition, *ob/ob* mice had much more sensitivity to enhanced lipolytic rates of adipocytes by leptin treatment than did the wild type mice (24). Leptin appears to inhibit lipogenesis by changing the activity of a few key enzymes in lipid metabolism (25). For example, leptin suppresses the activity of carboxylase, therefore inhibiting the subsequent process of triglyceride formation in the fat cells (26). Leptin also decreases the mRNA expression of fatty acid synthase, and increases the expression levels of lipolytic

enzymes, including lipoprotein lipase (LPL) and hormone-sensitive lipase (HSL) in adipose tissue of wild type mice (27). Leptin treatment has been shown to induce lipolysis without a concomitant increase in plasma free fatty acids, as typically occurs during fasting-induced fat mobilization (28). Although the signaling pathway used by leptin to stimulate lipolysis is not clear, it is suggested that leptin promotes lipolysis by increasing the concentration of cAMP and stimulating adenylate cyclase protein levels (29).

Leptin and Adipose Tissue Apoptosis

In addition to regulating lipogenesis and lipolysis in adipocytes, leptin may also reduce adipose mass by inducing adipocyte apoptosis. Apoptosis, or programmed cell death is a normal physiological process involved in deleting unwanted cells such as aged cells, damaged cells, or precancerous cells, from living tissue during embryonic and adult tissue homeostasis (30). The process can be initiated through a number of pathways such as intrinsic (or mitochondrial) and extrinsic (death receptor) pathways (31), but ultimately involves the activation of cytoplasmic proteases that lead to DNA fragmentation, the hallmark of apoptosis (32). Adipose tissue apoptosis induced by leptin was first described by Qian et al. (33). Della-Fera and colleagues have shown that continuous 13-day i.p. infusion of leptin significantly increases adipocyte apoptosis in white adipose tissue in male and female mice, but only male mice developed reduced responsiveness to leptin-induced apoptosis after high-fat (45% fat) feeding for 5 or 15 weeks, thus extending the findings that leptin-induced apoptosis can occur with peripheral infusion in mice (34). The β-2 adrenergic agonist, clenbuterol, increased adipose tissue apoptosis *in vivo*, suggesting that activation of adrenergic receptors on adipocytes may be involved in adipocyte apoptosis (34). Central injections of 10 μg leptin for 4 days in Sprague Dawley rats reduced fat mass by both decreasing adipocyte volume and increasing apoptosis in

fat pads (35). Moreover, *ob/ob* mice are more sensitive to leptin-induced adipose tissue apoptosis than lean mice (36).

Leptin mediates lipid metabolism by different routes. Its effects are exerted both indirectly through the central nervous system and directly on the peripheral tissues. Both pathways end up with similar outcomes: a decrease in triglyceride synthesis, an increase in lipid oxidation, and adipocyte apoptosis. Of all peptides studied that affect food intake and body energy balance, leptin is unique in that it acts in the brain to induce loss of adipose tissue by both apoptosis and increased lipid metabolism.

Regulation of Bone Mass

Body Composition

Body weight is closely related to bone mass and bone mineral density in adult men and women (37-39). Obesity has protective effects on bone, and is associated with a reduction in the prevalence of osteoporosis. The protective effects of body weight on bone mass was primarily thought to result from mechanical loading effects, with the consequential increase in bone volume secondary to a requirement to provide a support for a greater weight. In line with this, lean body status is a known risk factor for low bone mineral density (BMD) and thus for fracture risk and the development of osteoporosis. Interestingly, several studies investigating the contribution of lean mass versus fat mass have reported fat mass to be a major correlate of bone density (39-42). However, the strong correlation between fat mass and bone mass was not entirely due to mechanical loading, leading to the hypothesis that adipose itself, or an adipose-released factor may influence bone mass.

Secretion of leptin has been demonstrated by extramedullary adipose tissue and by marrow adipocytes (43), indicating it may directly affect bone cell activity by local interactions

within the bone microenvironment. Indeed, several lines of evidence suggest this peptide is able to directly modulate the activity of osteoblasts, osteoclasts, and chondrocytes, and therefore may play an important role in bone remodeling. Recent evidence suggests that receptors (Ob-Rb) located in the hypothalamus not only regulate leptin's mediation of energy balance and food intake but also may modulate the action of leptin on bone cell activity, indicating the actions of leptin on bone physiology may be more complex than initially thought. Therefore, before the role of leptin in bone metabolism is discussed further, it is important to review the physiology of bone and the process of bone remodeling.

Bone Function and Bone Cells

Bone is a highly specialized form of connective tissue with a mineralized extracellular matrix providing strength and rigidity to the skeleton. It has properties that are required for the skeleton's role as an internal mechanical support system and its protective role physically shielding soft inner organs and bone marrow. Moreover, the skeleton also provides sites for the attachment of muscles, allowing locomotion, and acts as a reservoir of ions for metabolic processes, including the maintenance of calcium and phosphate homeostasis (44-45). Its main constituents, like other connective tissue, are matrices and cells. The former is composed of mainly collagen fiber and non-collagen fiber, while the latter is composed of three types of bone cells: osteoclasts, osteoblasts, and osteocytes.

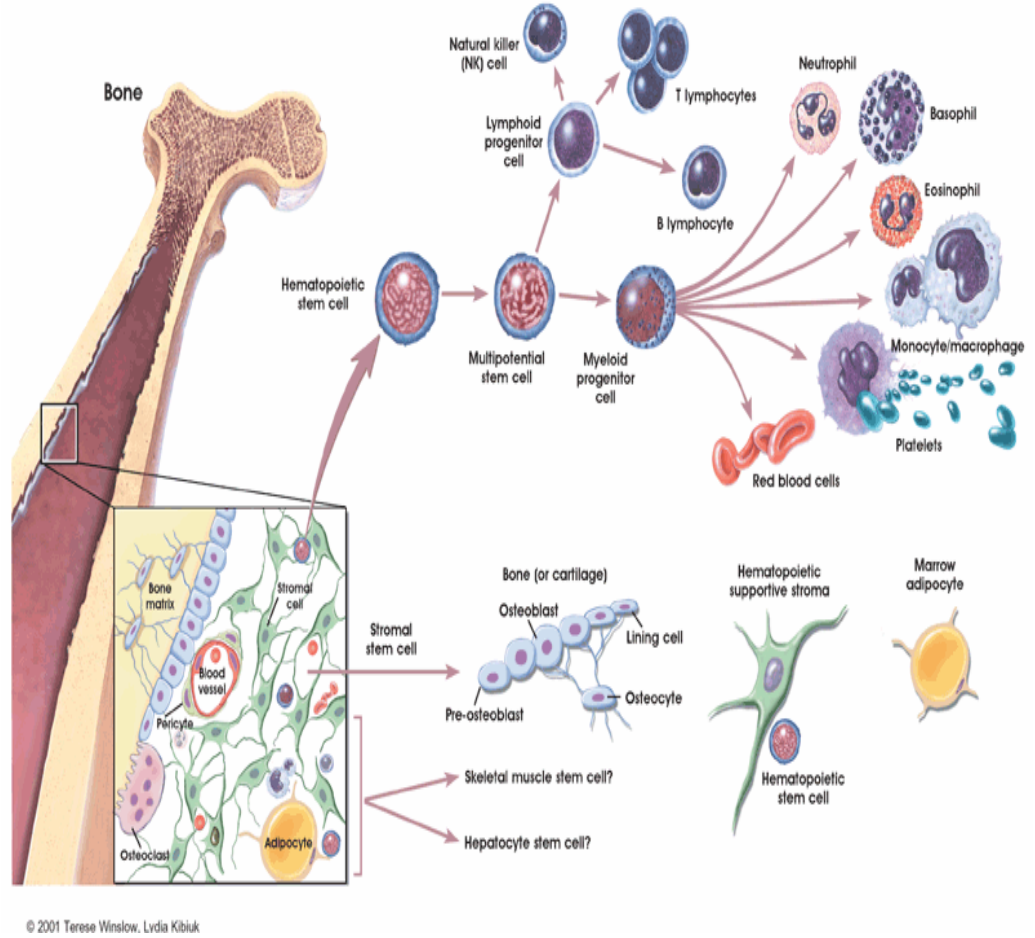

Figure 2.2 Cell types in the bone marrow

Osteoclasts are the bone cells responsible for bone resorption. They are multinucleated cells that are located on the calcified bone. These multinucleated cells are derived from the differentiation of haemotopoietic precursor cells of the marrow (46). The differentiation of osteoclast precursors is regulated by a number of local factors. Two haematopoietic factors essential for inducing osteoclast differentiation are receptor activator for nuclear factor κ B ligand (RANK-L) and macrophage colony stimulating factor (M-CSF) (47). M-CSF and RANK-L alone are sufficient to induce the proliferation and differentiation of haematopoietic

precursor cells into osteoclasts (47-48). Together these factors regulate terminal osteoclast development and function. The osteoclasts will eventually undergo apoptosis.

Osteoblasts are the bone-lining cells responsible for bone formation. They originate from the local stromal cells under the influence of local growth factors and require transcription factors such as runt homology domain protein Runx2/Cbfa1 (Runx2). Stromal cells differentiate into pre-osteoblasts, then mature osteoblasts, followed by a mineralization process. Osteoblasts will then transform either into bone-lining cells, or will become trapped in matrix and calcify to form osteocytes. Osteoblasts line the osteoid, which is bone matrix prior to mineralization. The osteoblast's cell membrane is rich in alkaline phosphatase (the concentration of which is an index for level of bone formation). It also has receptors for parathyroid hormone, steroid-like hormones, vitamin D3, and various cytokines. It also expresses an array of cytokines such as RANK-L, which has been shown to serve important functions during osteoclast differentiation. Additionally, osteoblasts express osteoprotegerin (OPG), a decoy receptor for RANK-L, with antiosteoclastogenic effects by affecting the interaction of receptor activator of nuclear factor κ B (RANK) with RANK-L. In experimental animals over-expression of OPG leads to osteopetrosis (49), while deletion of OPG results in increased bone remodeling and osteoporosis (49). Thus, by adjusting the expression of RANK-L and OPG, and the activation state of RANK on the osteoclast, the body can manage the positive and negative influence over bone absorption and BMD.

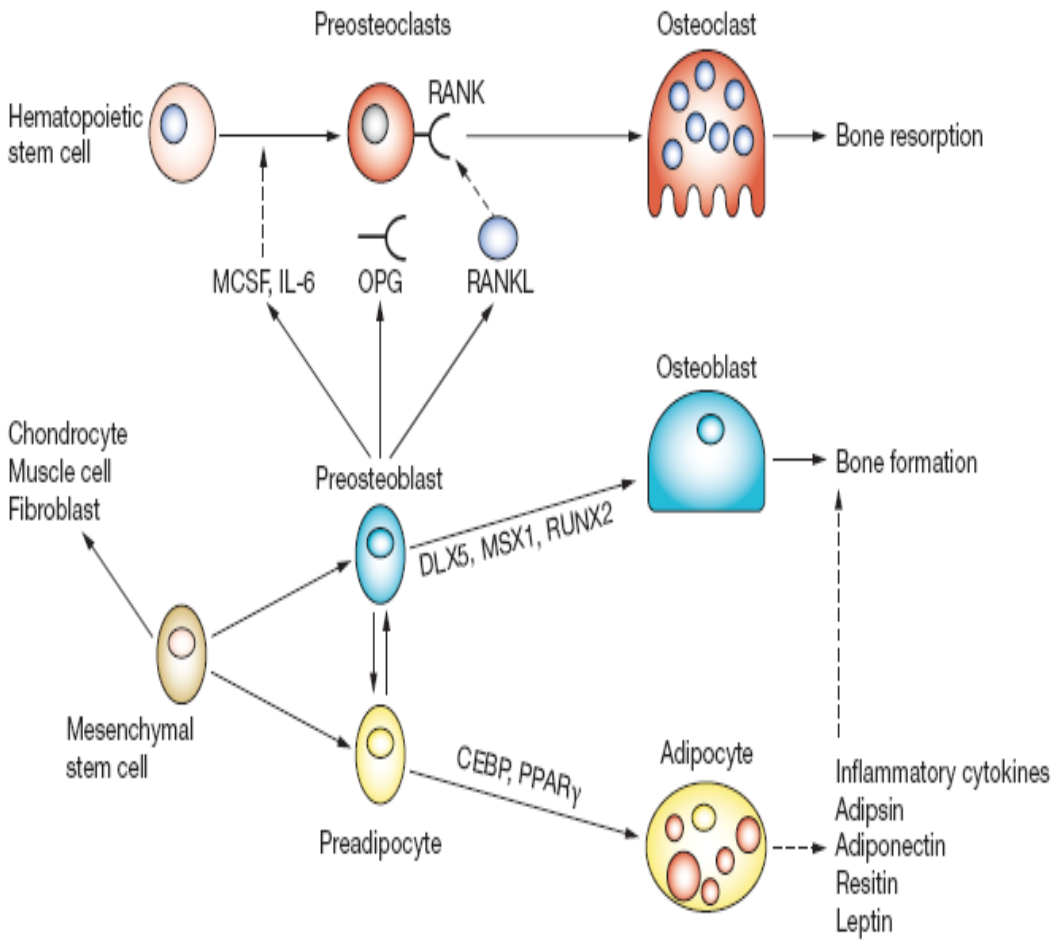

Figure 2.3 The schematic process of bone marrow cell differentiation (50)

The osteoblast and the adipocyte share a common mesenchymal stem cell progenitor, and the transcriptional mechanisms which determine the differentiation down either of these lineages are of much interest. A mouse model of accelerated aging suggests that aging is associated with decreased osteoblastogenesis and increased numbers of adipocytes (51). These findings are consistent with those of a number of studies suggesting an increase in the ratio of adipocyte to osteoblast progenitors with age (52). Clinical observations in humans have also shown that the number and size of bone marrow adipocytes increases with age and with the development of

osteoporosis (53-56). Co-culture studies of primary human osteoblastic cells have also demonstrated the ability of factors secreted by mature adipocytes and by polyunsatured fatty acids to inhibit osteoblast proliferation (57-58).

These findings suggest that adipogenic differentiation of stem cells occurs at the expense of the osteogenic differentiation; however, little is known about the factors determining lineage fate *in vivo.* Two different possibilities underlying lineage commitment of mesenchymal stem cells have been proposed: the first is the possibility of an expanding hierarchy of increasingly restricted progeny. The second is that there is a non-random, single step process, whereby multipotential progenitors become exclusively restricted to a single lineage (59). It is estimated that only a small percentage (15%) of proliferating cells obtained from flushed marrow have multipotential stem cell-like properties, with only a proportion of these able to undergo osteoblastic differentiation (60-61).

The numbers of adipocytes present in the whole body is not fixed, as clearly demonstrated by the expansion of adipocyte cell number or hypertrophic growth that occurs with overfeeding. Interestingly, high fat feeding in rats has been associated with an increase in the percentage of bone marrow fat due to increased adipocyte size, but not number (62). Differential adipose deposition also occurs in differing locations in males and females (63), suggesting that adipocyte differentiation is regulated in response to different levels of regulatory hormones or nutrient signaling.

Osteocytes originate from bone forming cells (osteoblasts), which become trapped in the bone matrix that they produce and later become calcified. However, they are not metabolically inert. Between the osteocyte plasma membrane and the bone matrix, the space is filled with extracellular fluid. Osteocytes play an important role as a mechanosensor and in local activation

of bone turnover. Due to the challenges of studying this cell, the effects of osteocytes on bone metabolism remain largely unknown.

Bone Remodeling

Bone is a dynamic, metabolically active tissue, which in the normal adult is constantly remodeled. Remodelling is a process involving the resorption and subsequent formation of new bone, and is regulated by numerous factors such as mechanical loading, locally acting factors, and systemically derived hormones, together maintaining whole body mineral homeostasis while maintaining the structural integrity of the skeleton.

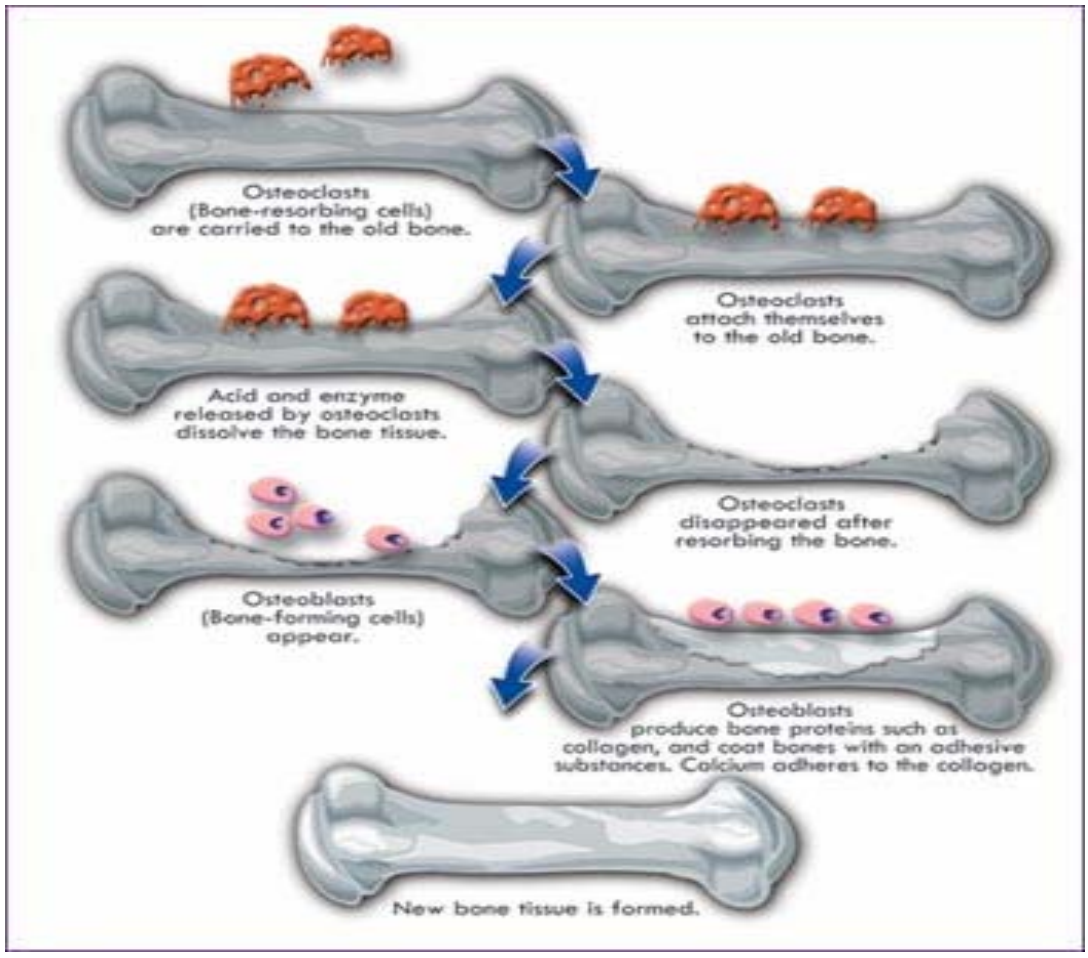

Figure 2.4 The bone remodeling process

Bone cell activity varies considerably throughout life during development and in the mature skeleton, including bone formation and resorption in both the process of *de novo* bone growth, and in bone remodeling, in which skeletal integrity is maintained. There are fundamental differences between the development of the growing skeleton and remodeling of the adult skeleton. Throughout growth, new bone formation occurs with growth in length and diameter following the cessation of growth, the adult skeleton through the actions of bone resorbing osteoclasts and bone forming osteoblasts in a tightly controlled process known as remodeling, allowing the mature skeleton to respond and adapt to mechanical stress, repair damaged bone, and participate in the maintenance of mineral homeostasis (64-65).

Under physiological conditions the process of bone remodeling is in equilibrium, such that localized resorption occurring in discrete areas is followed by an equal amount of bone formation, maintaining a constant bone mass. The mechanisms that determine the remodeling site or the initiation of remodeling or the signals coupling bone formation to resorption are not yet known. It has been proposed that coupling is mediated by factors released from the matrix during resorption, for example insulin-like growth factor 1 (IGF-1) or transforming growth factor β (TGF-β), stimulate the recruitment of osteoblasts to the resoption pit (66-69). However, there are numerous hormones, cytokines, and other factors that regulate the activity of osteoclasts and osteoblasts, and which together are likely to modulate the remodeling process. Structural proteins such as collagen or osteocalcin also have chemotactic effects and may therefore also be involved in the recruitment of osteoblasts (68). Moreover, osteoclastogenesis is highly dependent on the presence of osteoblasts, with essential factors for osteoclast proliferation and differentiation released from, and presented on, the surface of osteoblasts.

Osteoporosis

Disturbances in the balance between bone resorption and bone formation result in a change in net bone turnover and is responsible for many bone diseases including osteoporosis.

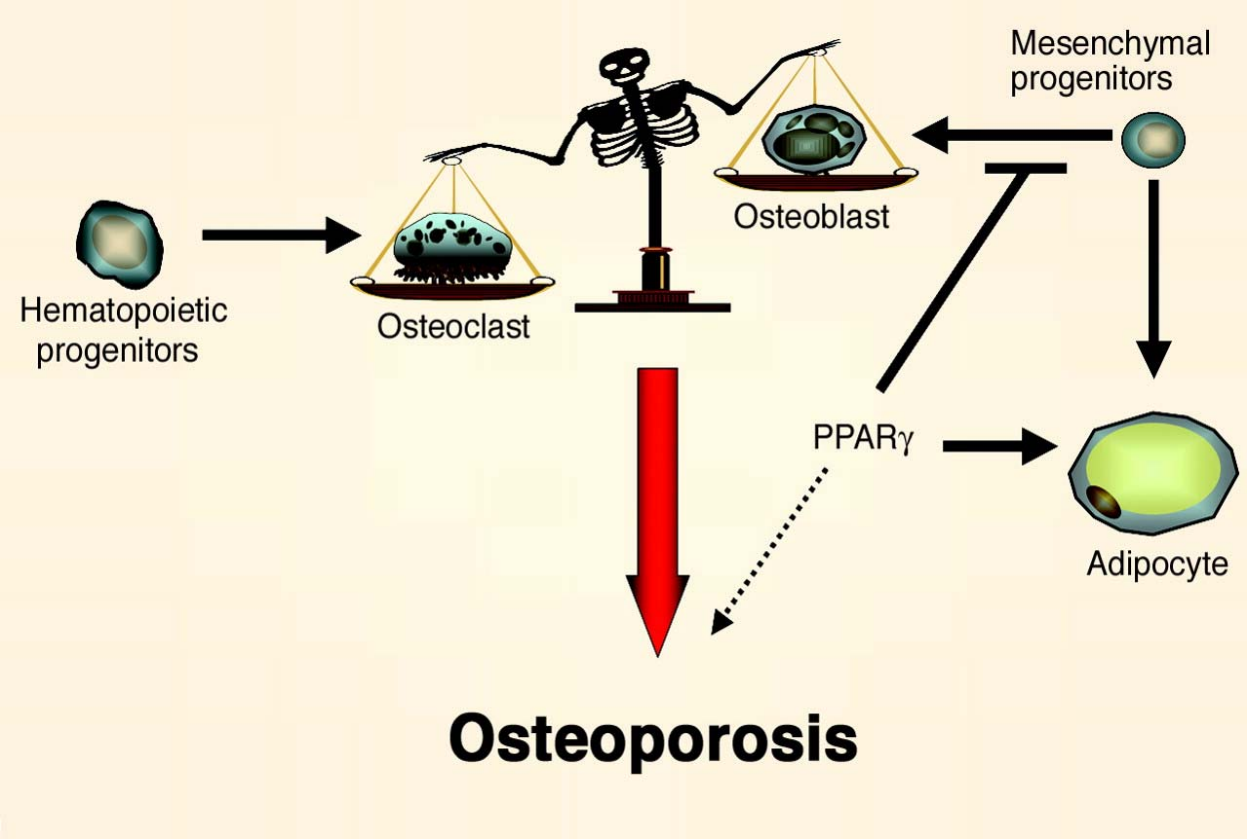

Figure 2.5 The balance between osteoblastic bone formation and osteoclastic bone resorption with an imbalance between the two being associated with osteoporosis (70)

Osteoporosis is a common disease in which low bone mass and deterioration of bone microarchitecture lead to increased risk of fracture with minimal trauma (71), resulting in significant morbidity and mortality in both men and women (72-73).

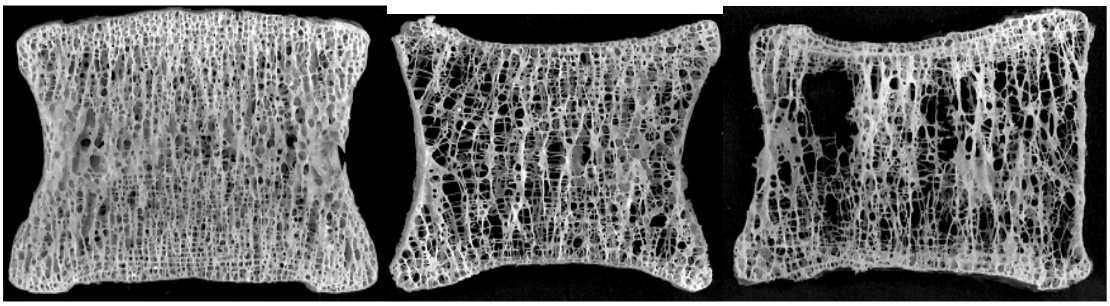

Figure 2.6 The bone microarchitecture of normal bone and a mild to severe case of osteoporosis

The loss of protective estrogenic effects on bone either with natural or surgical menopause, following ovariectomy, results in increased bone turnover, with elevations in both osteoclast and osteoblast activity. While the mechanisms behind this loss of bone are complex and involve many factors, the loss of bone results from an increase in the prevalence of osteoclasts through increased proliferation and differentiation of progenitors, increased support of osteoclast formation through increased osteoblastic numbers, and a decrease in osteoclastic apoptosis. Thus, an apparent increase in osteoclast activity exceeds the increase of the osteoblastic population, and an overall net increase in bone resorption ensues resulting in ongoing and at times, rapid bone loss.

Although obesity is a major risk factor for many disease processes and has become a heavy burden in western society, it at least bears one virtue--it may prevent osteoporosis. Studies have shown that heavier people tend to have higher bone mass, and that obese, menopausal women have decreased bone turnover. Mechanical loading is an important factor mediating the protective effects of adipocytes in bone marrow on BMD. This effect exists even at non-weight bearing sites, suggesting that factors other than skeletal loading might mediate this protective effect. Certain evidence has suggested that hormonal factors are involved in

regulating the mechanical loading process (74). Most studies suggest that insulin and estrogen may be the operative hormones.

However, in overweight women, this effect exists even after adjusting for estrogen levels (75). In patients with NIDDM (non-insulin dependent diabetes mellitus), BMD is not fixed, with some patients showing increased, unchanged or even decreased BMD (76). Thus, other hormones may be involved in this process. Recently, leptin has emerged as the best explanation for the protective effects of fat mass on BMD because serum leptin levels are directly correlated with BMD (77-78).

Leptin and Bone Metabolism

The recent discovery of the effects of leptin on bone through the sympathetic nervous system has sparked a new wave of research concerning the regulation of bone (79). The regulation of bone metabolism mediated by leptin is a complex process that is not clearly understood. The findings of increased trabecular bone formation and high bone mass within the proximal tibia and lumbar vertebrae of *ob/ob* mice (80), with similar observations in *db/db* mice, led to the conclusion that leptin signals through its known receptor (Ob-Rb) to control bone mass. Intracerebroventricular (ICV) infusion of leptin into the third ventricle resulted in bone loss in both *ob/ob* and wild-type mice, suggesting that leptin acts via the central nervous system to inhibit bone formation (80). However, Hamrick et al. showed that peripheral administration of leptin to *ob/ob* mice significantly increased whole body BMD and bone mineral content (BMC), decreased tibial bone marrow adipocyte number and increased tibial bone formation (81). The absence of detectable leptin in the serum of ICV-administered *ob/ob* mice also supports a central mode of leptin action (80). A parabiosis model in which only one *ob/ob* mouse of a parabiosed pair received ICV leptin was used to confirm the key role hypothalamic

leptin plays in regulating bone formation. Correction of the *ob/ob* skeletal phenotype by loss of bone mass was only achieved in the leptin recipient and not in the contralateral mouse (82), clearly demonstrating that similar to its control of energy homeostasis by central mediation, the action of leptin on bone is also regulated through a similar hypothalamic relay.

There are, however, contradictory findings when assessing leptin treatment on bone mass in rodents. Some studies have described *ob/ob* mice (leptin-deficient) as having a "high bone mass" (80,83-84), while Steppan et al. (85) observed that leptin-deficient mice had lower bone mass than normal mice. Hamrick et al., also showed that *ob/ob* mice had significantly shorter femora, lower femoral BMC, BMD, cortical thickness, and trabecular bone volume compared to lean mice, but had significantly increased vertebral length, lumbar BMC, lumbar BMD, and trabecular bone volume compared to lean mice (86).

One explanation of the discrepancies of results reported may be due to mode of leptin administration. SQ administration of leptin increased bone growth and indices of bone formation (81,85,87), whereas ICV infusion of leptin led to rapid bone loss in the limbs (80), revealing that leptin can regulate bone mass through alternative pathways: one involving a direct stimulatory effect on bone growth when administered peripherally and another that is indirect, involving a hypothalamic relay that suppresses bone formation when administered centrally (88).

It is also possible that differences in osteoblastic responses to leptin signaling are the result of species, strain, or age differences. Similar to the *db/db* mouse, the leptin-resistant Zucker (*fa/fa*) rats have an inactivating mutation in their Ob-Rb leptin receptor gene (89), but in contrast to the *ob/ob* or *db/db* models, these rats have reduced femoral BMD and trabecular volume and calcium content (90). Other studies have also demonstrated that leptin action in cortical bone may be distinct from its effects in trabecular bone, with reduced BMD and BMC in

4-week old and skeletally mature *ob/ob* mice (85-86,91). These effects were reversed in young mice following i.p. leptin injections (85). Some studies have also reported decreased femoral length in *ob/ob* mice (85-86). However, this observation is not consistent across studies (91) and may be related to the strain or age of rodent observed. Interestingly, some studies have also reported reduced distal and proximal femoral trabecular bone volume in *ob/ob* mice (85-86), contradicting findings from other studies of increased trabecular bone volume in the distal femur, proximal tibia, and lumbar spine (80,92). The reason for these inconsistencies is not clear but may relate to the trabecular bone volume measurement differences. One study utilized peripheral quantitative computerized tomography (pQCT) to measure trabecular bone volume (85). Others measured the bone volume with histomorphometry. Hamrick et al. (86) used hematoxylin and eosin staining on decalcified sections and would therefore be difficult to distinguish mineralized bone from osteoid, while others measured mineralized trabecular bone volume by von Kossa staining of un-decalcified sections (80,92). Discrepancies could also be because of the location of sampling observed in the studies. Reduced trabecular bone volume was reported in the proximal femur (86), while Baldock et al. (91) observed increased volume in the distal femur of *ob/ob* mice. Hamrick (81) reported reduced femoral BMD and BMC in *ob/ob* mice, while vertebral length and lumbar BMD and BMC were increased, suggesting distinct actions of leptin not only on cortical and trabecular bone compartments but also differential responses to leptin signaling between appendicular and axial skeletal sites. Hamrick (93) further suggested that leptin exerts opposite effects on the skeleton: peripherally, leptin may directly induce bone formation and increase cortical bone mass, while centrally, these anabolic effects may be attenuated by stimulation of sympathetic nervous system output to bone resulting

in increased β-1 AR stimulation, which prompts remodeling of trabecular bone. Leptin's CNS effects on bone may also be mediated in part through stimulation of the GH-IGF-1 axis

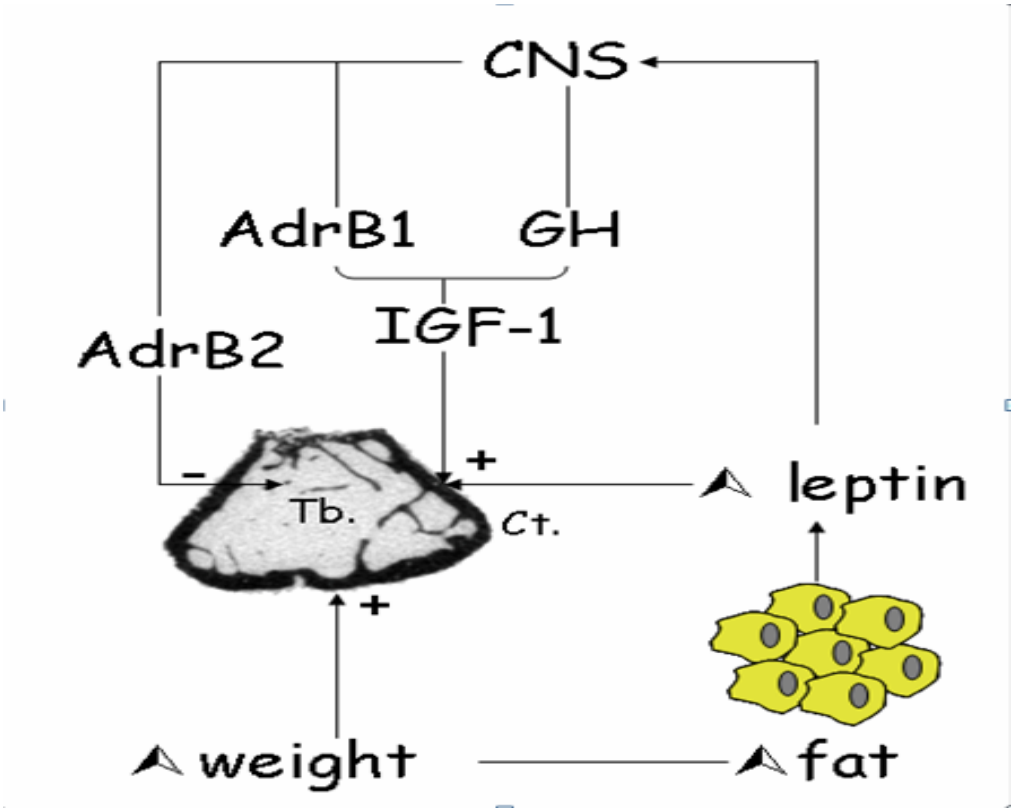

Figure 2.7 The schematic illustration of increasing leptin effects on bone(93)

The contradictions between centrally administered leptin in *ob*/ob mice findings could also be a result of the effects of positive peripheral leptin signaling at elevated leptin levels dominating the antiosteogenic effects of central leptin signaling (94).

Leptin and its neuronal co-factors in bone metabolism

Because of the apparent complexity of leptin's actions on the skeleton, recent studies have been conducted with several neuronal co-factors including beta-adrenergic receptors (β-AR), NPY, and CART that interact with leptin within the hypothalamus. These studies have

provided evidence of the coordinated efforts of these factors along with leptin and their roles in the regulation of energy homeostasis and bone metabolism. Therefore, changes in peripheral adiposity could affect bone mass by altering expression or activity of these neuronal factors.

Studies involving β-AR agonists and antagonists demonstrate that the central actions of leptin are mediated via the sympathetic nervous system (SNS). A mutant mouse strain deficient in β-AR was found to have high bone mass that was not reduced by ICV leptin infusion (82) indicating that the requirement for functional sympathetic signaling for leptin function. Sympathetic tone is reduced in leptin deficient mice (95). Pierroz et al. demonstrated that mice lacking β-AR show increased trabecular bone but decreased cortical bone, similar to *ob/ob* mice (96). Administration of β-AR antagonists increased bone mass in vertebrae and long bones of *ob/ob* and wild-type mice (82). Conversely, administration of β-AR agonists restored sympathetic activity in *ob/ob* mice, and decreased bone mass in both the *ob/ob* and wild-type mice without affecting body weight (82), demonstrating that the modulation of SNS activity can affect bone remodeling.

Another neuronal co-factor that interacts with leptin is NPY, which is a target of leptin signaling within the hypothalamus to regulate energy balance, modulating feeding behavior and bone metabolism through at least five receptor subtypes (Y1, Y2, Y4, Y5, and Y6) (97). Strongly supporting a role of NPY receptor signaling in the regulation of bone formation are findings that trabecular and cortical bone formation and volume are increased in $NPY^{-/-}$ mice (97) and that long bone trabecular bone mass was increased by the deletion of Y2 receptors in the hypothalamus (98). This evidence has pointed to a link between NPY and leptin for the regulation of bone mass.

Through recent studies, CART has been revealed to be a significant neuronal co-factor when combined with leptin. CART is a neuropeptide expressed at high levels in the mouse hypothalamus (99). CART peptide also increases food intake after ICV injection in rats, implicating CART as a potential regulator of feeding, satiety, and body weight (100). CART expression in the hypothalamus is also upregulated by the leptin (99). Although CART may play a role in mediating leptin's effects on bone marrow adipogenesis and apoptosis, it does not appear to be involved in linking body weight with bone mass (101). It has been shown that CART-deficient mice have low bone mass in their spine, suggesting that the effects of leptin on bone mass in the axial skeleton may be mediated at least in part by CART signaling, acting through a central, neuroendocrine signaling pathway (102). However, the effects of leptin on cortical bone mass do not appear to be mediated by CART (101). It has been proposed that while leptin may increase bone resorption by stimulating RANK-ligand expression in osteoblasts, leptin can also inhibit osteoclast activity by increasing CART expression, which decreases RANK ligand expression by osteoblasts (81,85).

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CHAPTER 3

INCREASING CONCENTRATIONS OF INTRACEREBROVENTRICULAR LEPTIN INJECTIONS PROMOTE BONE GROWTH AND ENHANCE BONE MARROW CELL DIFFERENTIATION IN *ob/ob* MICE[1]

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Abstract

Leptin injected intracerebroventricularly (ICV) reduces body weight (BW) and body fat. However, there are contradictory findings when assessing central leptin treatment on bone mass in rodents. The objective of this study was to determine the effect of increasing concentrations of central leptin administration on bone formation and bone marrow gene expression in leptin deficient ob/ob mice (15-wk old, init BW=60.6g). Leptin (1.5 μg/d or 0.38 μg/d) or control was continuously delivered ICV via osmotic pumps for 12 days (n=6). RNA was extracted from the bone marrow adherent cells of the left femur. Leptin decreased BW, body fat, and blood glucose and insulin concentrations and increased osteocalcin, OPG, IGF-1 and RANK-L concentrations, whole body bone mineral density (BMD) and bone mineral content (BMC) , distal tibial BMD and BMC, lumbar BMD and BMC and tibial mineral apposition rate (MAR). mRNA expression levels of genes associated with osteogenesis (Runx2, Sp7, Ccl27) were increased, while those associated with osteoclastogenesis (RANK, Csf1) were decreased, which is consistent with the observed leptin-stimulated bone growth and inclination of cells to differentiate into osteoblasts. Expression levels of genes involved in adipogenesis, adipocyte lipid storage and cell survival (eg., PPARγ, Bcl-2, Retn, Aebp1) were decreased, demonstrating an enhancement in the sensitivity to leptin-stimulated adipocyte apoptosis in the bone marrow. The reduction in bone marrow adipocytes and increase in the bone marrow osteoblasts, serum bone markers, BMD and BMC indicates a positive effect on bone formation. These results show that increasing concentrations of ICV leptin injections promote expression of pro-osteogenic factors in the bone marrow which enhance bone formation in *ob/ob* mice.

Keywords: leptin; osteoporosis; bone; gene expression; *ob/ob* mice

Introduction

Leptin, a cytokine-like hormone secreted by adipocytes, regulates food intake, energy expenditure, and bone metabolism by binding to its receptor in the hypothalamus. Unfortunately, resistance to leptin's effects develops rapidly. An important component in leptin resistance appears to be the development of defective leptin uptake into the brain, leptin receptors being down-regulated, and leptin sensitivity being decreased.

Leptin's regulation of bone mass occurs through a central, neuroendocrine signaling pathway (1). Marrow tissue in bones is known to accumulate adipocytes with age. Leptin can act directly on bone marrow cells to enhance their differentiation to osteoblasts and inhibit their differentiation to adipocytes (2).

There is, however, a controversy in the assessment of leptin's effects on bone metabolism in rodents. The contradictory findings are centered on the central administration of leptin. There are studies that have described *ob/ob* mice (leptin-deficient) that were administered leptin as having a "high bone mass"(3-5), while Steppan et al. (6) and Cornish et al. (7) observed that leptin-deficient mice had lower bone mass than normal mice. One explanation of the discrepancies of results reported may be due to the dose of leptin administered. Central leptin (ICV) administration at a lower dose increases bone growth and indices of bone formation (4), whereas higher doses of ICV infusion of leptin might lead to bone loss (3) suggesting that leptin can regulate bone mass through alternative pathways. One pathway involves a direct stimulatory effect on bone growth when administered peripherally and another indirect pathway, involves a hypothalamic relay that suppresses bone formation (8). However, in a study conducted by Iwaniec et al. in which *ob/ob* mice were injected in the hypothalamus with a recombinant adenovirus expressing leptin, femoral bone mass and length increased while trabecular bone

volume declined (9). Thus, based on current evidence, it is unclear how altered leptin signaling with varying doses of intracerebroventricular leptin injections affect bone modeling in vivo. Therefore, the objective of the study was to determine the effect of varying concentrations of central leptin administration on bone marrow cell differentiation and bone growth in *ob/ob* mice.

Materials and methods

Animals and Design

All experimental and surgical procedures in this study were approved by the Animal Care and Use Committee for The University of Georgia. Eighteen female leptin deficient (*ob/ob*) mice (15 wk old; 60.6g initial body weight) on the C57BL6 background were purchased from Jackson Laboratories, Inc. (Bar Harbor, ME). The mice were individually housed in plastic shoebox cages in a room with a 12-/12-hour light/dark cycle, 22 ± 1 ºC ambient temperature, and 50% humidity. Mice had ad libitum access to pelleted standard lab chow (LabDiet 5001, PMI Nutritional International, Brentwood MO) and water throughout the study.

All mice were surgically implanted with osmotic minipumps (model 1002, 0.25 µL/h; Alzet Corp., Cupertino, CA) for injection of treatment solutions. Mice were anesthetized with 0.5% oxygen/isoflurane and prefilled and primed pumps were inserted into a SQ pocket. Mice were surgically prepared with a unilateral lateral ventricular (ICV) guide cannula (model 3280P/spc; Plastics One Inc., Roanoke, VA), which included an infusion port that was attached to the osmotic pump, as previously described(10).

Treatments

Treatments were randomly assigned and included, ICV control (aCSF) 6 µl/day (treatment 1), ICV leptin 0.38 µg/day (treatment 2), and ICV leptin 1.5 µg/day (treatment 3).

Recombinant mouse leptin (R&D Systems, Minneapolis, MN) was dissolved in either an artificial cerebrospinal fluid (aCSF), which consisted of (in g/l): NaCl , 8.66; KCl, 0.224; $CaCl_2 \cdot 2H_2O$, 0.206; $MgCl_2 \cdot 6H_2O$, 0.163; $Na_2HPO_4 \cdot 7H_2O$, 0.214; $NaH_2PO_4 \cdot H_2O$, 0.027 for ICV delivery.

Design and Procedures

Mice had an ICV cannula with an osmotic pump attached for continuous ICV injection of control and test solutions. Osmotic mini-pumps remained in place for 14 days. All mice were allowed to recover for 2 days after surgery; during this time 6 µl of aCSF were infused prior to the treatment infusion, which occurred for the next 12 days. The mice were injected (intraperitoneal) with calcein (Sigma C-0875; 20 mg/kg BW) on the first day of treatment injections and again on the day before they were to be killed to label active bone forming surfaces. The health of the mice was monitored and BW and FI were measured and recorded daily.

The mice were killed by decapitation using a guillotine after sedation in a CO_2 chamber at the end of the 12^{th} day of treatment injection. The soleus and gastrocnemius (GC) muscles were collected, weighed, and then frozen in liquid nitrogen before storing at -80 °C. Trunk blood was collected for measurement of blood glucose. Serum RANK-L concentrations were determined using the Luminex100™ instrumentation and a single-plex assay kit (catalog # MBN-41K-1RANKL, Millipore Corp., St. Charles, MO). Serum IGF-1 concentrations were determined using the Luminex100™ instrumentation and a single-plex assay kit (Catalog No. RMIGF1-87K) manufactured by Millipore. The Mouse Bone Panel from Millipore (Catalog No. MBN-1B-41K) was used to determine the serum insulin, osteocalcin, and osteoprotegerin (OPG) concentrations using the Luminex100™ instrumentation.

Body composition was analyzed by PIXImus densitometry (GE Lunar Corp., Waukesha, WI). Fat mass, lean mass, percentage fat, bone mineral density (BMD), and bone mineral content (BMC) were measured for each animal. The left tibia and femur and lumbar spine of each mouse were dissected free of soft tissue. The left tibia was fixed in 70% ethanol for bone densitometry measurements and embedding in methyl-methacrylate. DEXA densitometry (PIXImus system) was used to measure BMD and BMC from the left tibia and lumbar spine (L2-L3). The PIXImus dual-energy X-ray absorptiometry system allows accurate measurement BMD and BMC from small lab animals using a relatively low X-ray energy (80/35 kVp) and ultra-high resolution (0.18 x 0.18 mm pixel size) to achieve contrast in low-density mouse bone. Replicability data indicate an excellent correlation (0.99) between PIXImus BMC and total ashed weight (11).

Bone histomorphometry

The left tibia was fixed in 70% ethyl alcohol, dehydrated, embedded in methyl methacrylate, and sectioned at 30μm across the proximal third of the shaft. These thicker sections are preferred because they provide excellent visualization of fluorochrome labels (1). The mineral apposition rate (MAR) was derived from fluorochrome interlabel distances.

Gene expression

Bone marrow was taken from the left femur. The left femur was flushed of its bone marrow, plated for 24h, before extracting for mRNA using the Qiagen RNeasy mini kit in accordance with the manufacturer's protocol. One μl of the 15 μl sample was used as an integrity check and for quantification by the Agilent 2100 bioanalyzer and RNA 6000 Nano Assay (Agilent Technologies, Foster City, CA). One hundred ng of total RNA in a 20-μl reaction was reverse-transcribed using

the cDNA Archive Kit (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer's protocol using MultiScribeTM Reverse Transcriptase. Reactions were incubated initially at 25 ºC for ten minutes and subsequently at 37 ºC for 120 min. Quantitative PCR (Taqman^{TM}) assays were chosen for the transcripts to be evaluated from Assays-On-Demand^{TM} (ABI), a pre-validated library of QPCR assays incorporated into 384-well MicroFluidic cards^{TM}. All of the oligonucleotide primer and fluorogenic probe sets for Taqman^{TM} real time PCR were from ABI. Two µl of the cDNA samples, along with 50 µl of 2X PCR master mixes were loaded into respective channels on the microfluidic card followed by a brief centrifugation (3000 rpm for three minutes). The card was then sealed and real-time PCR and relative quantification was carried out on the ABI PRISM 7900 Sequence Detection System. The cycle conditions were: 94.5 ºC for 15 minutes, followed by 40 cycles of 97ºC for 30 s and 59.7ºC for one minute. Data were expressed as relative quantification (RQ), which presents the fold difference of mRNA level in treatment groups relative to the aCSF control group, and were analyzed using sequence detection systems software. The expression of mRNA was normalized by using 18S RNA as an endogenous control. The ΔCT values were first calculated by using CT for a specific gene mRNA minus CT for 18S RNA mRNA in the sample. Then the mean mRNA expression from the treatment groups were compared with the aCSF control group using the formula: relative quantification = 2-ΔΔCT (ΔΔCT is the average aCSF control group ΔCT values minus the average experimental group ΔCT values, and ΔΔCT of one equates to a twofold difference in cDNA added into the PCR reaction).

Statistical analysis

All statistical analyses were conducted with SAS (Version 9.0) (SAS Institute, Cary, NC). Significance of treatment effects was determined by one or two way ANOVA. Significance of

differences among means was determined by Fisher's LSD. Significance was established at $p < 0.05$.

Results

Food intake, body weight, body composition

Regardless of dose, there were significant effects on BW due to leptin. By the end of the 12-day injection period, high dose and low dose of ICV leptin-treated mice had lost 17.2 ± 1.9 g and 5.2 ± 1.6 g BW, respectively ($p < 0.0001$) while the aCSF-injected mice gained 4.3 ± 2.7 g. The high dose of leptin decreased daily food intake by 86.6% and the low dose of leptin, by 58.2% ($p < 0.001$). (Figure 3.1)

DEXA results showed that the high dose of leptin administered mice had a significant decrease in fat mass and % body fat, and an increase in % lean mass. Both groups of leptin treated mice had an increase in whole body BMD but only the high dose of leptin mice had an increase in whole body BMC (Table 3.1). The GC and soleus muscle weights were significantly increased in the highest dose of leptin treated mice (Figure 3.2). The muscle mass of the mice was positively correlated with BMD ($r=0.49$, $p=0.004$) and BMC ($r=0.73$, $p<0.0001$).

Serum hormone concentrations

The high dose of leptin administration significantly reduced the blood glucose concentrations. Both groups of leptin treated mice had significantly lower concentrations of serum insulin and higher concentrations of osteocalcin and OPG, markers associated with bone formation, as compared to the control mice in a dose dependent manner. Likewise, both high and low dose of leptin treated mice had significantly dose dependent higher concentrations of RANK-L, a marker that indicates bone turnover. Serum IGF-1 concentrations were significantly higher in the high dose of leptin administration compared to the other treatment groups. (Figure

3.3) The serum IGF-1 levels were positively correlated with muscle mass (r=0.49, p=0.02), BMD (r=0.63, p=0.0001), and osteocalcin concentrations (r=0.57, p=0.009).

Bone BMD, BMC and histomorphometry

The highest dose of leptin administration increased the relative BMD and BMC in the left tibia and L2-L3 vertebrae (Table 3.2). However tibial length was not increased (data not shown). This can be a result of an increase in the way that leptin was administered but it needs to be determined in a future study where leptin is administered at varying lengths/periods of time. The tibial MAR was significantly higher in the high dose of leptin treated mice compared to the control and low dose of leptin treated mice by at least +3.5 micron/d (Figure 3.7).

Expression of genes associated with osteoclastogenesis (RANK, Csf1) in femoral bone marrow tissue in ob/ob mice

Regardless of dose, leptin treatment decreased the mRNA level of RANK as compared to the control. The high dose of leptin treated mice had significantly decreased expression of Csf1, which is required for osteoclast differentiation. (Figure 3.4).

Osteogenic-related genes (Runx2, Sp7, Ccl27) mRNA in femoral bone marrow tissue in ob/ob mice

The highest dose of leptin treatment increased the mRNA of Runx2, Sp7, and Ccl27 as compared to the control and the low dose of leptin administration (Figure 3.5).

Expression of genes involved in adipogenesis, adipocyte lipid storage, and cell survival (PPAR-γ, Bcl-2, Aebp1, Dlk1, GATA3, Retn) in femoral bone marrow tissue in ob/ob mice

Leptin treatment decreased the expression of PPAR-γ, Bcl-2, Aebp1, Dlk1, GATA3, and Retn as compared to the control and low dose of leptin administration (Figure 3.6).

Discussion

Leptin reduced food intake, resulting in a decrease in body weight through the loss of fat mass in a dose dependent manner. Lean mass regulates local bone formation by muscle-derived mechanical stimuli (12). Muscle mass is directly correlated to the amount of bone mass (13). Compared to lean mice, genetically obese *ob/ob* mice have less skeletal muscle mass (1), which has been shown to be associated with low circulating levels of growth hormone (14) and resistance to IGF-1 (15). Our results show that only leptin treated mice at the highest dose had increased serum IGF-1 levels, which likely contributed to increased muscle growth and bone formation via muscle-derived mechanical stimuli in these mice. These results appear to be dependent upon the dose of leptin administered. The stimulation of muscle growth may also have been a result of increased physical activity, since leptin has been shown especially in *ob/ob* mice during the loss of adipose tissue to increase physical activity (16).

RANK (Osteoclast Differentiation Factor Receptor) is an osteoclast differentiation factor that mediates an essential signal for osteoclastogenesis. It is also a ligand for OPG, a secreted protein that inhibits osteoclastogenesis (17). The increase in the expression of RANK may be why an increase in serum RANK-L and OPG concentrations in the low dose of leptin treated mice was observed. The decrease in RANK expression in the high dose of leptin treated mice indicate an inhibition of osteoclastogenesis in these mice.

Dobbins et al. suggests that Csf1 (Colony Stimulating Factor 1) is a growth factor required for osteoclast differentiation and activation (18). It is produced by osteoblasts to inhibit osteoclast apoptosis and may increase bone resorption by elongating the lifespan of osteoclasts. The increase in Csf1 expression in the low dose of leptin treated mice may help to explain the increase observed in serum RANK-L concentrations, since there is a need to increase

osteoclastogenesis and thus increase bone turnover for bone formation to occur (9). The decrease in Csf1 expression in the high dose of leptin treated mice indicate an inhibition of osteoclastogenesis in these mice.

Ducy's laboratory showed that Runx2 (Runt Related Transcription Factor 2) is an osteoblast specific transcription factor and a regulator of osteoblastic differentiation (19). Komori and colleagues demonstrated that without Runx2 expression there is a lack of complete bone formation and ossification of the skeleton and is involved in states of osteoclastic bone resorption (20). Sp7 (Specific Protein 7 or Osterix) is expressed in all developing bones and Sp7-null mice were found to have no occurrence of bone formation (21). The increase observed in the osteogenic genes in the high dose of leptin treatment translated into increased bone indices and thus greater bone formation. The response was dependent upon dose of leptin administered since the increase was only observed in the high dose of leptin treatment.

PPAR-γ (Peroxisome Proliferator-Activated Receptor Gamma) regulates adipocyte expression and may be indicative of an autoregulatory mechanism holding the expansion of individual adipocytes in states of positive energy balance (22). Cells lacking PPAR-γ fail to differentiate into adipocytes (23). PPAR-γ +/- mice displayed high bone mass due to increased osteoblastogenesis with normal osteoblast and osteoclast function (23). The decrease in the presence of mRNA PPAR-γ in the bone marrow cells with high dose of leptin treatment may help to explain the propensity of the cells to differentiate into osteoblasts rather than adipocytes thus resulting in increased bone mass in only the high dose of leptin treated mice.

The observation of bone marrow cells differentiating into osteoblasts versus adipocytes with only the high dose of leptin was further substantiated by the increased expression of Bcl-2, Aebp1, Dlk1, GATA3, and Retn. Bcl-2 (B-cell leukemia) expression provides a prevention of

apoptosis. Apoptosis is integral to physiological bone turnover. The balance of osteoblast proliferation, differentiation, and apoptosis determines the size of the osteoblast population at any given time (24). Zhang et al. found that Aebp1 (AE Binding Protein 1) expression is terminated in terminally differentiated, non-proliferated adipocytes and overexpression of the gene induces massive obesity (25). Dlk1 (Delta-drosophila Like Factor 1 or Preadipocyte Factor 1) is a regulator of adipocyte differentiation. Its expression is high in preadipocytes, but was not expressed during differentiation of preadipocytes to adipocytes (26). Tong and colleagues found that cells lacking GATA3 had an enhanced capacity to differentiate into adipocytes, while defective GATA3 expression is associated with obesity (27). Retn (Resistin) is a signaling molecule secreted from adipocytes and is expressed during adipocyte differentiation but is downregulated in mature adipocytes (28). The results demonstrate that the concentration during leptin administration enhance the differentiation of cells into osteoblasts but not adipocytes, since the boost of osteogenesis only occurred with the high level of leptin treatment. Furthermore, the decreased presence of adipocytes should have resulted in reduced induced production of inflammatory cytokine release that promotes osteoclastogenesis.

Martin and colleagues have suggested that leptin has a bimodal effect on bone, with low levels of leptin promoting bone formation and high levels resulting in bone resorption secondary to decreased serum IGF-1 levels caused by reduced energy intake (29). In the present study, the high dose of leptin treated mice had a reduction of food intake and body weight, yet their serum IGF-1 levels were increased compared to the control mice. Whether even higher or possibly even lower (lower doses that inhibit bone formation without loss of body weight (3)) doses of leptin would have resulted in suppression of IGF-1 levels and increased bone resorption is not known but could be tested in a future study. However, we believe our study is the first to

investigate the effects of increasing concentrations of centrally administered leptin on how *in vivo* bone marrow cell differentiation affects bone growth.

This study shows that the high dose of leptin administration can promote osteogenesis and reduce bone marrow adiposity, in agreement with previous reports (1,30). In a study conducted by Maness and colleagues, it was found that 3.64% of intact protein from the centrally injected dose of leptin is cleared from the brain to enter the circulation with blood levels rising over time (31). The effect of continuous centrally administered leptin may be partly due to its direct effect on bone marrow cells, enhancing their differentiation to osteoblasts and inhibiting their differentiation into adipocytes (2). Since the marrow tissue is known to accumulate adipocytes with age (2), the decrease in the bone marrow adipogenesis observed in this study in the high dose of leptin treated mice, while increasing the bone indices, may indicate that a reduction in marrow adipocytes has a positive effect on bone formation.

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Figure 3.1. Body weight changes and daily food intake after 12 days of low and high doses of ICV leptin injections in *ob/ob* mice (top). Photographs of the dose responses in mice as it affected their body composition (bottom). Means which don't share a common letter are significantly different: ^{a,b} $p < 0.05$.

Figure 3.2. The muscle weights of ICV leptin treated *ob/ob* mice injected for 12 days. Means which don't share a common letter are significantly different: ^{a,b} $p < 0.05$.

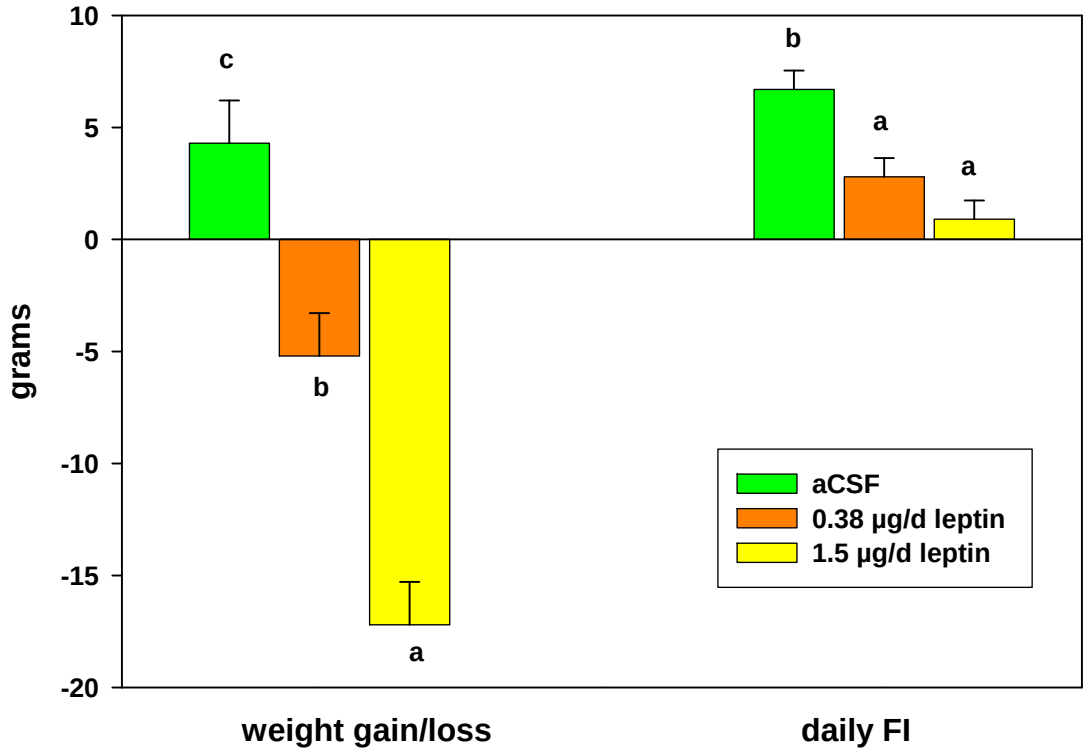
Figure 3.3. The effect of 12 days of low and high doses of ICV leptin injections on blood glucose and serum insulin, osteocalcin, OPG, IGF-1, and RANK-L concentrations. Means which don't share a common letter are significantly different: ^{a,b} $p < 0.05$.

Figure 3.4. Histograms showing the osteoclastic-related gene expression in leptin treated mice. Means which don't share a common letter are significantly different: ^{a,b} $p < 0.05$.

Figure 3.5. Histograms showing the expression of genes involved in osteogenesis in leptin treated mice. Means which don't share a common letter are significantly different: ^{a,b} $p < 0.05$.

Figure 3.6. Histograms of gene expression involved in adipogenesis, adipocyte lipid storage, and cell survival in leptin treated mice. Means which don't share a common letter are significantly different: ^{a,b} $p < 0.05$.

Figure 3.7. Images of fluorochrome-labeled bone forming surfaces in tibia (bottom). Histograms showing tibial MAR (top). Means which don't share a common letter are significantly different: ^{a,b} $p < 0.05$.


10
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-10
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grams
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aCSF
0.38 µg/d leptin
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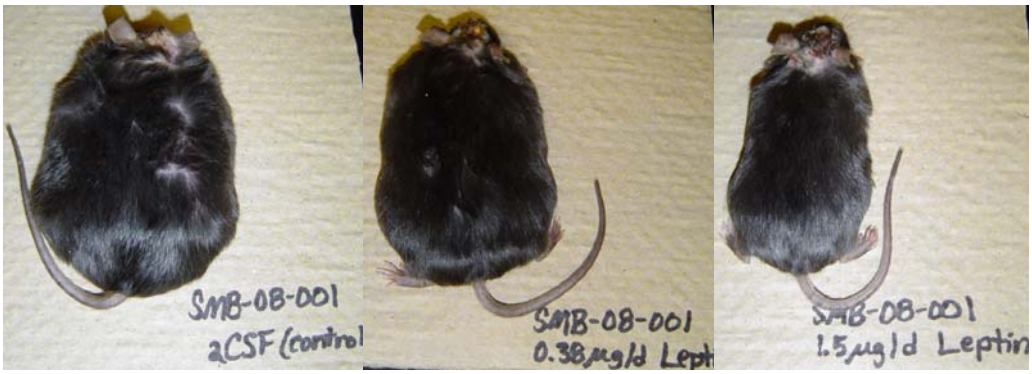
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SMB-08-001
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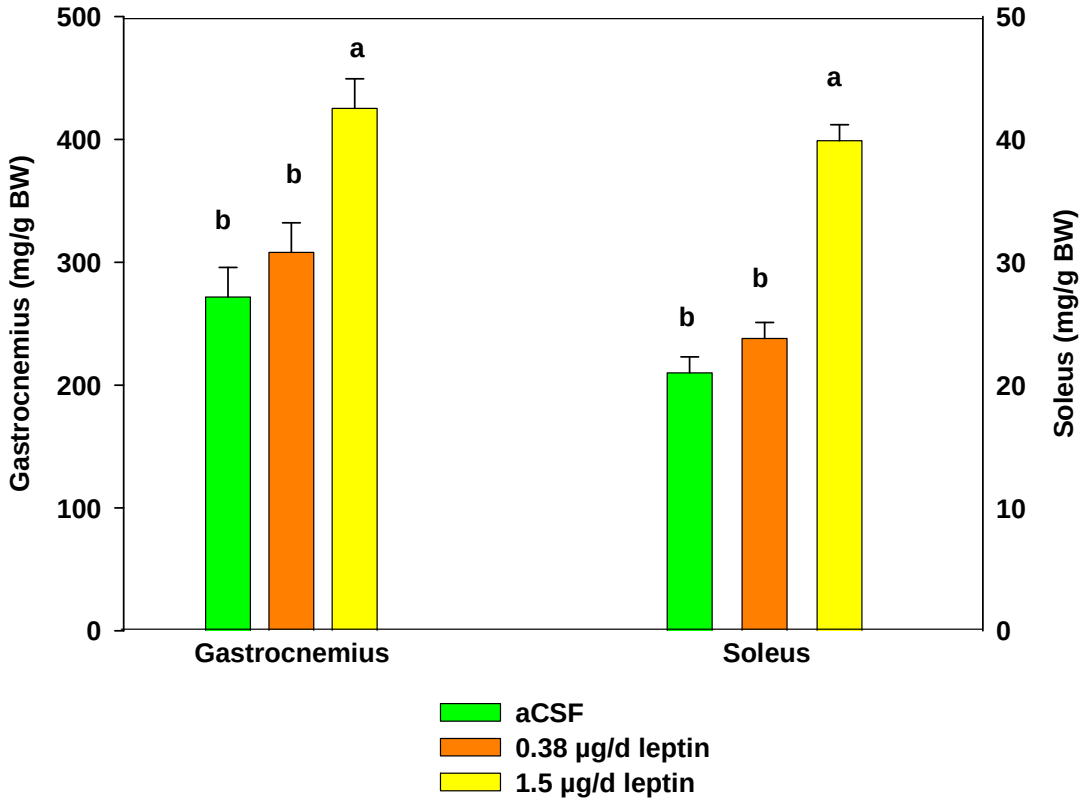
500
400
300
200
100
0
50
40
30
20
10
0
Gastrocnemius (mg/g BW)
Soleus (mg/g BW)
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b
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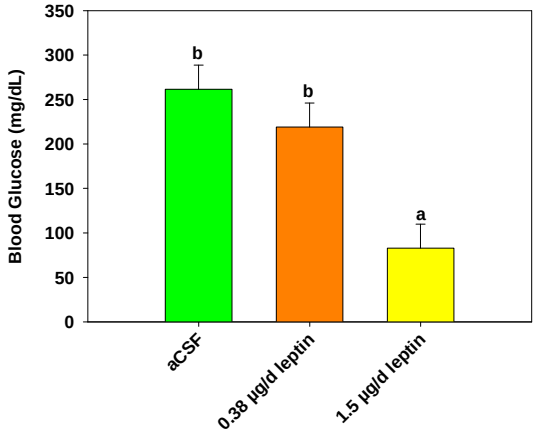
a
b
b
Gastrocnemius
Soleus
aCSF
0.38 µg/d leptin
1.5 µg/d leptin


Blood Glucose (mg/dL)
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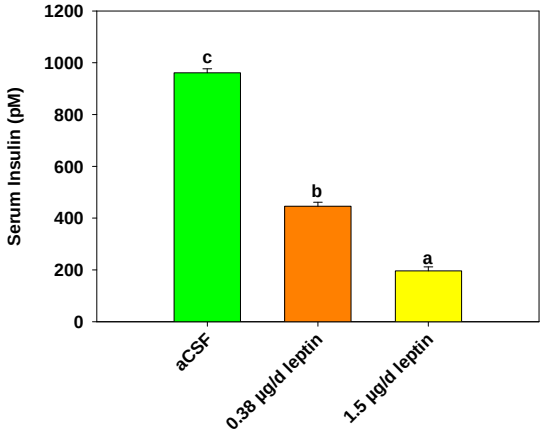
200
150
100
50
0
b
b
a
aCSF
0.38 µg/d leptin
1.5 µg/d leptin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1000
800
600
400
200
0
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b
a
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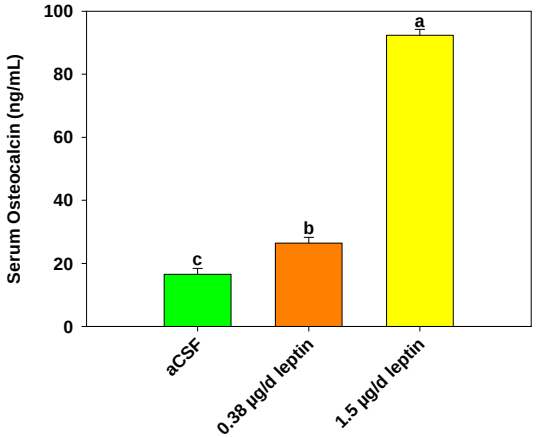
1.5 µg/d leptin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0
c
b
a
aCSF
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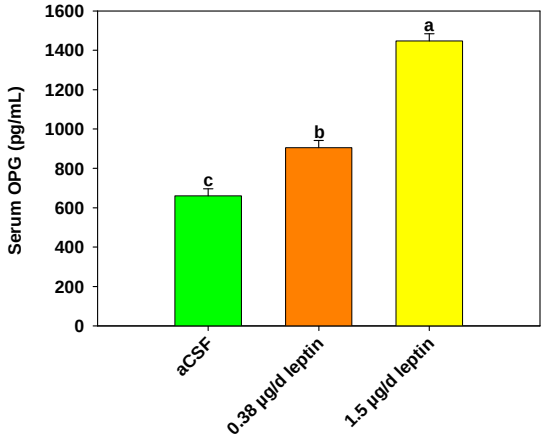
1.5 µg/d leptin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c
b
a
aCSF
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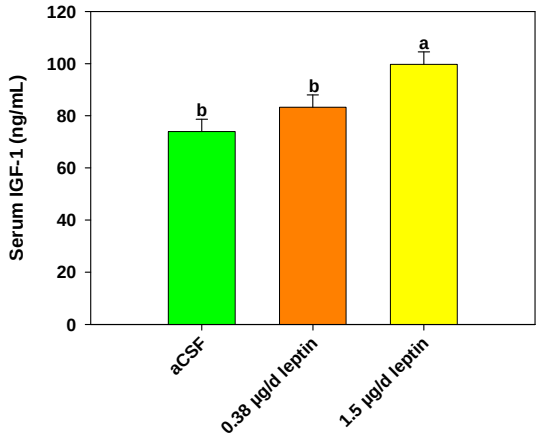
1.5 µg/d leptin


Serum IGF-1 (ng/mL)
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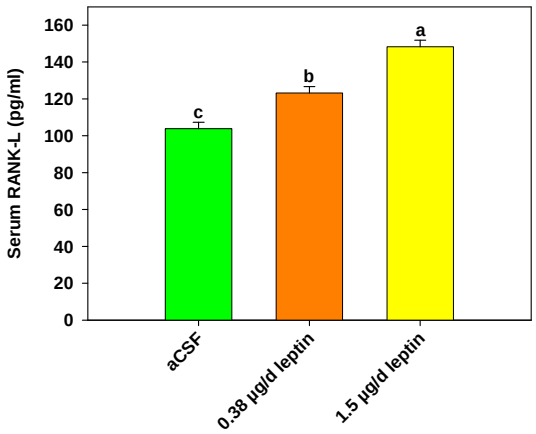
100
80
60
40
20
0
b
b
a
aCSF
0.38 µg/d leptin
1.5 µg/d leptin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60
40
20
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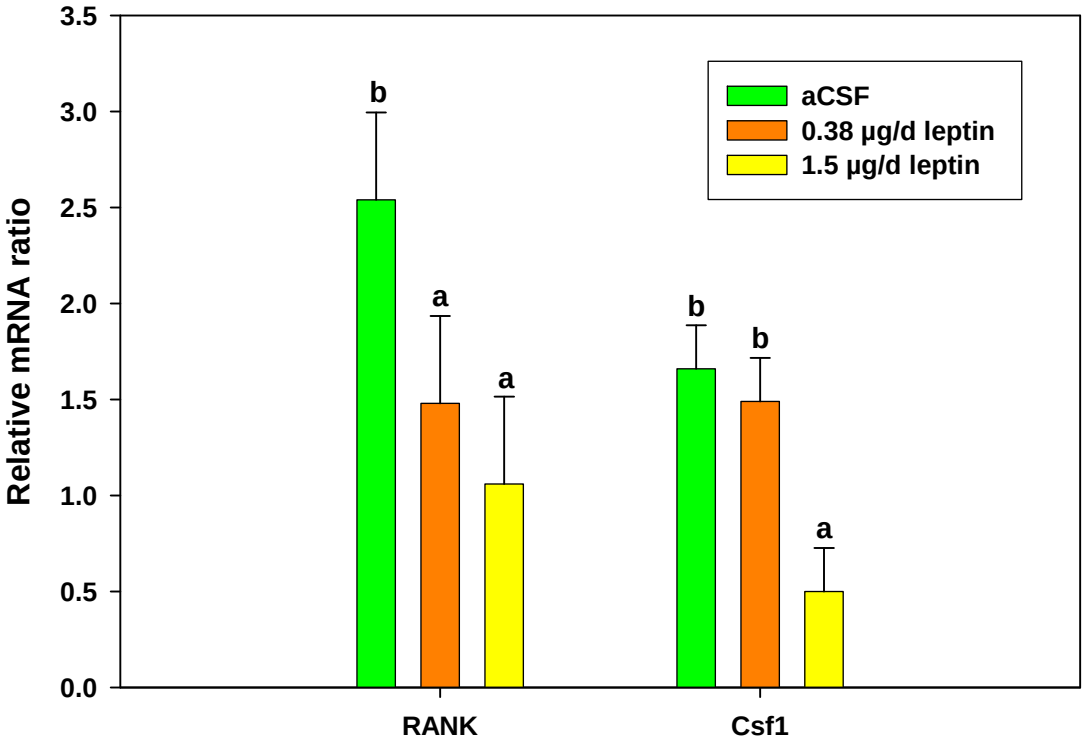
a
aCSF
0.38 µg/d leptin
1.5 µg/d leptin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3.0
2.5
2.0
1.5
1.0
0.5
0.0
aCSF
0.38 µg/d leptin
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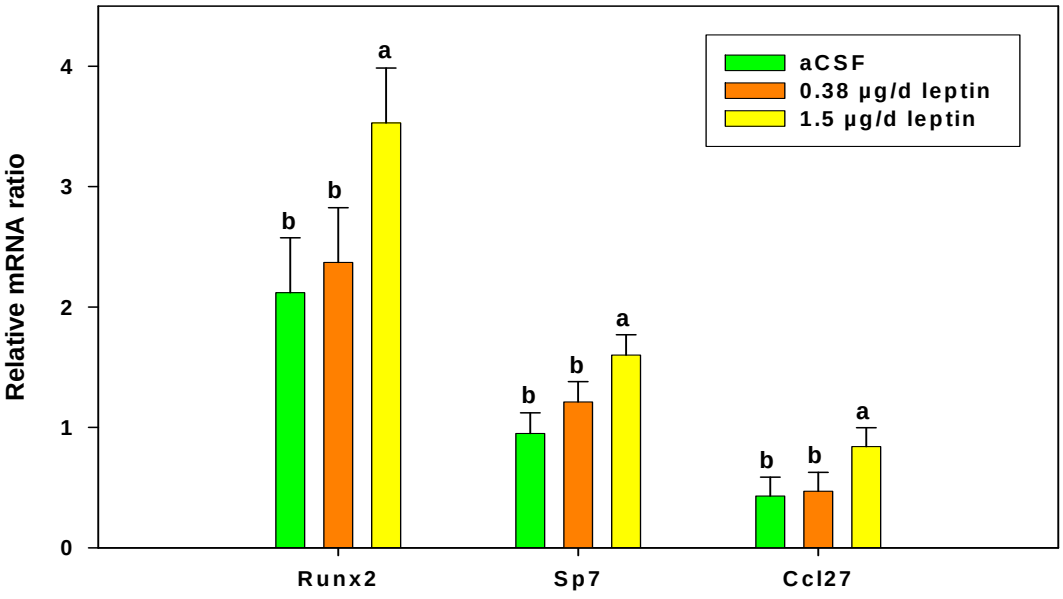
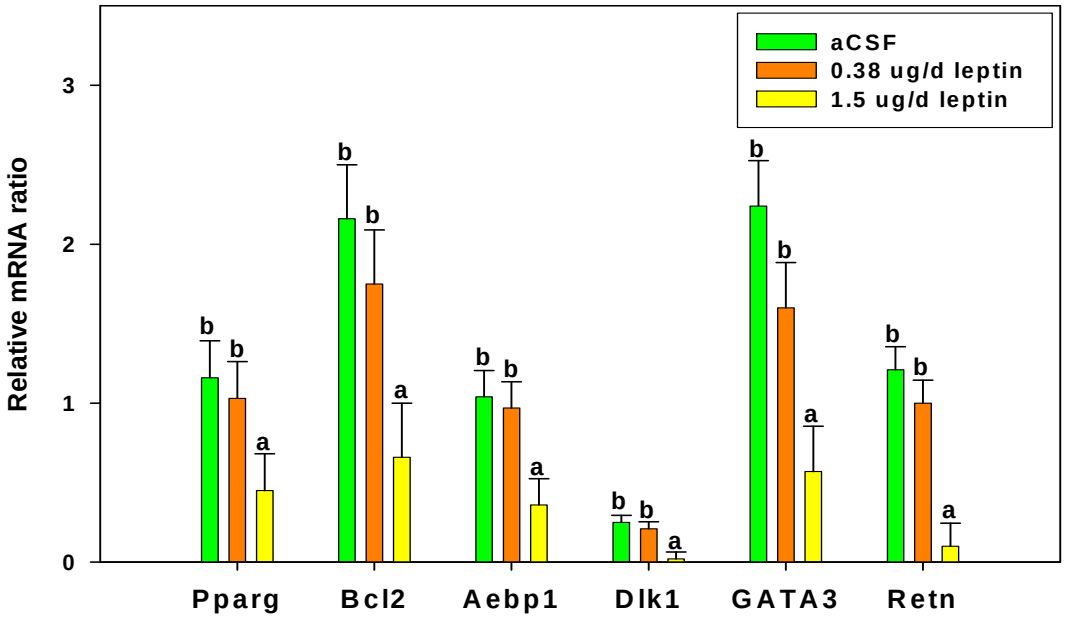
b
a
a
b
b
a
RANK
Csf1


Relative mRNA ratio
4
3
2
1
0
aCSF
0.38 µg/d leptin
1.5 µg/d leptin
b
b
a
b
b
a
b
b
a
Runx2
Sp7
Ccl27


aCSF
0.38 ug/d leptin
1.5 ug/d leptin
Relative mRNA ratio
0
1
2
3
b
b
a
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Pparg
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Aebp1
Dlk1
GATA3
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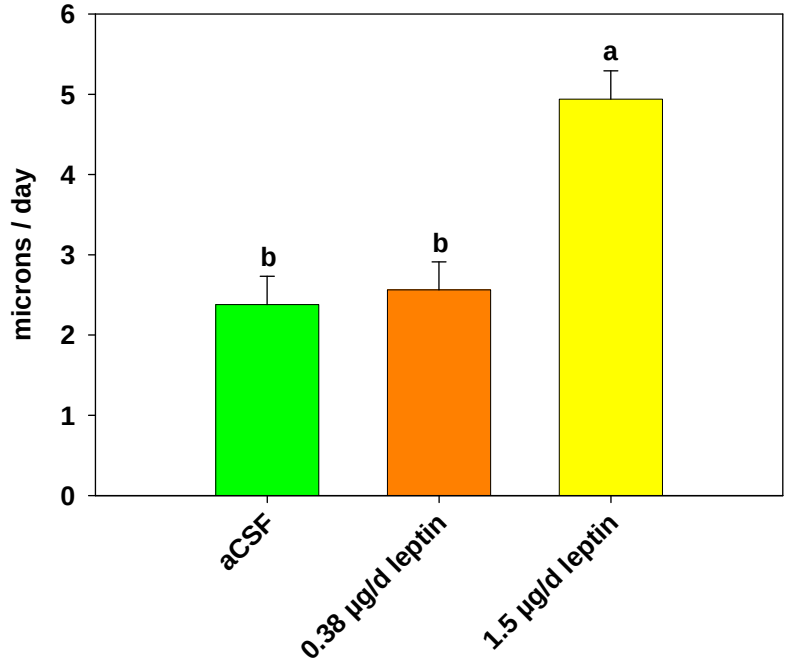
microns / day
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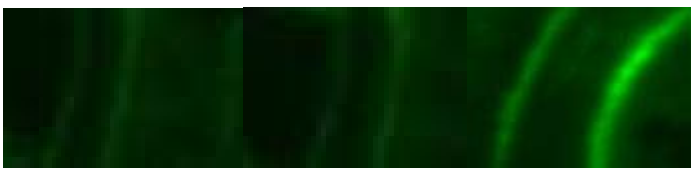

Table 3.1. Body composition and densitometric properties of high and low doses of leptin treated and control *ob/ob* mice.

	aCSF	0.38 µg/d Leptin	1.5 µg/d Leptin	SEM	p-value
BMD, g/cm^2	0.0536^b	0.0624^a	0.0628^a	0.002	0.01
BMC, g	0.399^b	0.486^b	0.677^a	0.06	0.02
Area, cm^2	7.37^b	7.77^b	10.72^a	0.88	0.03
Lean, g	21.9^b	20.3^b	16.6^a	1.01	0.006
Fat, g	35.0^b	30.6^b	22.3^a	0.86	0.001
Total, g	57.8^c	50.9^b	38.4^a	2.62	0.0006
% Fat, g	60.6^b	57.6^{ab}	54.1^a	1.45	0.01
% Lean, g	34.2^b	38.2^{ab}	40.3^a	1.45	0.01

Means without a common letter are significantly different: ^{a,b,c} $p < 0.05$.

Table 3.2. Densitometric properties of the tibia and vertebrae in leptin treated and control *ob/ob* mice.

	aCSF	0.38 µg/d Leptin	1.5 µg/d Leptin	SEM	p-value
Lf. Tibia BMD, g/cm^2	0.0406	0.0401	0.0410	0.001	Ns
Relative Tibia BMD, g/cm^2/g BW	0.0687^{b}	0.0726^{b}	0.0999^{a}	0.005	<0.0001
Lf. Tibia BMC, g	0.021	0.020	0.021	0.002	Ns
Relative Tibia BMC, g/g BW	0.035^{b}	0.036^{b}	0.051^{a}	0.004	0.006
Lf. Tibia Area, cm^2	0.51	0.50	0.51	0.03	Ns
Relative Tibia Area, cm^2/g BW	0.86^{b}	0.90^{b}	1.23^{a}	0.07	0.001
L2-L3 Spine BMD, g/cm^2	0.0406	0.0398	0.0416	0.001	Ns
Relative Spine BMD, g/cm^2/g BW	0.0688^{b}	0.0717^{b}	0.1014^{a}	0.004	<0.0001
L2-L3 Spine BMC, g	0.009	0.009	0.009	0.0006	Ns
Relative Spine BMC, g/g BW	0.015^{b}	0.016^{b}	0.022^{a}	0.002	0.004
L2-L3 Spine Area, cm^2	0.22	0.23	0.21	0.01	Ns
Relative Spine Area, cm^2/g BW	0.37^{b}	0.41^{b}	0.52^{a}	0.04	0.01

Means with different letters are significantly different: ^{a,b} $p < 0.05$.

CHAPTER 4

ADIPOSE TISSUE RESPONSES TO ICV AND SYSTEMIC LEPTIN INJECTIONS IN *ob/ob* MICE[1]

[1]S.M. Bartell, S. Ambati, S. Rayalam, D.R. Gaddam, J.Y. Yang, D.L. Hartzell, M. Hamrick, J. She, M.A. Della-Fera, C.A. Baile. To be submitted to *Journal of American Physiology.*

Abstract

Central or peripheral leptin administration reduces body weight (BW) and body fat. Due to its profound effect, leptin has been targeted as a potential treatment for obesity. In addition to regulating fat metabolism, leptin may reduce fat mass by inducing adipocyte apoptosis. It is unclear, however, if central and systemic administrations are acting through the same receptors, i.e., the higher doses of leptin given systemically, compared to those given centrally, could invoke responses from peripheral leptin receptors in addition to those in the CNS. Thus, one mode of leptin administration could have a greater effect on the sensitivity of leptin-induced adipose tissue apoptosis and/or induction of greater fat loss. The objective of this study was to compare the effects of peripheral and central leptin administration on adipose tissue gene mRNA responses, apoptosis and body composition in leptin deficient *ob/ob* mice (15 wk old, init. BW=61.3g). Leptin was continuously administered subcutaneously (SQ, 0 or 10µg/d) or intracerebroventricularly (ICV, 0 or 1.5µg/d) at doses selected that were expected to give a near maximum response via osmotic pumps for 12 days (n=10). Leptin decreased BW, food intake, body fat, and serum insulin and glucagon concentrations and increased relative gastrocnemius muscle wt, relative muscle mass, and adipose apoptosis, and the effects were equivalent between SQ and ICV modes of administration. However, the level of adipose tissue apoptosis was significantly greater after ICV leptin administration yielded a greater sensitivity to adipose tissue apoptosis rather than after SQ leptin administration, although there was no difference in gene expression among the modes or inducement of adipose tissue loss. These results indicate that SQ and ICV ($1/7^{th}$ the SQ dose) leptin administration decreased fat mass and increased muscle & gene expression equally in *ob/ob* mice.

Keywords: adipose tissue; apoptosis; leptin; obesity; *ob/ob* mice

Introduction

The understanding of the biological roles of fat, specifically white adipose tissue (WAT) has changed over the past few years. From being viewed primarily as a tissue for lipid storage, with the sympathetic nervous system being the major regulator of fat metabolism and energy homeostasis, to WAT being a major endocrine organ secreting the hormone leptin, which acts both locally and systemically (1).

Leptin is a cytokine-like hormone that is expressed primarily in adipose tissue. Its expression in adipose tissue is up-regulated by a variety of factors, such as pro-inflammatory cytokines and is down-regulated by adrenergic stimulation and growth hormone, and plasma levels of leptin are positively associated with increases in adiposity. Leptin was originally discovered as the protein whose absence was primarily responsible for the massive obesity in the genetically obese *ob/ob* mouse. It clearly plays a role in the regulation of body fat content in part through its effects on food intake, body temperature, physical activity, lipid mobilization, and adipose tissue apoptosis. Leptin appears to exert its effects on body weight and energy balance through the hypothalamus (2) and these effects are mediated through its receptor OB-R (3). Since the OB-R in the blood-brain barrier (BBB) is easily saturated, Burguerta and colleagues suggested that this reduced leptin transport at the BBB and thus resulted in obesity (4-5). The amount of adipose tissue is defined by the number and size of adipocytes. Changes in the triglyceride content determine the changes that occur within the fat mass. Leptin has a direct action on the rates of synthesis and degradation of lipids as an autocrine and/or paracrine signal.

In addition to regulating lipogenesis and lipolysis in adipocytes primarily through CNS receptors, leptin may also reduce adipose tissue mass by inducing adipocyte apoptosis. Apoptosis is considered the principal mechanism of "programmed cell death" in mammalian

tissues. Apoptosis is a coordinated and energy-dependent process involving a cascade of molecular events. Our studies have shown that leptin is capable of triggering adipose tissue apoptosis via a central nervous system (CNS) pathway, but not by acting directly on adipocytes (6-7). Even though the process is initiated in the CNS, both central (ICV) and peripheral (SQ) leptin treatment have been shown to stimulate adipose tissue apoptosis (6-7).

Although both modes of leptin administration induce adipocyte apoptosis, no studies have been conducted to compare the effectiveness of centrally- vs peripherally-administered leptin. Therefore, the objective of this study was to determine whether central and peripheral leptin doses, selected to produce a near maximum response for each mode of injection and have comparable effects on body weight and food intake, vary in their effectiveness in eliciting adipose tissue apoptosis that induces fat loss and how it affects the body composition.

Materials and methods

Animals and Design

All experimental and surgical procedures in this study were approved by the Animal Care and Use Committee for The University of Georgia. Forty female leptin deficient (*ob/ob*) mice (15 wk old; 61.3g initial body weight) on the C57BL6 background were purchased from Jackson Laboratories, Inc. (Bar Harbor, ME). The mice were individually housed in plastic shoebox cages in a room with a 12-/12-hour light/dark cycle, 22 ± 1 ºC ambient temperature, and 50% humidity. Mice had ad libitum access to pelleted standard lab chow (LabDiet 5001, PMI Nutritional International, Brentwood MO) and water throughout the study.

All mice were surgically implanted with osmotic minipumps (model 1002, 0.25 μL/h; Alzet Corp., Cupertino, CA) for injection of treatment solutions. Mice were anesthetized with 0.5% oxygen/isoflurane and prefilled and primed pumps were inserted into a subcutaneous (SQ)

pocket. Twenty mice were surgically prepared with a unilateral lateral ventricular (ICV) guide cannula (model 3280P/spc; Plastics One Inc., Roanoke, VA), which included an infusion port that was attached to the osmotic pump, as previously described (8).

Treatments

Treatments were randomly assigned and included SQ control (saline) 6 µl/day (treatment 1), SQ leptin 10 µg/day (treatment 2), ICV control (aCSF) 6 µl/day (treatment 3), and ICV leptin 1.5 µg/day (treatment 4). Previous studies found that leptin produced a maximal suppression of food intake (FI) and body weight (BW) reduction at 10 µg/day in a dose range of 2.5-10 µg/day when administered SQ (7) and at 1.5 µg/day in a dose range of 0.38-1.5 µg/day when administered ICV (9).

Recombinant mouse leptin (R&D Systems, Minneapolis, MN) was dissolved in either an artificial cerebrospinal fluid (aCSF) which consisted of (in g/l): NaCl, 8.66; KCl, 0.224; $CaCl_2 \cdot 2H_2O$, 0.206; $MgCl_2 \cdot 6H_2O$, 0.163; $Na_2HPO_4 \cdot 7H_2O$, 0.214; $NaH_2PO_4 \cdot H_2O$, 0.027 for ICV delivery or 0.9% phosphate-buffered saline solution for SQ delivery.

Design and Procedures

Mice assigned to treatments 1 and 2 had an osmotic pump for SQ delivery of the control and test solutions; whereas, mice assigned to treatments 3 and 4 had an ICV cannula with an osmotic pump attached for continuous ICV injection of control and test solutions. Osmotic mini-pumps remained in place for 14 days. All mice were allowed to recover for 2 days after surgery; during this time 6 µl of saline (for SQ delivery in treatments 1 and 2) or 6 µl of aCSF (for ICV delivery in treatments 3 and 4) were infused prior to the treatment infusion, which occurred for the next 12 days. The health of the mice was monitored and BW and FI were measured and recorded daily.

The mice were killed by decapitation using a guillotine after sedation in a CO_2 chamber at the end of the 12th day of treatment injection. Trunk blood was collected for measurement of serum concentrations of glucagon and insulin using the Luminex100™ instrumentation and a multiplex assay kit (Mouse Endocrine Immunoassay Panel MENDO-75K, Millipore Corp., St. Charles, MO). Retroperitoneal (RP) and parametrial (PM) fat pads, interscapular brown adipose tissue (BAT), liver, and gastrocnemius (GC) and soleus muscles were collected, weighed, and then frozen in liquid nitrogen before storing in -80 °C. Half of the RP and PM tissue samples were fixed in 4% paraformaldehyde for tissue apoptosis assay. Body composition was analyzed by PIXImus densitometry (GE Lunar Corp., Waukesha, WI). Fat mass, lean mass, and percentage fat were measured for each animal.

TUNEL Apoptosis Assay

Apoptosis of the RP and PM adipose tissue depots was detected by immunohistochemical TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling). Fragmental DNA in apoptotic cells results in multiple 3'-hydroxyl ends in the DNA. Thus, by labeling the 3'-hydroxyl ends of the DNA with bromolated deoxyuridine triphosphate nucleotides (Br-dUTP) followed by antibodies linked with fluorescent signals, apoptotic cells could be identified. The enzyme terminal deoxynucleotidyl transferase (TdT) catalyzes this template independent addition of deoxyribonucleotide triphosphates to the 3'-hydroxyl ends of double- or single-stranded DNA with either blunt, recessed or overhanging ends. Non-apoptotic cells do not incorporate significant amounts of the Br-dUTP, owing to the lack of exposed 3'-hydroxyl DNA ends. In this way, apoptotic cells can be identified and analyzed. Briefly, sections of formalin-fixed and paraffin-embedded adipose tissue were de-waxed by washing in xylene and re-hydrated through a gradual series of ethanol and distilled water. Proteinase K-permeabilized

sections were subjected to enzymatic *in situ* labeling of DNA strand breaks using the TUNEL technique of the Molecules Probes TUNEL cell kit (Molecular Probes, Inc., Eugene, OR). The number of TUNEL-positive cells was counted 9 sections per sample. A TUNEL-positive cell was defined by a red color.

Gene Expression

Total RNA was isolated from eWAT, using Trizol reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's protocol. One hundred mg of frozen adipose tissue from each sample was homogenized by adding 1 ml of Trizol reagent. The samples were centrifuged at 12,000 x g for 15 min at 4º C and the aqueous phase was removed and mixed with 0.5 ml of isopropyl alcohol. After centrifugation at 12,000 x g for 10 min at 4º, the pellet was washed with 1 ml of 75% ethanol and then dissolved in 12 µl RNase -free water. One µl of the 12 µl sample was used as an integrity check and for quantification by the Agilent 2100 bioanalyzer and RNA 6000 Nano Assay (Agilent Technologies, Foster City, CA). One hundred ng of total RNA in a 20-µl reaction was reverse-transcribed using the cDNA Archive Kit (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer's protocol using MultiScribeTM Reverse Transcriptase. Reactions were incubated initially at 25 ºC for ten minutes and subsequently at 37 ºC for 120 min. Quantitative PCR (Taqman^{TM}) assays were chosen for the transcripts to be evaluated from Assays-On-Demand^{TM} (ABI), a pre-validated library of QPCR assays incorporated into 384-well Micro Fluidic cards^{TM}. All of the oligonucleotide primer and fluorogenic probe sets for Taqman^{TM} real time PCR were from ABI. Two µl of the cDNA samples, along with 50 µl of 2X PCR master mixes were loaded into respective channels on the microfluidic card followed by a brief centrifugation (3000 rpm for three minutes). The card was

then sealed and real-time PCR and relative quantification was carried out on the ABI PRISM 7900 Sequence Detection System. The cycle conditions were: 94.5 ºC for 15 minutes, followed by 40 cycles of 97ºC for 30 s and 59.7ºC for one minute. Data were expressed as relative quantification (RQ), which presents the fold difference of mRNA level in treatment groups relative to the aCSF control group, and were analyzed using sequence detection systems software. mRNA expression was normalized by using 18S RNA as an endogenous control. The ΔCT values were first calculated by using CT for a specific gene mRNA minus CT for 18S RNA mRNA in the sample. Then the mean mRNA expression from the treatment groups were compared with the aCSF control group using the formula: relative quantification = 2-ΔΔCT (ΔΔCT is the average aCSF control group ΔCT values minus the average experimental group ΔCT values, and ΔΔCT of one equates to a twofold difference in cDNA added into the PCR reaction).

Statistical analysis

All statistical analyses were conducted with SAS (Version 9.0) (SAS Institute, Cary, NC). Significance of treatment effects was determined by one or two way ANOVA. Significance of differences among means was determined by Fisher's LSD. Significance was established at $p < 0.05$.

Results

Body weight and food intake

Leptin treatment significantly reduced BW, and there was no difference between modes of administration. By the end of the 12-day injection period, SQ and ICV leptin-treated mice had lost 8.8 ± 3.2 g and 8.4 ± 3.2 g BW, respectively while the saline and aCSF-injected mice gained

2.7 ± 3.2 g and 2.5 ± 3.2 g ($p = 0.0001$). SQ leptin decreased daily food intake by 36.9% and ICV leptin by 27.5% ($p < 0.04$). (Figure 4.1)

Endocrine serum concentrations (INSULIN, GLUCAGON)

There were significant decreases in serum insulin and glucagons concentrations with leptin administration, but there were no differences between modes of leptin administration (Figure 4.2).

Tissues and liver weights

There were no significant differences between modes of leptin administration. Both SQ and ICV leptin treated mice had smaller PM and RP fat pads as compared to the control mice. There was no change in BAT weights or BAT relative weights with leptin treatment (Table 4.1). Leptin treatment increased lean tissue mass and GC and soleus muscle weights (Figure 4.3), with no differences between the modes of administration. Liver weight was reduced in mice treated with SQ leptin (4.1 ± 0.3 g/g BW) compared to the controls (5.7 ± 0.3 g/g BW). The ICV leptin treatment had the largest liver relative weights of the treatment groups (6.2 ± 0.4 g/g BW).

Body composition (PIXImus DATA)

There were no significant differences between modes of leptin administration. Leptin-treated mice had significantly higher lean mass and lower fat mass (Figure 4.4) and percentage fat than the control mice (56 ± 1.2% leptin treated mice to 60 ± 1.2% control mice; $p = 0.001$).

Adipose tissue apoptosis assay

Leptin significantly increased apoptosis in the RP and PM fat pads. Adipose tissue apoptosis was significantly greater after ICV leptin than after SQ leptin administration in both of the adipose depots (Figure 4.5).

Expression of apoptosis-related genes (Bax, Bcl-2, Casp3) in RP and PM adipose tissues in ob/ob mice

Leptin treatment increased the expression of the apoptosis-related- Bax and Casp3 genes in both the RP and PM fat pads and decreased mRNA expression of Bcl-2 in the RP adipose depot, as compared to the control with no significant differences between modes of leptin administration (Figures 4.6 and 4.7).

Energy expenditure genes (Ucp-2, Ucp-3) mRNA in RP and PM adipose tissues in ob/ob mice

Leptin treatment increased the mRNA of Ucp-2 and Ucp-3 in the RP and PM fat pads as compared to the control with no significant differences between modes of leptin administration (Figures 4.8 and 4.9).

Expression of key lipid biosynthesis and metabolism genes (Fasn, Hsl, Lpl, PPAR-γ) in RP and PM adipose tissues in ob/ob mice

Leptin treatment increased expression of genes for the lipolytic enzymes HSL and LPL in the RP fat pad, while decreasing the expression of the gene for the lipid synthesis enzyme FASN (in both fat pads) as compared to the control, and there were no significant differences between modes of leptin administration. mRNA expression of the transcriptional factor PPAR-γ was decreased with both leptin treatment groups in both adipose depots as compared to the controls (Figures 4.10 and 4.11).

Expression of cell differentiation and proliferation related genes (Cebp-α, Fabp4, Pdia3, Phb;Fyb) in RP and PM adipose tissues in ob/ob mice

There were no significant differences between modes of leptin administration. Leptin treatment decreased the expression of Fabp4 in the RP fat pad, while increasing the expression of Pdia3 and Phb/Fyb in both the RP and PM fat pads as compared to the control. mRNA

expression of the transcriptional factor Cebp-α was decreased in the leptin treated mice as compared to the control mice in the RP adipose depot. (Figures 4.12 and 4.13)

Expression of adipocyte based cytokines and inflammation factors genes (Adiponectin, Leptin, Srebf1, TNF-α) in RP and PM adipose tissues in ob/ob mice

Leptin treatment decreased the mRNA level of leptin (in both fat pads) and Srebf1 (in the RP fat pad) as compared to the control with no significant differences between modes of leptin administration. In both the RP and PM adipose depots, leptin treated mice had increased expression of adiponectin, a cytokine involved in glucose and lipid metabolism. As compared to the control treatments, leptin administration decreased the mRNA level of TNF-α in the RP fat pad but increased the expression of TNF-α in the PM fat depot. (Figures 4.14 and 4.15)

Expression of glucose metabolism genes (C3, Rbp4, Slc2a4) in RP and PM adipose tissues in ob/ob mice

Leptin treatment increased the expression of C3, a cytokine in glucose metabolism that is involved with insulin sensitivity, in both the RP and PM fat pads, as compared to the control. The expression of Rbp4, a mediator associated with decreasing glucose transportation and impairing insulin function was decreased, while the expression of Slc2a4, a glucose transporter, was increased with leptin administration in only the RP fat pad. There were no significant differences between modes of leptin administration (Figures 4.16 and 4.17).

Correlations

The percent of adipose tissue apoptosis that occurred was not related to the body weight or the amount of fat loss that occurred. The weight of the fat depots was correlated to the percent of adipose tissue apoptosis ($r=0.72$, $p=0.0001$).

Discussion

The reason for the lack of differences between SQ and ICV leptin treatments in all of the parameters except inducement of apoptosis was most likely due to the fact that we selected doses for both routes of administration that caused maximal suppression of food intake and body weight. We and others have previously shown that *ob/ob* mice are very sensitive to the effects of exogenous leptin. In contrast, diet-induced obesity has been shown to result in receptor defects that reduce transport of leptin across the blood brain barrier (4). At the doses tested in this study, it is likely that peripherally administered leptin reached brain receptors at approximately the same concentration as the centrally administered leptin, thus accounting for the similarity of effects.

Even though we suspect that the amount of SQ administered leptin that reached the leptin receptors in the hypothalamus was similar to that of the ICV leptin, there was increased sensitivity to apoptosis of the centrally administered leptin. Our results demonstrate that the delivery of centrally administered leptin itself was enough to increase the sensitivity of adipose tissue apoptosis, although this did not result in increased fat mass loss or increased adipose tissue gene expression. The reason why the increased rate of apoptosis did not result in increased fat loss should be determined in a future study, but we believe that the answer lies in some aspect of lipid metabolism due to the fact that multiple factors determine adipose tissue mass, such as the coordinated regulation between lipid synthesis and oxidation, adipocyte differentiation, and apoptosis. Contrary to the increased apoptosis rate in the ICV leptin treatment, there were no differences between modes of leptin administration in regards to expression of apoptosis-related genes. This may be because mRNA levels are just a "snap-shot" look at what is occurring and needs to be further substantiated with other findings. It may also be that the increased apoptosis

rate in the ICV leptin treatment may have caused an increase in the circulation of free FA, which resulted in the re-esterification of FA or lipid accumulation in the liver and may help to explain the increased liver size that was observed in these mice. The difference in the liver size may also be due to the mode of leptin administration affecting the hepatic receptors differently by possibly suppressing lipogenesis and promoting proportional FA intake (10-11). This effect on the liver will be further investigated in a future study.

While there were no alterations in the gene expression among the leptin treatments in either adipose tissue depot, it lends insight into possible mechanisms in leptin's mediation of fat metabolism and these may be specific to the tissue assayed. Ucp-2 and Ucp-3 are thought to be involved in transporting fatty acids (12). It is assumed that the up-regulation of these genes after leptin administration would increase fatty acid transport and enhance lipid β-oxidation. Even though there was no significant increase in BAT, the increased Ucp-2 and Ucp-3 expression and greater muscle mass in the leptin-treated mice may be indicative of increased potential for thermogenesis and enhanced sympathetic tone, which agrees with previous reported data from our laboratory (13). To further determine if there was an increase of thermogenesis, it would be beneficial to know what the expression of these genes were in the BAT. Increased Hsl expression levels may indicate enhanced triglycerides (TG) hydrolysis and fatty acid mobilization in adipose tissue, while decreased Fas levels demonstrate that a reduction in lipogenesis and lack of de novo lipid synthesis in the cytosol by converting acetyl-CoA to the long-chain fatty acid palmitate, which is further synthesized into TG and stored in adipose tissue with leptin treatment. The decreased mRNA levels of PPAR-γ could indicate an absence of adipocyte differentiation and lipid storage in the adipose tissue. Decreased Srebf1 levels with leptin treatment is consistent with findings that show that Srebf1 regulates several key genes

related to lipid metabolism, including Fas (14) and is associated with a reduction in lipogenic activity (15).

TNF-α is a cytokine that stimulates lipolysis and induces apoptosis. The contrary gene profiles of TNF-α observed in this study may be explained by differing reports of TNF-α action and sensitivity dependent upon adipocyte location (16). The apoptosis-inducing effect of TNF-α varies in adipocytes due to their differing sensitivities. The apoptosis process in this study appears to be a mixture of paracrine and autocrine factors derived from the adipocytes and not the regulation of TNF-α action. Leptin administration appears to generate different gene expression profiles depending upon the adipose tissue depot assayed as this study demonstrates in the RP and PM fat pads along and also as shown in previous reports published by our laboratory in the inguinal fat pad (13). It is the potential net efforts of a decrease in both adipocyte volume (insulin resistance and lipolytic effects) and adipocyte number (anti-differentiation, dedifferentiation, and apoptosis effects) that affect the overall body composition of the animal. Adipose tissue reduction in is a coordinated effort between the induction of lipolysis, inhibition of lipogenesis, induction of insulin resistance, and impairment of adipocyte differentiation.

Not only was an insight into fat metabolism gained, but also into insulin function. Leptin administration induced adiponectin expression levels. In addition to its regulation of energy homeostasis and lipid metabolism, adiponectin is an abundant adipokine that plays an important role in modulating insulin action and inhibiting gluconeogenesis (17). In conjunction, the increased expression levels of adiponectin, the improved serum insulin concentrations, and increased expression levels of C3, Slc2a4, a glucose transporter, and decreased expression of

Rbp4, which is associated with impaired glucose regulation that results in insulin resistance, it appears that insulin sensitivity may have also been improved with leptin treatment.

In conclusion, although ICV leptin administration had a greater effect on the sensitivity to adipose tissue apoptosis rather than SQ leptin, it did not result in greater overall fat mass loss or adipose tissue gene expression. This study demonstrates that leptin's effect on adipose tissue and resulting fat mass loss is a coordinated effort involving increasing energy expenditure, insulin sensitivity, lipid oxidation, etc. not just regulating adipocyte apoptosis.

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Figure 4.1. Body weight changes and daily food intake after 12 days of SQ and ICV leptin injections in *ob/ob* mice. Means which don't share a common letter are significantly different: [a,b] $p < 0.05$.

Figure 4.2. The effect of 12 days of SQ or ICV leptin injections on the serum concentrations of insulin and glucagon. Means which don't share a common letter are significantly different: [a,b] $p < 0.05$.

Figure 4.3. The GC and soleus muscle weights in *ob/ob* mice injected with SQ or ICV leptin after 12 days. Means which don't share a common letter are significantly different: [a,b] $p < 0.05$.

Figure 4.4. The amount of lean and fat mass in *ob/ob* mice after 12 days of SQ or ICV leptin injections. Means which don't share a common letter are significantly different: [a,b] $p < 0.05$.

Figure 4.5. The effect of 12 days of SQ or ICV leptin injections on RP (top) and PM (bottom) fat depots apoptosis (percentage fragmented DNA) in *ob/ob* mice. Means which don't share a common letter are significantly different: [a,b,c] $p < 0.05$.

Figure 4.6. The effect of 12 days of SQ or ICV leptin injections on expression of apoptosis-related genes (Bax, Bcl-2, Casp3) in RP adipose tissue in ob/ob mice. Means which don't share a common letter are significantly different: [a,b] $p < 0.05$.

Figure 4.7. The effect of 12 days of SQ or ICV leptin injections on expression of apoptosis-related genes (Bax, Casp3) in PM adipose tissue in ob/ob mice. Means which don't share a common letter are significantly different: [a,b] $p < 0.05$.

Figure 4.8. The effect of 12 days of SQ or ICV leptin injections on energy expenditure genes (Ucp-2, Ucp-3) mRNA in RP adipose tissue in ob/ob mice. Means which don't share a common letter are significantly different: [a,b] $p < 0.05$.

Figure 4.9. The effect of 12 days of SQ or ICV leptin injections on energy expenditure genes (Ucp-2, Ucp-3) mRNA in PM adipose tissue in ob/ob mice. Means which don't share a common letter are significantly different: ^{a,b} $p < 0.05$.

Figure 4.10. The effect of 12 days of SQ or ICV leptin injections on e The effect of 12 days of SQ or ICV leptin injections on expression of key lipid biosynthesis and metabolism genes (Fasn, Hsl, Lpl, Ppar-γ) in RP adipose tissue in ob/ob mice. Means which don't share a common letter are significantly different: ^{a,b} $p < 0.05$.

Figure 4.11. The effect of 12 days of SQ or ICV leptin injections on expression of key lipid biosynthesis and metabolism genes (Fasn, Ppar-γ) in PM adipose tissue in ob/ob mice. Means which don't share a common letter are significantly different: ^{a,b} $p < 0.05$.

Figure 4.12. The effect of 12 days of SQ or ICV leptin injections on expression of cell differentiation and proliferation related genes (Cebp-α, Fabp4, Pdia3, Phb/Fyb) in RP adipose tissue in ob/ob mice. Means which don't share a common letter are significantly different: ^{a,b} $p < 0.05$.

Figure 4.13. The effect of 12 days of SQ or ICV leptin injections on expression of cell differentiation and proliferation related genes (Pdia3, Phb/Fyb) in PM adipose tissue in ob/ob mice. Means which don't share a common letter are significantly different: ^{a,b} $p < 0.05$.

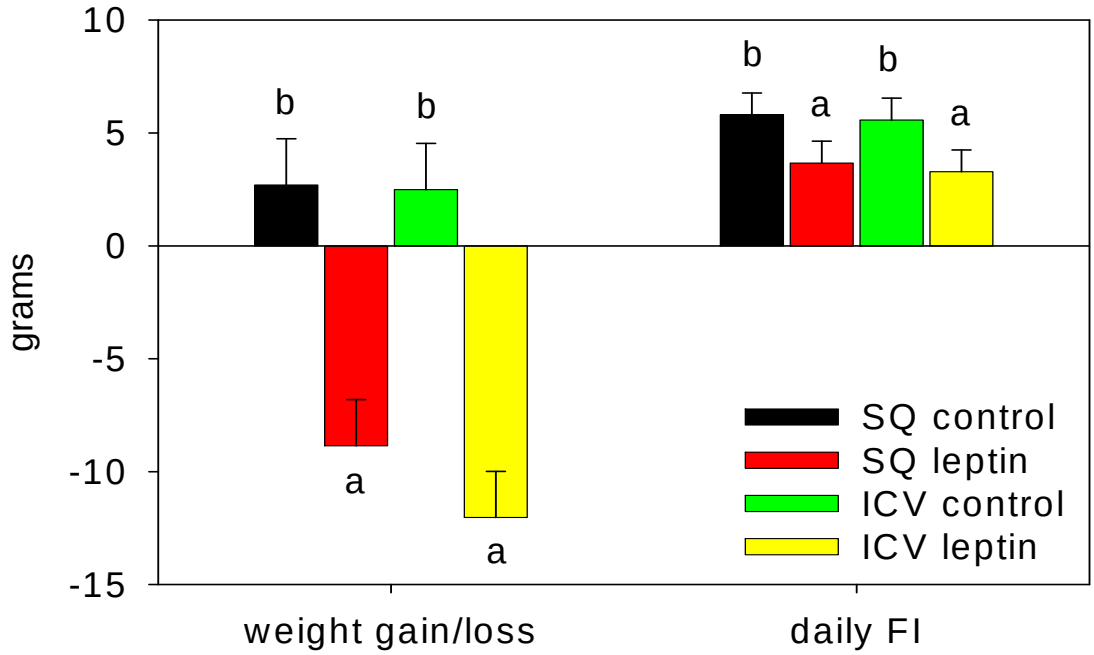
Figure 4.14. The effect of 12 days of SQ or ICV leptin injections on expression of adipocyte based cytokines and inflammation factors genes (Adiponectin, Leptin, Srebf1, TNF-α) in RP adipose tissue in ob/ob mice. Means which don't share a common letter are significantly different: ^{a,b} $p < 0.05$.

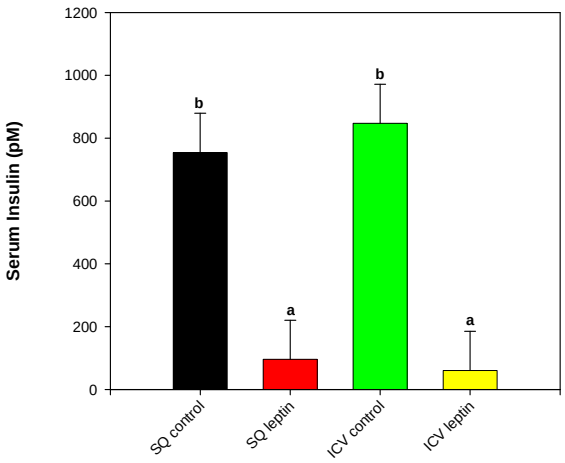
Figure 4.15. The effect of 12 days of SQ or ICV leptin injections on expression of adipocyte based cytokines and inflammation factors genes (Adiponectin, Leptin, TNF-α) in PM adipose

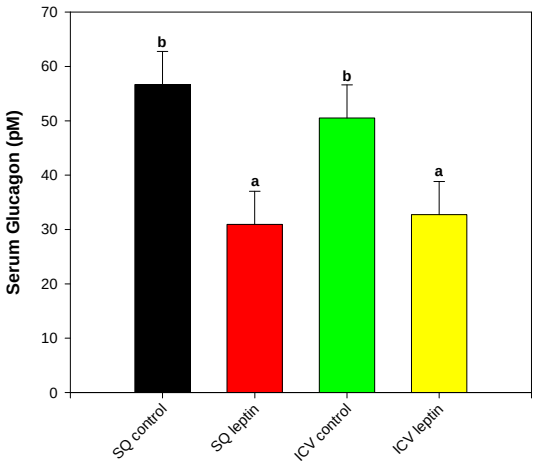
tissue in ob/ob mice. Means which don't share a common letter are significantly different: ^{a,b} $p < 0.05$.

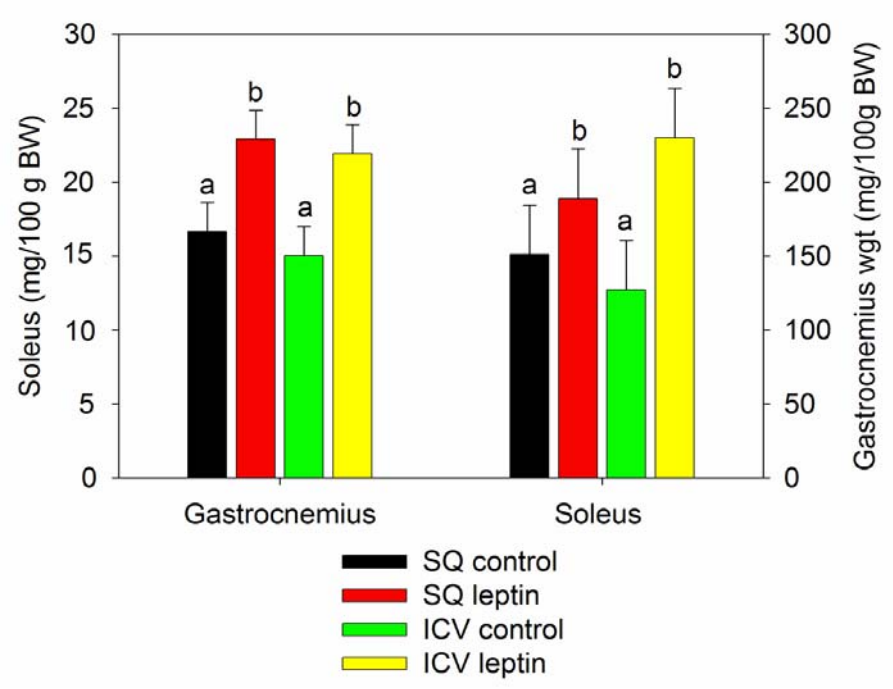
Figure 4.16. The effect of 12 days of SQ or ICV leptin injections on expression for glucose metabolism genes (C3, Rbp4, Slc2a4) in RP adipose tissue in ob/ob mice. Means which don't share a common letter are significantly different: ^{a,b} $p < 0.05$.

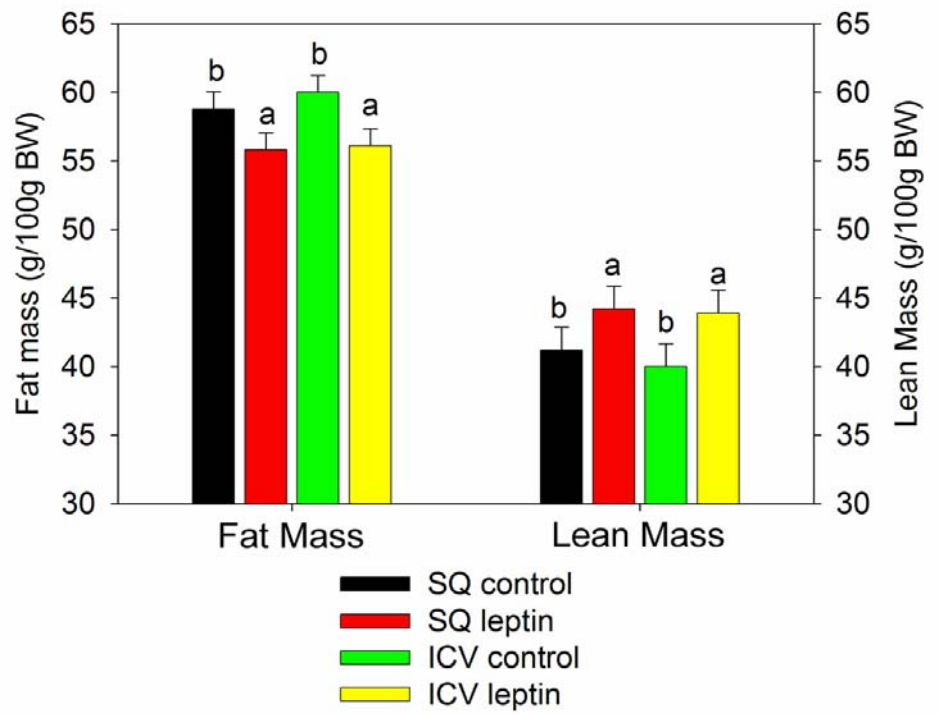
Figure 4.17. The effect of 12 days of SQ or ICV leptin injections on expression for glucose metabolism gene, C3 in PM adipose tissue in ob/ob mice. Means which don't share a common letter are significantly different: ^{a,b} $p < 0.05$.

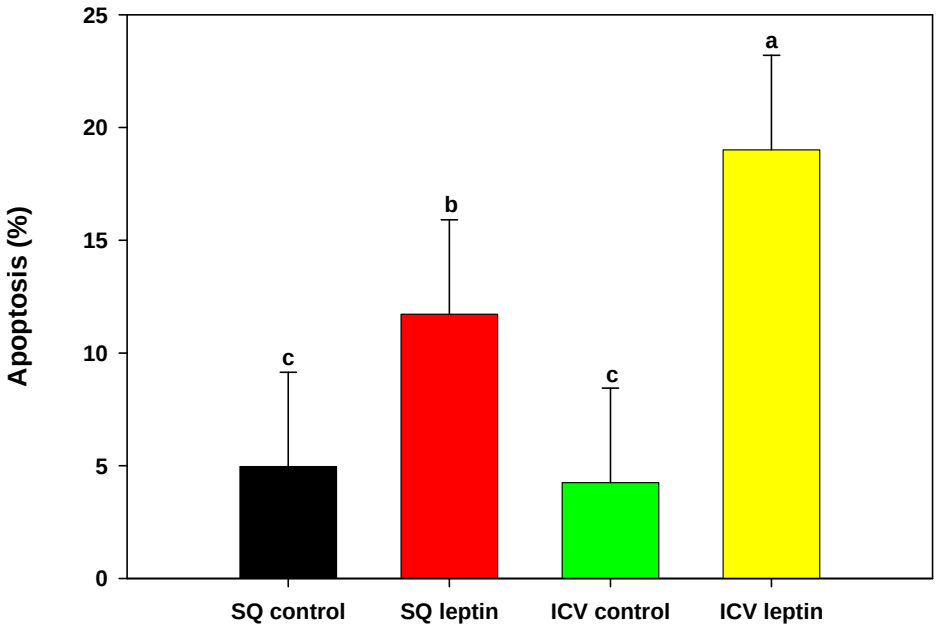

10
5
0
-5
-10
-15
grams
b
b
a
a
b
a
b
a
SQ control
SQ leptin
ICV control
ICV leptin
weight gain/loss
daily FI

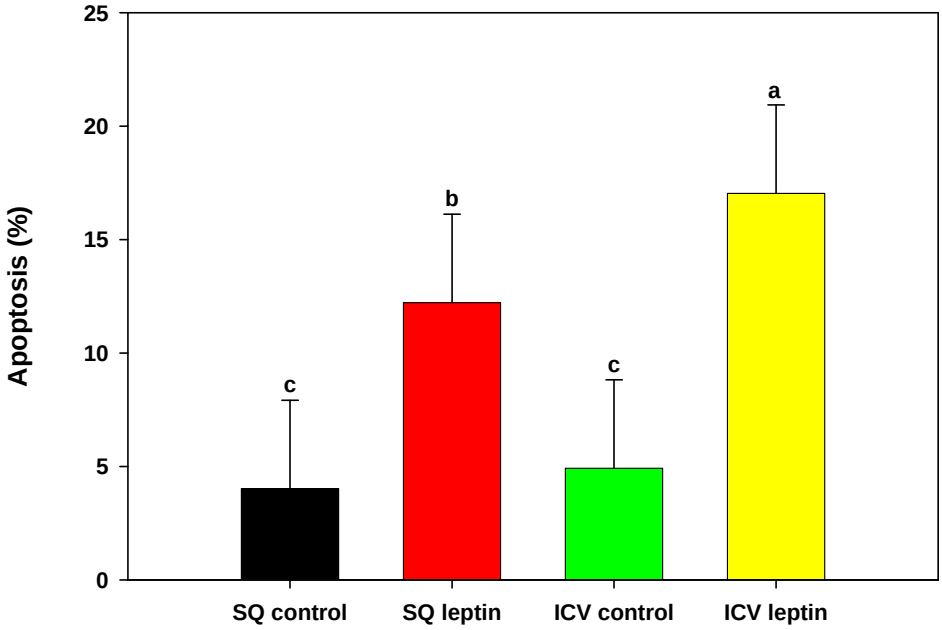

Serum Insulin (pM)
1200
1000
800
600
400
200
0
b
a
b
a
SQ control
SQ leptin
ICV control
ICV leptin

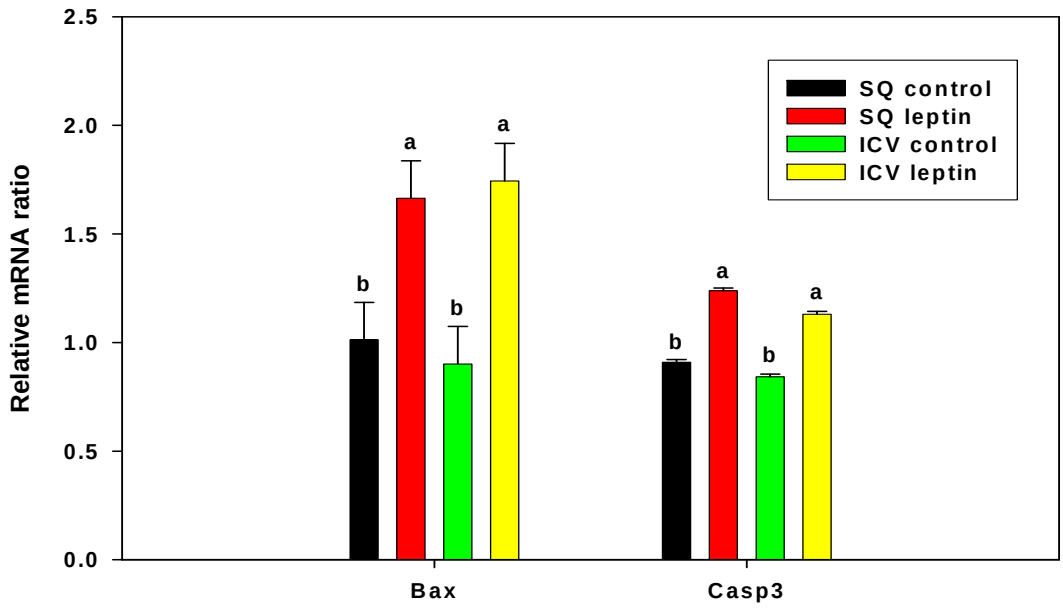

Serum Glucagon (pM)
70
60
50
40
30
20
10
0
b
a
b
a
SQ control
SQ leptin
ICV control
ICV leptin

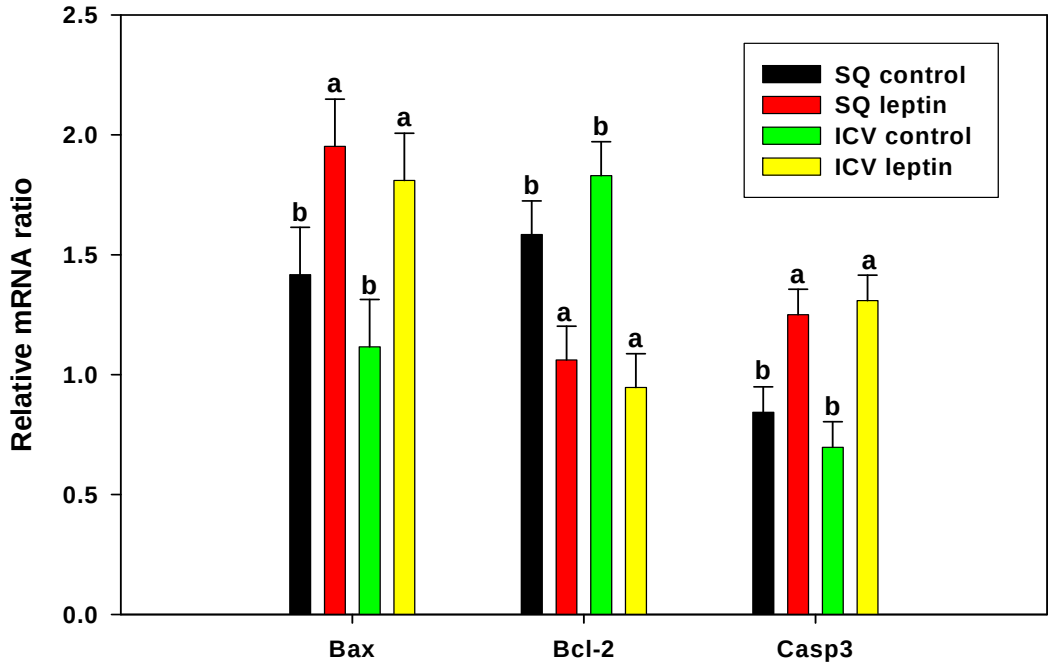

Soleus (mg/100 g BW)
Gastrocnemius wgt (mg/100g BW)
Gastrocnemius
Soleus
SQ control
SQ leptin
ICV control
ICV leptin


Fat mass (g/100g BW)
Lean Mass (g/100g BW)
Fat Mass
Lean Mass
SQ control
SQ leptin
ICV control
ICV leptin


Apoptosis (%)
25
20
15
10
5
0
a
b
c
c
SQ control
SQ leptin
ICV control
ICV leptin


Apoptosis (%)
25
20
15
10
5
0
a
b
c
c
SQ control
SQ leptin
ICV control
ICV leptin


Relative mRNA ratio
2.5
2.0
1.5
1.0
0.5
0.0
SQ control
SQ leptin
ICV control
ICV leptin
b
a
b
a
b
a
b
a
Bax
Casp3


Relative mRNA ratio
2.5
2.0
1.5
1.0
0.5
0.0
SQ control
SQ leptin
ICV control
ICV leptin
b
a
b
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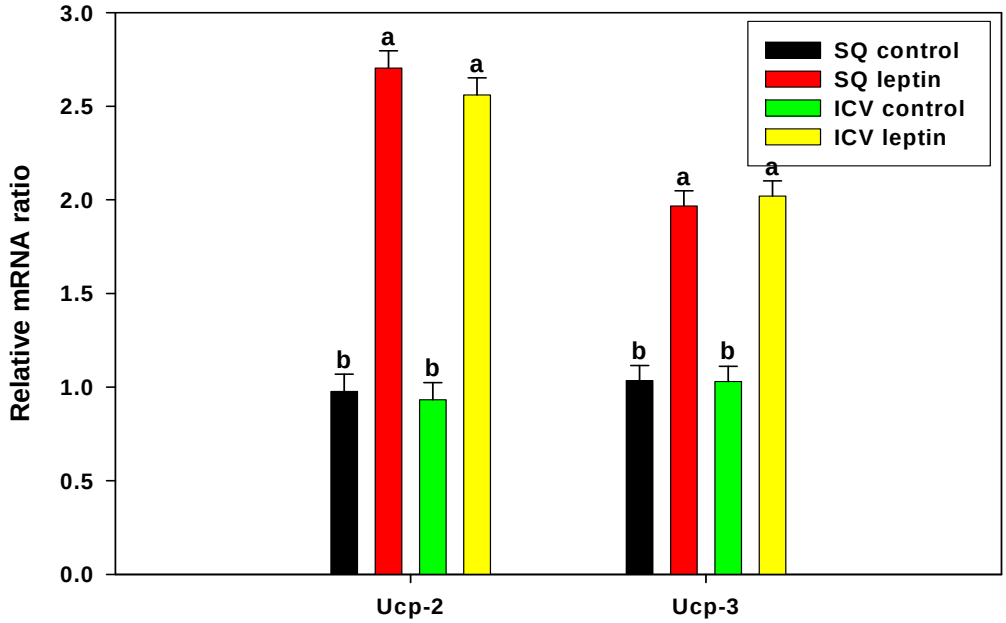
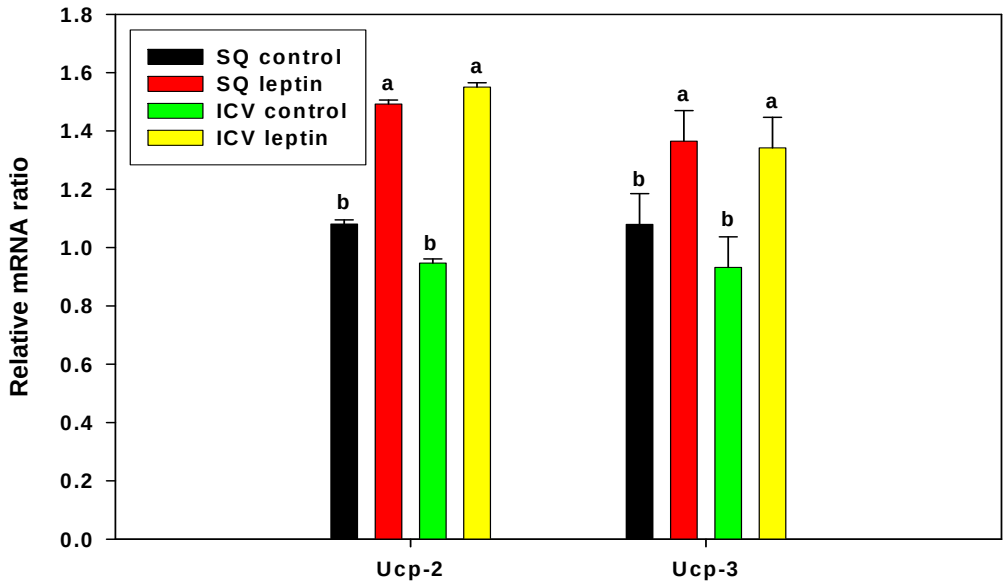
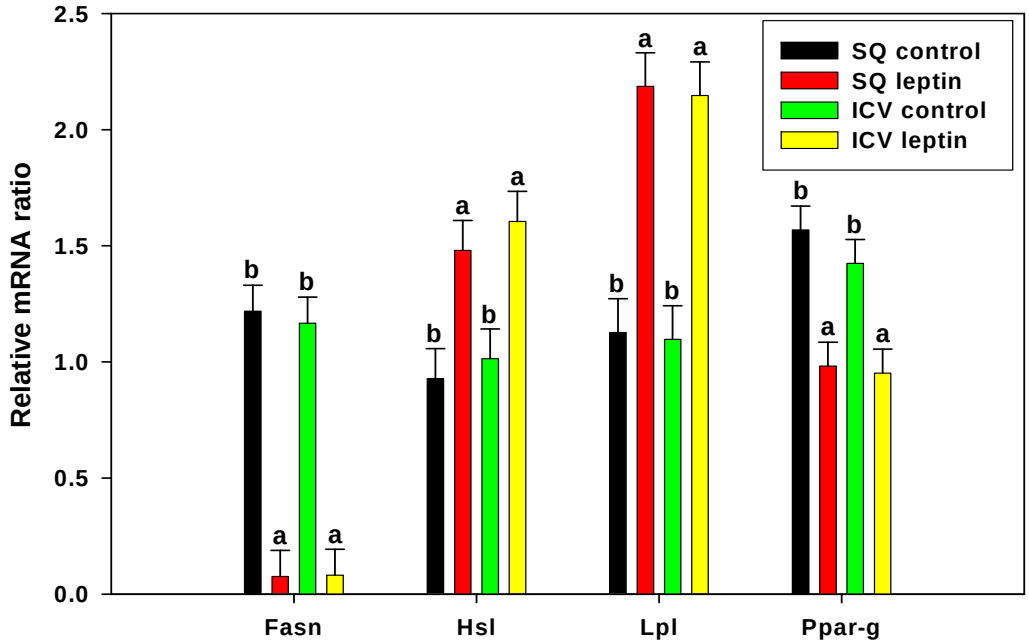
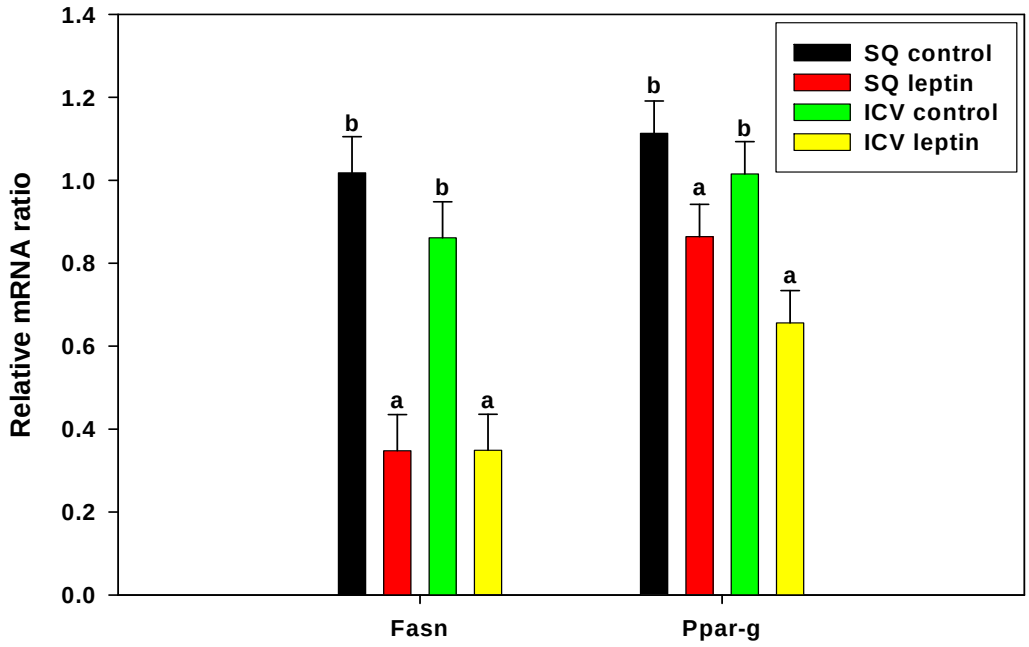
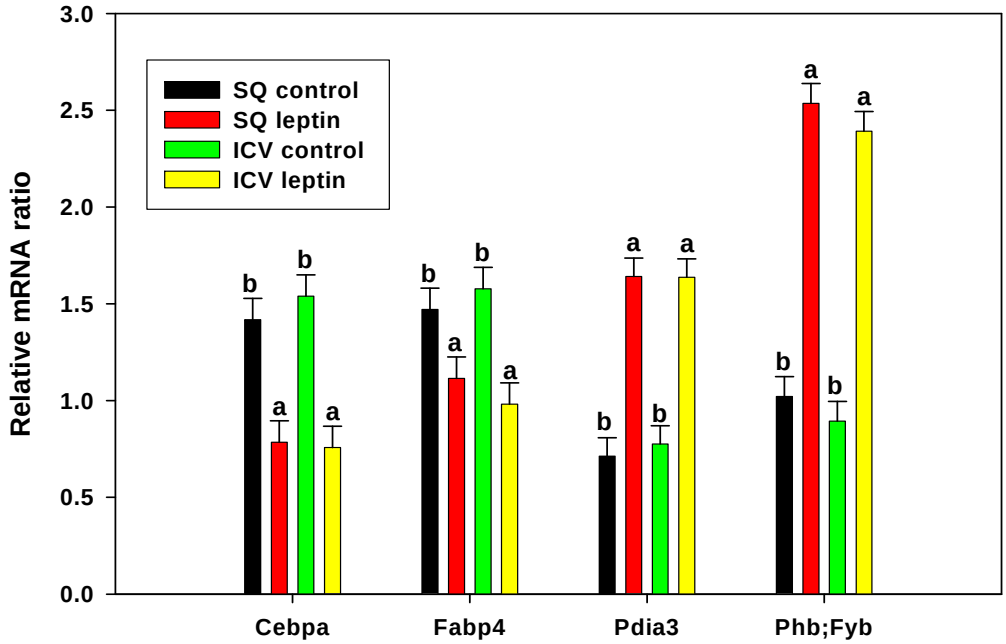
b
a
b
a
b
a
b
a
Bax
Bcl-2
Casp3

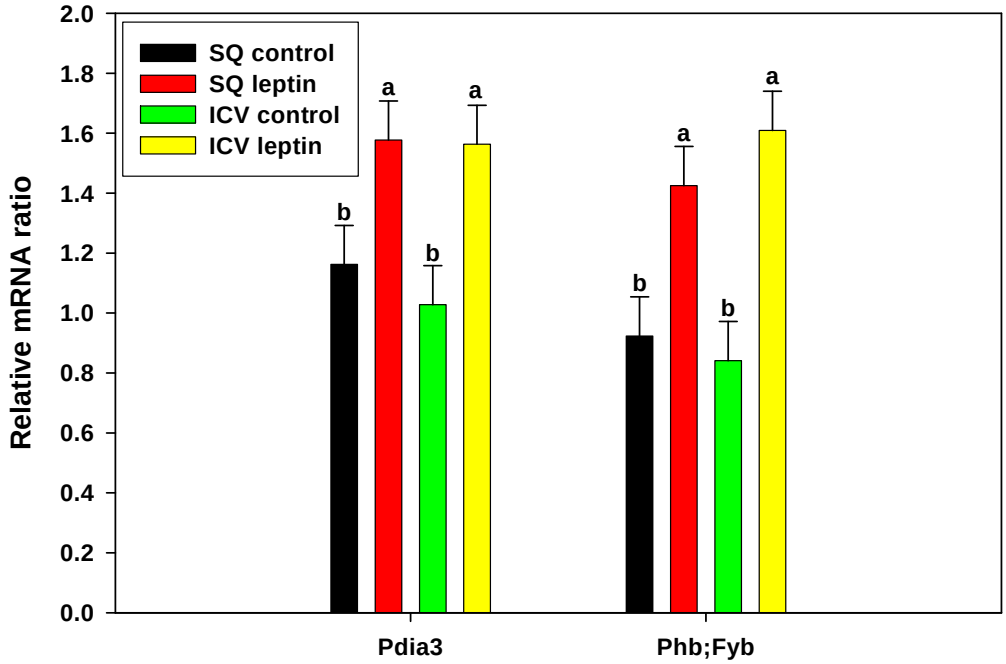

Relative mRNA ratio
3.0
2.5
2.0
1.5
1.0
0.5
0.0
SQ control
SQ leptin
ICV control
ICV leptin
a
a
b
b
a
a
b
b
Ucp-2
Ucp-3

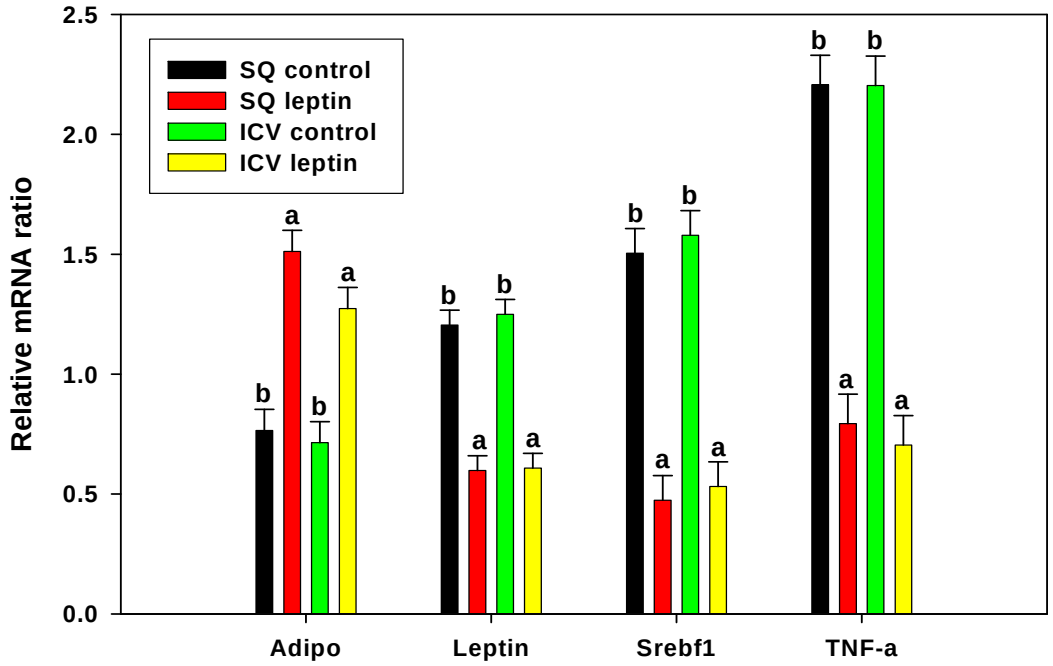

Relative mRNA ratio
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
SQ control
SQ leptin
ICV control
ICV leptin
a
a
b
b
a
a
b
b
Ucp-2
Ucp-3

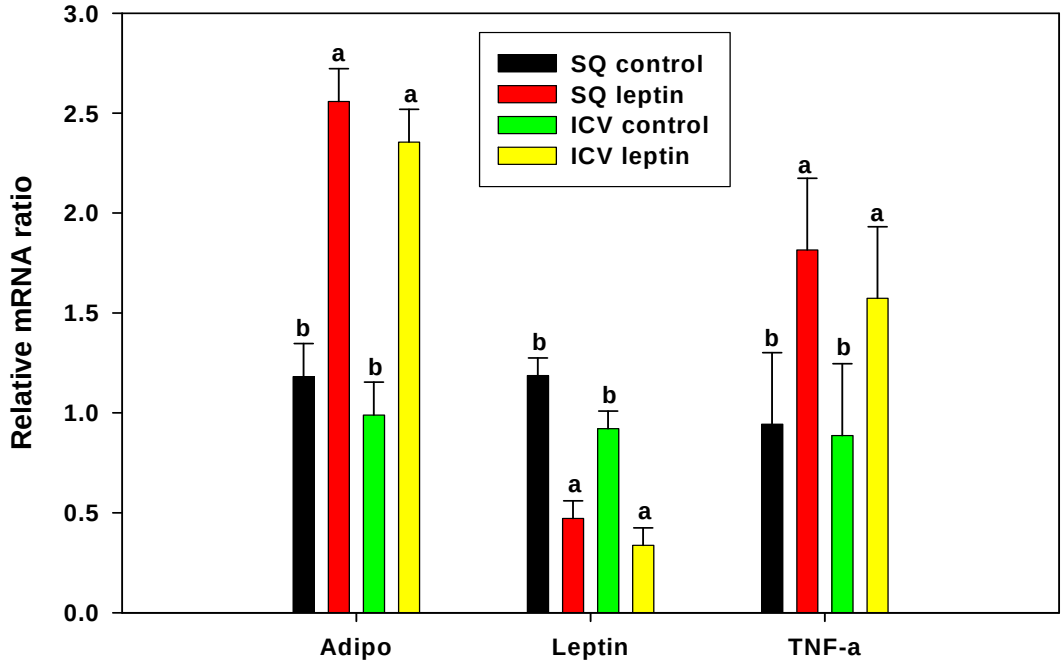

Relative mRNA ratio
2.5
2.0
1.5
1.0
0.5
0.0
SQ control
SQ leptin
ICV control
ICV leptin
Fasn
Hsl
Lpl
Ppar-g


Relative mRNA ratio
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
SQ control
SQ leptin
ICV control
ICV leptin
Fasn
Ppar-g


SQ control
SQ leptin
ICV control
ICV leptin
Relative mRNA ratio
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Cebpa
Fabp4
Pdia3
Phb;Fyb


SQ control
SQ leptin
ICV control
ICV leptin
Relative mRNA ratio
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pdia3
Phb;Fyb


SQ control
SQ leptin
ICV control
ICV leptin
Relative mRNA ratio
2.5
2.0
1.5
1.0
0.5
0.0
Adipo
Leptin
Srebf1
TNF-a


SQ control
SQ leptin
ICV control
ICV leptin
Relative mRNA ratio
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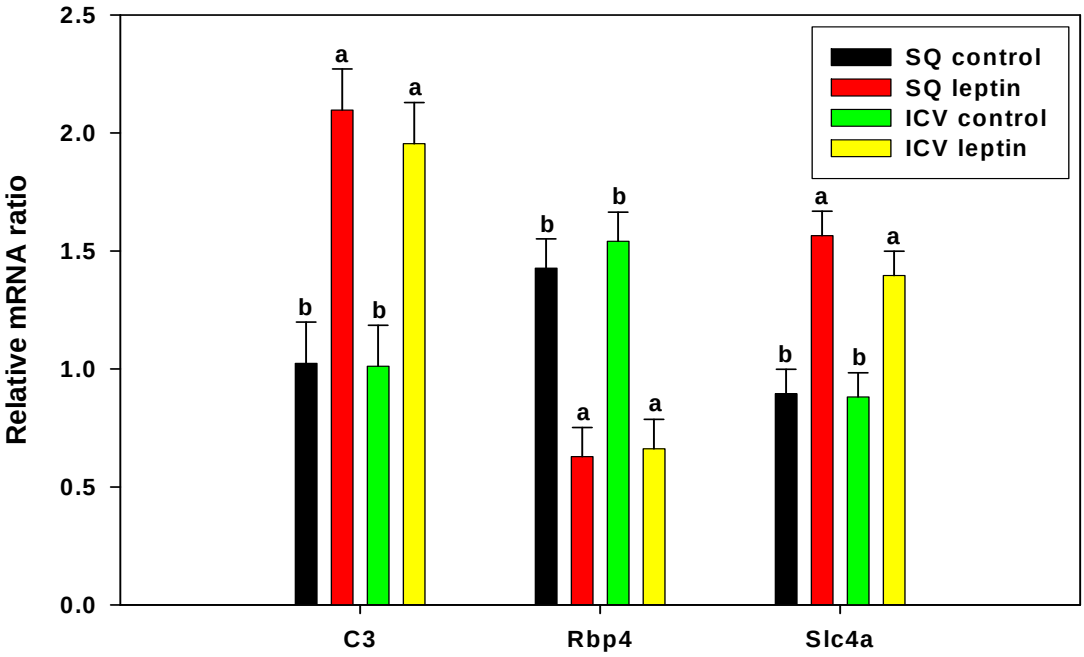
2.5
2.0
1.5
1.0
0.5
0.0
Adipo
Leptin
TNF-a


Relative mRNA ratio
2.5
2.0
1.5
1.0
0.5
0.0
SQ control
SQ leptin
ICV control
ICV leptin
a
a
b
b
b
a
b
a
b
a
a
b
C3
Rbp4
Slc4a

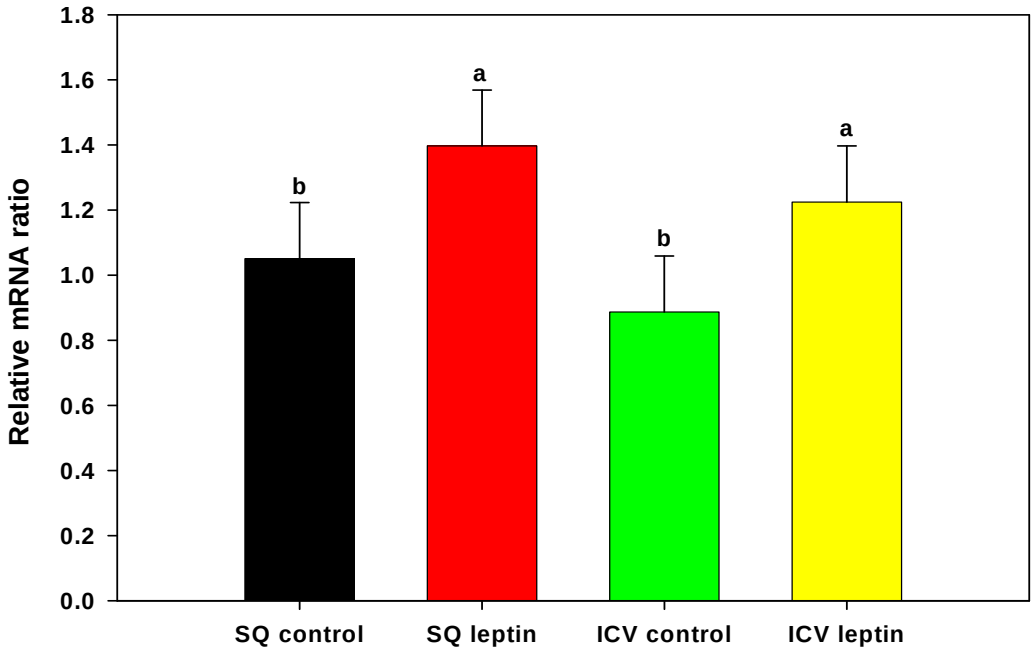

Relative mRNA ratio
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
b
a
SQ control
SQ leptin
ICV control
ICV leptin

Table 4.1. Carcass Characteristics of Mice injected SQ with Saline or Leptin or ICV with aCSF or Leptin

	SQ Injection		ICV Injection		SEM	p-value
	saline	Leptin	aCSF	Leptin		
Final Body Weight, g	63.1^{b}	50.8^{a}	61.3^{b}	51.1^{a}	3.21	0.0001
BAT, mg	120.0	95.1	121.9	95.8	16.07	Ns
Relative, mg/g BW	192.6	187.2	198.6	187.9	26.62	Ns
Parametrial, mg	5464.4^{bc}	4750.6^{b}	6010.8^{c}	3497.3^{a}	386.19	<0.0001
Relative, mg/g BW	8600.8^{ab}	9640.3^{bc}	9808.1^{c}	8189.3^{a}	582.45	0.04
Retroperitoneal, mg	2518.3^{b}	1634.9^{a}	2331.2^{b}	1301.9^{a}	193.42	<0.0001
Relative, mg/g BW	4022.6^{b}	3248.7^{a}	3463.2^{ab}	2828.6^{a}	373.67	0.04
Total Fat Pads, mg	8102.8^{c}	6480.2^{b}	8241.6^{c}	4884.5^{a}	327.71	<0.0001
Relative Total Fat Wt., mg/g BW	12816^{b}	13076^{b}	13470^{b}	11209^{a}	454.38	0.001

CHAPTER 5

CENTRAL AND PERIPHERAL LEPTIN TREATMENT PRODUCE SIMILAR INCREASES IN BONE MASS IN *ob/ob* MICE [1]

[1]S.M. Bartell, S. Rayalam, S. Ambati, J. Mumaw, D.R. Gaddam, D.L. Hartzell, M. Hamrick, J. She, M.A. Della-Fera, C.A. Baile. To be submitted to *Journal of Bone and Mineral Research.*

Abstract

Central or peripheral leptin administration reduces body weight, food intake, & body fat in leptin deficient ob/ob mice. However, conflicting reports assessing the effects of leptin on bone mass in rodents have been published. The objective of this study was to determine the effects of central and peripheral administration of leptin on bone metabolism in appendicular and axial skeleton in ob/ob mice (15 wk old, init. BW=61.3g). Leptin was continuously delivered intracerebroventricularly (ICV, 0 or 1.5μg/d) or subcutaneously (SQ, 0 or 10μg/d) via osmotic pumps for 12 days (n=10). Mice were injected intraperitoneally with calcein, a fluorochrome label, to measure bone formation during the experimental period. There were no significant differences in bone parameters between the modes of leptin administration. Regardless of mode of administration, leptin decreased BW, food intake, and body fat and increased relative gastrocnemius muscle wt, relative muscle mass, whole body bone mineral density (BMD), bone mineral content (BMC), and bone area, lumbar BMD & BMC, proximal femoral BMD & BMC, distal tibial BMD, tibial, femoral, & spinal mineral apposition rates, serum insulin, glucagon, osteocalcin, IGF-1,OPG, PYD, & RANK-L concentrations. Both ICV and SQ leptin administration decreased bone marrow adipocyte number and size while increasing BMD, suggesting that a reduction in marrow adipocytes has a positive effect on bone formation. These observed increases in BMD, BMC, bone formation and bone marker serum concentrations indicate that both ICV and SQ leptin administration increased bone growth equally in ob/ob mice.

Keywords: leptin; osteoporosis; bone; *ob/ob* mice

Introduction

Leptin, a cytokine-like hormone secreted by adipocytes, was first discovered in 1994 by Friedman and colleagues (1). Leptin was originally discovered as the missing protein in the genetically obese *ob/ob* mouse. Its expression is up-regulated by a variety of factors, such as pro-inflammatory cytokines, and is down-regulated by adrenergic stimulation and growth hormone. Leptin receptors are expressed in peripheral tissues including skeletal muscle, bone and cartilage, but the primary target of leptin binding is the brain, specifically the hypothalamus (2). Leptin's binding in the hypothalamus results in the regulation of food intake, energy expenditure, and bone metabolism. Leptin's profound effect on body weight, fat loss, and bone growth has been targeted as a potential treatment for obesity and recently also for osteoporosis. Central and peripheral administration of leptin reduces food intake, body weight, and body fat content in rodents without functional leptin (3). Unfortunately, most obese humans have a resistance to leptin's effects. An important component in leptin resistance appears to be the development of defective leptin uptake into the brain, leptin receptors being down regulated, and leptin sensitivity being decreased.

The complexity of bone metabolism regulation mediated by leptin is not clearly understood. The findings of increased trabecular bone formation and high bone mass within the proximal tibia and lumbar vertebrae of *ob/ob* mice (4), with similar observations in the *db/db* mouse, led to the conclusion that leptin signals through its known receptors to control bone mass. Intracerebroventricular (ICV) infusion of leptin into the third ventricle resulted in vertebral bone loss in both *ob/ob* and wild-type ovariectomized female mice, demonstrating that leptin acts via the central nervous system to inhibit axial bone formation under certain conditions (4). The absence of detectable leptin in the serum of *ob/ob* mice treated ICV with leptin also supports a

central mode of leptin action. A parabiosis model in which only one *ob/ob* mouse of a parabiosed pair received ICV leptin was used to confirm the key role hypothalamic leptin plays in regulating bone formation. Correction of the *ob/ob* skeletal phenotype by loss of bone mass was only achieved in the leptin recipient and not in the contralateral mouse(5), clearly demonstrating that similar to its control of energy homeostasis by central mediation, the action of leptin on bone is also regulated through a hypothalamic relay.

Leptin can also act directly on bone marrow cells to enhance their differentiation to osteoblasts and inhibit their differentiation to adipocytes (6). Marrow tissue in the limb bones is known to accumulate adipocytes with age, whereas marrow remains much lower in adipocytes in the sternum and spine much later in life. However, it is not known whether the different modes of leptin administration in the absence of leptin signaling affect the bone marrow cells in the spine and limb similarly.

There are contradictory published data, regarding the effects of leptin on bone mass in rodents. Some studies have described *ob/ob* mice (leptin-deficient) as having a "high bone mass" (4,7-8) while Steppan et al. (9) observed that leptin-deficient mice had lower bone mass than normal mice. In a study conducted by Hamrick et al. (10), leptin-deficient mice were shown to have higher bone mass in the axial skeleton than in the appendicular skeleton. One explanation of the discrepancies of results reported may be due to mode of leptin administration. Peripheral (SQ) administration of leptin increased bone growth and indices of bone formation (9,11). While central (ICV) infusion of leptin led to rapid bone loss in the vertebrae (4), suggests that leptin can regulate bone mass through alternative pathways: one involving a direct stimulatory effect on bone growth when administered peripherally and another that is indirect, involving a hypothalamic relay that suppresses bone formation when administered centrally (12).

The location of bone that was observed may be another explanation of the contradictory findings. Hamrick et al. (10) reported that trabecular bone volume was reduced in the proximal femur, but elevated in the distal femur of *ob/ob* mice (13). In a separate study Hamrick et. al. (14) reported reduced femoral bone mineral content (BMC) and bone mineral density (BMD) in *ob/ob* mice, reduced cortical thickness of lumbar vertebrae, increased vertebral length, and increased lumbar BMC and BMD. The observations suggest not only differential responses to leptin signaling between the appendicular and axial skeletal sites, but also different actions of leptin on cortical and trabecular bone. Because of these varied reports regarding the effects of leptin treatment and leptin deficiency on bone growth and metabolism, our objective was to determine the effects of differing modes of leptin administration on both the appendicular and axial skeleton.

Materials and methods

Animals & Design

All experimental and surgical procedures in this study were approved by the Animal Care and Use Committee for The University of Georgia. Forty female leptin deficient (*ob/ob*) mice (15 wk old; 61.3g initial body weight) on the C57BL6 background were purchased from Jackson Laboratories, Inc. (Bar Harbor, ME). The mice were individually housed in plastic shoebox cages in a room with a 12-/12-hour light/dark cycle, 22 ± 1 ºC ambient temperature, and 50% humidity. Mice had ad libitum access to pelleted standard lab chow (LabDiet 5001, PMI Nutritional International, Brentwood MO) and water throughout the study.

All mice were surgically implanted with osmotic minipumps (model 1002, 0.25 μL/h; Alzet Corp., Cupertino, CA) for injection of treatment solutions. Mice were anesthetized with 0.5% oxygen/isoflurane and prefilled and primed pumps were inserted into a SQ pocket. Twenty mice were surgically prepared with a unilateral lateral ventricular (ICV) guide cannula (model

3280P/spc; Plastics One Inc., Roanoke, VA), which included an infusion port that was attached to the osmotic pump, as previously described (15).

Treatments

Treatments were randomly assigned and included SQ control (saline) 6 µl/day (treatment 1), SQ leptin 10 µg/day (treatment 2), ICV control (aCSF) 6 µl/day (treatment 3), and ICV leptin 1.5 µg/day (treatment 4). Previous studies found that leptin produced a maximal suppression of food intake (FI) and body weight (BW) reduction at 10 µg/day in a dose range of 2.5-10 µg/day when administered SQ (16) and at 1.5 µg/day in a dose range of 0.38-1.5 µg/day when administered ICV (17).

Recombinant mouse leptin (R&D Systems, Minneapolis, MN) was dissolved in either an artificial cerebrospinal fluid (aCSF), which consisted of (in g/l): NaCl, 8.66; KCl, 0.224; $CaCl_2 \cdot 2H_2O$, 0.206; $MgCl_2 \cdot 6H_2O$, 0.163; $Na_2HPO_4 \cdot 7H_2O$, 0.214; $NaH_2PO_4 \cdot H_2O$, 0.027 for ICV delivery, or 0.9% phosphate-buffered saline solution for SQ delivery.

Design and Procedures

Mice assigned to treatments 1 and 2 had an osmotic pump for SQ delivery of the control and test solutions; whereas, mice assigned to treatments 3 and 4 had an ICV cannula with an osmotic pump attached for continuous ICV injection of control and test solutions. Osmotic mini-pumps remained in place for 14 days. All mice were allowed to recover for 2 days after surgery; during this time 6 µl of saline (for SQ delivery in treatments 1 and 2) or 6 µl of aCSF (for ICV delivery in treatments 3 and 4) were infused prior to the treatment infusion, which occurred for the next 12 days. The mice were injected (intraperitoneal) with calcein (Sigma C-0875; 20 mg/kg BW) on the first day of treatment injections and again on the day before they were to be

killed to label active bone forming surfaces. The health of the mice was monitored and BW and FI were measured and recorded daily.

The mice were killed by decapitation using a guillotine after sedation in a CO_2 chamber at the end of the 12th day of treatment injection. Retroperitoneal (RP) fat pads, interscapular brown adipose tissue (BAT), and the soleus and gastrocnemius muscles (GC) were collected, weighed, and then frozen in liquid nitrogen before storing at -80 °C. Half of the RP tissue sample was fixed in 4% paraformaldehyde for tissue apoptosis assay. Trunk blood was collected for measurement of serum concentrations of glucagon and insulin using the Luminex100™ instrumentation and a multiplex assay kit (Mouse Endocrine Immunoassay Panel MENDO-75K, Millipore Corp., St. Charles, MO). Osteocalcin concentrations were determined by the BTI Mouse Osteocalcin EIA Kit (Biomedical Technologies, Inc., Stougton, MA; Catalog No: BT-470). Pyridinoline (PYD) concentrations were determined by the Metra® Serum PYD kit (Quidel Corp., San Diego, CA; Catalog number 8019). RANK-L concentrations were determined using the Luminex100™ instrumentation and a single-plex assay kit (catalog # MBN-41K-1RANKL). Serum IGF-1 concentrations were determined using the Luminex100™ instrumentation and a single-plex assay kit (Catalog No. RMIGF1-87K) manufactured by LINCO (St. Charles, MO). The Mouse Bone Panel from LINCO (Catolog No. MBN-1B-41K) was used to determine the serum osteoprotegerin (OPG) concentrations using the Luminex100™ instrumentation.

Body composition was analyzed by PIXImus densitometry (GE Lunar Corp., Waukesha, WI). Fat mass, lean mass, percentage fat, bone mineral density (BMD), and bone mineral content (BMC) were measured for each animal. The right and left tibia and lumbar spine of each mouse were dissected free of soft tissue. The right tibia and lumbar spine (L4-L5) were fixed in

10% buffered formalin for 48h at room temperature, and then stored in 70% ETOH for bone histomorphometry (see below). Later, the proximal third of the tibia was removed and the spine was cut across the L4-L5 disc. Both were decalcified in 4% EDTA, embedded in paraffin, and sectioned on the transverse plane. The left tibia and femur and lumbar spine (L2-L3) were fixed in 70% ethanol for bone densitometry measurements and embedding in methyl-methacrylate. DEXA densitometry (PIXImus system) was used to measure BMD and BMC from the left tibia and femur and lumbar spine (L2-L3). The PIXImus dual-energy X-ray absorptiometry system allows accurate measurement BMD and BMC from small lab animals using a relatively low X-ray energy (80/35 kVp) and ultra-high resolution (0.18 x 0.18 mm pixel size) to achieve contrast in low-density mouse bone. Replicability data indicate an excellent correlation (0.99) between PIXImus BMC and total ashed weight (18).

Bone histomorphometry

As noted above, the right tibia was cut across the proximal third of the shaft and the L4 vertebrae was cut across the horizontal plane, decalcified in EDTA, embedded in paraffin, and sectioned at ~5μm. Sections were stained with H&E to visualize adipocytes. Adipocytes were counted over a 0.10mm^2 area. Adipocyte size was measured as the cross-sectional area of each adipocyte by digitizing the border of each cell (four to five cells per section) using ImagePro analysis software.

The left tibia and femur and lumbar spine (L3) were fixed in 70% ethyl alcohol, dehydrated, embedded in methyl methacrylate, and sectioned at 30μm across the proximal third of the shaft for the tibia, distal third for the femur, and across the horizontal plane for the L3 vertebrae. These thicker sections are preferred because they provide excellent visualization of

fluorochrome labels (10). The mineral apposition rate (MAR) was derived from fluorochrome interlabel distances.

Statistical analysis

All statistical analyses were conducted with SAS (Version 9.0) (SAS Institute, Cary, NC). Significance of treatment effects was determined by one or two way ANOVA. Significance of differences among means was determined by Fisher's LSD. Significance was established at $p < 0.05$.

Results

Food intake, body weight, body composition

Regardless of mode of administration, there were significant effects on BW due to leptin. By the end of the 12-day injection period, SQ and ICV leptin-treated mice had lost 8.8 ± 2.8 g and 12.0 ± 5.6 g BW, respectively ($p < 0.0001$) while the saline and aCSF-injected mice gained 2.7 ± 2.1 g and 2.5 ± 1.8 g. SQ leptin decreased daily food intake by 36.9% and ICV leptin, by 37.5% ($p < 0.03$). (Figure 5.1)

As compared to their respective controls, the mice treated with leptin had smaller RP fat depots relative to body weight by at least 25% ($p = 0.04$). There was no change in the BAT weight between the leptin administration groups and the controls (data not shown). The GC and soleus muscle weights were increased in the leptin treated mice (Figure 5.2).

DEXA results showed that both the SQ and ICV leptin administered mice had a significant decrease in fat mass and % body fat, and an increase in % lean mass. Both groups of leptin treated mice had an increase in whole body BMD, BMC, and area (Table 5.1). The

muscle mass of the leptin treated mice was positively correlated with BMD ($r=0.49$, $p=0.004$) and BMC ($r=0.73$, $p<0.0001$).

Serum hormone concentrations

Both SQ and ICV leptin treated mice had significantly higher concentrations of osteocalcin and OPG, markers associated with bone formation, as compared to the control mice (Figure 5.3). Likewise, both SQ and ICV leptin treated mice had significantly higher concentrations of PYD and RANK-L, markers that indicate bone turnover.

Regardless of mode of administration, leptin significantly decreased insulin and glucagon concentrations. Both SQ and ICV leptin treated mice had significantly higher IGF-1 concentrations as compared to their respective control groups. Mice treated with SQ leptin had slightly, but significantly, higher serum IGF-1 and RANK-L levels compared to mice treated with ICV leptin (Figure 5.3). The serum IGF-1 levels were positively correlated with muscle mass ($r=0.51$, $p=0.002$), BMD ($r=0.62$, $p=0.0001$), and osteocalcin concentrations ($r=0.45$, $p=0.007$).

Bone BMD, BMC and histomorphometry

Regardless of mode of administration, leptin increased the relative BMD in the left tibia, femur and L2-L3 vertebrae and increased relative BMC in the left femur and L2-L3 vertebrae (Table 5.2), however tibial and femoral length were not increased (data not shown). The tibial and trabecular bone of the vertebrae MAR was significantly higher in the leptin treated mice compared to the control mice by at least +2.5 microns/d and +3.5 micron/d respectively (Figure 5.4). The femoral MAR was significantly higher with ICV (2.9 ± 0.2 microns/d) and SQ (3.3 ± 0.3 microns/d) leptin administration than in the ICV (1.4 ± 0.2 microns/d) and SQ (1.7 ± 0.1 microns/d) controls ($p < 0.01$). Both modes of leptin administration significantly decreased the

bone marrow adipocyte number by at least100 cells/0.1 mm^2 (45%) and adipocyte area by more than 50% in both the left tibia and L4 vertebra as compared to the control mice (Figure 5.5). There was an increase in the number of osteoblasts per $area^2$ with leptin treatment although there was no difference among the modes of administration. There was, however, no increase in bone volume with leptin administration (data not shown). This could be a result of an increase in the way that leptin was administered but it needs to be determined in a future study where leptin is administered at varying lengths/periods of time.

Discussion

The changes in BW, FI, adiposity and bone indices observed in this study demonstrate that leptin administered either SQ or ICV had essentially similar effects. These similarities were at doses selected to generate a near maximum effect of previously tested doses on body weight reduction and restriction in food intake (16-17). Regardless of mode of administration, leptin reduced food intake, resulting in a decrease in body weight through the loss of fat mass. Contrary to the findings by Martin et al., this reduction in energy intake was not associated with a dramatic decrease in serum IGF-1 levels in the leptin treated mice (19). The results suggest that the pathways of bone regulation affected by leptin are not dependent on the equilibrium between food intake and energy expenditure but may be on leptin's increase in sympathetic tone in *ob/ob* mice, since Karsenty and colleagues demonstrated that the sympathetic tone mediates only leptin's regulation of bone mass and the regulation of the bone mass by the sympathetic nervous system is independent of energy expenditure (20). Leptin treated mice had increased bone turnover with increased bone formation. The bone growth was observed in both the tibia, femur, and trabecular bone of the vertebrae. The increased trabecular bone formation observed in the study agrees with reports of bone formation in the axial skeleton (10). The increased tibial

and femoral bone formation by leptin administration may be explained by the increase in the serum IGF-1 levels observed in the study. Increased IGF-1 levels may be indicative of the stimulation of beta 1-adrenergic receptors and the GH-IGF-1 axis (21), which have been shown to stimulate bone growth. Furthermore, leptin has been shown to suppress hypothalamic NPY expression and both the suppression of NPY expression and the stimulation of the GH-IGF-1 axis increase tibial and femoral bone formation (21-22). Moreover, leptin injected into the brain at a rate that did not result in any detectable leak of leptin into the general circulation (4,20) induced bone resorption. Thus, bone formation was inhibited in these studies at lower doses of leptin than those necessary to cause the loss of body weight.

This study shows that both peripherally and centrally administered leptin can promote osteogenesis and reduce bone marrow adiposity, in agreement with previous reports (10,23). The effect of peripherally administered leptin may be partly due to its direct effect on bone marrow cells, enhancing their differentiation to osteoblasts and inhibiting their differentiation into adipocytes (6). Since the marrow tissue is known to accumulate adipocytes with age (6), the decrease in the bone marrow adipocyte size and number in both the limbs and spine observed in this study in the leptin treated mice, while increasing the bone indices may indicate that a reduction in marrow adipocytes has a positive effect on bone formation.

Lean mass regulates local bone formation by muscle-derived mechanical stimuli (20). Muscle mass is directly correlated to the amount of bone mass (24). Compared to lean mice, genetically obese *ob/ob* mice have less skeletal muscle mass (10), which is associated with low circulating levels of growth hormone (25) and resistance to IGF-1 (26). Our results show that leptin administered either peripherally or centrally increased serum IGF-1 levels, which likely contributed to increased muscle growth and bone formation via muscle-derived mechanical

stimuli. The stimulation of muscle growth may also have been a result of increased physical activity, since leptin has been shown to increase physical activity (16).

Martin and colleagues have suggested that leptin has a bimodal effect on bone, with low levels of leptin promoting bone formation and high levels resulting in bone resorption secondary to decreased serum IGF-1 levels caused by reduced energy intake (19). In our study, both groups of leptin-treated mice had equivalent reduction of food intake and body weight, yet their serum IGF-1 levels were increased compared to the controls. Whether even higher doses of leptin would have resulted in suppression of IGF-1 levels and increased bone resorption is not known but could be tested in a future study. However, we believe our study is the first to investigate the effects of centrally and peripherally administered leptin on both the appendicular and axial skeleton.

The lack of differences between SQ and ICV leptin treatments was most likely due to the fact that we selected doses for both routes of administration that caused maximal suppression of food intake and body weight. We and others have previously shown that *ob/ob* mice are very sensitive to the effects of exogenous leptin. In contrast, diet-induced obesity has been shown to result in receptor defects that reduce transport of leptin across the blood brain barrier (27). At the doses tested in this study, it is likely that peripherally administered leptin reached brain receptors at approximately the same concentration as the centrally administered leptin, thus accounting for the similarity of effects.

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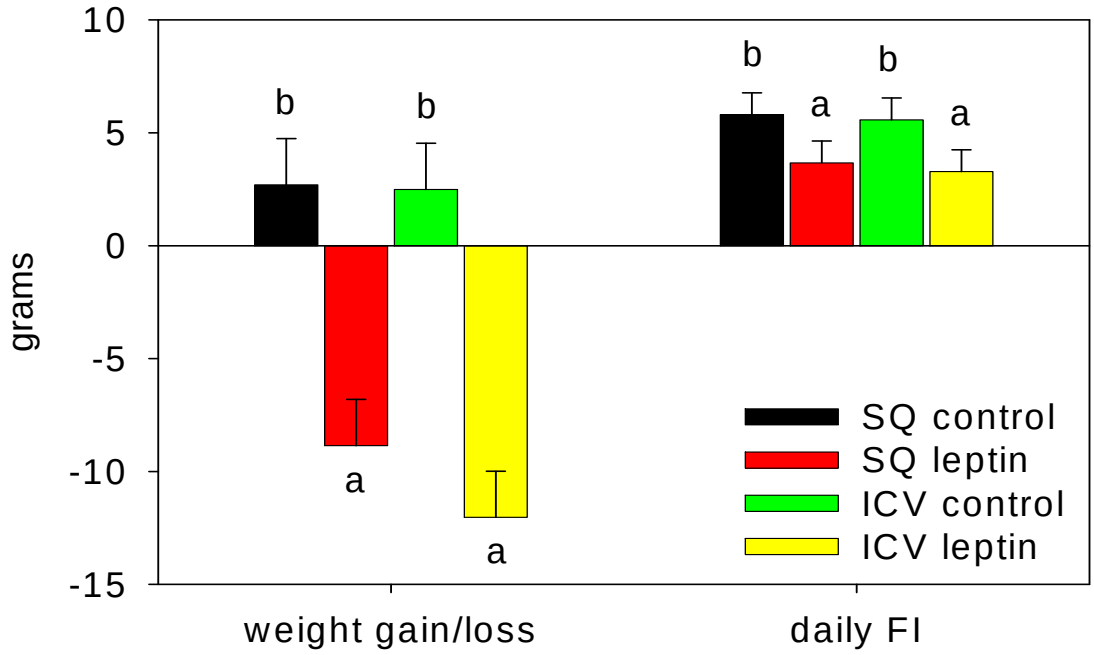
Figure 5.1. Body weight changes and daily food intake after 12 days of SQ and ICV leptin injections in *ob/ob* mice. Means which don't share a common letter are significantly different: ^{a,b} $p < 0.05$.

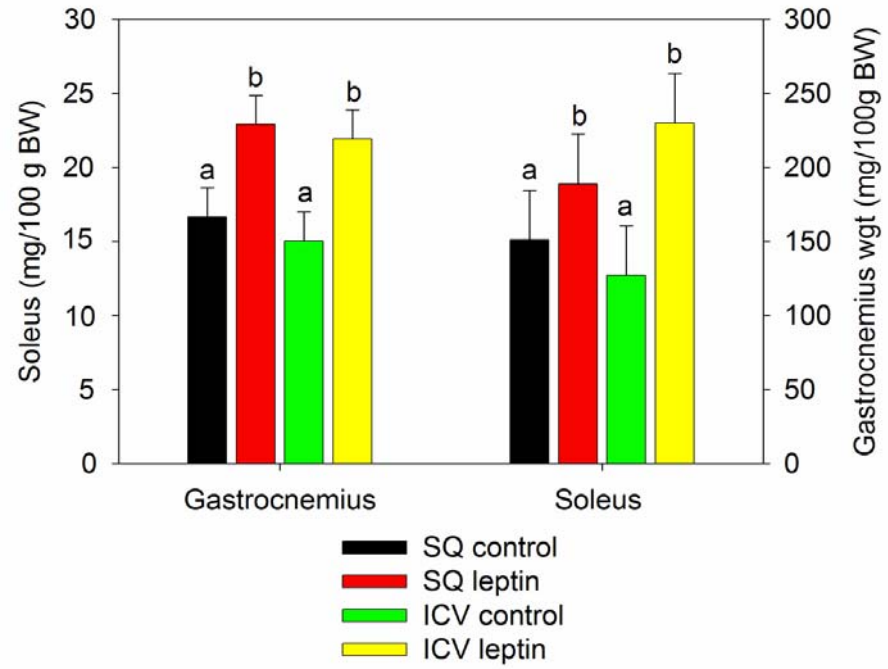
Figure 5.2. The muscle weights of SQ and ICV leptin treated *ob/ob* mice injected for 12 days. Means which don't share a common letter are significantly different: ^{a,b} $p < 0.05$.

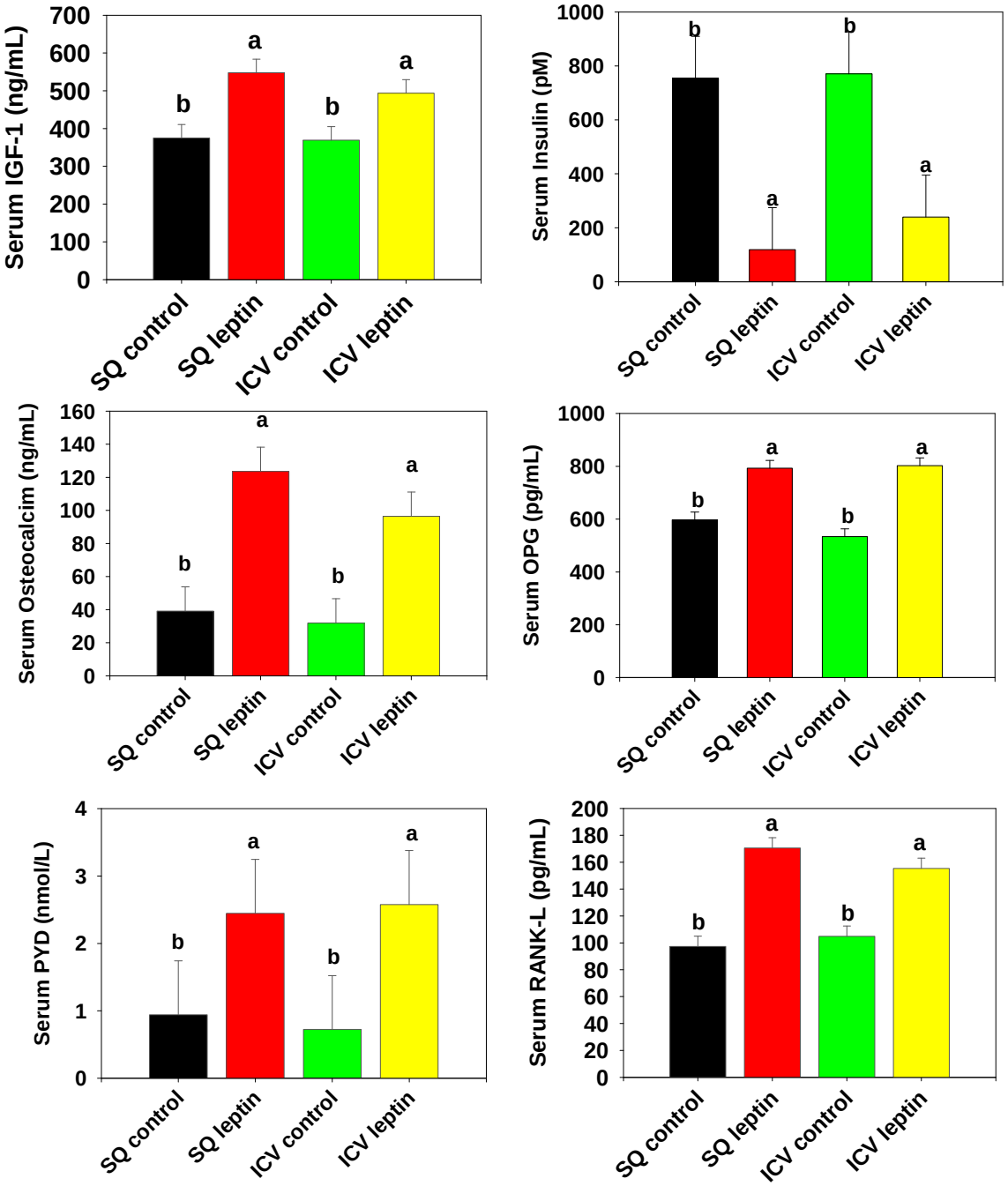
Figure 5.3. The effect of 12 days of SQ or ICV leptin injections on serum IGF-1, insulin, osteocalcin, OPG, PYD, and RANK-L concentrations. Means which don't share a common letter are significantly different: ^{a,b} $p < 0.05$.

Figure 5.4. Images of fluorochrome-labeled bone forming surfaces in tibial cortical bone (right top) and vertebral trabecular bone (left top). Histograms showing cortical mineral apposition rate (MAR) in the tibia (left top) and trabecular MAR in the L4 vertebrae (left bottom). Means which don't share a common letter are significantly different: ^{a,b} $p < 0.05$.

Figure 5.5. Histograms showing tibial bone marrow adipocyte number (left) and size (right). Means which don't share a common letter are significantly different: ^{a,b} $p < 0.05$.
H&E stained tibial cross-sections (right).


10
5
0
-5
-10
-15
grams
b
b
a
a
b
a
b
a
SQ control
SQ leptin
ICV control
ICV leptin
weight gain/loss
daily FI


30
25
20
15
10
5
0
Soleus (mg/100 g BW)
300
250
200
150
100
50
0
Gastrocnemius wgt (mg/100g BW)
a
b
a
b
a
b
a
b
Gastrocnemius
Soleus
SQ control
SQ leptin
ICV control
ICV leptin


Serum IGF-1 (ng/mL)
700
600
500
400
300
200
100
0
b
a
b
a
SQ control
SQ leptin
ICV control
ICV leptin
Serum Insulin (pM)
1000
800
600
400
200
0
b
a
b
a
SQ control
SQ leptin
ICV control
ICV leptin
Serum Osteocalcim (ng/mL)
160
140
120
100
80
60
40
20
0
b
a
b
a
SQ control
SQ leptin
ICV control
ICV leptin
Serum OPG (pg/mL)
1000
800
600
400
200
0
b
a
b
a
SQ control
SQ leptin
ICV control
ICV leptin
Serum PYD (nmol/L)
4
3
2
1
0
b
a
b
a
SQ control
SQ leptin
ICV control
ICV leptin
Serum RANK-L (pg/mL)
200
180
160
140
120
100
80
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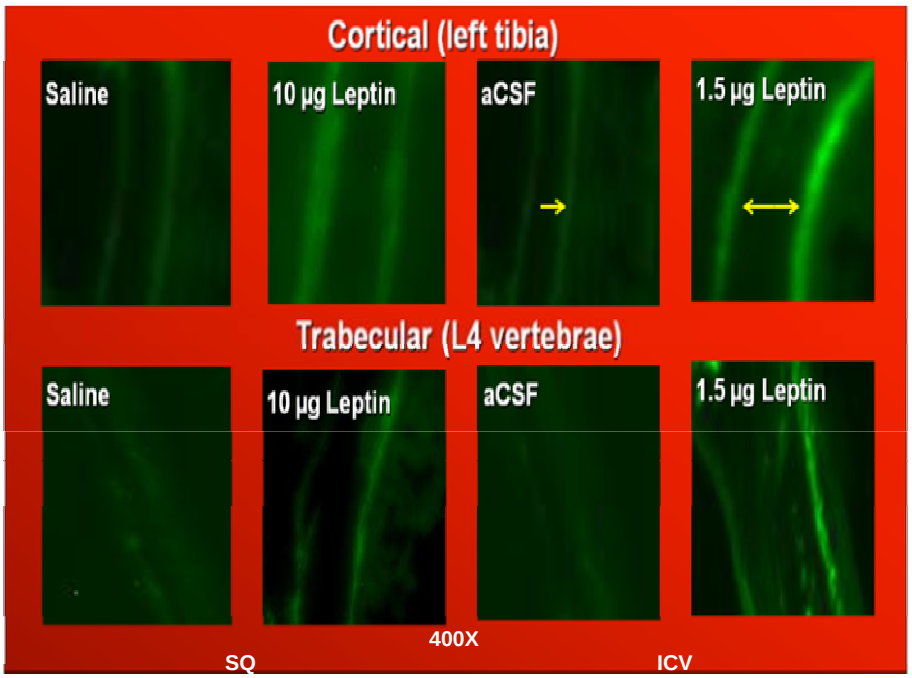
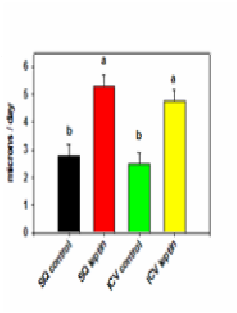
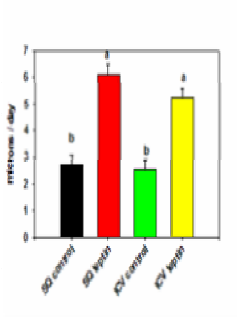
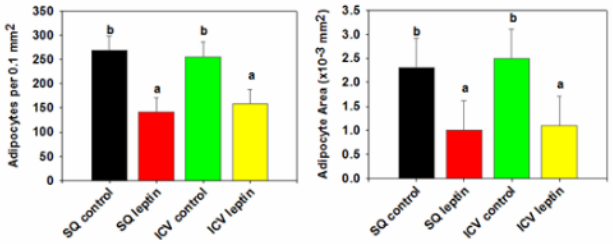
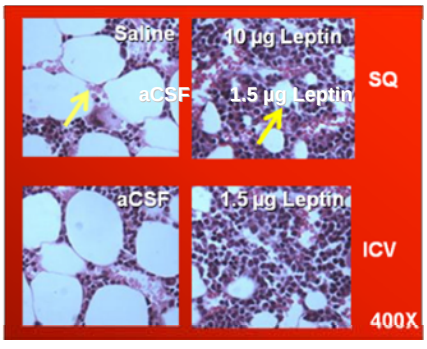
b
a
b
a
SQ control
SQ leptin
ICV control
ICV leptin

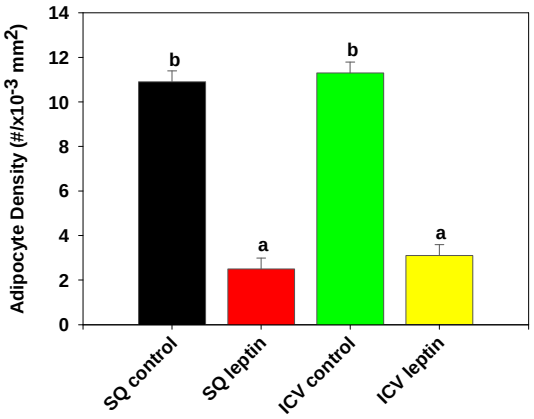

Cortical (left tibia)
Saline
10 µg Leptin
aCSF
1.5 µg Leptin
Trabecular (L4 vertebrae)
Saline
10 µg Leptin
aCSF
1.5 µg Leptin
400X
SQ
ICV






Adipocytes per 0.1 mm2
SQ control
SQ leptin
ICV control
ICV leptin
Adipocyte Area (x10-3 mm2)
SQ control
SQ leptin
ICV control
ICV leptin


Saline
10 µg Leptin
aCSF
1.5 µg Leptin
SQ
aCSF
1.5 µg Leptin
ICV
400X


Adipocyte Density (#/x10-3 mm2)
14
12
10
8
6
4
2
0
b
a
b
a
SQ control
SQ leptin
ICV control
ICV leptin

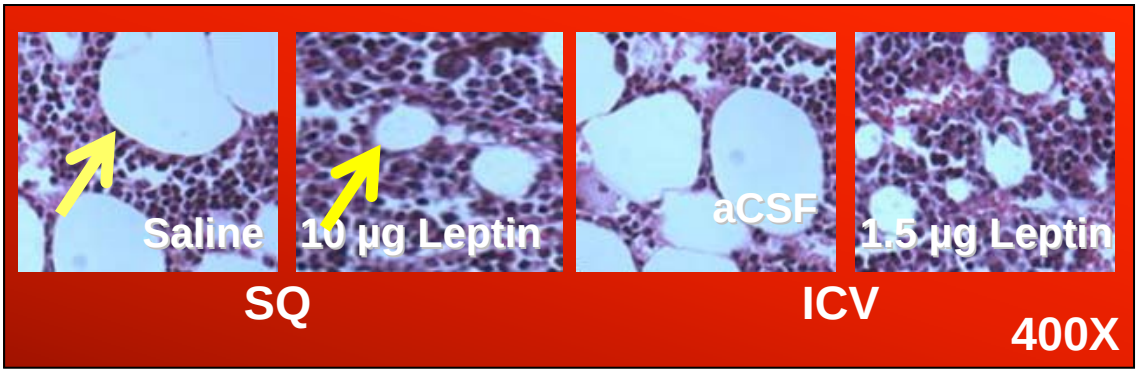

Saline
10 µg Leptin
aCSF
1.5 µg Leptin
SQ
ICV
400X

Table 5.1. Body composition and densitometric properties of leptin treated and control *ob/ob* mice.

	SQ Injection		ICV Injection		SEM	p-value
	saline	Leptin	aCSF	Leptin		
BMD, g/cm^2	.0492^{c}	.0545^{ab}	.0513^{bc}	.0553^{a}	0.002	0.006
BMC, g	.387^{b}	.483^{a}	.406^{b}	.518^{a}	0.02	0.001
Area, cm^2	7.14^{b}	7.79^{a}	7.09^{b}	8.30^{a}	0.30	0.004
Lean, g	23.3^{a}	20.4^{b}	22.3^{a}	20.3^{b}	1.20	0.03
Fat, g	33.3^{b}	25.6^{a}	33.4^{b}	26.2^{a}	1.93	<0.0001
% Fat, g	58.8^{b}	55.8^{a}	60.0^{b}	56.1^{a}	1.23	0.001
Fat : Lean, g:g	1.43^{b}	1.25^{a}	1.50^{b}	1.29^{a}	8.08	0.006

Means which don't share a common letter are significantly different: ^{a,b,c} $p < 0.05$.

Table 5.2. Densitometric properties of the femur, tibia, and vertebrae in leptin treated and control *ob/ob* mice.

	SQ Injection		ICV Injection		SEM	p-value
	saline	Leptin	aCSF	Leptin		
Lf. Tibia BMD, g/cm^2	.0419	.0417	.0405	.0430	0.001	Ns
Relative Tibia BMD, g/cm^2/g BW	.0667^b	.0825^a	.0682^b	.0856^a	0.01	0.0009
Lf. Tibia BMC, g	.020	.019	.020	.020	0.001	Ns
Relative Tibia BMC, g/g BW	.032	.037	.033	.039	0.01	Ns
Lf. Tibia Area, cm^2	.48	.47	.48	.47	0.04	Ns
Relative Tibia Area, cm^2/g BW	.76^c	.92^{ab}	.79^{bc}	.94^a	0.07	0.02
L2-L3 Spine BMD, g/cm^2	.0450	.0451	.0446	.0457	0.001	Ns
Relative Spine BMD, g/cm^2/g BW	.0716^b	.0891^a	.0730^b	.0934^a	0.006	0.0002
L2-L3 Spine BMC, g	.014	.016	.015	.017	0.001	Ns
Relative Spine BMC, g/g BW	.023^b	.031^a	.025^b	.034^a	0.002	<0.0001
L2-L3 Spine Area, cm^2	.32	.35	.34	.36	0.02	Ns
Relative Spine Area, cm^2/g BW	.51^b	.70^a	.56^b	.73^a	0.05	<0.0001
Lf. Femur BMD, g/cm^2	.0467^{ab}	.0491^a	.0440^b	.0481^a	0.002	0.009
Relative Femur BMD, g/cm^2/g BW	.0744^b	.0973^a	.0719^b	.0995^a	0.007	0.0002
Lf. Femur BMC, g	.025	.026	.023	.025	0.001	Ns
Relative Femur BMC, g/g BW	.040^b	.052^a	.037^b	.051^a	0.004	0.0006
Lf. Femur Area, cm^2	.54	.53	.52	.52	0.02	Ns
Relative Femur Area, cm^2/g BW	.85^b	1.05^a	.85^b	1.06^a	0.07	0.002

Means which don't share a common letter are significantly different: ^{a,b} $p < 0.05$.

CHAPTER 6

CONCLUSION

The aim of this research was to evaluate leptin's roles in bone growth, throughout the skeleton, and adipose tissue in *ob/ob* mice and their relationship to osteoporosis. The studies utilized a model of an aged (15-wk) obese *ob/ob* mice, who cannot produce leptin due to an inactivating mutation in the leptin gene. These mice have the skeletal abnormalities observed in an individual suffering from osteoporosis. Compared to wild-type mice, *ob/ob* mice have reduced bone mass and strength, suggesting that leptin is required for optimal bone growth and quality. The precise mechanisms of leptin action on the skeleton are not fully understood, in part, due to the hormonal actions on bone through multiple pathways: one or more indirect pathways involving central/hypothalamic relays, and a direct pathway involving the binding of leptin to its receptor on cartilage and bone cells. It was hypothesized that leptin administered to *ob/ob* mice would have activity in preventing all three mechanisms causing osteoporosis.

Experiment 1 was conducted to gain insight into on the bone marrow cell environment and bone growth, increasing concentrations of central leptin were injected and bone marrow gene expression was analyzed. Leptin decreased body weight (BW) and body fat and increased distal tibial and lumbar bone mineral density (BMD) and bone mineral content (BMC) in a dose dependent manner. mRNA expression levels of genes involved in adipogenesis and cell survival (eg., PPARγ, Bcl-2) were decreased, demonstrating an enhanced sensitivity to leptin-stimulated adipocyte apoptosis in the bone marrow. Expression levels of genes associated with

osteogenesis (Runx2) were increased, while those associated with osteoclastogenesis (RANK) were decreased, which is consistent with the observed leptin-stimulated bone growth as demonstrated by the increased mineral apposition rate (MAR) and inclination of cells to differentiate into osteoblasts. These results indicate that increasing concentrations of leptin promote expression of pro-osteogenic factors in the bone marrow and enhanced bone formation in *ob/ob* mice.

Central or peripheral leptin administration reduces body weight, food intake, & body fat in leptin deficient *ob/ob* mice. Experiment 2 was conducted to determine the effects of leptin on body composition, since body composition has been shown to be directly related to bone mass and density. Prior SQ and ICV leptin dose studies were conducted to determine the concentration of leptin administration used in this study. The doses were deemed optimal based upon maximum weight loss and reduction of food intake. Leptin decreased BW, food intake, body fat, serum insulin and glucagon concentrations and increased relative gastrocnemius muscle wt, relative muscle mass and adipose apoptosis, and the effects were equivalent between SQ and ICV modes of administration. However, the level of adipose tissue apoptosis was significantly greater after ICV leptin administration yielded a greater sensitivity to adipose tissue apoptosis rather than after SQ leptin administration, although there was no difference in gene expression among the modes or inducement of adipose tissue loss. These results indicate that SQ and ICV ($1/7^{th}$ the SQ dose) leptin administration decreased fat mass and increased muscle mass & gene expression equally in *ob/ob* mice.

Knowing the relationship between body composition and bone mass and density, it was still unknown how the fat loss observed in the previous study would affect bone metabolism. Also, complicating the issue was the varying reports on leptin administration and its effect on

bone metabolism and how the absence of leptin signaling affects *in vivo* bone growth throughout the skeleton. Therefore, the objective of experiment 3 was to determine the effects of central & peripheral administration of leptin on bone metabolism throughout the skeleton. There were no significant differences in bone parameters between the modes of leptin administration. Regardless of mode of administration, leptin increased lumbar, proximal femoral, and distal tibial BMD and BMC. The tibial, femoral, and lumbar spine MARs were increased with leptin administration. Both ICV and SQ leptin administration decreased bone marrow adipocyte number and size, while increasing BMD indicating that a reduction in marrow adipocytes has a positive effect on bone formation and possibly bone strength and integrity. The presence of adipocytes in the bone marrow would have been detrimental to bone, since fat cells may not only suppress osteoblastogenesis, but also promote bone resorption because fat cells secrete inflammatory cytokines capable of recruiting osteoclasts. Serum osteoprotegerin (OPG), a secretory marker produced by the osteoblasts that inhibits osteoclastogenesis, was increased by leptin administration, regardless of mode. Serum osteocalcin, a marker for bone formation, was increased with both modes of leptin administration. These observed amplifications in BMD, BMC, bone formation, bone marker serum concentrations, and reduction of bone marrow adipocyte size and number indicate that both ICV and SQ leptin administration increased bone growth equally in *ob/ob* mice and was not affected by fat mass loss.

The results of this research demonstrate that leptin has characteristics that make it a good model for future development of pharmaceuticals for prevention and treatment of osteoporosis. Leptin induced bone marrow adipocyte apoptosis, reduced cell differentiation into osteoclasts and adipocytes, and promoted osteoblast differentiation as demonstrated by changes in gene expression, bone histology, serum marker concentrations, and body composition. Thus, leptin

preserved muscle mass, bone mass and bone integrity, which are important for the prevention of osteoporosis.