BONE LOSS DURING ENERGY RESTRICTION:

MECHANISTIC ROLE OF LEPTIN

A Dissertation

by

KYUNG HWA BAEK

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2007

Major Subject: Nutrition

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Approved by:

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ABSTRACT

Bone Loss During Energy Restriction: Mechanistic Role of Leptin.

(December 2007)

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Mechanical unloading and food restriction (FR) are leading causes of bone loss, which increase the risk of fracture later in life. Leptin, a 16*kDa* cytokine like hormone principally produced by white adipocytes, may be involved in bone metabolism with physiological or mechanical changes causing bone loss. The hypotheses of the first study were aimed at determining if serum leptin is reduced by unloading or FR. The serum leptin level reduced by unloading or by global FR, is associated with the decline in bone formation rate. It was conjectured that decreased serum leptin may be due to reduced adipocyte number/size and/or sympathetic nervous system (SNS) activation of beta-adrenoreceptors with unloading or FR, inhibiting the release of leptin from adipocytes. In the second experiment, we tested whether leptin or beta-adrenoreceptor blockade attenuates bone loss during unloading and whether such an effect due to beta blockade is associated with changes in serum leptin level. Beta-blockade mitigated unloading induced reduction in serum leptin and also beta blockade was as effective as leptin administration in mitigating a reduction in cancellous bone mineral density with unloading through both stimulation of bone formation and suppression of resorption. It was previously demonstrated that energy restriction (ER) is a major contributor to the

bone loss during global FR. In the third study, we tested whether beta- blockade attenuates bone loss during ER and whether such an effect is associated with changes in serum leptin level and leptin localization in bone tissues. Beta blockade attenuated the ER induced reduction in serum leptin level, cancellous bone mineral density and bone formation rate, and also abolished the ER induced increase in bone resorption. Reduction in leptin expression in bone marrow adipocytes observed with ER was attenuated by beta-blockade. Reduction in the number of cells (bone lining cells, osteocytes and chondrocytes in cartilage) which are stained positive for leptin was also attenuated by beta-blockade. Collectively, these data identify circulating leptin effects on preventing bone loss during mechanical unloading or energy restriction. Also beta blockade is associated with mitigating reduction in serum leptin and subsequently with mitigating reduction in bone mass with unloading or ER.

DEDICATION

To my parents, husband and

The sacrificed animals

ACKNOWLEDGMENTS

First and foremost, thanks should be given to my advisor, Dr. Susan Bloomfield. Her insight, help, and guidance were great through this research. She has not only provided me with invaluable lessons in scientific and professional conduct but also presented warm hearted care and consideration for the last six years. I would also like to thank my other committee members, Dr. Harry Hogan for introducing me to a whole new world of mechanical engineering and for cheering me up with his sense of humor and support, and Dr. Joanne Lupton and Dr Robert Chapkin for their great teaching and helpful advice throughout this effort.

Certainly not to be forgotten are all the members of the bone biology lab group. Thanks to Mats, Josh and Sibyl for their friendship and advice. Thanks especially to Jan Stallone; I am grateful to her for sharing her knowledge with me about all the techniques and valuable tips for experiments and for always providing a listening ear on good days or bad days.

Most importantly, I would like to thank my mom and dad, my greatest role models and the ones who instilled the desire in me to work in science. I still remember the early mornings that I and my sister were studying math with dad when I was young. Also thanks to my mom for being a listening ear almost everyday, talking over the phone and

giving me precious advice and keeping me grounded. The same thanks and love go to my sister and brother who always made me smile and cheered me up though all of this.

Last, but definitely not the least, most special thanks should be given to my husband, Sungwook. He's been my best friend, the largest supporter and sometimes a constructive commentator on my work as well as the greatest husband. He always kept me motivated and gave me more love than I expected. Without his support, this body of work would never have been possible. I pray that I can provide the same support and love for his dreams throughout all the days of our life together.

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CHAPTER I

INTRODUCTION

Mechanical unloading and restricted dietary intake are leading causes of bone loss, which increase the risk of fracture in later in life. Mechanical unloading due to spaceflight or bed rest induce decrements in bone health. About 1% of bone mineral density (BMD) is lost per month while in microgravity (1, 2) although the magnitude of loss is highly variable among subjects and among anatomical sites. A significant percentage of pre-menopausal women in modern society utilizes food restriction in their attempts to lose weight (3). Dieting and weight cycling are known to result in clinical concerns, leading to menstrual cycle disturbances and decreased bone mass (4-7). An association between a history of weight loss in pre-menopausal years and increased risk of hip fracture later in life has been reported (8). On average, a 1% decrease in bone mass (9) and an increase in bone resorption (10, 11) is associated with each 10% decrement in body weight.

Leptin, a 16 kDa cytokine-like hormone principally produced by white adipocytes, may also be involved in the bone response to microgravity and/or restricted food intake. Its principal function is the regulation of energy balance and body composition through negative feedback mechanisms at the hypothalamic nuclei. Leptin is now known to have

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This dissertation follows the style and format of the Journal of Nutrition.

numerous biological effects on the immune system (12), reproduction (13), development (14), hemopoiesis (15), angiogenesis (16) and, most recently, on bone metabolism. However, there is controversy about the nature of leptin's effects on bone. Early studies demonstrated anti-osteogenic effects of leptin via the sympathetic nervous system when delivered directly to the brain's hypothalamus (17,18), but several more recent studies have demonstrated a bone-protective effect of leptin during hindlimb unloading or caloric restriction when administered to the peripheral circulation (19,20).

Activation of the sympathethic nervous system (SNS) is increased with stressful conditions, such as food restriction (21, 22) and simulated microgravity (23-25), which conditions also result in increased bone resorption and/or decreased bone formation. Beta-adrenergic receptor activation is also known to inhibit leptin release from adipocytes (21, 26). Pharmacological blockade of beta-adrenergic SNS signaling mitigates loss of cancellous bone with hindlimb unloading rats (27), but the effect of beta-adrenergic blockade on serum leptin and on bone during caloric restriction has not been demonstrated.

In the first study described in Chapter III, I investigated the individual and combined effects of food restriction and simulated microgravity in adult male rats and the contribution of altered serum leptin to changes in bone strength, density, and turnover status. With the result from the first study, we conjectured that changes in serum leptin during hindlimb unloading (simulating microgravity) in rats may result from the

inhibition of leptin synthesis or secretion due to SNS activation of adipocyte β-adrenoreceptors. In the second study (Chapter IV), we pharmacologically blocked β-adrenoreceptors to examine the impact of the sympathetic nervous system on changes in important bone parameters during unloading. In addition, we replaced leptin during hindlimb unloading to determine if leptin deficiency is an important factor in the loss of bone mass with unloading**.**

In a-preliminary study quantifying the effect of restricting individual nutrients (calcium, protein, energy) to bone loss in a side-by-side comparison (28), we demonstrated that reduced energy intake is the major contributor to the impact of restricting all food intake (global food restriction) on reductions in bone mineral density. This dissertation's first and second studies demonstrate that hindlimb unloading and food restriction each independently produce a decrease in serum leptin. Further, blockade of beta-adrenergic SNS signaling alleviated reductions in serum leptin, bone mineral density, and bone formation rate in hindlimb unloading rats. In the third study (chapter V), we tested the mechanistic role of leptin in bone loss during another stressful condition, dietary energy restriction. We characterized the effect of β-blockade on serum leptin levels and on bone parameters during energy restriction. However, yet unproven in the second study was whether elevating circulating leptin levels actually resulted in more leptin reaching the critical bone cells responsible for bone formation or resorption. Therefore, in the third study, we also confirmed whether increased serum leptin levels actually results in more

leptin reaching the critical bone cells responsible for bone formation/resorption by staining histological sections of bone with antibodies specific to leptin protein.

Taken together, the results of these studies illustrate important role of "peripheral" leptin mechanisms in regulation of bone mass during mechanical unloading or energy restriction. Also, these data illustrate an association between beta-adrenergic signaling and leptin pathways in regulating bone cell activity in the context of mechanical unloading or energy restriction.

CHAPTER II

REVIEW OF LITERATURE

Bone remodeling

Mammalian bone has the ability to constantly rejuvenate itself through the process of remodeling, which continues until death. The major functions of bone remodeling are, first, the maintenance of mechanical strength by constantly replacing old bone by new, mechanically healthy bone, and second, assisting in mineral homeostasis as a store of calcium and phosphorus. Remodeling is performed by a group of cells, which are termed bone remodeling unit (BRU), that act on bone surfaces in a close and sequential collaboration.

There are four phase in the remodeling cycle: activation, resorption, reversal, and formation. During activation, osteoclast precursor cells, which initiate remodeling on quiescent bone, are recruited. The pre-osteoclasts stick to the bone matrix via binding between integrin receptors on the osteoclast surface and RGD- containing peptides in the organic matrix, creating a unique sealed microenvironment between itself and bone matrix. During the resorption phase, protons transferred by a specific pump on the multinucleated osteoclast surface acidify the resorbing area. A number of lysosomal enzymes that are active at low pH are also secreted (29). The acidic solution, accompanied by lysosomal enzymes, dissolves and digests the bone matrix and mineral.

With osteoclast apoptosis, the resorption phase ends and is followed by a reversal phase. In the reversal phase, coupling between osteoclasts and osteoblasts takes place; that is, signals activating osteoblasts to replace resorbed matrix couple or link activity of these two cell types to a specific bone surface site. The origin of coupling signals or the exact mechanism of the coupling process is not fully understood. One major hypothesis is that during the resorption process, osteoclasts release growth factors such as TGF-β, IGF-I, and bone morphogenetic proteins (BMP's), which act as chemotatic factors attracting osteoblasts and stimulating osteoblast differentiation and proliferation (30-32). During the formation phase, osteoblasts initiate the synthesis of organic bone matrix, which is termed osteoid, and regulate its mineralization.

There are three stages in the formation phase. First, collagen synthesized and secreted by osteoblasts is deposited and an osteoid seam, the area of unmineralized matrix between the osteoblasts and pre-existing mineralized bone, is produced. Then, osteoblasts trigger mineralization, which is the process of hydroxylapatite crystal growth within osteoid, by releasing matrix vesicles. Matirix vesicles set up suitable conditions for initial mineral deposition by concentrating calcium and phosphate ions and enzymatically degrading pyrophosphate and proteoglycans which are inhibitors of mineralization. Finally, the rate of collagen synthesis decreases and the mineralization continues until the osteoid seam disappears. After completion of their bone formation function, osteoblasts die by apoptosis or are incorporated into the mineralizing matrix as osteocytes or remain on the surface as bone lining cells. Osteocytes located deep inside

of the bone matrix are in a close connection with each other and osteoblasts on the bone surface through the numerous cellular extensions that lie within canaliculi.

Healthy , young adults, there is virtually no bone loss in most regions of the skeleton, because the coupling of bone formation to previous bone resorption effectively replaces all bone resorbed. Various physiological changes or stressors, such as menopausal estrogen deficiency, reduced energy intake, or chronic disuse can induce "uncoupling" of this balance between formation and resorption. The activation frequency of bone remodeling sites increases and the resorption phase becomes prolonged, leading to net bone loss (33). Cancellous bone loss due to these physiological and/or environmental changes is more dramatic than cortical bone loss for several reasons. Cancellous bone has a greater bone surface per unit volume of bone upon which bone cells can act and also has a better developed contact with blood vessels.

Bone and disuse/tail suspension

Mechanical loading is an essential factor for maintaining skeletal integrity. Decrements in bone health result from spaceflight and other periods of prolonged skeletal disuse. Major skeletal losses during spaceflight have been well documented in both humans and animals. Although this is highly variable among subjects and among bone sites, about 1% of bone mineral density is lost per month while in microgravity. This is a rate is about 12 times faster than the average 1% loss that normal postmenopausal women

experience per year (2,34). Astronauts tend to lose bone only in weight-bearing sites, such as the distal tibia and femoral neck, although vertebral bone is also affected. Losses are much greater in cancellous bone than in cortical bone, at least over 6 months (2). Whether this loss eventually plateaus is not known, because very few astronauts or experimental subjects have been exposed to microgravity or strict bed rest for more than 6 months. Microgravity-induced changes in bone turnover, bone mineral density (BMC), bone mineral density (BMD), and mechanical strength have been measured via biochemical markers, quantitative computed tomography (QCT), dual energy X-ray absorptiometry (DEXA) and mechanical testing.

As a whole body measure, biochemical markers of bone turnover can be used to quantify changes in bone formation (e.g., osteocalcin, OC) and bone resorption (e.g., deoxypyridonoline, DPD) to imposed changes in the mechanical environment. Decreases in OC concentration have been measured in growing rats subjected to simulated microgravity by hindlimb unloading (HU) (35). Serum OC transiently decreases by 25% in 6-week old rats after one week of HU, then returns to almost normal levels after 28 days of HU (36). Consistent and dramatic increases in DPD have been demonstrated in humans exposed to space missions lasting 4 -6 months. DPD measured at post-flight increased 55% above preflight levels (37), suggesting that bone remodeling becomes uncoupled during disuse, resulting in net bone loss.

In Mir crew members, significant reductions in cancellous volumetric bone mineral density (vBMD) (by peripheral QCT) in 1 month and significant reductions in cortical vBMD in distal tibia after 2 months has been reported, along with substantial inter-individual variations in this response. . In this study, tibial bone loss persisted during 6 months of recovery, suggesting that the time needed to recover is longer than the mission duration (2). In a recent study in 2006 with crew members of the International Space Station (ISS) a comparison of pre- and post flight image measurement by using clinical QCT demonstrated that the crew members lost roughly 11% of the total bone mineral content from their proximal femora. The cancellous bone mineral content and density declined by 14.4-16.5% over the 4.5 to 6 months of the mission. Consistent with previous reports, measures of vBMD and estimated bone strength of proximal femoral indicate only partial recovery, although bone mineral content was recovered in the year after spaceflight. An increase of bone volume and cross sectional area at the femoral neck during the recovery period has been also reported (34). Small declines in cancellous vBMD of cosmonaut's distal tibia have been reported starting at 1month of spaceflight and even greater decreases in cancellous and cortical vBMD are observed by 6 months of flight (38).

More data exist from microgravity studies using DEXA. Bone mineral content, measured pre- and post-flight using DEXA, declines by 1 to 1.6% per month at the spine, femoral neck, trochanter, pelvis, and calcaneus (39). Femoral neck BMD declines roughly 22.5% on average in astronauts after 14.4 months of flight (40). The most

abundant spaceflight animal data is derived from rodents, particularly rats. A number of studies have demonstrated lower cancellous bone volume in the proximal tibia (41-43), vertebrae (43), and pelvic bone (44) of space flown rats as compared to ground controls. But the most rats utilized in spaceflight and ground-based simulation studies were relatively young, rapidly growing rats, making interpretation relevant to the mature skeleton difficult, because a major effect of microgravity on growing bone is growth retardation. The skeleton of all the human astronauts who are exposed to microgravity is mature, hence the major effect of disuse in this case is altered remodeling.

The precise mechanism of microgravity-induced bone loss isn't yet fully understood. To investigate the alteration in skeletal cell signaling, hormonal effect, bone blood flow, fluid shifts, or any other mechanism which induce bone loss in microgravity, invasive methods must be utilized. Researchers cannot easily perform such methods on humans, so rat models have been developed to provide more mechanistic information about the skeleton's adaptations to unloading. With several exceptions, the rat skeleton has numerous structural and physiological similarities to human skeleton, making it an effective model of human bone responses. A ground-based model to study the effects of microgravity on the rats was developed by Emily Morey-Holton in the early 1980's. This model, called hindlimb unloading (HU) has been widely used and accepted among microgravity researchers. In HU model, the rat's hindlimb is elevated around 30° from the ground by a tail harness, creating a head down tilt position. The rat can move its rear legs without being able to push against the ground. Rat's forearms experience normal

weightbearing on the ground, thus can act as an internal control. Rats subjected to HU demonstrate similar physiological adaptations similar to those observed in rats and humans flown in space, including a cephalic fluid shift, a decline in weight bearing bone and muscle mass, and negative calcium and nitrogen balance (45).

Decreases in osteoblast number, bone formation rate and cancellous bone volume also occur in HU rats, and all of these changes are specific to the unweighted hindlimbs. Adult rats suspended for 14 days demonstrate decreased bone formation. Alkaline phosphatase activity in the femoral and tibial diaphysis of HU rats is reduced as much as two times below the level in control rats (46). Six-mo-old rats suspended for 14 days demonstrate a significantly decreased tibial ash weight, calcium content, BMC, and BMD versus controls (47). HU results in gender-independent decreases in cancellous bone volume (BV/TV) compared with baseline values, accompanied by architectural changes, such as decreased trabecular number and corresponding increases in trabecular separation. Histomorphometric measurement reveal decreases in BV/TV in both genders that are associated with decreased bone formation and increased bone resorption (48).Twenty-eight days of HU results in ~ 20% decline in cancellous BMD at the proximal tibia and femoral neck compared to controls. Bone formation rate at tibial midshaft is lower (by 90%) vs. baseline controls after 21 days of HU (49). Consistent with human studies, bone loss in HU rats is site-specific. Bone mineral is lost primarily from the cancellous bone compartments in unweighted bones of tail-suspended rats.

With some exception (50), bone loss during HU likely occurs predominantly in cancellous bone because that is the site where remodeling is most active.

Exposing humans or animals to slightly stressful conditions can increase heart rate and blood pressure through activation of the autonomic nervous system and elicit the release of catecholamines. Several studies have demonstrated that sympathetic nervous activity during microgravity is increased compared with ground-based values. Plasma norepinephrine values are elevated in space flight above values observed in the seated position in ground-based experiments (51). Platelet norepinephrine and epinephrine increased in four of the five cosmonauts during two weeks of microgravity (Platelet norepinephrine and epinephrine are a more reliable measurement for detection of changes in epinephrine release than comparable measurements of epinephrine in venous blood, because the platelet epinephrine concentration does not depend on the extraction ratio in tissues) (52). Platelet norepinephrine concentrations were 153 ± 28% (mean ± SE) of pre-flight values. Baseline sympathetic neural outflow is increased moderately in spaceflight, as is norepinephrine spillover rate (53). Plasma corticosterone is higher after short (5-7 days) and long (> 14 days) term flights, and catecholamine levels in plasma increase after 14 days or more in microgravity (54). In simulated microgravity experiments with tail suspended rats, plasma norepinephrine and epinephrine increase by 53 and 42% after 7 days, but only epinephrine returns to baseline after 14 days (24). This generalized activation of the sympathetic nervous system and increased circulating catecholamines may be involved in the bone loss during spaceflight. Potential

relationships between elevated level of catecholamines, especially norepinephrine, and bone during exposure to microgravity or to other conditions causing bone loss will be discussed in a later section of this chapter.

Food restriction and bone health

Individuals with a history of weight loss, weight cycling or low body weight have an increased risk of osteoporosis since bone mineral density may be compromised in these scenarios (55,56). Crash dieting, weight cycling and disordered eating are known to result in clinical concerns, including a negative impact on reproductive hormone profiles, resulting in menstrual cycle disturbances and decreased bone mass (4-7).

The prevalence of dieting and weight loss efforts at any given time amongst U.S. women exceeds 50% (3). The 1990 National Health Interview Survey reported that nearly 44 million persons age 25 years and older were attempting to lose weight, which was a significant increase from the 1985 version of the survey (57). Weight loss attempts are not restricted to adults (58). It was reported that 46% of college undergraduates were trying to lose weight in 1995 National College Health Risk Behavior Survey (59); 44% of female and 15% of male high school students admitted that they were trying to lose weight in 1990 Youth Risk Behavior Survey (60). The gap between the proportion of female and male students attempting to lose weight has increased (58) and women are also outnumber men in the quantity of their weight-loss attempts (61).

Body mass is positively correlated with BMD (62,63). On average a 1% decrease in bone mass (9) and increased bone resorption (10, 11) is associated with each 10% decrement in body weight. A negative correlation between the number of times that dieters cycle their weight and their BMC values has also been reported (64). Hip fracture risk is significantly increased with weight variability in middle-aged women over a twelve-year period, which was further exacerbated by the association between weight loss and hip fractures (8). Energy restriction diet induced weight loss results in rapid loss in women's total body BMD (65); even general reductions in body mass can decrease bone density as demonstrated a 17% reduction in body mass over 10 weeks yielding an average 2.5% total body BMD decrement.

Global food restriction in the attempt to lose weight results in reduced dietary intake of many individual nutrients such as energy, protein, calcium and vitamin D that are important to maintain bone health. There exists a rich literature examining the effect of individual nutrient restriction such as calcium, vitamin D, protein, or energy on bone, with most of these studies indicating negative effects on bone turnover and bone mass.

Calcium

Calcium is the most studied nutrient important for optimal bone health. Almost 99% of the total body calcium is stored in the bones in the form of hydroxyapatite crystal; therefore, bone serves as the primary source of calcium when dietary intake is

insufficient and blood calcium level is low. Almost 80-90% of hydroxyapatite in bone is composed of calcium and phosphorus (66).

Dietary calcium restriction results in reduced BMD and CSMI in femoral and vertebral bone in female rats (67). Dietary calcium deficiency also impairs normal bone remodeling as it uncouples bone formation from bone resorption. In a study testing 90% reductions in calcium intake over one month, turnover markers osteocalcin (+24%) and pyridinoline (+48%) were elevated in mature female rats. In the same study, reductions in cancellous bone mass (-26%) and trabecular connectivity (-54%) were observed (68).

In general, the effect of dietary calcium on bone is more dramatic if superimposed on estrogen deficiency, as observed after menopause. BMD is maintained or increased in postmenopausal women when additional dietary calcium or calcium supplement was given (69-74). If estrogen is treated combined with dietary calcium, the preventive effect was more pronounced than either treatment alone in late postmenopausal women (75), particularly if Ca intake is low (76). Bone loss is exacerbated in late postmenopausal women whose calcium intake is low (76).

Vitamin D

Vitamin D is one of the essential nutrients to maintaining bone health because its active form, 1,25-dihydroxyvitamin D [1,25-$(OH)_2$D, or calcitriol) influences calcium and phosphorus metabolism by affecting the target organs: intestine, bone and kidney. The

homeostasis of extracellular ionized calcium is tightly regulated by a number of hormones, among which parathyroid hormone and vitmin D play a major role. Vitamin D derived from dietary sources or synthesized from skin by ultraviolet radiation of 7-dehydrocholestrerol is hydroxylated to 25(OH)D in the liver and further hydroxylated to $1,25(OH)_2D$ in the kidney. The $1,25\text{-}(OH)_2D$ facilitates active calcium absorption in the intestine by stimulating the synthesis of calcium binding protein (calbindin), the reabsorption of calcium in kidney and the release of calcium from bone. Parathyroid hormone is the major regulator of $1,25\text{-}(OH)_2D$ and also maintains extracellular calcium homeostasis through its effect on kidney and bone. It is well known that vitamin D deficiency may cause rickets in children and osteomalacia in adults. It's been reported of vitamin D inadequacy is prevalent amongst women with osteoporosis (77), and a substantial proportion of patients with hip fractures also have osteomalacia, caused by vitamin D deficiency (78).

Protein

Generally, increases in dietary protein result in increased urinary calcium excretion even though the long term implications of high protein diet for skeletal health are uncertain (79). Higher protein intake could bring about net calcium loss, resulting in negative calcium balance. Calcium mobilization via bone resorption may be stimulated to counterbalance the net calcium loss, and the prevalence of osteopenia or osteoporosis might be increased.

On the other hand, adequate intake of dietary protein stimulates the release of IGF-1, which promotes bone formation and mineralization by accelerating osteoblastic cell differentiation (80). Research data addressing the effects of high or low protein diets on bone are controversial. An uncoupling of bone resorption and formation was observed in 8-month-old male rats with isocaloric low-protein diet (2.5% casein vs 15% casein in control diet)(81). It has also been demonstrated dietary protein restriction lowers plasma insulin-like growth factor-1, impairs cortical bone formation, and induces osteoblastic resistance to insulin-like growth factor-1 in adult female rats (82). In epidemiological studies, most data demonstrate a positive association between protein intake and BMD (83-85), but not all. Many (86-88), but not all (89), report higher fracture rates in groups consuming a high protein diet. Clinical intervention studies generally support the negative effect of high protein on bone hypothesis. Many studies report increases in bone resorption when animals or humans are fed a high protein diet (90-92), but some do not (93-96). Bourrin et al. (81) reported that an isocaloric low protein (2.5% casein) diet in male rats significantly decreased proximal tibial BMD and mechanical strength. Moderate (40%) protein restriction, when combined with aerobic exercise, appears to strengthen the femoral neck more than those in the exercising control (97). Kerstetter et al (98) reported that the status of PTH is affected by various protein intake, and secondary hyperparathyroidism induced by low protein diet is attributed to a reduction in intestinal calcium absorption.

Energy

As previously described, restricting calories is known to have negative consequences on bone integrity. Bone loss is associated with energy restriction in both animals (99-101) and humans (102,103). When mature (aged 20 weeks) female rats were fed an 40% energy-restricted diet for nine weeks, significant reductions were found in their femoral BMD and cortical area, tibial BMD, and uterine weights (99). In human studies, the effects of graded energy availability decreased from 40 kcal/kg/lean body mass to 10 kcal/kg/lean body mass in young exercising women were assessed with bone resorption/formation markers. The bone resorption marker NTX increased as dietary intake was reduced from 20- to 10/kcal/LBM/day. The bone formation marker serum PICP decreased as the degree of energy restriction increased from 40- to 30/kcal/LBM/day and continued as restriction was increased. Estradiol levels increased at 30/kcal/LBM/day initially, but its level steadily declined as energy restriction increased (102).

In the study to find out relationship between calcium and energy intake on bone (100), bone mineral density is compromised by calcium restriction in both older and younger female rats, whereas only older rats are negatively influenced by energy restriction, suggesting an adverse effect not only of low calcium but also low energy on skeletal health in mature animals. In this study, adverse effect of calcium restriction on bone mineral density in older rats was slightly greater than that of energy restriction, but not significant. A few data are available on a secondary deficiency of another bone-relevant

nutrient that is a consequence of restricted caloric intake. Restricting energy by 40% for 10 weeks reduces fractional calcium absorption in mature obese and lean rats, from 30% to 24%, compared with 25% to 29% in 100% fed control rats (99). In a study trying to quantify the magnitude of each individual nutrients restriction's contributions to bone loss in a side-by-side comparison in adult female exercising rats, 40 % energy restriction resulted in the greatest negative effects on bone health versus 40% calcium or 40% protein restriction (28).

There are several potential mechanisms by which restricted energy intake may contribute to lower bone mass, including the decreased mechanical loading on the skeleton with reduced body mass, the reduction in number and size of adipocytes that secrete bone-active hormones, and alterations in secretion of bone- related hormones from the gut or pancreas. With energy restriction, there is a decrease in circulating adipocyte-derived leptin that normally promotes bone mass increase. But the effect of leptin on bone is still controversial and it likely varies depending on the bone site. [The review for leptin and its effect on bone will be described in more detail in the last section of this chapter.] Consequent to restricted energy intake (and subsequent weight reduction) serum levels of circulating estrogen and other sex hormones that may play a role in osteoblast/osteoclast activity decrease. Estrogen receptors are present on both osteoblasts and osteoclasts. Estrogen promotes the differentiation of bone marrow stromal cells (BMSC) to osteoblasts rather than to adipocytes (104), increases osteoblast proliferation (105), and increases production by osteoblasts of proteins such as IGF-1,

transforming growth factor-beta (TGF-β), and bone morphogenetic protein-6 (BMP-6) (106-108). Estrogen normally suppresses bone resorption by increasing osteoclast apoptosis and by contributing to increased osteoprotegerin (OPG) relative to another regulator of osteoclast activity, receptor activator of nuclear factor-kappaB ligand (RANKL), thereby increasing the OPG/RANKL ratio (109). Exposure of BMSC's to estrogen also reduces their production of cytokines such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor-α (TNF-α), which are potent stimulators of osteoclast recruitment and activity (110).

IGF-I stimulates proliferation of osteoblast precursor cells and independently promotes matrix production by mature osteoblasts; during energy restriction, serum IGF-I is suppressed (31). In addition, energy restriction induces a rise in the calcium-PTH axis; serum PTH rise, in women consuming low/normal calcium (0.6-1.0g/d), resulting in reduced fractional calcium absorption (99).

Noradrenaline and/or epinephrine release is increased with fasting or weight reduction (111,112) that also results in loss of bone mass. Mammalian bones are widely innervated by sympathetic and sensory nerves, which are particularly abundant in regions of high osteogenic activity (113,114). Chemical denervation of sympathetic and/or sensory nerves has been demonstrated to impair bone resorption in rats by decreasing the number of osteoclasts (115,116). Relatively recent studies confirmed that osteoblasts express β-adrenergic receptors (117). Sympathetic activation or increased sympathetic tonic output

reduces osteoblast proliferation and stimulates bone resorption (117). It is well known that beta-adrenergic agonists can stimulate bone resorption in the intact mouse calvaria (118). This may be mediated by the activation of osteoclastic cells and/or the production of osteotrophic factors by osteoblastic cells. Activation of beta adrenoreceptors by epinephrine increases the expression of osteotrophic factors such as receptor activator of NF- kappa B ligand (RANKL), interleukin (IL)-6, IL-11 and prostaglandin E2 (PGE2), as well as the formation of osteoclast-like cells from mouse bone marrow cells (119). Beta-adrenergic blockade, on the other hand, blunts the inhibition of alkaline phosphatase activity by isoproterenol (a beta agonist) in an osteoblast-like cell line, which suggests that beta blockers may enhance bone formation by preserving osteoblastic activity in the face of beta adrenergic receptor stimulation (120).

Supporting these in vitro studies, in vivo studies also have demonstrated that adrenergic stimulation modulates osteoblastic activity and osteoblast-mediated osteoclastogenesis. Administration of lipopolysaccharide (LPS) increases the norepinephrine (NE) turnover rate in various brain areas and peripheral tissues (121,122). Intracerebroventricular injection of LPS induces an increase in IL-6 serum levels and in IL-6 mRNA expression in the brain and peripheral organs (123,124). Treatment of mouse calvaria with NE increases IL-6 synthesis in an organ culture system. (125). Pretreatment with a beta-blocker inhibits both stress- and LPS-induced increases in the level of IL-6 mRNA, but pretreatment with an alpha-blocker did not inhibit them in mouse calvaria . Recently, it was reported that an increase in bone norepinephrine levels triggered by depression is

associated with bone loss, suggesting the sympathetic nervous system mediates even psychological stress-induced effect on skeleton (126).

Energy restriction in human clinical studies

Studies have demonstrated beneficial effects of chronic energy restriction on slowing the aging process and increasing the maximal lifespan in nonhuman primates (127,128). Little is known regarding the effects of long term energy restriction in humans. Feasibility studies for human ER, referred to as CALERIE (Comprehensive Assessment of Long-term Effect of Reducing Intake of Energy), were completed recently. At CALERIE phase 1, effects of 20% ER with those of a 20% increase in energy expenditure induced by exercise with caloric intake kept constant (EX) were compared. Reductions in body weight and abdominal fat, induced by ER or by EX, improve insulin action and glucose tolerance (129).

But the fact is, many dieters lose and regain weight many times in their lifetime. Weight cycling dieters, especially in female athletes, are at risk of compromising long-term bone health due to nutritional deficits with weight loss and related menstrual irregularities (130). One-time diet-induced weight loss is accompanied by a significant decrease in bone mineral density (BMD); for every kg fat loss, 16.5 g of bone mineral loss was observed in eight energy -restricting women volunteers before and 10 weeks after a very-low-calorie diet [405 kcal/day] (131). In another more liberal diet regime, which prescribed energy intake up to 4.2 MJ (1000kcal) and moderate weight losses,

significant losses in total body bone mineral and lumbar bone mineral density were documented.

Generally, low-calorie diets (1000-1200 kcal/d for women, 1200-1400 kcal/d for men) are considered as a safe strategy for weight loss (132,133) and have been selected for several diet education programs (134,135) and some commercial programs (132). This low-calorie diets represents 40% or more energy restriction compared to the 2006 DRI energy requirements for moderately active young men and women. Sometimes very-low-calorie diets (~800kcal/day) are recommended to people with a body mass index over 30 kg/m^2, who face major health risks (136). Very-low-calorie diets result in an average weekly weight loss of 1.5–2.5 kg, compared with 0.4–0.5 kg with low-calorie diets (137). Even though conclusions from human epidemiological studies are limited by the inability to measure the common degree of voluntary dieters and by the lack of data regarding plausible biological mechanisms underlying the effects of voluntary weight loss, bone loss in weight losers is an unwanted effect of very low calorie diets, and it can be avoided by using regimes with a higher energy content (138).

Bone and leptin

Leptin

Fourteen years ago, a study identifying the "obese" (Ob) was reported (139), launching a new era in obesity research. Leptin (Ob), a 16 -kDa cytokine-like hormone principally produced by adipocytes, has emerged as a candidate signaling molecule to link energy metabolism and regulation of bone mass. White adipose tissue is the primary site of

leptin synthesis and secretory regulation, but recent studies demonstrate that leptin is produced in placenta, skeletal muscle, fetal bone/cartilage and primary cultures of human osteoblasts (140-143). Leptin interacts with the central nervous system by binding to its own receptor in the hypothalamus, with its main functions being the regulation of appetite and energy metabolism. Leptin also exerts effects on cells in peripheral tissues via high-affinity leptin receptors (140-145). In terms of its structure, leptin is a member of the growth hormone four-helical cytokine subfamily.

Even though serum leptin level in humans and rodents are primarily regulated by the relative size of body fat stores (146), there are several other factors that affect circulating leptin levels independently of alterations in adipocity. Studies in human and rodent models demonstrate that stimulation of sympathetic nervous system output and activation of β-adrenergic receptors decrease serum leptin levels over a short time frame (147-149). β-adrenergic agonist administration decreases serum leptin and subcutaneous adipose tissue interstitial leptin concentration in humans within 3 hrs (150). Activation of the β-adrenergic receptors, combined with a decrease in serum insulin , is thought to be responsible for the decrease in leptin levels with fasting. In addition to short-term fasting, long-term food restriction (85%, 70% and 50% of ad libitum energy intake for one month) in rats produces a substantial fall in serum leptin and leptin mRNA levels in epididymal white adipose tissue (151).

Leptin receptor

Leptin receptor (db) is classified as a member of the class I cytokine receptor superfamily due to strong homology in signal transducing subunits and a shared signaling pathway with IL-6, G-CSG, and LIF. Five isoforms (OBRa, OBRb, OBRc, OBRd and OBRe) are known to exist. The OBRa, OBRb, OBRc and OBRd have a membrane-spanning domain that anchors the cell membrane, but OBRe does not (152). Leptin circulates in plasma in a free form and a protein-bound form, and OBRe functions as a major binding component for plasma leptin (153). Overexpression of OBRe in ob/ob mice produces phenotypic changes suggesting that OBRe serves to delay leptin clearance, increasing the available leptin in circulation (154). OBRb, the longest form of receptor, is thought to be the only isoform that is capable of full signal transduction because it contains cytoplasmic domain with specific sequence that binds to intracellular signaling molecules. The db/db (leptin receptor-deficient) mouse, whose phenotype is obese with high bone mass at select bone sites, has a db locus mutation that eliminates OBRb (152). OBRb is primarily expressed in the hypothalamus. Expression of OBRb in peripheral tissue such as bone, skeletal muscle, adrenals, pancreatic β-cells adipocytes, immune cells, kidneys, and liver also has been reported (12,155-158) . The cellular effects of leptin are dependent on the receptor subtypes that cell expresses and the fate of endocytosed leptin inside the cells. Endocytosed leptin can remain intact for at least 1 h. This stability is further enhanced by inhibition of lysosomal activity. Thus, the intracellular pool of intact leptin may allow for prolonged biological functions for this adipokine (159).

Leptin transport/Leptin resistance

Blood brain barrier

Nutrients and drugs diffuse freely between the blood stream and tissues in the body, but this is not so between the blood and the brain. Between blood and brain, precise control over the substances that leave or enter the brain is required, because the brain's neuronal tissue must be protected from potential toxic compounds in the bloodstream and from the chemical or hormonal fluctuations that occur after a meal or exercise that might disrupt optimal function. Simultaneously, glucose, oxygen, and other nutrients should be in constant supply to meet the high metabolic needs of central nervous system (CNS) tissue. To meet tboth requirements; the need for constant supply of nutrients and protection from potentially harmful compounds or sudden chemical fluctuation, blood vessels in the brain are coated with a tight-knit layer of endothelial cells, which are largely responsible for the blood-brain barrier (BBB) (160,161). These cells coat the 400 miles of capillaries and blood vessels in the brain, creating a barricade. An elaborate network of complex tight junctions between the endothelial cells forms the structural basis of the BBB and restricts the paracellular diffusion of hydrophilic molecules (161). Also, transcellular passage of molecules across the barrier is also blocked due to the very low pinocytotic activity and the scarcity of fenestrae in BBB endothelial cells (162). The structures located in the midline of the ventricular system (160) and that lack an endothelial BBB are collectively referred to as circumventricular organs (CVO's). CVO's are responsible for monitoring hormonal stimuli and other substances within the bloodstream or secrete neuroendocrine factors into the peripheral circulation (163).

Molecules have to enter BBB endothelial cells via membrane-embedded protein tranporters or by slipping directly through its outer membrane. Hypothalamus is one of the CVO's and it's been suggested the existence of leptin specific transporter for 16 kDa size of leptin to pass the BBB (164). Expression of leptin transporter may be regualated with serum leptin level (164). Once passing the outer membrane of the BBB, foreign compounds must avert various protein pumps trying to evict any foreign molecules and also lots of metabolic enzymes that may able to digest the compound. Having avoided these obstacles, foreign molecules must then pass through the inner membrane of a BBB cell to finally reach the brain. In terms of drug delivery to heal brain-related disease, BBB is considered more of an obstacle than safeguard. Molecules that can slip across the BBB membrane cells are typically small (under 500 daltons), are lipid-soluble, and have a low polar surface area (160).

Leptin resistance

Leptin needs to cross the blood brain barrier to reach to its receptor in the hypothalamus, especially arcuate nucleus and ventromedial nucleus to regulate food intake. The ratio of leptin in cerebrospinal fluid to plasma in obese human is low comparing to nonobese subjects, suggesting that obesity may arise from an impaired capacity for leptin transport into the brain (165). Rats made obese rats due to overfeeding successfully lose body weight with leptin infused directly into the brain's ventricle, but do not respond to peripheral (outside the CNS) leptin administration, serving to support this transport hypothesis (166). In human clinical studies, peripheral leptin injections have a limited

ability to reduce food intake and body weight (167). Leptin resistance, which is caused by impaired leptin transport to brain even with high serum leptin, appears to the major cause of human obesity.

A suppressor of cytokine signaling, (SOCS-3), has been identified as a negative feedback regulator of leptin signaling which diminishes leptin sensitivity, throwing an additional hypothesis in leptin resistance. SOCS-3 knockout mice exhibit enhanced tyrosine residue phosphorylation in JAK/STAT3 leptin signaling pathway, associated with a decrease in food intake and weight loss (168,169). Whether a specific leptin transporter molecule exists, and the details of its specific function, needs to be investigated further.

Leptin signal transduction

After leptin binds to and activates its receptor, signal transduction takes place to the hypothalamic genes activating signals regulating food intake, lipid metabolism and bone metabolism. Activated leptin receptor activates JAK2 kinase, resulting in tyrosine phosphorylation of Tyr1138 residue. Signal transducer and activator of transcription 3 (STAT3) binds to phosphorylated Tyr1138 residue, resulting in its own phosphorylation and dimerization. The STAT3 dimer eventually translocates to the nucleus, where transcriptional activity of several target genes are modulated (170). Genes whose expression is modulated by Janus kinase (JAK)/STAT3 pathway are thought to be pro-opiomelanocortin (POMC) and Agouti related peptide (AgRP) (170,171), which are

important for food intake and body weight regulation and Thyrotropin-releasing hormone (TRH), which plays a role in thermogenesis (172). AMP-activated protein kinase (AMPK) is an enzyme that works as a fuel gauge which becomes activated in situations of energy consumption. Leptin in skeletal muscle stimulates the phosphorylation of AMPK, reducing acetyl CoA carboxylase (ACC) activity. Inhibition of ACC lowers intracellular malonyl CoA, eventually increasing fatty acid oxidation (173). The leptin-induced signal transduction pathway in bone cells is not yet fully understood. Leptin may increase bone mass by stimulating osteoblast proliferation through activation of PI3K and MAPK signaling pathway in human osteosarcoma cells (174).

Leptin as an endocrine factor (central vs peripheral actions on bone)

Leptin's principal function is the regulation of energy stores and body composition through negative feedback at the hypothalamic arcuate nuclei. Leptin is now known to have numerous biological effects in the immune system (12), reproduction (13), development (14), hemopoiesis (15), angiogenesis (16) and, most recently, in bone metabolism. The nature of leptin's effects on bone has not been fully determined. Early studies claimed to demonstrate an anti-osteogenic effect of leptin signaled via the sympathetic nervous system when leptin was administered intracerebroventricularly (17,18), but several studies later demonstrated an osteoprotective effect of leptin during hindlimb unloading or caloric restriction when administered into the systemic circulation (19,20). Peripheral administration of leptin has a stimulatory effect on bone growth and bone formation via regulation of osteoblastic function (175) or, possibly, via preferential

differentiation of bone marrow stromal cells into osteoblasts rather than to adipocytes (176). Inhibition of osteoclast generation (177) and positive effects on angiogenesis with peripheral leptin administration also has been demonstrated (178-180). By contrast, intracerebroventricular leptin administration, in other words, leptin signaling that is mediated by the central nervous system (CNS), results in a negative effect on bone mass. It has been proposed that centrally infused leptin suppresses bone formation via a hypothalamic relay. Neuropeptides upregulated by leptin in the hypothalamus activate SNS pathways, resulting in stimulation of β-adrenergic receptors on osteoblasts and inhibition of bone formation. However, leptin deficiency produces contrasting phenotypes in bones of the limb and spine (181). Also, injections of leptin into rat ventromedial hypothalamus increase apoptosis of bone marrow adipocytes; loss of these adipocyte populations may be a factor contributing to age-related bone loss (182). Together, these studies elucidate that the effect of leptin on bone is dependent on bone site (perhaps varying with the degree of sympathetic innervation), leptin transport into the hypothalamus, and the heterogeneity in bone-marrow composition. Studies utilizing pharmacological blockade of sympathetic nervous signaling can reveal important clues about the relative importance of SNS signaling to a particular cell population.

Beta-adrenergic signaling blockade

β-blocker pharmacological agents

β blockers are a class of drugs used for various indications. Originally developed as a medication to treat hypertension, β blockers have also become essential therapies for patients with cardiovascular disease, acute myocardial infarction and those with tachyarrythmias (183-190). β blockers inhibit the action of endogenous catecholamines (epinephrine and norepinephrine in particular), on β-adrenergic receptors, key elements of the sympathetic nervous system. Three types of β receptors are known to exist: β_1, β_2 and β_3. β_1-adrenergic receptors are located mainly in the heart and in the kidneys. β_2-adrenergic receptors are located mainly in the lungs, gastrointestinal tract, liver, uterus, bone, vascular smooth muscle, and skeletal muscle, whereas β_3 receptors are located on adipocytes (191).

β-adrenergic receptor activation

An activated β-adrenergic receptor couples to heterodimeric guanine-nucleotide-biding protein (Gs protein), which stimulates adenyl cyclase to increase intracellular cAMP level. cAMP activates cAMP-dependent kinase (PKA), which phosphorylates cellular and nuclear targets. Stimulation of β receptors by catecholamines induces various results depending on the cell type affected. β_1 receptor stimulation of myocardial cells in the heart causes increases cardiac conduction velocity and automaticity. Activated β_1 receptors in the kidney induce renin release. Reduction in renin release in kidney due to

β blockade results in reduced serum aldosterone via the renin-angiotensin-aldosterone system, with a resultant decrease in blood pressure due to decreased sodium and water retention (191). In smooth muscle, stimulation of β_2 receptors induces smooth muscle relaxation, resulting in vasodilation and bronchodilation. Glycogenolysis in the liver and skeletal muscle is increased also with β_2 receptor stimulation, while stimulation of β_3 receptors induces lipolysis in the fat cells (191).

Activation of β_2-adrenergic activity in osteoblastic and osteoclastic cells are associated with increased bone resorption and decreased bone formation. The mechanistic signaling pathway in osteoblast has been demonstrated. Following binding to β_2-adrenergic receptor, norepinephrine increases RANKL expression and bone resorption via a signaling pathway involving Gs protein, adenylyl cyclase, the phosphorylation of activating transcription factor 4 (ATF4) by activated PKA, and the binding of ATF4 to a cAMP response element (CRE)-like site in the RANKL promoter (192).
Adrenergic signaling requires b2AR and ATF4, which are both mainly expressed in immature osteoblasts, while PTH signaling requires PTHR1 and CREB, which are expressed in mature, fully differentiated osteoblasts (193). The distinct signaling pathways, and the fact that b2AR and PTHR are expressed at different stages of osteoblast differentiation, suggest that PTH and adrenergic signaling act on different stages of osteoblast to regulate the RANKL expression and bone resorption. Increased expression of interlenkin (IL)-6 and IL11, two cytokines stimulating osteoclast differentiation, in epinephrine-treated osteoblasts has been reported. This effect of

epinephrine appeared to involve PKA and P38 MAPK (194,195). The signaling pathway leading to reduced bone formation has not yet been fully characterized. Such findings indicate that β-blockers may be effective against osteoporosis, in which case there is increased sympathetic activity stimulating the bone loss.

Reduction in dilation of blood vessels, that is, vasoconstriction induced by β blockers seems to somewhat contradictory to its antihypertensive effect. It is likely that antihypertenstive effect of β blockers is related to reduction in cardiac output, reduction in renin release in kidney, and a central nervous system effect to reduce sympathetic activity.

Propranolol

Propranolol is a non-selective β blocker mainly used for antihypertensive purpose. It was the first successful β blocker developed in the late 1950s. It blocks the action of epinephrine on all β_1-, β_2- and β_3 adrenergic receptors. Absorption of propranolol is rapid and complete; the peak level in plasma is achieved in 1-3 hours after ingestion. Propranolol is a highly lipophilic drug, which is able to cross the blood brain barrier into the CNS, achieving high concentrations in the brain. The duration of action of a single oral dose may be up to 12 hours. Effective plasma concentrations are between 10–100 ng/mL. Toxic effects are associated with plasma concentrations above 2000 ng/ml. Propranolol is clinically used for the management of hypertension ,tachyarrhythmias, myocardial infarction, control of tachycardia/tremor associated with anxiety and

hyperthyroidism, essential tremor, and migraine prophylaxis. Propranolol is currently being investigated as a potential treatment for post-traumatic stress disorder (191,196). In fact, there are some data indicating a statistical link between the use of propranolol and a reduced risk of fractures (179), although this potential beneficial effect on bone health remains controversial.

CHAPTER III

FOOD RESTRICTION AND SIMULATED MICROGRAVITY: EFFECTS ON BONE AND SERUM LEPTIN

Introduction

The effects of microgravity or disuse on weight bearing bones have well been documented. About 1% of bone mineral density (BMD) is lost per month while in microgravity, although the magnitude of loss is highly variable among subjects and among anatomical sites (1, 2). Whether this loss eventually plateaus is unknown, because very few humans have been exposed to microgravity or strict bedrest for more than 6 months. The rodent model of hindlimb unloading by tail suspension effectively mimics the microgravity environment and produces significant bone loss, allowing for ground-based, invasive studies to be performed (49,197).

Crew members frequently undereat during space flight missions (198-200). Food intake has been observed to be as low as 50% of a flight member's estimated required amount, and even as low as 25% in one instance (the latter assuming the adult male needs 2500 calories) (200,201). Reduced food intake results in decreased availability of nutrients important for maintaining bone health, but may also independently affect bone status. When these effects are combined with the reduced mechanical loading of the microgravity environment, the deleterious effect on the skeleton could be augmented.

This change of bone status due to space flight and/or negative energy balance involves numerous endocrine factors. Hormones such as estrogen, growth hormone, insulin-like growth factor-1, insulin, T_4, thyroid-stimulating hormone, calcitonin, active D_3, and parathyroid hormone have been shown to play a role in the deleterious skeletal adaptation to actual or simulated microgravity and/or to negative energy balance (202-210). Leptin, a 16 kDa cytokine-like hormone principally produced by white adipocytes, may also be involved in the bone response to microgravity and/or restricted food intake. Its principal function is the regulation of energy stores and body composition through negative feedback at the hypothalamic nuclei. Leptin is now known to have numerous biological effects on the immune system (12), reproduction (13), development (14), hemopoiesis (15), angiogenesis (16) and, most recently, on bone metabolism. However, there is controversy about the nature of leptin's effects on bone. Early studies demonstrated antiosteogenic effects of leptin via the sympathetic nervous system when administered centrally (17,18), but several more recent studies have demonstrated a bone-protective effect of leptin during hindlimb unloading or caloric restriction when administered peripherally (19,20).

No published data, to our knowledge, test a side-by-side comparison of the effect of microgravity and/or food restriction on serum leptin level and on bone outcomes. Our purpose, then, was to investigate the individual and combined effects of food restriction and simulated microgravity in adult male rats and to investigate the contribution of altered serum leptin to changes in bone strength, density, and turnover

status. Our primary hypotheses were that food restriction and hindlimb unloading independently impair skeletal integrity via decreased bone formation and/or increased bone resorption and, if rats are subjected to both treatments, even greater decrements and changes in the above variables would result.

Materials and methods

Animals and experimental design

Forty-eight 6-month-old adult male Sprague Dawley rats (Harlan; Indianapolis, IN) were used in this experiment lasting six weeks. The chow utilized by our animal facility (8604 Harlan Teklad) provides excess densities of vitamins and minerals; providing 70% of usual intake of this chow would not result in deficiencies in any key vitamins/ minerals. Therefore, we chose to use for this experiment, purified diet AIN93-M, a casein- based purified diet that provides 100% of NRC- determined requirement levels of vitamins and minerals for rats. Table 1.1 illustrates details on nutrient content in the 8604 Harlan Teklad chow and the AIN93-M diet.

During the first two weeks, all rats were fed *ad libitium* and food intake was measured daily to establish each rat's usual food requirement (average grams chow eaten per day). Then rats were randomly assigned to four groups. One group (CC100) was allowed regular cage activity with each rat receiving 100% of its usual food intake. The second

Table 1.1 Comparison of feed relative to rat requirements

Nntrient*	Rat requirement[1]	AIN-93M[2]	8604 Harlan Teklad[3]
Protein (g)	50	125	244
Calcium (g)	5	5	13.6
Magnesium (g)	0.5	0.5	2.8
Phosphorus (g)	3	3	10.1
Vitamin D (IU)	1000	1000	2400
Vitamin K (mg)	0.9	0.86	4.11
Energy (Kcal/g)		3.50	3.40

* Amounts of nutrients are given as unit of nutrient/kg of feed
1. (211); 2.(212);
3. 8604 Harlan Teklad Rodent diet (W) fed by vendor to rats first 5 months of life.

group (CC70) was also allowed regular cage activity, but each rat was provided 70% of its usual food intake. The remaining rats were subjected to hindlimb unloading by tail suspension, using a tail harness as previously described (213), and received 100% (HU100) or 70% (HU70) of usual food intake. The treatment period lasted for 28 days. During the entire experiment, the rats were housed in a light – controlled room (12:12 light: dark cycle) maintained at 70-72º F in a AAALAC- accredited animal care facility. All procedures in this study were approved by Texas A&M University Lab Animal Care Committee.

On day 1 of treatment and on sacrifice day, peripheral computed tomography (pQCT) scans were performed and urine and serum samples were collected while the rats were anesthetized. For the hindlimb unloading experiment, bone mineral density and cross-sectional geometry were measured both *in vivo* (tibia) on experiment days 0 and 28 and after sacrifice *ex vivo* (humerus and femur). Blood and urine collections were performed on both day 1 and 28 of the treatment at the same time of the day and before 10 AM to minimize contribution of diurnal variation to leptin and bone turnover markers. On days 9 and 2 before sacrifice, animals were given subcutaneous injections of calcein (25 mg/kg) to label mineralizing bone for histo-morphometric analysis. HU animals were anesthetized before removal from tail suspension to prevent any weight bearing by the hindlimbs. Right tibia and humeri were removed, cleaned of soft tissue and stored at −80°C in PBS-soaked gauze for ex-vivo pQCT scan and/or mechanical testing, whereas left femora were stored in 70% ethanol at 4°C for histology.

Peripheral computed tomography (pQCT)

The XCT Research M (Stratec; Norland Corp., Fort Atkinson, WI) scanner has a minimum voxel size of 0.07 mm, a scanning beam thickness of 0.50 mm, and is calibrated daily using a standard hydroxyapatite phantom. *In vivo* scans were taken of the proximal metaphysis of the right tibia on days 0 and 28 with the animal anesthetized. Transverse images were scanned at 5.0, 5.5, and 6.0 mm from the proximal tibia plateau. *Ex vivo* scans were taken at the proximal humerus metaphysis (5.0, 5.5, 6.0 mm from

proximal end) and at the distal femur (6.0, 6.5, 7.0 mm from the distal condylar edge). Bones for *ex vivo* scans were thawed and placed in a vial filled with 1× phosphate-buffered saline for scanning to ensure standard hydration. A standardized analysis for either metaphyseal bone (contour mode 3, peel mode 2, outer threshold of 0.214 g/cm^3, inner threshold of 0.605 g/cm^3) or diaphyseal bone (separation 1, threshold of 0.605 g/cm^3) was applied to each section. The same contour and peel modes and thresholds were used by our laboratory to successfully differentiate cortical and cancellous bone in skeletally mature unloaded animals (49) and are explained in detail elsewhere (214). Values of total, cortical shell, and cancellous volumetric bone mineral density (vBMD), cross-sectional area, cortical area, and marrow area (A) were averaged across 3 slices at each bone tissue to yield a mean value for each site. In addition, middiaphyseal cross-sectional moment of inertia (CSMI) was obtained with respect to the neutral bending axis during three-point bending for later calculation of material properties. Machine precision (based on manufacturer's data) is ± 3 mg/cm^3 for cancellous BMD and ± 9 mg/cm^3 for cortical BMD. Reproducibility in our laboratory for both *in vivo* and *ex vivo* measures was determined from five repeat scans with repositioning of the animal or bone between scans. Coefficients of variation for these measurements were 1.24, 2.13, and 1.95% for *in vivo* proximal tibia total BMD, cancellous BMD, and total area, respectively. *Ex vivo* distal metaphysis coefficients of variation for the same variables were 0.37, 1.43, and 0.28%, respectively.

Biochemical analyses

A rat osteocalcin ELISA immunoassay kit was used (Biomedical Technologies, INC.; Stoughton, MA) to measure the concentration of osteocalcin in the animals' serum. Osteocalcin is reported as ng/ml serum. Precision C.V. within-run was ± 7% and C.V. between run was ± 10.5%. The concentration of urinary deoxypyridinoline (DPD) crosslinks, one of the pyridinium crosslinks, was assessed to estimate changes in bone resorption, using a competitive enzyme immunoassay (Pyrilinks-D; Quidel, Mountain View, CA). Results were normalized to urine creatinine, determined by a colorimetric assay (Quidel). DPD is reported as nmol DPD/mmol creatinine. Precision C.V. within-run was ± 3.5% and C.V. between run was ± 7.0%. A rat leptin ELISA immunoassay kit (Crystal Chem, Chicago, IL) was used to measure the concentration of leptin in serum and reported as ng/ml serum. Precision C.V. intra-assay was ± 3.1% and C.V. inter-assay run was ± 6.4%.

Cancellous histomorphometry

Undemineralized distal left femora were subjected to serial dehydration and embedded in methylmethacrylate (Aldrich M5, 590-9). Serial frontal sections were cut 8μm thick and left unstained for fluorochrome label measurements, and cut at 4μm sections for Von Kossa staining for measurement of cancellous bone volume normalized to tissue volume, % BV/TV and quantification of osteoid and osteoclast surfaces as a % of total cancellous surface.

At 20X, a defined region of interest was established ~0.8 mm from the growth plate and within the endocortical edges encompassing 8-9 mm^2. Total bone surface, single-labeled surface, and double-labeled surface were measured at 100x and interlabel distances, bone volume and osteoid/osteoclast surface were measured at 200x magnification. Mineral apposition rate (MAR, μm/day) was calculated by dividing the average interlabel width by the time between labels (7 days), and mineralizing surface (MS/BS) for cancellous bone surfaces was calculated by using the formula MS/BS = {[(single labeled surface/2) + double label surface]/surface perimeter} x 100. BFR was calculated as MAR × MS/BS. Histomorphometric analyses were performed with BioQuant True Color Windows image-analysis software (BQTCW98, Version 3.05.6, R&M Biometrics) interfaced with Optronics 3-chip color camera and an Olympus BX60 Microscope with epifluorescent light (Leeds Instruments, Inc. Irving, TX). All nomenclature for cancellous histomorphometry follows standard usage (215).

Mechanical testing of bone

An Instron 1125 machine in the TEES material testing laboratory was used to perform 3-point bending to failure on tibiae and humeri. Excised bones were allowed to thaw to room temperature prior to mechanical testing. Anterior-posterior (AP) and medial-lateral (ML) surface diameters at the mid –shaft were measured at the same midshaft location as scanned on by pQCT. The tibia was then set medial side down on two metal supports 18 mm apart. The humerus was loaded posterior side down, on supports 12mm apart. Bone was loaded at mid-shaft in 3-point bending with a 50-lb load cell using quasi-static

loading rate of 2.5 mm/min until the bone fractured. Force and displacement data were obtained with a sampling rate of 10Hz using a linear variable differential transformer (LVDT) interfaced with Gardener Systems software. Values obtained from this data were ultimate load, defined as the maximum load prior to breaking, and stiffness, which is the change in load/change in displacement during the pre-yield portion of the load-deformation curve. Elastic modulus and ultimate stress are material properties calculated from stiffness and ultimate load, respectively, by normalizing for cross-sectional moment of inertia at the midshaft derived from the automated analysis of the pQCT data. The equations used were same as used in a previous study (50).

Statistical analysis

To analyze pre- and post- treatment values of tibia pQCT and blood/urine variables, a 3-way ANOVA with repeated measures was used. In addition, a simple main effects analysis was performed on any 2-way or 3-way interactions and, when appropriate, Duncan post-hoc tests were used within the simple main effects analyses. For end point measures (e.g. mechanical testing variables, soleus weight, and histomorphometry data), 2-way ANOVA was performed, with appropriate post-hoc tests. Linear associations between change of serum leptin level to the bone formation rate were described with Pearson correlation coefficients. All values reported are means ± standard errors.

Results

Food intake/body weight

Actual food intake over the 28 days' experiment was low for all groups during week1, but during the remainder of the experiment rats ate close to 100% of their assigned food, achieving intended food intake (Table 1.2). By week 4, mean body weights for CC70, HU100, and HU70 groups were significantly lower than that observed in CC100 (Figure 1.1). The HU 100 and CC70 rats exhibited a significant drop of body weight during first 7 days, but maintained body weight thereafter till the end of the experiment. The CC100 group had the highest mean body weight at sacrifice (519 ±14 g), followed with progressively lower body weights by the HU100 group (448 ± 12 g), the CC70 group (439 ±12 g) , and then the HU70 group (388 ± 17 g), who weighed the least.

Average soleus weight in weight bearing CC rats was 199.5 ± 6g and in HU rats, 84.5 ± 4 g (on average, 60% lower than pooled CC value, $p<0.0001$), confirming that tail suspension effectively unloaded rats' hindlimbs.

pQCT data

Proximal tibial metaphysis

Hindlimb unloading and food restriction caused a reduction in total vBMD (cortical shell with cancellous core) (Figure 1.2). Progressively greater reductions of total vBMD over time were observed in CC70 rats (4.7%) and HU100 rats (8.1%), with this

reduction exacerbated in combined treatment (HU70) rats (9.3%) Cancellous vBMD at this site decreased an average of 20% in all groups over time (Table 1.3); there were no significant independent effects of loading condition or food restriction on this bone compartment.

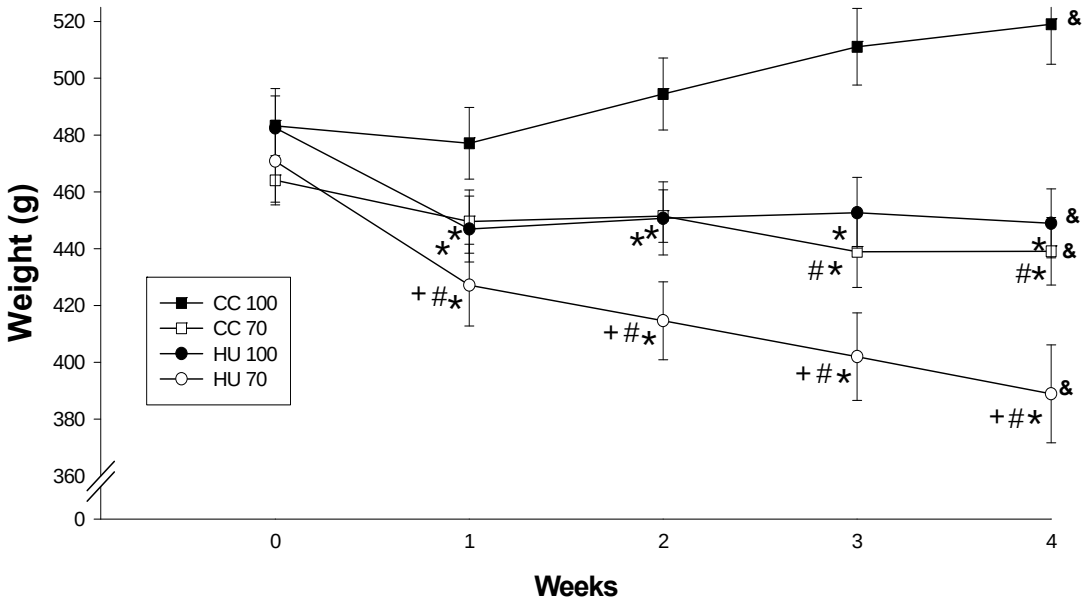


Figure 1.1 Body weights over 28 days of hindlimb unloading (HU) or cage activity (CC) in rats consuming 100% (100) or 70% (70) of usual intake. All groups are n=11 except for HU100 in which n=12. $p \leq 0.05$ vs *CC100, #HU100, +CC70; **&** $p<0.05$ **vs** initial body weight within group.

Midshaft tibia

Tibial midshaft vBMD and geometry variables were not affected by HU or FR (Table 1.3). However, almost all measured parameters increased over time (total area, cortical area, marrow area, cross-sectional moment of inertia) in all groups. Cortical density did

not change in any group. These data suggest a uniform continued growth of midshaft cortical bone in all animals, unaffected by loading condition or food intake.

Midshaf humerus

No significant differences in vBMD or bone geometry were noted at the humerus midshaft with food restriction or hindlimb unloading (data not shown).

Table 1.2 Food intake

Group	Baseline Intake Average(g)	70% of Baseline Intake	Actual Food Intake During Experiment			
			Week1*	Week2	Week3	Week4
CC100	23±0.6	--	17±1.0	23±0.6	23±0.6	22±0.7
CC70	21±0.6	15	13±1.5	14±0.7	15±0.4	15±0.4
HU100	23±0.8	--	17±0.9	21±0.6	22±0.7	22±0.6
HU70	23±0.9	16	13±0.8	16±0.6	16±0.7	16±0.7

CC: age matched cage controls, HU: hindlimb unloading. Baseline intake assessed for 5 days prior to actual experiment. Values presented as mean ± SE.
* Within all groups, week1 intake is less than that of all other weeks, $p<0.05$

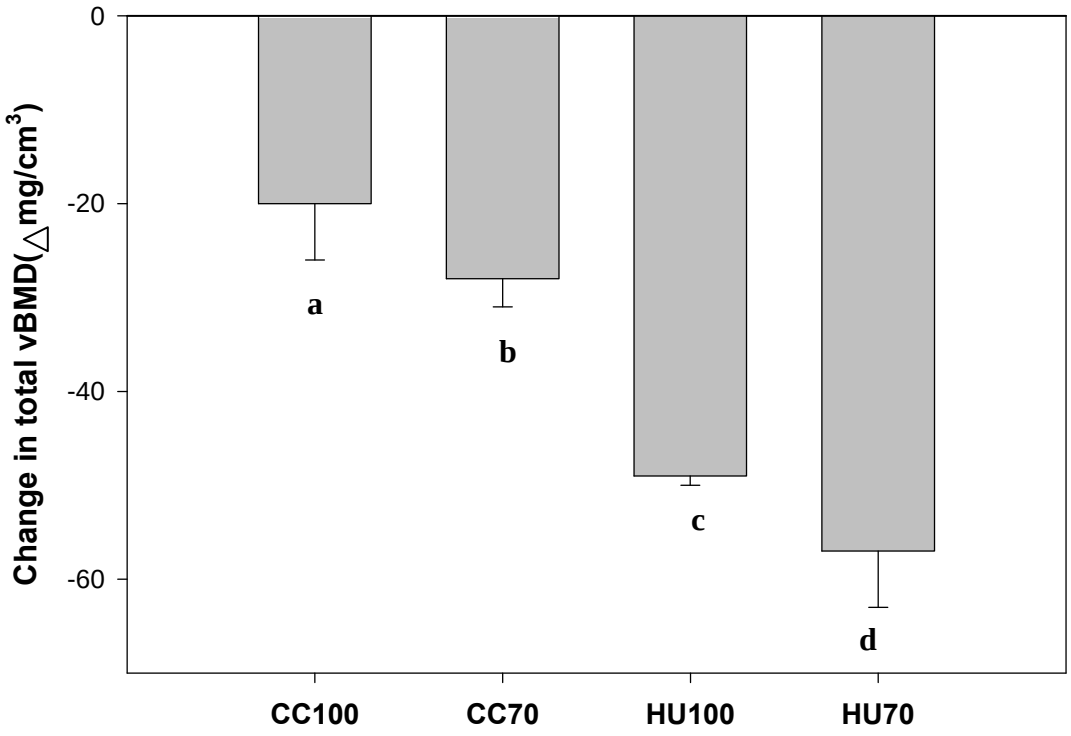


Figure 1.2 Changes in Proximal tibia total volumetric bone mineral density (vBMD) over 28 days of hindlimb unloading (HU) or cage activity (CC) in rats consuming 100% (100) or 70% (70) of usual intake (Δ mg/cm^3). Bars with different letters are significantly different, $p<0.01$.

Table 1.3 In vivo PQCT measures of FR and/or HU effects on tibia density and geometry

	CC100		CC70		HU100		HU70	
	Day 0	Day 28	Day 0	Day 28	Day 0	Day 28	Day 0	Day 28
Proximal Tibia								
Cancellous vBMD ‡ (mg/cm^3)	203 ±7	166 ±6	204 ±7	167± 5	214 ±7	161± 5	221 ±8	180 ±5
Marrow Area (cm^2) †	10.3 ±0.5	10.8 ±0.7	10.0 ±0.4	10.5 ±0.5	11.3 ±0.5	11.8 ±0.5	10.5 ±0.5	11.2 ±0.6
Total Area (cm^2)	19.0 ±0.5	19.5 ±0.8	18.5 ±0.6	18.9 ±0.6	20.1 ±0.7	20.1 ±0.6	19.1 ±0.8	19.1 ±0.8
Tibia Diaphysis								
Cortical vBMD(mg/cm^3)	1324 ±4	1326 ±7	1324 ±4	1317 ±15	1312 ±6	1328 ±6	1315 ±4	1329 ±2
Cortical Area (cm^2) §	6.2 ±0.2	6.2 ±0.2	6.2± 0.1	6.2 ±0.2	6.2 ±0.1	6.3 ±0.1	6.0 ±0.1	6.1 ±0.1
Total Area (cm^2) §	8.4 ±0.3	8.6 ±0.3	8.5 ±0.2	8.7 ±0.3	8.9 ±0.2	9.0 ±0.2	8.4 ±0.2	8.5 ±0.2
Marrow Area (cm^2) §	2.3 ±0.1	2.3 ±0.2	2.3 ±0.1	2.4 ±0.1	2.6 ± 0.1	2.7 ±0.1	2.3 ±0.1	2.4 ±0.1
CSMI (mm^4) §	11.2 ±0.7	12.3 ±0.8	11.3 ±0.5	12.1 ±0.7	12.1 ±0.4	12.8 ±0.4	11.0 ±0.6	11.5 ±0.6

CC: age-matched cage controls, HU: hindlimb unloading. 100 and 70 = 100% and 70% of usual intake, respectively. For all experimental groups, n=11, except for HU 100, in which n=12. †All groups' means increased over 28 days vs day 0, $p \leq 0.01$; ‡All groups' means decreased over 28 days vs day 0, $p \leq 0.0001$. § All group's values increased over time, $p \leq 0.005$.

Table 1.4 Mechanical properties of tibial mid-shaft

	Ultimate Load (N)	Ultimate Stress (MPa)	Stiffness (N/mm)	Elastic Modulus(GPa)
CC100	164 ±8	235 ±13	299 ±14	6.1 ±0.4
CC70	159 ±6	225 ±7	308 ±13	6.3 ± 0.3
HU100	157 ±7	217 ±12	315 ±16	6.8 ±0.3 *
HU70	155 ±6	229 ±4	319 ±18	7.6 ±0.4 *

CC: age-matched cage controls, HU: hindlimb unloading.100 and 70 = 100% and 70% of usual intake, respectively. For all experimental groups, n=11, except for HU 100, in which n=12. * $p \leq 0.01$ for pooled HU groups vs pooled CC groups

Mechanical testing

When collapsed over food intake, elastic modulus of tibial midshaft was higher in HU vs CC rats (Table 1.4). No independent effect of food restriction on material or structural properties at mid-shaft tibia was detected. No changes of mechanical properties were observed with food restriction and/or hindlimb unloading in humeri of any group (data not shown).

Distal femur cancellous bone histomorphometry

Due to technical error, 4-5 rats in each treatment group did not receive fluorochrome labels previous to sacrifice; hence, histological analyses for % MS/BS, MAR and BFR were performed on femurs from 20 animals spread across all groups. Decreases in mineral apposition rate due to FR (CC70) and to unloading (HU100) were similar (-25%), as were the decreases in % mineralizing surface (%MS/BS) for both groups (-59%) (Figure1.3). Reduction in MAR and %MS observed in the combined treatment group (HU70) were similar to that of CC70 and HU100, which implies no additive effect of FR and HU on MAR and %MS. These reductions in MAR and %MS/BS contributed to the 70% reduction in bone formation rate observed in all three treatment groups. Osteoid surface was 33% lower in the CC70 and the HU100 groups as compared to the control group (CC100). In this case, an additive effect of food restriction and hindlimb unloading was observed; a much larger decrement of % osteoid surface was observed in HU70 rats (60%). The % BV/TV in HU rats was lower than that of CC rats by 28.5%, but not statistically significant. Cancellous % osteoclast surface did not vary among the 4 groups (Fig 1.3E, 1.3F).

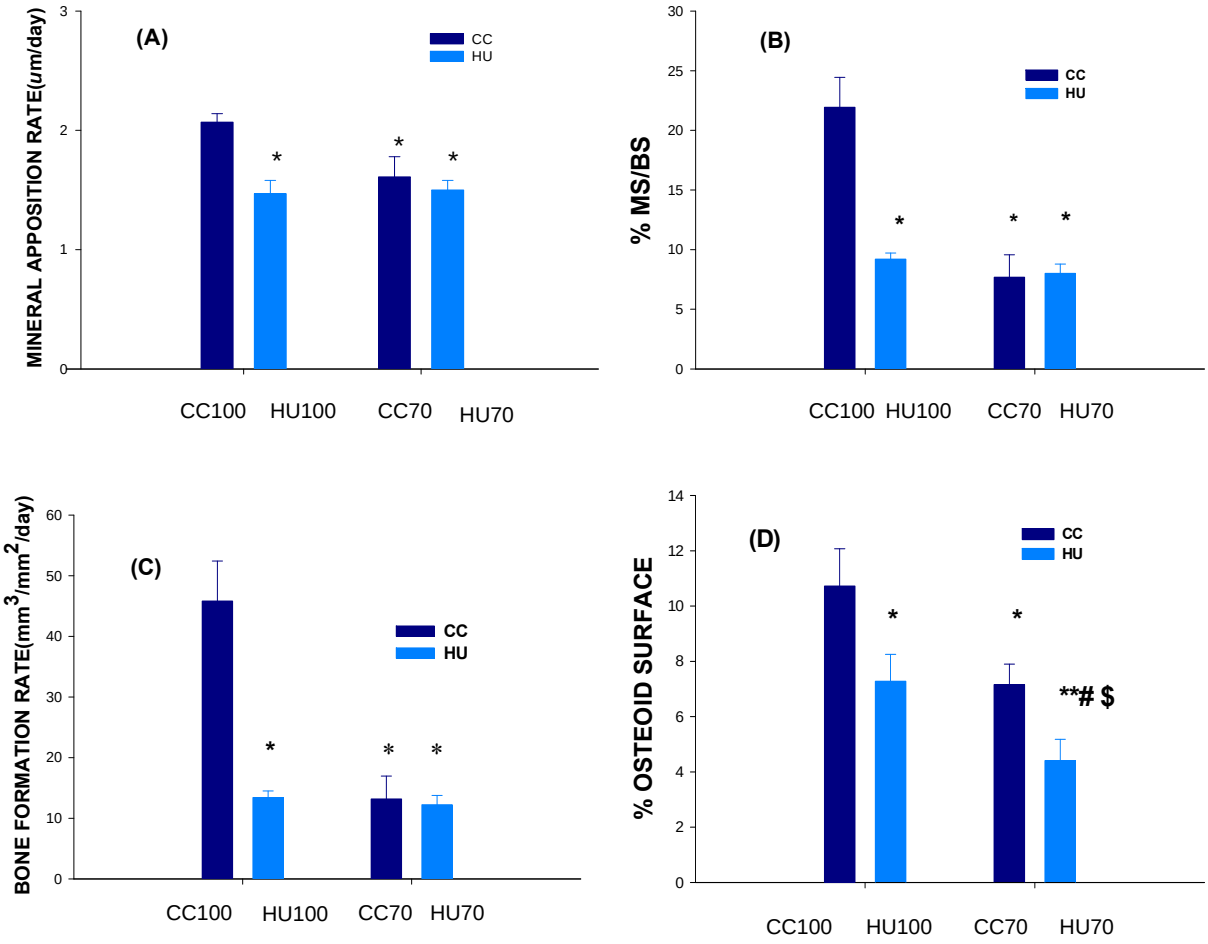


Figure 1.3 Effect of hindlimb unloading (HU) or cage activity (CC) in rats consuming 100% (100) or 70% (70) of usual intake on histomorphometric indicator.

Due to technical error, 4-5 rats in each treatment group did not receive fluorochrome labels previous to sacrifice; hence, histological analyses for % MS/BS, MAR and BFR were performed on femurs from 20 animals spread across all groups.

(A) mineral apposition rate (MAR), *$p<0.03$ compared with values in CC100.

(B) % mineralizing surface (% MS/BS), *$p<0.0001$ compared with values in CC100.

(C) bone formation rate (BFR), *$p<0.0001$ compared with values in CC100.

(D) % osteoid surface, * $p<0.02$ compared with values in CC100; ** $p<0.0001$ compared with values in CC100; # $p<0.06$ compared with values in CC70 ; $ $p<0.05$ compared with values in HU100. Values are means ± SE.

(E) % Bone volume/ Tissue Voulme (BV/TV), No significant difference among groups.

(F) % Osteoclast surface, No significant difference among groups.

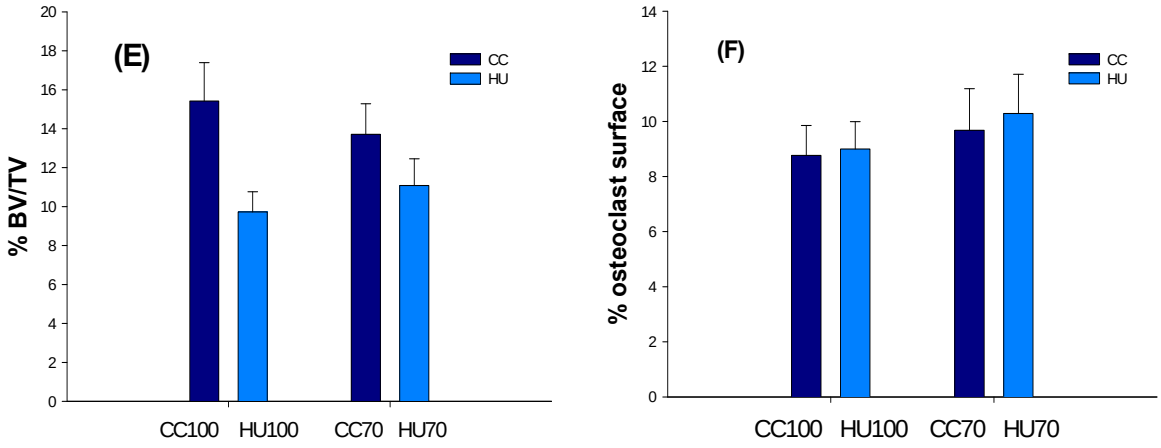


Figure 1.3 Continued

Leptin and turnover markers

As compared to day 0 values, decreases in serum leptin levels (Figure 1.4A) were observed by 28 days in all groups except CC100, with 27% and 60% reductions observed in CC70 and HU100 groups, respectively. Serum leptin was not detectable after 28 days in HU70 rats. Assays for this group were repeated with increasing serum volumes (5µl, 10µl and 20µl), but serum leptin remained undetectable. The change in serum leptin level over the experimental period correlated well with bone formation rate. Those rats exhibiting the greatest decline in serum leptin had the lowest bone formation rates in distal femur cancellous bone (Fig 1.4B).

The mean serum osteocalcin for the all food-restricted rats (pooled 70% intake groups) was 32% lower at the end of the experimental period than that of the pooled 100%

groups (Fig 1.5). When collapsed over food intake levels, HU animals exhibited a greater decline in serum osteocalcin (-23 %) versus that observed in the CC animals (-10%) ($p< 0.01$) . At the end of the experimental period, the mean serum osteocalcin for pooled HU groups was 14% lower than that of the CC groups. The average value of DPD, the resorption marker urinary, at day 0 was 21.23nmol/mmol creatinine and increased in all groups over the experimental period. Urinary DPD rose by 17% (CC100), 24% (CC70) 29% (HU100) and 46% (HU70) over 28days (data not shown). However, these magnitudes of increases were not statistically different among groups.

Discussion

This experiment is the first to demonstrate the independent and combined effects of food restriction and hindlimb unloading on skeletal integrity. Our data confirm our hypothesis that food restriction and hindlimb unloading independently impair skeletal integrity via decreased bone formation and/or increased bone resorption and, if rats are subjected to both treatments, even greater decrements and changes in the above variables would result. Food restriction and hindlimb unloading independently reduced total vBMD in the proximal tibia after 28 days. The impact of hindlimb unloading was significantly greater than that of food restriction and some additive effects of the combined treatment were also observed.

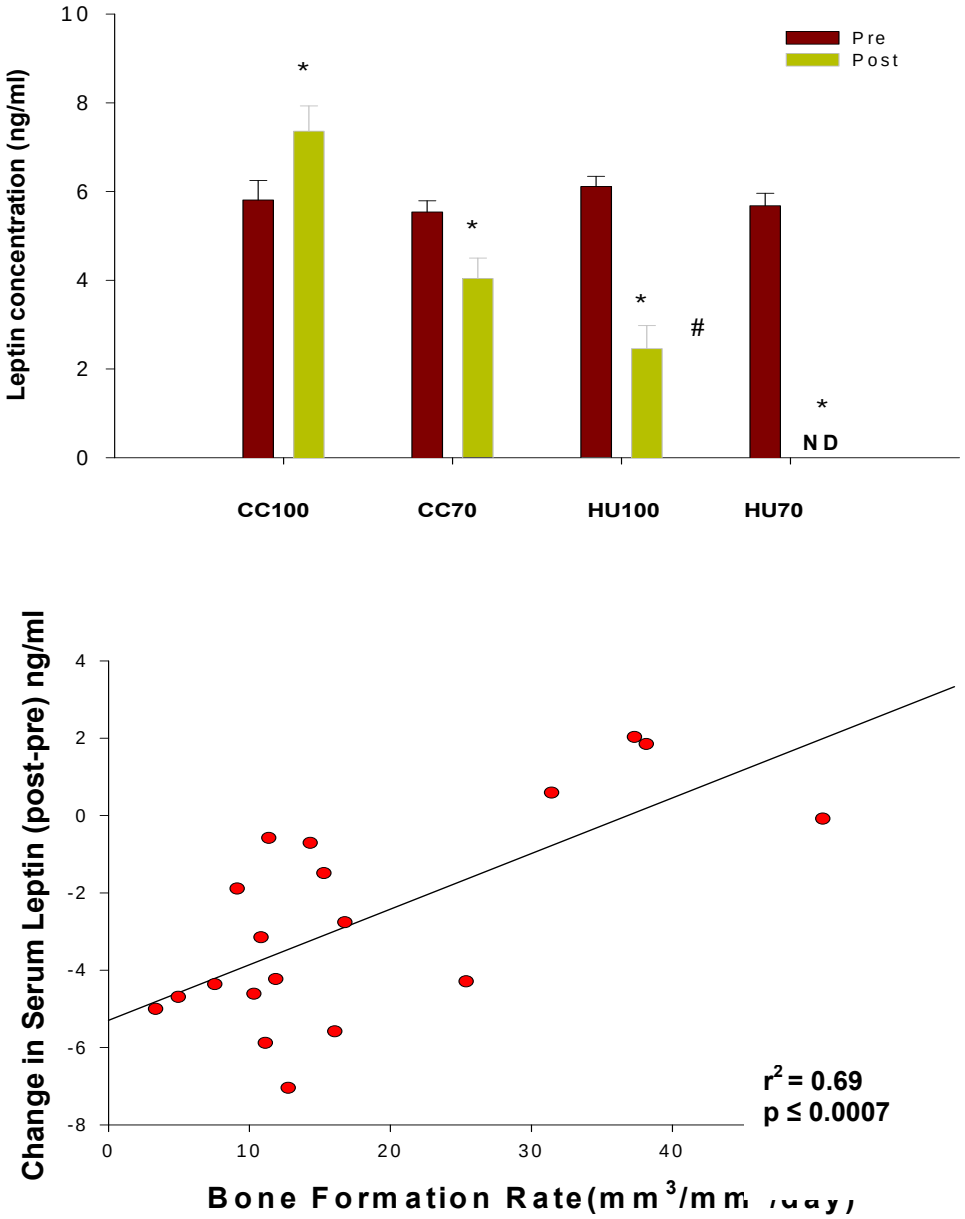


Figure 1.4 (A) Serum leptin levels after 28 days of food restriction (FR) or hindlimb unloading (HU) in rats consuming 100% (100) or 70% (70) of usual food intake. All groups are n=11 except for HU100 in which n=12.* $p<0.0001$ vs pre-value, # $p<0.05$ vs post-value in CC70. (B) Correlation between change in serum leptin value and bone formation rate (BFR) measured at 28 days for 20 animals

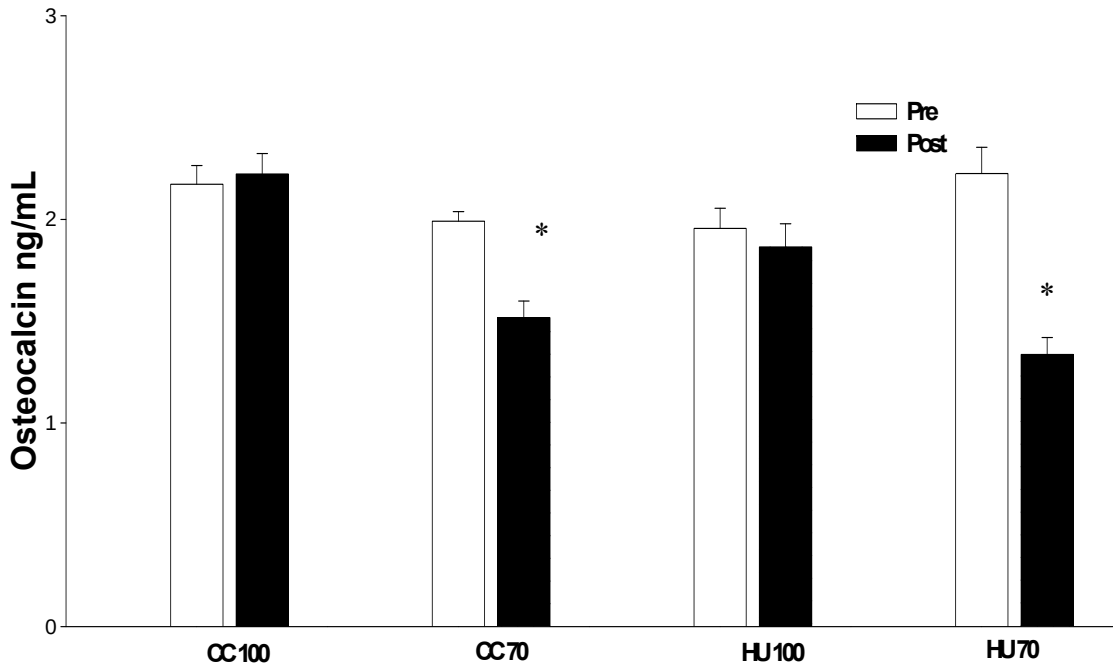


Figure 1.5 Effect of hindlimb unloading (HU) or cage activity (CC) in rats consuming 100% (100) or 70% (70) of usual intake on serum osteocalcin over 28 days. Values are means ± SE. All groups are n=11 except for HU100 in which n=12. The decline in the pooled HU groups is greater than the decline in the CC groups, $p \leq 0.05$. The decline in the pooled 70% group is greater than the decline in the pooled100% group, $p \leq 0.05$. * $p<0.0001$ vs pre-value

These declines in vBMD are due to consistent decreases in mineral apposition rate and especially mineralized surface/bone surface, leading to significant reductions in bone formation rate in cancellous bone observed in both food restriction and hindlimb-unloaded rats. Osteoid surface was the only histomorphometric variable exhibiting an additive effect when both treatments were applied. Osteoid deposition is an early step

in formation of new bone, which must precede mineralization. This additive effect of restricted food intake and hindlimb unloading was not observed in the mineralization-dependent variables after 28 days. It is reasonable to surmise that a longer duration of unloading combined with food restriction would eventually yield greater decrement in mineralization and bone formation rates.

We could not confirm an additive effect of unloading and food restriction on proximal tibial cancellous vBMD in this study. Proximal tibial cancellous vBMD after 28 days was not significantly different among the 4 groups; a significant 18% reduction in cancellous vBMD was observed even in weight bearing control (CC100) rats. It is important to note that rats were switched from 8604 Harlan Teklad Rodent diet (fed by vendor till rats were supplied at 5 months of age) to AIN-93M 5 days before the experiment started. The 8604 chow has a high content of minerals and vitamins, providing about three times the nutrient requirement for laboratory rats established by the NRC (212). In this study, the use of a purified diet containing the minimum amount of nutrients for good rodent health was required in order to effectively restrict nutrient intake below those NRC- recommended intakes. Even though purified diet AIN93-M meets 100% rats' nutrient and energy requirement, a switch to AIN93-M from 8604 chow results in reduced vitamin and mineral intake, which may explain the reduction in cancellous vBMD even in CC100. Recent studies have demonstrated that 40% food restriction causes significant increase in disuse-like bone turnover of endocortical bone in the tibial diaphysis of 13 month-old female rats (216) and that 20% food restriction

augments decrements in bone mineral content and vBMD in total tibia of 7 month-old ovariectomized rats (217), supporting our findings. Ideally, a more prolonged acclimatization period for rats to new chow should be utilized to allow changes in vBMD and other key variables to plateau.

In the bone microenvironment, there is a dynamic balance between resorption and formation that maintains skeletal homeostasis. In the present study, we demonstrated reduced % osteoid surface with food restriction and/or hindlimb unloading, and no differences in % osteoclast surface among the groups, as measured at the proximal tibia. As a whole body measure, biochemical markers of bone turnover showed more responsive changes in a marker of bone formation (osteocalcin) to imposed change of nutritional and/or mechanical environment than in a bone resorption marker (DPD). Decreases in osteocalcin concentration and bone formation activity in young rapidly growing rats have been measured (35). Serum OC transiently decreases by 25% in 6-week-old rats after one week of HU, then returns to almost normal levels after 28days of HU (36). However, there are few published data on the osteocalcin responses to HU in adult rats. The skeletally mature (6-month-old) male rats used in the present study subjected to HU experienced an average decline of 23% OC decline as measured after 28 days. To our knowledge, only one published study reports urinary DPD measurements on tail suspended rats, which has exhibited the same result as our study (218). By contract, consistent and dramatic increases in DPD have been demonstrated in humans exposed to space missions lasting 4 -6 months. With crew members on space

missions for 4-6 months, study showed deoxypyridinoline was increased 55% above preflight levels. (37)

It has been proposed that leptin is responsible for linking energy metabolism to bone mass and also may play a role in bone loss during HU. To our knowledge, our study's results provide the first documentation of the independent effect of microgravity and food restriction on serum leptin and skeletal integrity. There still exists some controversy about the putative effect of leptin on bone. Obesity and hypogonadison result in an increased bone mass in *ob/ob* (leptin deficient*)*- and *db/db (*leptin receptor deficient)-mice deficient mice (17). Evidence for a central (CNS) action for leptin has been provided by Takeda et al. demonstrating that leptin's anorexigenic and anti-osteogenic effects act via two distinct neuronal pathways involving the sympathetic nervous system (18). By contrast, peripherally administered leptin has an anabolic effect on bone metabolism in adult HU rat (27) and in *ob/ob* mice (219). In ob/ob mice altered leptin signaling affects bone differently in axial and appendicular regions of the skeleton (181). Mice in missing *ob/ob* gene have significantly shorter femora, lower femoral bone mineral content (BMC), BMD, cortical thickness, and trabecular bone volume compared to wildtype littermates. In contrast to the pattern observed in the femur, ob/ob mice have significantly increased vertebral length, lumbar BMC, lumbar BMD, and trabecular bone volume compared to lean controls. Interestingly, ob/ob (leptin deficient) mice demonstrate increased adipocyte numbers in femoral bone marrow but fewer

adipocytes in vertebral bone marrow, suggesting that leptin's impact on bone marrow stromal cell's differentiation varies by bone site.

In the present study, we found a significant decrease of serum leptin after 28 days in response to hindlimb unloading and to food restriction, with undetectable levels of serum leptin in rats exposed to both treatments, which suggests a striking effect of the combined treatments on serum leptin level. The decrement in serum leptin was strongly associated with the decline in bone formation rate. This finding is consistent with previous research demonstrating a positive effect of circulating leptin on bone (19). Interestingly, after 28 days hindlimb unloading had a greater suppressive effect on serum leptin that did restricting food intake. Given that rats exposed only to unloading had lower serum leptin but higher body weights than rats exposed to only food restriction by the end of the experiment, it may be that some other factor than body weight and food intake is associated with regulation of serum leptin. It is interesting to speculate that increased stress or increased sympathetic neural output may be another potential regulator of serum leptin levels, which then may impact on bone outcomes (21, 23-25,220).

In summary, moderate caloric restriction caused nearly as much bone loss at the proximal tibia as did the unloading effect of simulated microgravity; osteoblast activity was more responsive than that of osteoclasts to changes in the nutritional and mechanical environment. Deleterious effect of food restriction and/or hindlimb unloading on bone

was observed only in metaphyseal bone but not in diaphyseal bone. Serum leptin level was reduced more by hindlimb unloading than with food restriction, and was associated with the decline in bone formation rate. Many outcomes in the present study provide evidence for an additional negative effect of poor nutrition in the context of disuse bone loss. We conclude that bone loss during spaceflight could be aggravated by consistent reductions in food intake as is frequently observed during short-term shuttle missions (200). These results may also have serious implications for bed-rest patients who restrict food intake or for those individuals who utilize food restriction in attempts to lose weight. Serum leptin may be an important endocrine regulator contributing to this change in bone metabolism.

CHAPTER IV

β-BLOCKADE MITIGATES BONE LOSS DURING HINDLIMB UNLOADING

Introduction

Decrements in bone health result from spaceflight and other periods of skeletal disuse. About 1% of bone mineral density is lost per month while in microgravity, although this is highly variable among subjects (2, 34). Astronauts tend to lose bone only in weight-bearing sites, such as the distal tibia and femoral neck, although vertebral bone is also affected. Losses are much greater in cancellous bone than in cortical bone- up to 24% and 5%, respectively, over 6 months (2). Whether this loss eventually plateaus isn't known, because very few humans have been exposed to microgravity or strict bedrest for more than 6 months.

This change of bone status due to microgravity may involve the sympathetic nervous system (SNS) and numerous endocrine factors. Hormones such as growth hormone, insulin-like growth factor-1, insulin, luteinizing hormone, T_4, thyroid stimulating hormone, calcitonin, active D_3 and parathyroid hormone have been shown to play a role in the deleterious skeletal adaptation to actual or simulated microgravity in rodents and humans (209, 221-224). Leptin, a 16*kDa* cytokine like hormone principally produced by white adipoytes, may also be involved in the bone response to microgravity. Leptin's

principal function is the regulation of energy stores and body composition through negative feedback at the hypothalamic nuclei. Leptin is now known to have numerous biological effects in the immune system (12), reproduction (13), development (14), hemopoiesis (15), angiogenesis (16) and, most recently, in bone metabolism. Early studies claimed to demonstrate an antiosteogenic effect of leptin via the sympathetic nervous system when leptin was administered intracerebroventricularly (17,18), but several studies later demonstrated an osteoprotective effect of leptin during hindlimb unloading or caloric restriction when administered peripherally (19,20). Peripheral administration of leptin has a stimulatory effect on bone growth and bone formation via regulation of osteoblastic function (175) or, possibly, via preferential differentiation of bone marrow stromal cells into osteoblasts rather than to adipocytes (176). Inhibition of osteoclast generation (177) and positive effects on angiogenesis by peripheral leptin administration also has been demonstrated (178-180).

Using the rodent hindlimb unloading model to mimic spaceflight effects on the hindlimb bones, we observed a decrease in serum leptin after 28 days of hindlimb unloading (225). Serum leptin also declined in cage-activity rats subjected to food restriction (70% of usual intake) for a similar time period. The magnitude of the decrease in serum leptin was greater with unloading than with food restriction (60% vs 27%, respectively), even though body weight decreased similarly in both conditions. If rats were subjected to both treatments (food restriction during hindlimb unloading), serum leptin decreased to non-detectable levels.

Evidence indicates that activation of β-adrenoreceptors inhibits leptin release from adipose tissue (21, 220). Exposing animals to slightly stressful conditions such as tail suspension could increase heart rate and blood pressure through activation of the autonomic nervous system (23, 24) and elicit the release of the catecholamine norepinephrine (25). We conjectured that the lower serum leptin in HU rats may be caused by the inhibition of leptin synthesis or secretion resulting from activation of adipocyte β-adrenoreceptor by circulating catecholamines. We pharmacologically blocked β-adrenoreceptors to examine the impact of the sympathetic nervous system on changes in serum important bone parameters during unloading. In addition, we replaced leptin during hindlimb unloading to determine if leptin deficiency is an important factor in the loss of bone mass with unloading**.**

Materials and methods

Animals and experimental design

After one week acclimatization, sixty-six adult male Sprague-Dawley rats (Harlan;Indianapolis,IN) were randomized into six groups of ten animals each: cage activity controls (n=10 each) treated with vehicle, leptin, or β- blocker, and 3 groups of hindlimb unloaded rats (HU) (n=12 each) treated with the same 3 agents. All animals were 6- mo-old at the beginning of the experimental period. Time-release osmotic pump

(Alzet osmotic pumps, Cupertino, CA) were implanted subcutaneously in all rats on day 0 to deliver either placebo vehicle (saline), propranolol (DL-propanolol, Sigma ; 250μg/kg·hr), or leptin (leptin analog, Lilly; 0.35mg/kg·day). Leptin was generously donated by Eli Lilly. Rats were singly housed and maintained under condition of 12h:12h light and dark cycle . On the day osmotic pumps were implanted, half the rats began a 28 day period of HU using tail suspension as previously described (49). Cage controls were allowed normal cage activity and were pair-fed with normal rat chow (2018 Harlan Teklad) to the HU animals in order to control for reduced food intake usually observed over the first 7 days of HU. On day 0 of treatment and on sacrifice day, peripheral computed tomography (pQCT) scans were performed by using a XCT Research M scanner (Stratec; Norland Corp., Fort Atkinson, WI). Urine was expressed and serum samples were collected from a leg vein while the rats were anesthetized. Volumetric bone mineral density (vBMD) and cross-sectional geometry of the proximal and mid-shaft tibia were measured *in vivo* on experiment days 0 and 28. On days 9 and *2* before sacrifice, animals were given subcutaneous injections of calcein (25 mg/kg) to label mineralizing bone for histomorphometric analyses. Right tibiae were removed, cleaned of soft tissue and stored in 70% ethanol at 4°C for histology. All procedures in this study were approved by Texas A&M University Lab Animal Care Committee.

Peripheral computed tomography

The XCT Research M (Stratec; Norland Corp., Fort Atkinson, WI) has a minimum voxel size of 0.07 mm, a scanning beam thickness of 0.50 mm, and is calibrated daily using a

standard hydroxyapatite phantom. *In vivo* measures were made of the proximal metaphysis of the right tibia by collecting transverse images at 5.0, 5.5, and 6.0 mm from the proximal tibia plateau and at the midshaft (50% of total bone length). A standardized analysis for either metaphyseal bone (contour mode 3, peel mode 2, outer threshold of 0.214 g/cm^3, inner threshold of 0.605 g/cm^3) or diaphyseal bone (separation 1, threshold of 0.605 g/cm^3) was applied to each section and is explained in detail elsewhere (214). Values of total (cortical shell + cancellous core) and cancellous volumetric bone mineral density (vBMD), total cross-sectional area, and marrow area (A) were averaged across 3 slices at each bone tissue to yield a mean value for each site. Machine precision (based on manufacturer's data) is ±3 mg/cm^3 for cancellous BMD and ±9 mg/cm^3 for cortical BMD. Reproducibility in our laboratory for both *in vivo* and *ex vivo* measures was determined from five repeat scans with repositioning of the animal or bone between scans. Coefficients of variation for these measurements were 1.24, 2.13, and 1.95% for in vivo proximal tibia total BMD, cancellous BMD, and total area, respectively.

Serum leptin analyses

A rat leptin ELISA immunoassay kit (Crystal Chem, Chicago,IL) was used to measure the concentration of leptin in animal's serum and reported as ng/ml serum. All tests were performed in duplicate and control serum tested with each assay. Precision C.V. within-run was ± 3.2% and C.V. between run was ± 6.4%.

Cancellous histomorphometry

Undemineralized proximal tibia were subjected to serial dehydration and embedded in methylmethacrylate (Aldrich M5, 590-9). Serial frontal sections were cut 8μm thick and left unstained for fluorochrome label measurements; 4μm sections with Von Kossa staining were used for measurement of trabecular bone volume and quantification of osteoid and osteoclast surfaces.

At 20X, a defined region of interest was established ~0.8 mm proximal from the growth plate and within the endocortical edges encompassing 8-9 mm^2. Total cancellous bone, single-labeled, and double-labeled surfaces were measured at 100X and interlabel distances, bone volume and osteoid/osteoclast surface were measured at 200X magnification. Mineral apposition rate (MAR, μm/day) was calculated by dividing the average interlabel width by the time between labels (7 days), and mineralizing surface (MS/BS) for cancellous bone surfaces was calculated by using the formula MS/BS = {[(single labeled surface/2) + double label surface]/surface perimeter} x 100. BFR was calculated as MAR × MS/BS. Histomorphometric analyses were performed with BioQuant True Color Windows image-analysis software (BQTCW98, Version 3.05.6, R&M Biometrics) interfaced with Optronics 3-chip color camera and an Olympus BX60 Microscope with epifluorescent light (Leeds Instruments, Inc. Irving, TX). All nomenclature for cancellous histomorphometry follows standard usage (215).

Statistical analysis

To analyze pre- and post- treatment values of tibia pQCT variables, and serum leptin variables, a 3-way ANOVA with repeated measures was used. To analyze the delta values (pre-post) of tibia pQCT variables, and serum leptin variables, 2-way ANOVA was used. For end point measures (e.g. body and tissue weights, and histomorphometry data), 2-way ANOVA was performed. In those cases where a significant interaction ($p<0.05$) was detected, appropriate post-hoc Duncan and LSD test was performed. Linear associations between change of serum leptin and bone formation rate were described with Pearson correlation coefficients. All values reported are means ± standard errors.

Results

Body weight and tissue weights

Mean body weight tended to decrease for all groups except BBCC over the first 7 days (Figure 2.1). However, by day 28, all groups' mean body weight except that of BBHU rats increased significantly compared to initial body weight. Mean body weight of BBHU rats gradually increased after day 14 and at day 28 was not significantly different from initial body weight. Daily food intake during week 1 was low (~17.5g) for all groups compared to that of remainder of the experiment (22.0~ 25.9g) (Table 2.1). Within each time point, there was no food intake difference among groups implying that beta blocker and leptin treatment didn't impact on food intake.

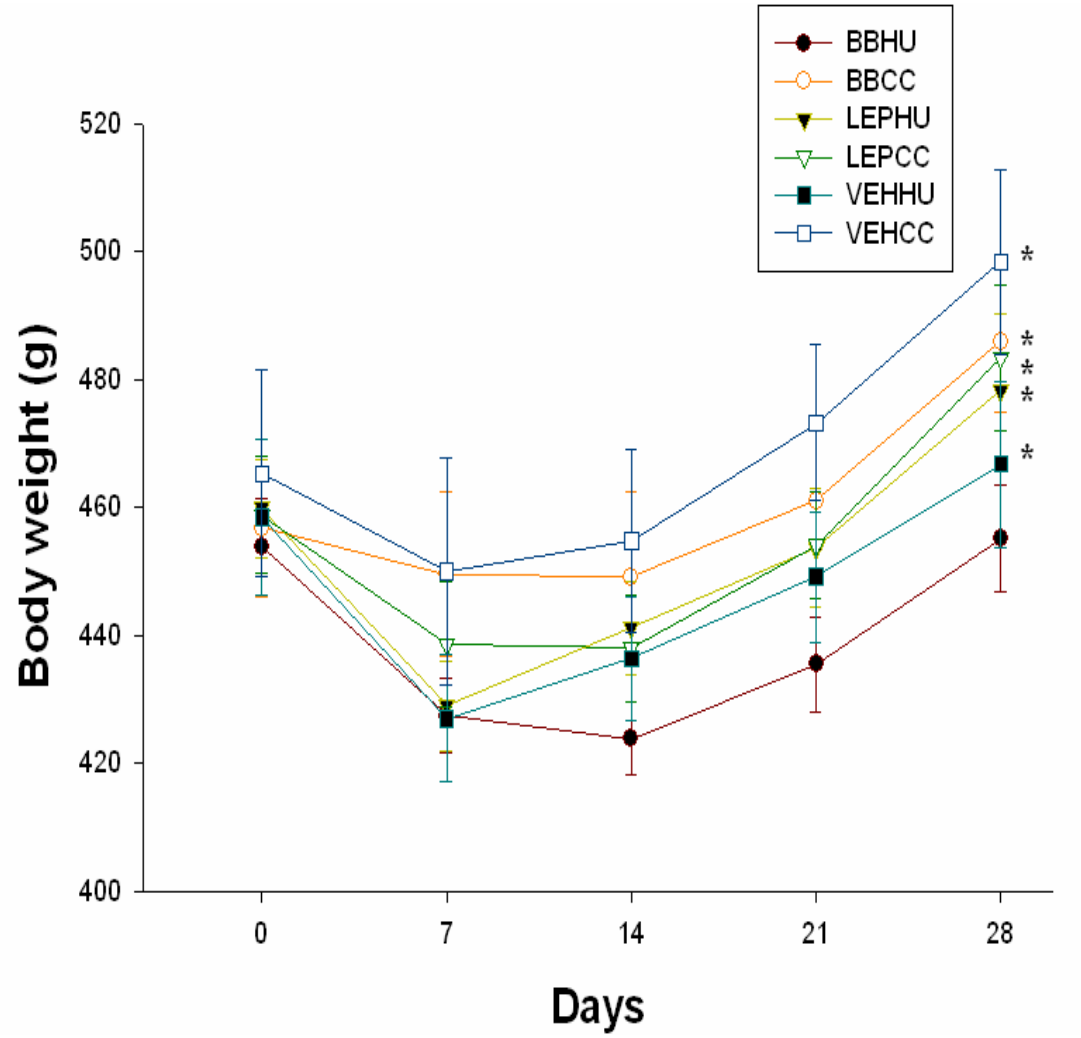


Figure 2.1 Body weights over 28days of hindlimb unloading (HU) or cage activity (CC) in rats administered vehicle (VEH), beta blocker (BB) or leptin (LEP). For all experiment groups, n=10. * $p \leq 0.05$ vs day 0.

Table 2.1 Food intake for 28 days

Group	Food Intake During Experiment			
	Week1	Week2	Week3	Week4
VEHCC	17.3±0.3*	20.9±0.7[#]	25.3±1.0	25.7±0.5
VEHHU	18.7±0.9*	22.9±0.8	26.2±1.2	26.1±0.6
BBCC	18.0±0.6*	22.3±0.8	25.3±0.8	25.7±0.6
BBHU	16.8±1.2*	21.5±1.2[#]	24.7±1.2	25.1±1.1
LEPCC	17.0±0.7*	21.4±0.9[$]	24.3±1.3	25.9±0.8
LEPHU	18.2±0.7*	23.2±1.4[#]	26.5±1.4	27.2±1.1

CC: age matched cage controls, HU: hindlimb unloading.
VEH: vehicle (saline) administration, BB: beta blocker administration, LEP: leptin administration.Values presented as mean ± SE.
* Within group, week 1 intake is less than that of all other weeks, $p<0.05$
[#] Within group, week 2 intake is less than that of week3 and week 4, $p<0.05$
[$] Within group, week 2 intake is less than that of week 4, $p<0.05$
Within each time point, there is no difference in food intake among groups.

Mean epididymal fat weights in BBCC rats were significantly greater than in VEHHU rats at days 28, but there were no significant difference among other groups (Table 2.2). Although not statistically significant, the decrease in epdidymal fat weight observed after HU in VEH and BB rats (21% and 15%, respectively), not seen in leptin treated rats. Soleus muscle weights of all HU groups (pooled average: 0.123 ± 0.017) were 43% lower than that observed in weight bearing CC groups (pooled average: 0.209 ± 0.025)($p<0.0001$), confirming that tail suspension provided effective unloading of rats' hindlimbs (Table 2.2).

Table 2.2 Soleus muscle and epididymal fat weights

	VEHCC	VEHHU	BBCC	BBHU	LEPCC	LEPHU
Epididymal fat weight (mg)	351 ± 20 ^{a,b}	276 ± 10 ^{b}	375 ± 30 ^{a}	322 ± 20 ^{a,b}	321 ± 10 ^{a,b}	332 ± 20 ^{a,b}
Soleus muscle weight (mg)	209 ± 9 ^{c}	121 ± 4 ^{d}	217 ± 10 ^{c}	121 ± 10 ^{d}	211 ± 8 ^{c}	127 ± 5 ^{d}

CC: age matched cage controls, HU: hindlimb unloading.
VEH: vehicle (saline) administration, BB: beta blocker administration, LEP: leptin administration.Values presented as mean ± SE.
a, b : Means sharing same letter are not different, $p<0.05$
c, d **:** Means sharing same letter are not different, $p<0.0001$

pQCT data

Proximal tibial metaphysis vBMD

Hindlimb unloading (HU) caused a reduction in total vBMD (cortical shell with cancellous core) and cancellous vBMD; these losses were attenuated by beta-blocker and leptin treatments (Fig 2.2A). The 11% decline in total vBMD observed in VEHHU rats was attenuated in βBHU rats (7%) and LEPHU rats (5%). Similarly, the 20% decline in cancellous vBMD observed in VEHHU rats was attenuated by half in βBHU (11%) and LEPHU rats (10%) (Fig 2.2B). Marrow area (area inside endocortical perimeter) tend to increase in both VEHHU (+9.3%) and in βBHU rats (+9.1%), but not in HU rats treated with leptin analog (+3%) ($p<0.1$) (Table 2.3).

Midshaft site, tibia

Tibial midshaft vBMD and geometry variables did not exhibit significant changes with unloading or with treatment (data not shown).

Proximal tibia cancellous bone histomorphometry

Mineral apposition rate in VEHHU rats was significantly lower than in VEHCC rats; however, beta-blockade and leptin treatments appear to prevent this decrease in MAR, since this value in BBHU and LEPHU rats is not significantly different from that in their respective control groups (Fig 2.3A). Percent osteoid surface in LEPHU and βBHU rats tended to be greater than in VEHHU (7.90 & 6.54 vs 4.39, respectively; $p< 0.09$) (Table 2.4). Percent resorbing surface (the surface covered by osteoclasts) in VEHHU rats were 3.5-fold higher than in VEHCC rats, but beta-blockade and leptin treatments

abolished this increase in % resorbing surface (Fig 2.3B). Significantly lower cancellous bone volume (%BV/TV) observed in VEHHU rats vs VEHCC rats was not observed in βBHU and LEPHU rats (Table 2.4). No significant differences in any histomorphometric values were observed among LEPCC, βBCC and VEHCC.

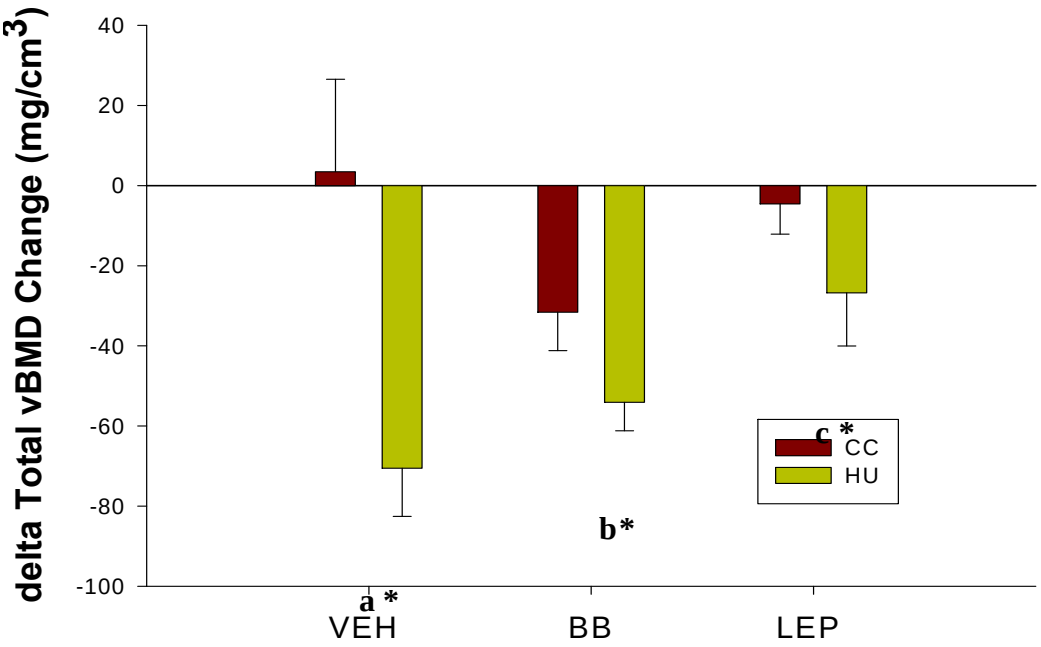


Figure 2.2 (A) Changes of proximal tibia total volumetric bone mineral density (vBMD) over 28 days of hindlimb unloading (HU) or cage activity (CC) in rats administered vehicle (VEH), beta blocker (BB) or leptin (LEP) (post value- pre value in Δ mg/cm^3). For all experiment groups, n=10.Bars with different letters are significantly different; * $p<0.01$ vs CC within treatment group. (B) Changes of proximal tibia cancellous volumetric bone mineral density (vBMD) over 28 days of hindlimb unloading (HU) or cage activity (CC) in rats administered vehicle (VEH), beta blocker (BB) or leptin (LEP) (post value- pre value in Δ mg/cm^3). For all experiment groups, n=10. Bars with different letters are significantly different; # $p<0.05$ vs CC within treatment groups.

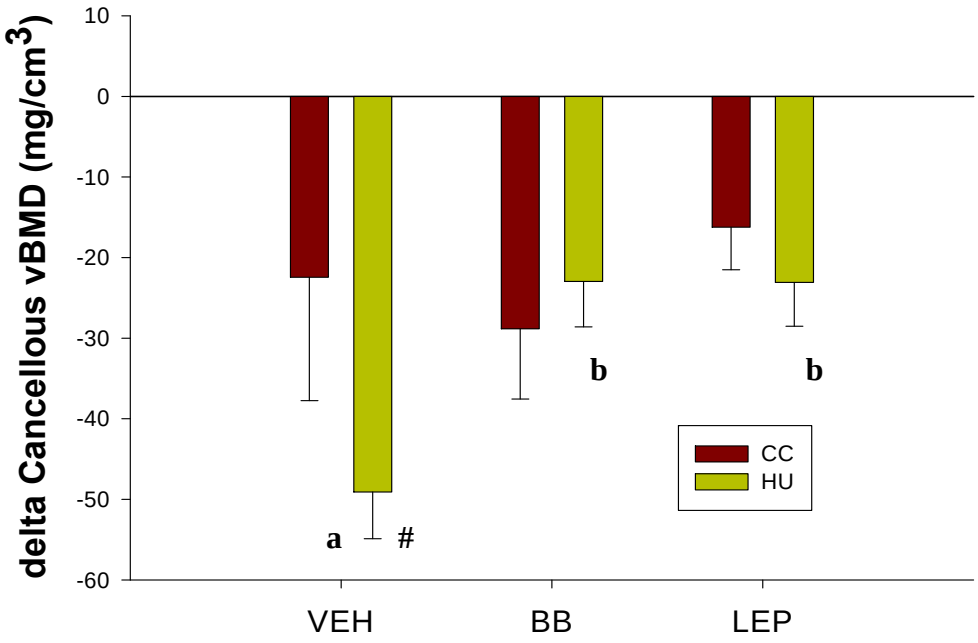


Figure 2.2 continued

Table 2.3 In vivo PQCT measures of proximal tibia cross-sectional geometry

	VEHCC		VEHHU		BBCC		BBHU		LEPCC		LEPHU	
	Day 0	Day 28	Day 0	Day 28	Day 0	Day 28	Day 0	Day 28	Day 0	Day 28	Day 0	Day 28
Marrow Area (cm^2)	55.1 ± 1.0	54.1 ± 2.2	55.9 ± 0.6	61.0 ± 1.5	53.8 ± 1.0	54.4 ± 1.2	53.3 ± 1.0	58.9 ± 0.9	54.7 ± 1.1	54.9 ± 1.0	53.9 ± 1.2	56.0±1.1
Total Area (cm^2)	20.6 ± 1.0	20.7 ± 1.7	19.3 ± 0.6	18.9 ± 1.0	17.7 ± 0.6	17.9 ± 0.5	18.1 ± 0.4	17.7± 0.4	19.2 ±1.2	18.6 ± 0.8	18.0 ± 0.6	17.3 ± 0.6

Table 2.4 Proximal Tibia cancellous bone histomorphometric data

		VEHCC	VEHHU	BBCC	BBHU	LEPCC	LEPHU
Static measures	% osteoid surface	7.7 ± 1.2	4.3 ± 0.8	7.3 ± 1.7	6.0 ± 1.3	8.7 ± 1.2	7.9 ± 1.3
	%BV/TV	22.2±1.6	13.4±1.3$^{\$}$	18.1 ±1.4	16.6±2.9	19.3±1.6	16.7±1.7
Dynamic measures	% Mineralizing surface	25.2 ± 2.8	17.3 ± 2.2	21.0 ± 2.6	20.1 ± 2.2	26.6 ± 3.6	16.5 ± 1.5
	Bone formation rate ($mm^3/mm^2/day$)	51.4 ±9.5	21.4 ± 5.6$^{*, £}$	44.5 ± 7.1	50.4 ± 7.3	64.3 ± 10.9	34.5 ± 4.6$^{\#}$

*p< 0.05 vs CC within treatment groups
#p<0.01vs CC within treatment groups
$p<0.001 vs CC within treatment groups
£ p<0.05 vs BBHU

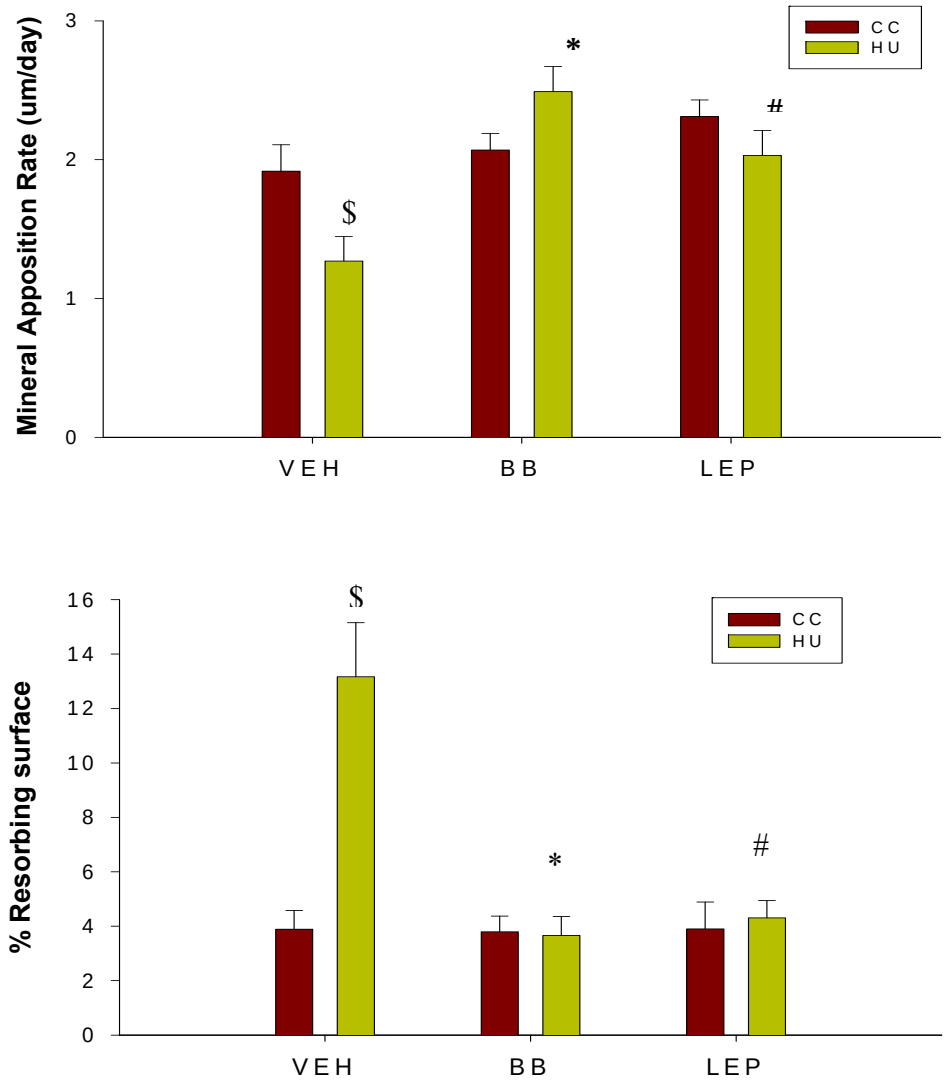


Figure 2.3 (A) Effect of vehicle (VEH), beta blocker (BB) or leptin (LEP) during hindlimb unloaded (HU) or cage activity (CC) rats on proximal tibia mineral apposition rate (MAR).Values are means ± SE. For all experiment groups, n=10. *p<0.0001 vs VEH HU, #p<0.004 vs VEH HU, $p< 0.05 vs CC within treatment group.
(B) Effect of vehicle (VEH), beta blocker (BB) or leptin (LEP) during hindlimb unloaded (HU) or cage activity (CC) rats on % resorbing surface. Values are means ± SE. For all experiment groups, n=10. * p<0.0001 vs VEH HU, # p<0.0003 vs VEH HU, $ p<0.0001 vs CC within treatment group.

Serum leptin

The reduction in serum leptin level was significantly less in βBHU(-25%) and in LEPHU (-31%) than in VEHHU (-62%) (Fig 2.4A) and did not correlate with changes in body weight. Changes in serum leptin among all CC groups were not significantly different. The change in serum leptin level over the experimental period correlated with BFR (r^2= 0.57, p< 0.0001). Those rats exhibiting the greatest decline in serum leptin had the lowest BFR in proximal tibial cancellous bone (Fig 2.4B).

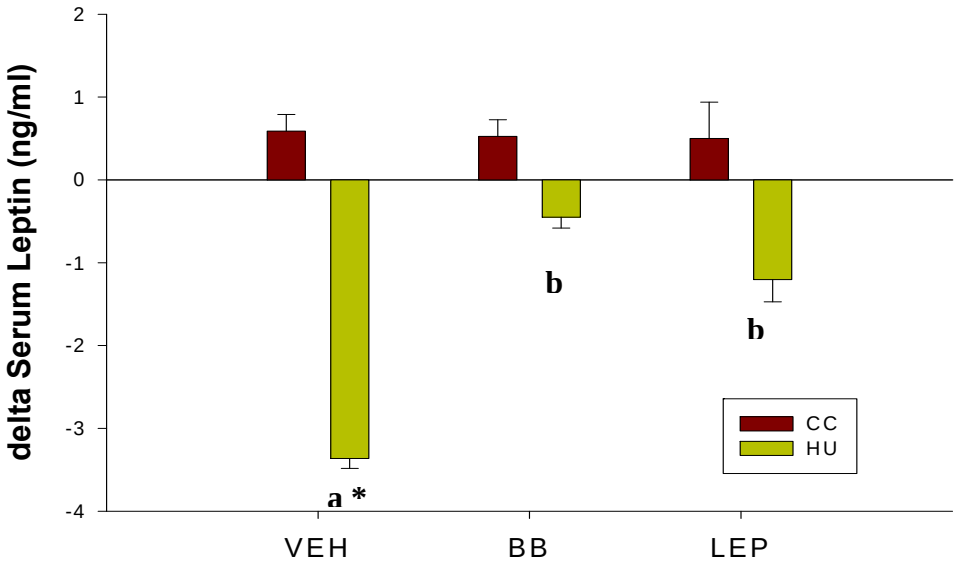


Figure 2.4 (A) Changes of serum leptin level over 28 days of hindlimb unloading (HU) or cage activity (CC) in rats administered vehicle (VEH), beta blocker (BB) or leptin (LEP) (post value- pre value in ng/ml). Bar sharing same letters are not significantly different.* p<0.0001 vs CC within treatment groups. (B) Correlation between change in serum leptin value (post value- pre value in ng/ml) and bone formation rate (BFR) measured at 28 days for all animals.

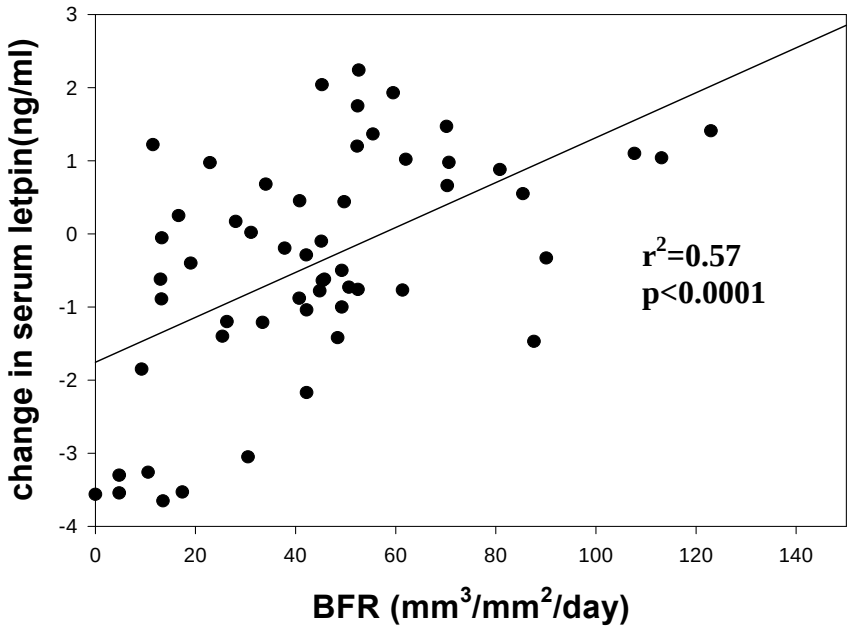


Fig 2.4 continued

Discussion

This experiment is the first to demonstrate the relationship between beta blocker treatment and serum leptin level in the context of simulated microgravity , modeled with hindlimb unloading of adult rats. Our primary hypotheses were that beta-blockade would attenuate the decrease of serum leptin and volumetric bone mineral density during hindlimb unloading and that replacing leptin during HU would also mitigate the

reduction in vBMD during HU, confirming that leptin is an important factor protecting against loss of bone mass during HU.

In this study, beta-adrenergic blockade resulted in significant mitigation of serum leptin reduction during HU. Reductions in total vBMD and cancellous vBMD in proximal tibia during HU were also significantly mitigated by beta-blocker treatment. These effect of beta-adrenergic blockade were as effective as replacing leptin. There exist numerous studies examining the individual effects of peripheral beta blocker treatment or peripheral leptin treatment on vBMD during HU or physiological changes resulting in bone loss. Martin et al. recently demonstrated that leptin modulates both resorption and formation while preventing disuse-induced bone loss in tail-suspended female rats (19). Ovariectomized adult female rats exhibit less loss of cancellous and cortical BMD when treated with propranolol (226). However, no investigation to our knowledge has simultaneously demonstrated the effect of beta-adrenergic blockade on serum leptin and its subsequent effect on vBMD.

Assuming that mitigated loss of vBMD by beta-adrenergic blockade or leptin may result from suppressed resorption or attenuated decrease of formation, we examined histomorphometric measures of formation and resorption. Consistent with vBMD data, the decrease of mineral apposition rate (MAR) during HU was abolished by beta blocker treatment and leptin replacement. Another indicator of osteoblast activity, percent osteoid surface, may also positively affected by beta-adrenergic blockade or by leptin

treatment, but did not reach statistical significance ($p<0.09$). As previously demonstrated *in vitro* (175,176), and *in vivo* (19,227), changes in circulating leptin were directly correlated with bone formation rate. The dramatic increase of bone resorbing surface during HU was successfully suppressed by beta blocker treatment and leptin replacement, which is also consistent with our cancellous vBMD data. We conjecture that the normalized serum leptin levels with beta-adrenergic blockade or peripheral leptin administration prevented the transient increase in osteoclast number with tail suspension. Regulation of osteoclast number by leptin or beta blocker could be mediated by the receptor activator of nuclear factor kappa B-ligand (RANKL)/osteoprotegerin (OPG) pathway. Martin et al. determined with RT-PCR analyses that the suspension-induced increase in RANKL gene expression was counterbalanced during exogenous leptin administration with a 3-fold increase in OPG expression, resulting in a RANKL to OPG ratio similar that observed in weight bearing rats (19). It would be desirable to verify this in our study model in the future.

Because adrenergic receptors also exist on osteoblasts, we cannot overlook the potential direct effect of beta-adrenergic blockade on osteoblast function. Takeda et al. confirmed that osteoblasts express β-adrenergic receptors (18). To determine if bone formation is regulated by the SNS, wildtype and overiectomized mice were administered a β-adrenergic blocker, which resulted in increase in bone mass. An earlier *in vitro* study demonstrated that beta-adrenergic blockade blunts the inhibition of alkaline phosphatase activity by isoproterenol in an osteoblast-like cell line, which suggests that propranolol

may enhance bone formation by preserving osteoblastic activity in the face of beta adrenergic receptor stimulation (120).

We speculate that the positive effects of beta-adrenergic blockade on mitigating bone loss with HU may derive from the synergistic effects of these two mechanistic pathways. That is, one pathway involves the direct effect of beta-adrenergic blockade on osteoblast function and the other the indirect effect of beta--adrenergic blockade on adipocyte function, resulting in rescue of suppressed leptin synthesis or leptin release. Therefore, further studies are needed to determine whether removal of beta-adrenergic signaling effectively restores normal leptin release from adipocytes and to confirm if this leptin actually reaches mature osteoblasts in bone tissue to impact on their bone formation activity during disuse or other stressors causing bone loss.

Leptin is also expressed in and secreted from primary cultures of human osteoblasts as well as from adipoctyes (143). Investigations testing the effect of beta-adrenergic blockade on expression, secretion and putative autocrine signaling of leptin in osteoblasts would be needed.

Our result showed mitigated reduction of vBMD during HU with leptin rescue by beta-adrenergic blockade or with leptin replacement, confirming that circulating leptin is an important factor protecting against loss of bone mass during HU. However, there is controversy about the nature of leptin's effects on bone. Intracerebroventricular leptin

administration, which is employed to demonstrate effects mediated by the central nervous system (CNS) results in a negative effect on bone mass (18). It has been proposed that centrally infused leptin suppresses bone formation via a hypothalamic relay. Neuropeptides upregulated by leptin in the hypothalamus stimulate SNS pathways, resulting in stimulation of beta-adrenergic receptors on osteoblasts and inhibition of bone formation. However, leptin deficiency (ob/ob) produces contrasting phenotypes in bones of the limb and spine, which is high bone mass in spine and low bone mass in limb (181). Injections of leptin into rat ventromedial hypothalamus increase the apoptosis of bone marrow adipocytes, which may be a factor contributing to age-related bone loss (182). All these studies taken together indicate that the effect of leptin on bone is dependent on condition and dose, and also may vary between axial/appendicular and bone shell/marrow regions.

In the present study, no significant differences in histomorphometric values were observed among CCVEH, CCBB and CCLEP, suggesting that leptin and beta-adrenergic blockade exert effects on bone in the context of reduced mechanical loading and/or serum leptin deficiency, but not during normal weight-bearing activity. Also, alterations in body weight were not the critical determinant regulating serum leptin and vBMD in this study, given that beta blocked HU rats experienced similar weight loss as VEHHU rats but exhibited little change of bone mass or serum leptin.

Interestingly, a slight decrement in cancellous vBMD was observed even in cage activity control groups over 28days. These cage activity rats were switched from double housing to single housing and from ad-lib feeding to pair-feeding to HU rats' food intake when the experiment started. In several previous studies, we have observed similar effect of pair-feeding and single housing on reduction in cancellous vBMD of cage activity control rats (23, and unpublished observations). Even though rats' food intake was reduced for the first several days of hindlimb unloading, it then returned to normal levels and was maintained for the rest of the experiment; however, the effect of this mild food restriction for the first week on bone may be important, given changes observed in normal weight-bearing rats. Also, reductions in spontaneous physical activity due to single housing may need to be considered as another factor contributing to cancellous bone loss in cage activity control rats.

In summary, beta-adrenergic blockade and leptin replacement mitigated the decrease in total vBMD, cancellous vBMD, MAR and cancellous bone volume in the tibia during hindlimb unloading and also attenuated the increase in resorption activity during hindlimb unloading. Beta-adrenergic blockade also attenuated the decrease in serum leptin during HU. We conclude that beta-adrenergic blockade is as effective as leptin analog administration in mitigating bone loss with hindlimb unloading through both stimulation of osteoblastic activity and suppression of osteoclastic activity, perhaps in part by disinhibition of leptin release from adipose.

CHAPTER V

BONE LOSS DURING ENERGY RESTRICTION: MECHANISTIC ROLE OF LEPTIN

Introduction

Significant percentage of premenopausal women, especially athletes and military personnel, utilize food restriction in their attempts to lose weight. The prevalence of dieting and weight loss efforts at any given time amongst U.S. women exceeds 50% (3).

Dieting, weight cycling are known to result in clinical concerns, leading to menstrual cycle disturbances and decreased bone mass (4-7). An association between a history of weight loss in premenopausal years and increase in hip fracture risk in later in life has been reported (8). It's been known that 1% decrease in bone mass (9) and an increase in bone resorption (10, 11) is associated with 10% weight loss.

Importance and underlying mechanisms of dietary calcium and vitamin D as critical nutrients for bone has been established through numerous studies. In contrast, our understanding of how energy restriction consequentially happens in the context of food restriction, affect skeletal metabolism is limited. Researches has been performed that suggest bone loss is associated with energy restriction in both animals (11,99) and humans (102,103). In our previous study trying to quantify the magnitude of each individual nutrient's (calcium, protein, energy) to bone loss in a side-by-side comparison

(28), we demonstrated that reduced energy intake is the major contributor to the impact of global food restriction on reductions in bone mineral density and serum estrogen levels in exercising rodents. Hence, one mechanism for bone loss subsequent to restricted energy intake may be via estrogen –dependent pathways.

Another important endocrine change with energy restriction is a decrease in circulating leptin. Leptin, a 16 kDa cytokine-like hormone principally produced by white adipocytes, is another endocrine factor that impacts on bone cell function by linking energy metabolism and bone metabolism. Its principal function is the regulation of energy stores and body composition through negative feedback at the hypothalamic nuclei. The nature of leptin's effects on bone is controversial. Leptin that binds to its receptors on hypothalamus demonstrates antiosteogenic effects via the sympathetic nervous system (18,117), but when administered peripherally (e.g. via osmotic pump), leptin demonstrates a bone protective effect with hindlimb unloading or caloric restriction or overiectomy (27, 20, 228). Expression of long and short forms of leptin receptors on the cells of osteoblastic lineage has been demonstrated, confirming that osteoblasts are targets for leptin action with the ability of signal transduction (17,176,229). In addition to a positive effect on osteoblastic differentiation (17), leptin inhibits the expression of RANKL, synthesized by oseteoblasts, an important inhibitor of bone resorption (230).

Noradrenaline release is increased with stressful conditions, such as food restriction (21, 22), simulated microgravity (23-25), which are normally associated with increased bone

resorption and/or decreased bone formation. Beta-blocker treatment mitigates loss of cancellous bone with hindlimb unloading rats (27), but the effect of beta blockade on serum leptin and/or bone during calrorie restriction hasn't been demonstrated. Several evidence indicate that beta-adrenergic receptor activation is known to one of the inhibition mechanism against leptin release from adipocyte (21, 6). We previously demonstrated that hindlimb unloading and food restriction each independently produce a decrease in serum leptin (225). Our data also indicated that beta blockade alleviated serum leptin decrement and also mitigated reduction in bone mineral density and bone formation rate in hindlimb unloading rats (27). However, yet unproven is whether elevating circulating leptin levels actually results in more leptin reaching the critical bone cells responsible for bone formation or resorption.

In the present study, we tested the mechanistic role of leptin in bone loss during another stressful condition, dietary energy restriction. First, we characterized the effect of β-blockade on blood leptin levels and on bone mass during energy restriction. With the result of bone loss mitigation by beta blockade, we then confirmed whether increased serum leptin levels actually results in more leptin reaching the critical bone cells responsible for bone formation by staining histological sections of bone with antibodies specific to leptin protein. Our primary hypotheses were, therefore, 1) beta-blockade would attenuate the decrease of serum leptin and bone mineral density during energy restriction and 2) the rescued serum leptin would result in more leptin localizing on the critical bone cells responsible for bone formation.

Materials and methods

Animals and experimental design

Adaptation period to achieve bone stabilization on new diet

Day 0 was defined as the start of the restrictive dietary and pharmaceutical interventions; time points before day 0 are designated as "minus" days. We used a specially formulated rat chow, AIN93-M, which most closely matches National Research Council (NRC) recommendations for adult rat dietary requirements (212,231). Because we had previously observed a significant loss in volumetric bone mineral density (vBMD) at the proximal tibia in rats switched from the vendor's rat chow (Harlan Teklad 2018) to this AIN93-M diet, we tested an extended period of acclimation to this diet. At least 7 weeks were required before a "stable" proximal tibia vBMD (defined by two consecutive average vBMD values that change by less than 10 mg/cm^3) was observed. In total, 40 Sprague-Dawley female rats, aged 4 months at purchase, were singly housed and allowed to eat AIN93-M chow for 8 weeks to assure complete adaptation to the new diet.

Experiment: day 0 to week12

On day 0, 40 adult female Sprague-Dawely rats (Harlan; Indianapolis, IN) was randomized into four groups of ten animals each by trabecular vBMD first and them by body weight : ad-lib fed controls (n=10 each) treated with vehicle (CONVEH), or β-blocker (CONBB), and 2 groups of energy restricted rats (n=10 each) treated with

vehicle (ERVEH), or β- blocker (ERBB). The dose of beta-blocker used was equivalent to that which in previous studies effectively mitigated loss of bone during disuse (hindlimb unloading). The CONVEH and the CONBB groups were fed *ad lib* for the twelve-week protocol. Daily food intake for the CONVEH rats was measured each day. To achieve the 40% energy restriction, but with all other nutrients at 100% levels, ERVEH and ERBB rats were fed 0.61 gm of the specially formulated diet (AIN-93M-E) for every 1 gm of AIN-93M of the CONVEH group average consumption (Table 3.1). Beta-blocker treated group was administered propranolol (DL-propanolol, Sigma, 6mg/kg), via drinking water. A graduated tube was installed in each cage 7 days prior to day 0 for measuring daily water intake. At day 0, based on daily water intake average (19ml /day), 94.7mg of propranolol/ 1L of water was distributed into the water tube in order to deliver 1.8mg/d (for a 300g rat) or 6mg/kg BW of propranolol to each rat. Over the 12 week experimental period, daily water intake of each rat was measured and concentration of propranolol in drinking water was adjusted from 1.8mg/16ml to 1.8mg/22ml inn order to deliver the goal dose. Over the 12 week experiment, Rats were singly housed and maintained under condition of a 12h:12h light and dark cycle. Dual X-Ray Absorptiometry (DEXA) Scans and peripheral quantitative computed tomography (pQCT) scans of the lower leg bone (tibia) were performed while rats were anesthetized with Ketamine/medetomidine (55 mg/kg (ket) + 0.3 mg/kg (med)) on days 0, week4 and week 12 to measure early and late changes in outcome variables. A blood sample was drawn from a leg vein at Days 0, week 4 and week 12 (maximum volume 1.5 ml) while the rat is anesthetized and saved at -80°C. On days 9 and 2 before sacrifice, animals

were given subcutaneous injections of calcein (25 mg/kg) to label mineralizing bone for histomorphometric analyes. Right femora were removed, cleaned of soft tissue and stored at −80°C in PBS-soaked gauze for ex-vivo pQCT scan and/or mechanical testing,

Table 3.1 AIN-93M mature rodent diet and modifications for 40% energy restriction diets

	AIN-93M		**AIN-93M-E (40% energy restriction)**	
	gm%	**kcal%**	**gm%**	**kcal%**
Protein	**14**	**15**	**23**	**24**
Carbohydrate	**73**	**76**	**56**	**60**
Fat	**4**	**9**	**6**	**16**
Total		**100**		**100**
kcal/gm	**3.85**		**3.76**	
Ingredient	**gm**	**kcal**	**gm**	**kcal**
Casein, 80 Mesh	**140**	**560**	**140**	**560**
L-cystine	**1.8**	**7.2**	**1.8**	**7.2**
Corn Starch	**495.692**	**1982.8**	**112.5**	**450**
Maltodextrin 10	**125**	**500**	**125**	**500**
Sucrose	**100**	**400**	**100**	**400**
Cellulose, BW200	**50**	**0**	**50**	**0**
Soybean Oil	**40**	**360**	**40**	**360**
t-Butylhydroquinone	**0.008**	**0**	**0.008**	**0**
Mineral Mix	**35**	**0**	**35**	**0**
Vitamin Mix	**10**	**40**	**10**	**40**
Choline Bitartrate	**2.5**	**0**	**2.5**	**0**
Total	**1000**	**3850**	**616.808**	**2317**
Phosphorus	3.1		3.1	
Calcium	5.0		5.0	
Potassium	3.6		3.6	
Magnesium	0.5		0.5	
Vitamin K	0.000750		0.000750	
Vitamin D	0.0100		0.0100	

whereas left femora were fixed in 4% paraform aldehyde for immunihistochemistry (IHC) staining. Left tibiae were stored in 70% ethanol at 4°C for histology. *Ex Vivo* CT scans of the femoral neck (near the hip joint) and mechanical testing of mid-shaft femur and of the femoral neck was performed. All procedures in this study were approved by Texas A&M University Lab Animal Care Committee.

Peripheral computed tomography

XCT Research M (Stratec; Norland Corp., Fort Atkinson, WI) model has a minimum voxel size of 0.1 mm, a scanning beam thickness of 0.50 mm, and was calibrated daily using a standard hydroxyapatite phantom. *In vivo* measures were made of the proximal metaphysis of the right tibia on day 0, week4 and week 12 with the animal anesthetized. Transverse images were scanned at 5.0, 5.5, and 6.0 mm from the proximal tibia plateau. A standardized analysis for either metaphyseal bone (contour mode 3, peel mode 2, outer threshold of 0.169 g/cm^3, inner threshold of 0.650 g/cm^3) or diaphyseal bone (separation 1, threshold of 0.650 g/cm^3) was applied to each section. Values of total, cortical shell, and cancellous volumetric bone mineral density (vBMD), cross-sectional area, cortical area, and marrow area (A) were averaged across 3 slices at each bone tissue to yield a mean value for each site. In addition, middiaphyseal cross-sectional moment of inertia (CSMI) was obtained with respect to the neutral bending axis during three-point bending. Machine precision (based on manufacturer's data) is ±3 mg/cm^3 for cancellous BMD and ±9 mg/cm^3 for cortical BMD. Reproducibility in our laboratory for both in vivo and ex vivo measures was determined from five repeat scans with repositioning of the animal or

bone between scans. Coefficients of variation for these measurements were 1.24, 2.13, and 1.95% for in vivo proximal tibia total BMD, cancellous BMD, and total area, respectively. Ex vivo distal metaphysis coefficients of variation for the same variables were 0.37, 1.43, and 0.28%, respectively.

Dual energy x-ray absorptionmetry (DEXA)

Total body BMD and BMC, total body fat mass and lean mass were measured by DEXA with a Lunar DPX-MD^{+} bone densitometer (GE Lunar, GE Medical Systems, Milwaukee, Wis), with software standardized for small animals. Anesthetized rats were laid prone on the platform and measurement was performed. BMD determinations are expressed in grams per square centimeter and BMC, fat and lean mass are expressed in gram. Percent fat mass was calculated as fat mass (g)*100/body weight (g). Daily densitometer standardization with a phantom spine showed variation from one day to the next of <0.1%.

Serum leptin analyses

A rat leptin ELISA immunoassay kit (Crystal Chem, Chicago, IL) was used to measure the concentration of leptin in animal's serum and reported as ng/ml serum. Assays were run on duplicate samples. Precision C.V. within-run was ± 3.2% and C.V. between run was ± 6.4%.

Cancellous histomorphometry

Undemineralized distal left femora were subjected to serial dehydration and embedded in methylmethacrylate (Aldrich M5, 590-9). Serial frontal sections were cut 8μm thick and left unstained for fluorochrome label measurements, and cut at 4μm sections for Von Kossa staining for measurement of cancellous bone volume and quantification of osteoblast, osteoid and osteoclast surfaces.

At 20x, a defined region of interest was established ~1.0 mm from the growth plate and within the endocortical edges encompassing 6-7 mm^2. Total bone surface, single-labeled surface, and double-labeled surface were measured at 100x and interlabel distances, bone volume and osteoid/osteoclast surface were measured at 200x magnification. Mineral apposition rate (MAR, μm/day) was calculated by dividing the average interlabel width by the time between labels (7 days), and mineralizing surface (MS/BS) for cancellous bone surfaces was calculated by using the formula MS/BS = {[(single labeled surface/2) + double label surface]/surface perimeter} x 100. BFR was calculated as MAR × MS/BS. Total bone surface, osteoid surface, osteoclast surface were obtained by manual tracing. The raw data were exported to an excel sheet and derived indices of bone volume (BV)/tissue volume referent, osteoid/bone surface (BS), osteoclast surface/BS, osteoid thickness were calculated using previously described formulae (27). Histomorphometric analyses were performed with OsteoMeasure system (OsteoMetrics, Inc., Atlanta, GA) and digitizing pad, interfaced with color video camera (DXC-390P, Sony, Japan) and an Olympus BX60 Microscope with epifluorescent light (Leeds

Instruments, Inc. Irving, TX). All nomenclature for cancellous histomorphometry follows standard usage (215).

Immunohistochemistry

Femur specimens from rats were fixed by immersion in 4% paraformaldenyde in 0.1M sodium phosphate buffer (PBS), pH 7.4. After 20~24 hours fixation, the samples were decalcified with 50% formic acid (by mixture with 20% sodium citrate, 1:1 ratio), dehydrated in a graded series of ethanol and embedded in paraffin (Paraplast). Sections (5μm thick) were immunostained by avidin-biotin technique (232). Tissue sections were deparaffinized, hydrated in PBS and incubated in 0.3% H_2O_2 in D.I. water for 30 min at room temperature to block endogenous peroxidase activity. After being washed in PBS, the sections were preincubated with avidin D solution and then biotin solution for 15 minutes each, then with normal serum from the same species as the secondary antibody for 20 min at room temperature (to minimize background staining). Then, they were incubated first overnight at 4°C with primary rabbit polyclonal anti-leptin antibody (Ob (A20): sc-842, Santa Cruz Biotech, CA, USA), diluted 1:500 in PBS/2% BSA, then with the corresponding biotinylated anti-rabbit IgG secondary antibody, made in goat, diluted by manufacturer's protocol (VECTOR) in PBS/2% BSA/5% NGS and finally with ABS complex (Vectastain elite ABC reagent, VECTOR). Peroxidase activity was revealed by developing sections with enzyme substrate (NovaRED substrate kit, VECTOR). Sections then counterstained with hematoxyline, mounted with Eukitt (Kindler, Germany) and observed under microscope. Method specificity was tested by omitting the primary antibody in the immunostaining procedures.

Statistical analysis

To analyze pre- and post- treatment values of tibia pQCT variables, and blood variables, a 3-way ANOVA with repeated measures was used. In addition, a simple main effects analysis was performed when 3-way interactions were significant and, when appropriate, Duncan post-hoc or LSD tests were used within the simple main effects analyses. For end point measures (e.g. histomorphometry data), 2-way ANOVA was performed, with appropriate post-hoc tests. Linear associations between change of serum leptin level to the bone formation rate were described with Pearson correlation coefficients. All values reported are means ± standard errors.

Results

Food intake/ water intake

Over the course of the 12-wk study, CON rats (ad-lib fed) consumed 15.3±2.7g /day of the AIN-93M diet in VEH and BB groups. ER rats consumed 9.3g±1.7g /day of the AIN-93M-E diet in VEH and BB groups. These intake provided 58.6±10.5 and 34.8±6.3 kcal/d, respectively for CON and ER rats. Food intake over 12 weeks' experiment was not different between VEH and BB rats (data not shown). Water intake over 12 weeks' experiment was not different among all groups (16-21.5ml/day); average propranolol dose delivered via water intake was 1.70mg ± 0.2 /day and 1.77 ±0.2 /day for CONBB and ERBB, respectively.

Body weight/body composition

ERVEH and ERBB rats had lower body weights than CONVEH and CONBB rats starting at week 2 of treatment until the end of the experiment (Fig 3.1). Over the 12-wk experiment period, ER reduced body weight body weight to 249 ± 12.63g in VEH treated (- 20.1%) and to 254 ± 3.73g in BB treated (-18%) rats ($p< 0.0001$), while CONBB rats increased body weight to 347 ± 6.44 g (+ 10.4%) ($p < 0.01$). Significant interaction between energy status and time was observed in fat mass and in lean mass variables (but no significant interaction with 3 factors: energy status, beta blocker treatment, and time), so 2 way ANOVA with repeated measure test in each energy status group (CON and ER) was performed seperately. At week 12, fat mass and lean mass in ER rats were significantly lower rats subjected to ad lib feeding. Over 12 weeks, control rats gained fat mass in VEH (+ 47 %) and BB (+ 64 %) rats and energy restricted rats lost body fat mass in VEH (-63%) and BB (-78%) rats ($p < 0.0001$). Lean body mass increased by 4 weeks in energy restricted rats, but not in control rats; by week 12, there was no difference in lean body mass among groups or versus week 0 value within group (Table 3.2).

Serum leptin

The reduction in serum leptin level was significantly less in ERBB (-1.14 ± 1.0ng/ml) than in ERVEH (-5.31± 1.1ng/ml) at week12 (Fig 3.2A). The change in serum leptin level over the experimental period correlated with bone formation rate (BFR) ($r= 0.62$, $p< 0.0001$) (Fig 3.2B). Those rats exhibiting the greatest decline in serum leptin had the

Table 3.2 In vivo DEXA measures of ER and/or BB effects on body composition and total body BMD, BMC

	CONVEH			CONBB			ERVEH			ERBB		
	wk0	wk4	wk12	wk0	wk4	wk12	wk0	wk4	wk12	wk0	wk4	wk12
Fat mass (g)$	65.0±4.5	86.1±7.0	96.1±11.8*	65.9±2.9	91.4±4.9	108.9±6.0*	66.4±4.2	41.1±5.7	24.2±7.8*	61.5±6.7	35.0±3.5	12.8±2.8*
Lean mass (g)&	213±6.3	214±5.8	212±5.9	212±3.7	215±3.3	212±4.8	220±8.4	243±5.7†	216±4.4	213±6.6	236±3.6†	216±2.2
Total body BMC$	9.6±0.2	10.0±0.2	10.3±0.4*	9.4±0.1	10.2±0.1	10.5±0.1§	9.6±0.2	9.5±0.2	9.0±0.3*	9.5±0.2	9.4±0.1	8.7±0.1*
Total body BMD$	0.179± 0.001	0.182± 0.001	0.180± 0.003	0.180± 0.001	0.183± 0.001	0.183± 0.001	0.182± 0.001	0.175± 0.002	0.174± 0.001£	0.182± 0.002	0.174± 0.001	0.172± 0.002£

CON: ad-lib fed, ER: energy restriction, VEH: vehicle administration, BB: beta blocker administration, respectively. For all experimental groups, n=10, except for CONVEH, in which n=9.

& ER groups' means lower at week 4 vs CON group's means, $p<0.0001$

† $p< 0.0001$ vs wk 0 within energy status group

$ ER groups' means lower at week 12 vs CON group's means, $p<0.0001$

* $p<0.05$ vs wk 0 within energy status group

£ $p<0.01$ vs wk 0 within energy status group

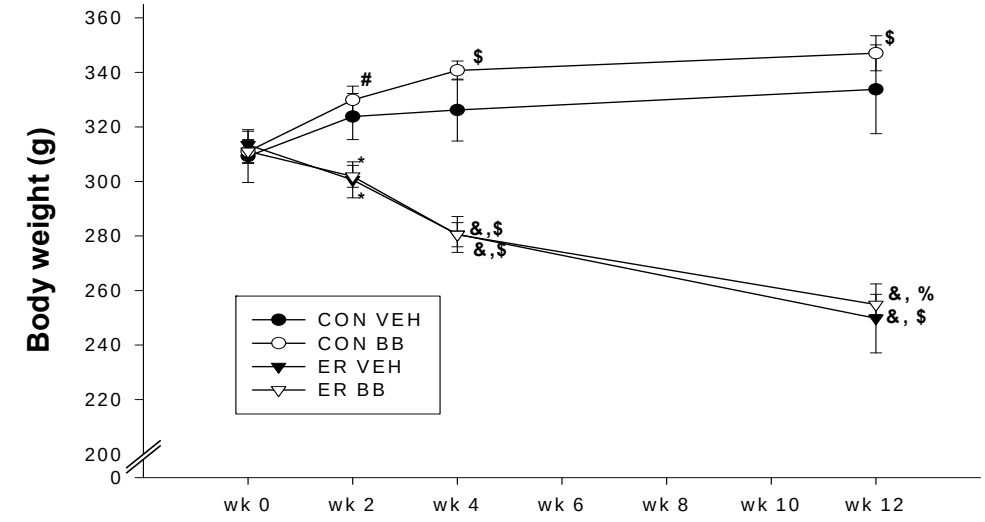
§ $p< 0.0001$ vs wk 0 within energy status group

lowest BFR in proximal tibial cancellous bone. The serum leptin level at week 12 correlated with bone formation rate ($r= 0.54$, $p<0.0001$) (data now shown). Serum leptin level at week 12 normalized by fat mass(g) in ERBB rats tended to be greater than that of ERVEH, but not statistically significant (Table 3.3).

Table 3.3

Serum leptin level normalized by fat mass from DEXA analysis at week 12

Group	Serum leptin (ng/ml)/g fat
CONVEH	0.127±0.04
CONBB	0.103±0.04
ERVEH	0.069±0.02
ERBB	0.167±0.01



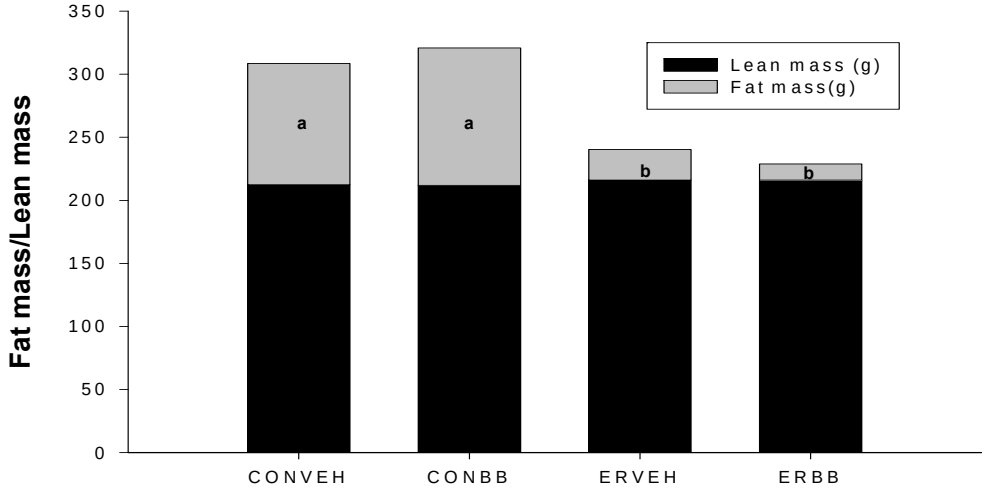
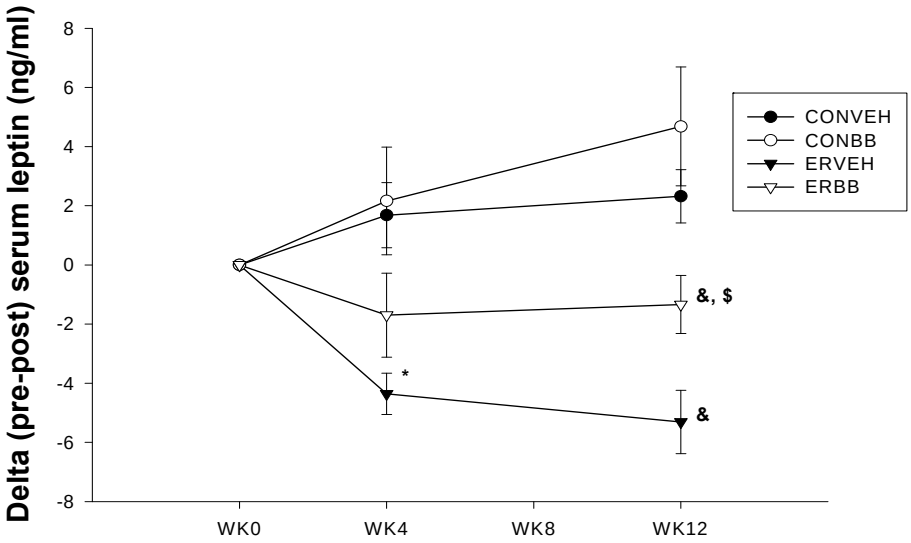


Figure 3.1 (A) Body weights over 12 weeks of energy restriction (ER) or ad-lib fed (CON) in rats administered vehicle (VEH) or beta blocker (BB).
For all experiment group, n=10 except CONVEH, in which n=9.
* p<0.01 vs CONVEH, & P<0.0001 VS CONVEH, # p<0.01 vs wk 0 within group
$ p<0.001 vs wk 0 within group, % p<0.0001 wk 0 within group
(B) Total body fat mass and lean mass at week12. Fat mass portion of bars sharing same letters are not significantly different.



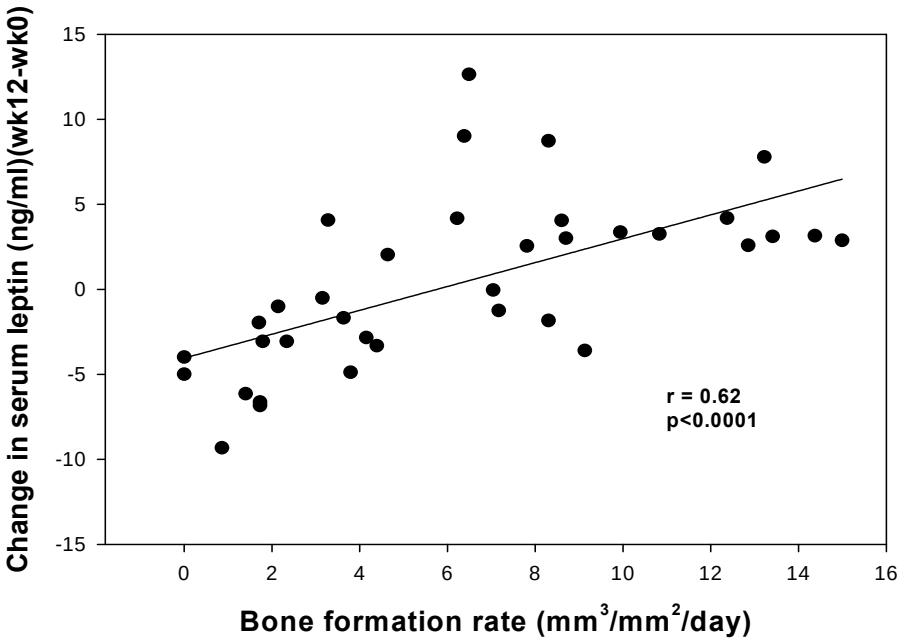


Fig 3.2 (A) Change of serum leptin over 4 weeks and over 12 weeks of energy restriction (ER) or ad-lib fed (CON) in rats administered vehicle (VEH) or beta blockade (BB) (post value- post value in ng/ml).* $p<0.05$ vs CONVEH at week 4, & $p<0.05$ vs CONVEH at week 12, $ $p<0.05$ ERVEH at week 12.
(B) Correlation between change in serum leptin value (post value- pre value in ng/ml) and bone formation rate (BFR) measured at 84 days for all animals.

Bone mass

Proximal tibial metaphysis and midshaft vBMD (pQCT)

Longitudinal vBMD measurement at the proximal tibia revealed a significant decrement in cancellous vBMD, compared to their initial value, in ERVEH rats starting at week 4 (-12%), which decrement increased by the end of the experiment (-22%)(Table 3.4). Even though the significant reduction in cancellous vBMD was also observed in ERBB rats at week12 (-16%) compared to their initial value, but the magnitude of decrement caused by ER over 12 weeks was significantly attenuated by BB treatment (-53.94 mg/cm^3 vs -85.24 mg/cm^3 in ERVEH rats) (Fig 3.3) ($p < 0.05$). Total vBMD (including cortical shell and cancellous core) decreased over the experimental period in all groups starting at week 4. ER alone (-91.34 mg/cm^3), but not ER rats given BB (-62.68 mg/cm^3), resulted in significantly greater reduction in total vBMD than in CONBB and CONVEH rats (-36.00, -33.28 mg/cm^3, respectively). Cortical shell vBMD in CONBB and ERBB rats (+17, +19 mg/cm^3, respectively) tended to increase over 12 weeks ($p < 0.07$), but this increment was not observed in VEH treated rats (data not shown). Longitudinal vBMD measurement at midshaft tibia revealed a significant and similar (~2.0%) increment in cortical vBMD for all groups except ERVEH rats. ($p<0.001$) (Table 3.4).

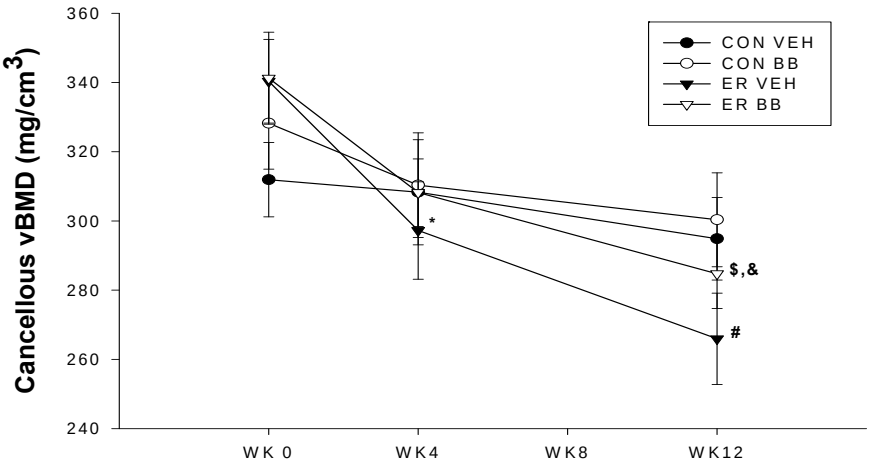


Fig 3.3
Proximal tibia cancellous volumetric bone mineral density (vBMD) over 12 weeks of energy restriction (ER) or ad-lib fed (CON) in rats administered vehicle (VEH) or beta blocker (BB). * $p<0.05$ vs wk 0 within group, $ $p<0.01$ vs wk 0 within group, # $p<0.001$ vs wk 0 within group, & $p<0.05$ vs ERBB at week 12.

Total body BMD and BMC (DEXA)

Significant interaction between energy status and time was observed in Total body BMD and BMC (but no significant interaction with 3 factors: energy status, beta blocker treatment, and time), so 2 way ANOVA with repeated measure test in each energy status group (CON and ER) was performed separately. At week 12, total body BMC and BMD in ER rats were significantly lower rats subjected to ad lib feeding. Total body BMD and BMC decreased in rats subjected to ER (Table 3.2). No difference between ERVEH and ERBB rats was observed in any of the DEXA variables.

Table 3.4 In vivo PQCT measures of ER and/or BB effects on tibia density and geometry

	CONVEH			CONBB			ERVEH			ERBB		
	wk0	wk4	wk12	wk0	wk4	wk12	wk0	wk4	wk12	wk0	wk4	wk12
Proximal Tibia												
Total vBMD (mg/cm^3)	699.2± 12	680.8± 12*	666.2± 14*	712.2± 14	679.6± 15*	676.2± 12*	712.4± 13	657.2± 16*	632.4± 17*	722.2± 11	680.4± 9*	663.9± 7*
Marrow Area (cm^2)	7.9±0.4	8.3±0.6	8.6±0.5	7.8±0.3	8.2±0.2	8.3±0.2	7.8±0.4	8.6±0.6	9.4±0.6	8.0±0.4	8.7±0.3	8.8±0.4
Total Area (cm^2)	15.1±0.5	15.4±0.8	15.6±0.6	15.1±0.4	15.3±0.3	15.3±0.3	15.1±0.6	15.6±0.8	16.3±0.9	15.7±0.5	15.8±0.3	15.8±0.4
Cortical Area (cm^2)	6.3 ±0.2	6.1±0.2	6.1±0.2	6.3±0.2	6.1±0.1	6.1±0.1	6.2±0.2	6.0±0.3	6.2±0.2	6.4±0.3	6.2±0.2	6.1±0.2
Tibia Diaphysis												
Cortical vBMD(mg/cm^3)	1334±3	1344±3	1360±4#	1333±5	1340±5	1357±4#	1341±3	1342±6	1357±6	1332±5	1342±5	1361±4#
Cortical Area (cm^2)	5.0±0.09	5.0±0.10	5.0±0.10	5.1±0.09	5.1±0.07	5.0±0.06	5.2±0.13	5.2±0.13	5.2±0.13	5.4±0.10	5.4±0.06	5.2±0.09
Total Area (cm^2)	5.8±0.11	5.8±0.11	5.7±0.10	5.8±0.10	5.8±0.10	5.7±0.10	6.0±0.15	6.0±0.17	6.0±0.20	6.1±0.15	6.1±0.10	5.9±0.11

CON: ad-lib fed, ER: energy restriction, VEH: vehicle administration, BB: beta blocker administration, respectively. For all experimental groups, n=10, except for CONVEH, in which n=9.
*$p<0.05$ vs wk 0 within group, # $p<0.001$ vs wk 0 within group

Proximal tibia cancellous bone histomorphometry

The decrease in % MS/BS observed in ERVEH rats vs CONVEH rats was abolished in ERBB rats (Fig 3.4A). The MAR observed in ERVEH was significantly lower, but the MAR observed in ERBB rats was not significantly lower than that of CONVEH rats (Fig 3.4B). These decreases in MAR and %MS/BS contributed to the 79% reduction in BFR (Fig 3.4C) in ERVEH rats; this reduction in BFR with energy restriction was significantly attenuated in ERBB rats. The reduction in % osteoid surface vs CONVEH was observed both in ERVEH and ERBB rats (Fig 3.4D). The 2-fold increase in % resorbing surface (the surface covered by osteoclasts) observed in ERVEH rats was abolished in ERBB rats (Fig 3.4E). Percent BV/TV in ERVEH rats tended to be lower than in ERBB rats (Fig 3.4F). No significant differences in any histomorphometric values were observed with beta blocker treatment in CON rats (vs CONVEH).

Immunohistochemistry

Reduction in leptin expression in bone marrow adipocytes observed in ERVEH rats was attenuated in ERBB rats (Fig 3.5). Reduction in the number of cells (bone lining cells, osteocytes and chondrocytes in cartilage) staining positive for leptin observed in ERVEH rats was also attenuated in ERBB rats. No background staining was observed in negative control studies when primary antibody was omitted.

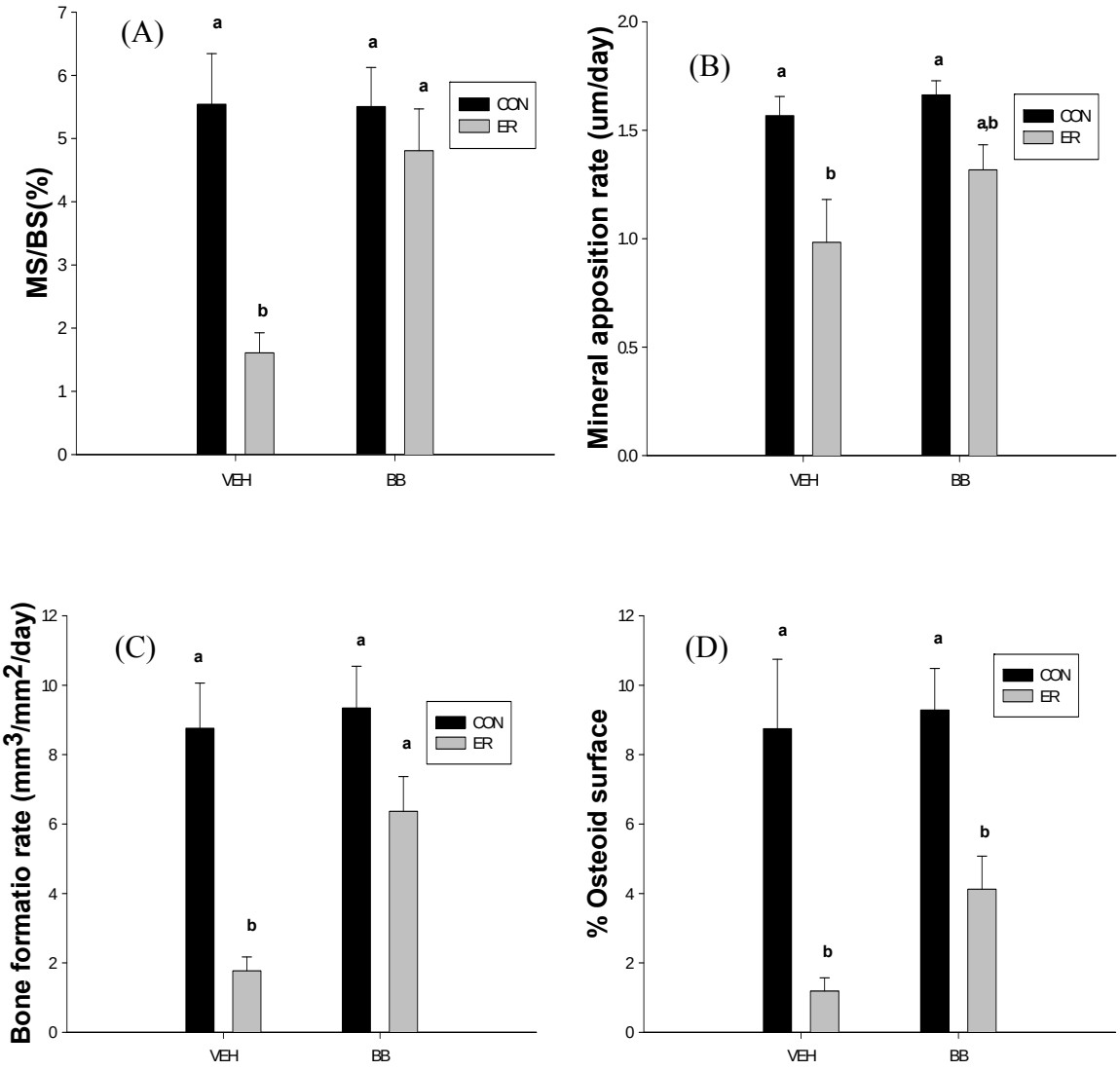


Fig 3.4 Proximal tibia histomorphometric indicators
Effect of vehicle (VEH), beta blocker (BB) during energy restriction (ER) or ad-lib fed (CON) rats on proximal tibia **(A)** mineral apposition rate (MAR), **(B)** % mineralizing surface (%MS/BS), **(C)** bone formation rate (BFR), **(D)** % Osteoid surface, **(E)** % osteoclast surface and **(F)** % bone volume/tissue volume (% BV/TV). Values are means ± SE. For all experiment groups, n=10 except CONVEH, ERVEH, in which n=9. Bar sharing same letters are not significantly different.

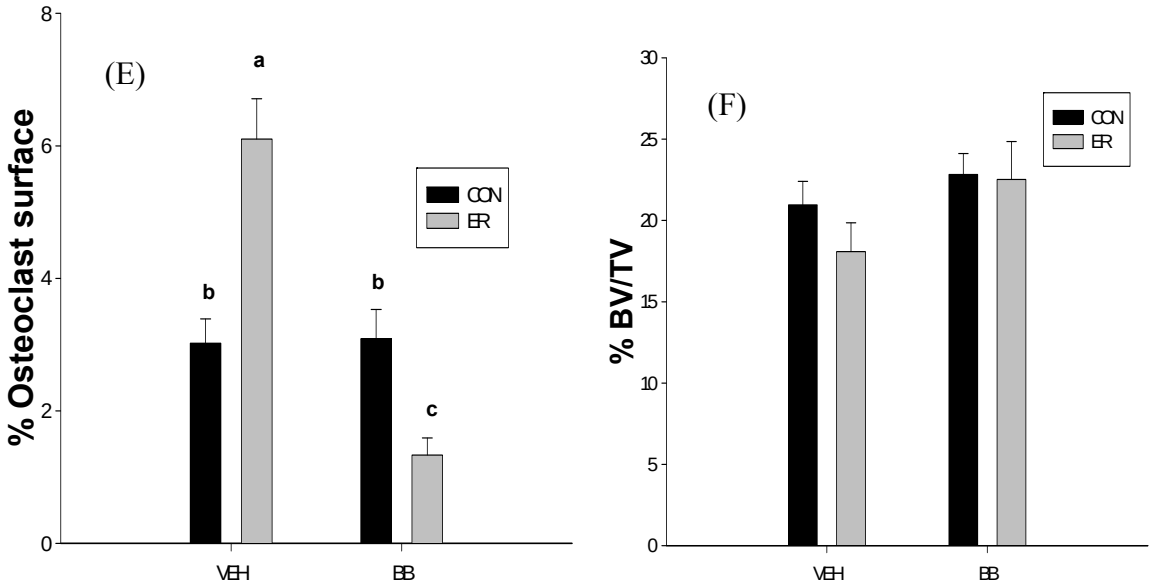


Fig 3.4 Continued

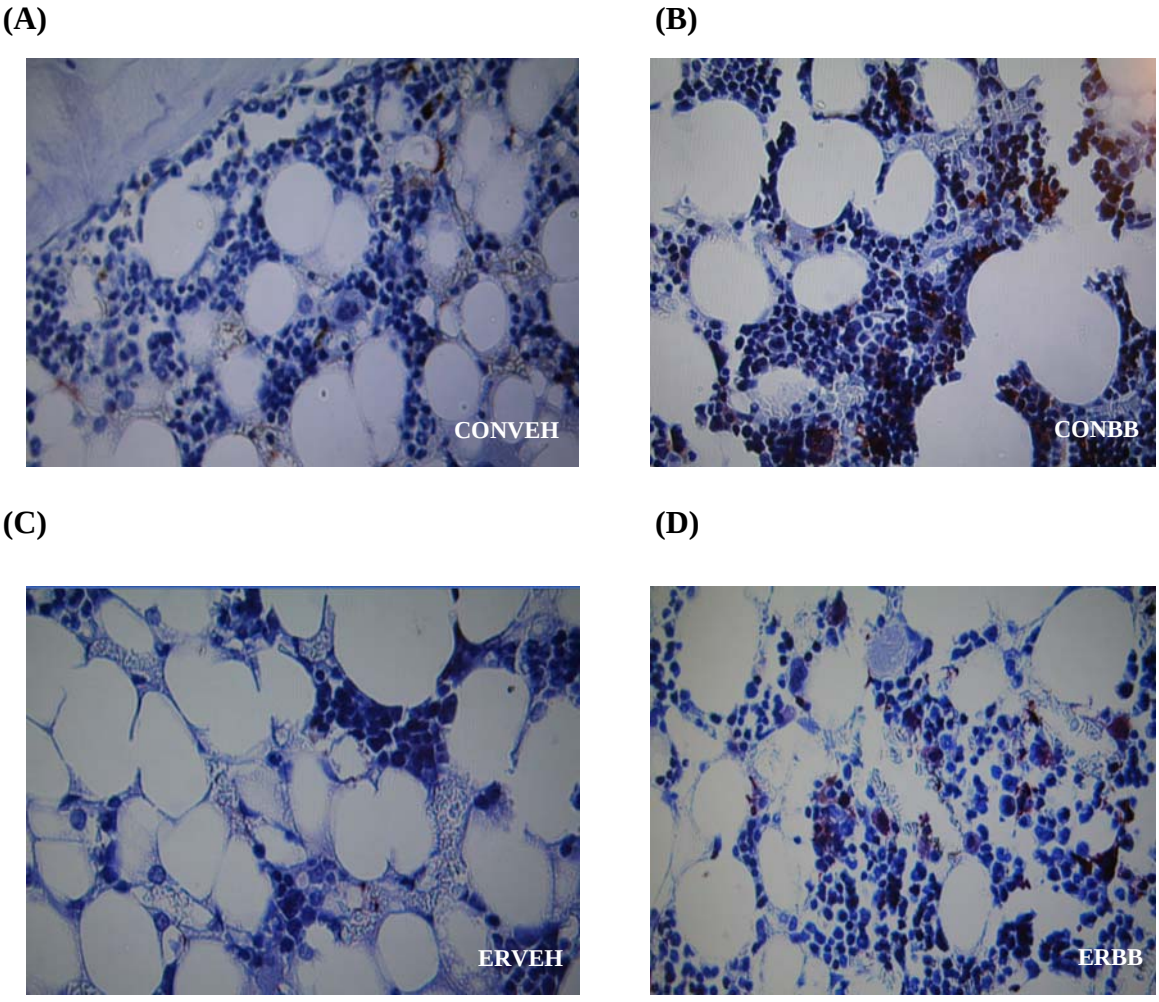
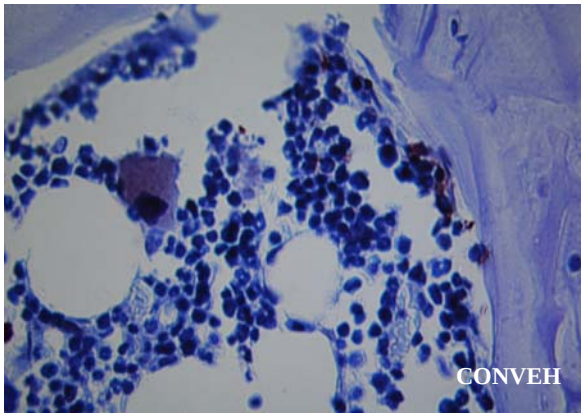
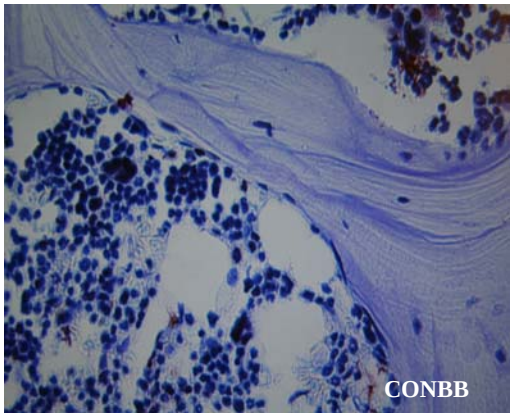


Fig 3.5
Adipocytes positive for leptin expression (red staining) in the marrow space of femur in CONVEH**(A),** CONBB**(B),** ERVEH**(C)** and ERBB**(D)**
Bone lining cells positive for leptin expression in femur CONVEH**(E),** CONBB**(F),** ERVEH**(G)** and ERBB**(H).** Magnification: x 400.

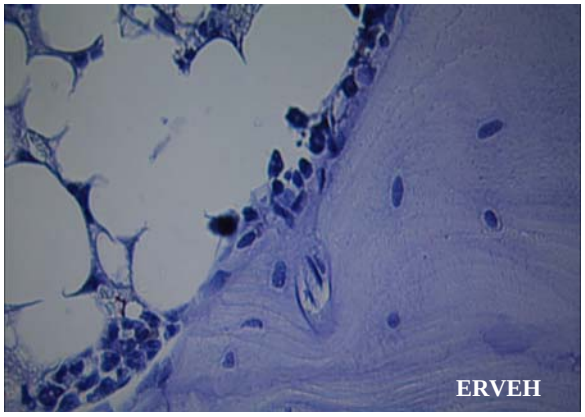
(E)



(F)



(G)



(H)

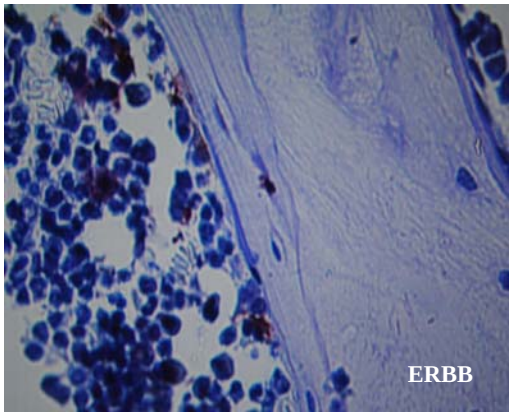


Fig 3.5 Continued

Discussion

These experiments extend upon previous work demonstrating interesting links between the sympathetic nervous system, regulation of serum leptin and/or bone mass. The primary hypotheses of our study were that beta-adrenergic blockade would attenuate the decrease of serum leptin and bone mineral density during energy restriction and that rescued serum leptin would result in more leptin localizing to the critical bone cells responsible for bone formation.

Our data demonstrate that beta- adrenergic blockade significantly mitigate the reduction in serum leptin during energy restriction in adult rats. Reductions in cancellous vBMD and bone formation rate in proximal tibia during restricted calorie intake was also significantly mitigated by beta-adrenergic blockade. No investigation to our knowledge has demonstrated the effect of beta blockade on bone mass with energy restriction. Furthermore, no study has demonstrated the effect of beta blockade on leptin expression and serum leptin level and its correlation with bone cell activity or bone formation rate with energy restriction.

In the present study, the reduction in cancellous vBMD in proximal tibia with 40% energy restriction was significantly attenuated by beta blockade. There exist literatures examining the effect of peripheral beta blockade on bone in the rats under the circumstances causing bone loss. We recently demonstrated that beta blocker modulates both resorption and formation while attenuating disuse-induced bone loss, which is

almost as effective as providing a leptin analog in tail-suspended male rats (27). Exposing animals to stressful conditions such as tail suspension could increase heart rate and blood pressure through activation of the sympathetic nervous system (23,24) and elicit the release of the catecholamines norepinephrine (25). It's been shown that noradrenaline release is increased with food restriction (21) which is associated with increased bone resorption or decreased bone formation.

Attenuated loss of vBMD by beta blockade may result from suppressed resorption or attenuated decrease of formation or both, so we examined histomorphometric measures of formation and resorption. The 71% decreases in mineralizing surface (%MS/BS), the 37% decrease in mineral apposition rate and the 79% decreases in bone formation rate during ER were successfully attenuated by beta blockade (-13%,-16% and -27%, respectively). Percent MS/BS indicates osteoblasts recruitments and MAR indicates the vigor of individual osteoblast teams. Reduction in bone formation rate was more related to reduction in % MS/BS rather than to mineralizing surface, suggesting that the change in osteoblast recruitment rather than osteoblast cell activity contributed more to the reduction in bone formation rate during ER. The dramatic increase of bone resorbing surface during ER was abolished by beta blocker treatment. By this result, we conjecture that rescued serum leptin level due to beta blockade would prevent the transient increase in osteoclast number with energy restriction. Regulation of osteoclast number by letpin or beta blocker could be mediated by the receptor activator of nuclear factor kappaB-ligand (RANKL)/osteoprotegerin (OPG) pathway, and Martin et al. showed in their

study by using RT-PCR that a suspension-induced increase in RANKL gene expression in proximal tibia, which was counterbalanced by leptin administration with a similar 3-fold increase in OPG expression and a RANKL to OPG ratio close to nonsuspended conditions (19). It would be desirable to verify this study model, which is beta blockade with energy restriction, in the future.

Even though serum leptin level in humans and rodents are mainly correlated with fat mass, there are several factors affecting circulating leptin levels independently of alterations in adipocity. Studies in humans and rodent model demonstrate that stimulation of sympathetic nervous system and activation of beta-adrenergic receptors decrease serum leptin levels (233). Activation of the beta-adrenergic receptors, combined with a decrease in insulin level, is thought to be responsible for the decrease in leptin levels with fasting (17).

Besides fasting, long-term food restriction (from 50% to 80% of ad libitum energy intake) in rats results in a substantial fall in serum leptin concentration and in white adipose leptin mRNA levels (151). In the present study, there was no difference in gram body fat at week 12 in ERBB and ERVEH rats. However, serum leptin level normalized by body fat tended to be greater in beta-blockade treated ER rats than that in ERVEH rats at the end of the study, although this comparison did not reach statistical significance. This observation suggests that the level of leptin synthesis or release per unit adipocyte was greater when beta-adrenergic signaling is blocked in energy restricted

rats. The exact mechanism for beta blockade rescue of leptin synthesis or release has not been fully investigated. There is agreement that elevated cyclic AMP inhibits leptin release by adipose tissue (21, 234). Stimulated adenylyl cyclase and subsequent PKA phosphorylation is thought to be the regulating mechanism of leptin release from adipose tissue. Measurement of adipocyte cyclic AMP level and/or adenylyl cyclase activity in beta blockade treated animals could help define the mechanism underlying the rescue of leptin release by beta blockade during energy restriction.

Although we confirmed that beta blockade attenuated the reduction in serum leptin, and bone mass in energy restricted rats, we cannot overlook the possibility that these effects may be due to blocking beta-adrenergic input to osteoblasts. Takeda et al. confirmed that osteoblasts express β-adrenergic receptors (18). To address bone formation is regulated by the SNS, Wildtype and overiectomized mice given a β-adrenergic antagonist experienced an increase in bone mass. Sympathetic activation reduces osteoblast proliferation (18) and it stimulates bone resorption by increasing RANKL expression (235). Beta-adrenergic blockade blunts the inhibition of alkaline phosphatase activity by isoproterenol (a beta agonist) in an osteoblast-like cell line, which suggests that propranolol may enhance bone formation by preserving osteoblastic activity in the face of beta adrenergic receptor stimulation (120). We speculate that the positive effects of beta-blockade on mitigating bone loss with ER may derive from the synergistic effects of these two mechanistic pathways, that is, one from the direct effect of beta blockade on

osteoblast and the other from the indirect effect of beta blockade on leptin release from adipocytes, resulting in rescue of suppressed leptin synthesis or leptin release.

White adipose tissue is the primary site of leptin synthesis and secretory regulation, but recent studies show that leptin is produced in placenta, skeletal muscle, fetal bone/cartilage and primary cultures of human osteoblasts (140-143). Our immunostaining results suggest greater leptin expression in bone marrow adipocytes in groups showing higher serum leptin level and higher cancellous vBMD. In the same groups, more bone lining cells and osteocytes also stained positive for leptin.The hypothesis that beta blockade would positively affect the leptin synthesis and/or release is supported by the mitigated reduction in serum leptin with energy restriction in the present study. But we cannot affirm the leptin-positive staining observed in osteocytes, and bone lining cells are the result of localization of leptin derived from the marrow adipocytes or derived from the other peripheral adipose tissue or the result of leptin synthesized and secreted from the cell itself. Further investigation on the effect of beta blockade on expression, secretion and autocrine signaling (if exists) of leptin among bone cells is justified.

The beta blocker dose used in this study was 6mg/ kg•d and was achieved by oral drinking throughout 24 hours. It has been previously demonstrated that 0.1 or 5 mg/kg of propranolol injection (5 days/week) prevents the deterioration of cancellous bone due to the different physiological challenge of estrogen deficiency (ovariectomy) (226) but

the high-dose treatments (20-mg) did not. Interestingly, the lower dose was more effective in attenuating the increase of ostoclastic cells after ovariectomy. When beta blockade is administered by oral drinking (0.5 g/L) in estrogen deficient (by ovariectomy) mice, loss of total body bone mineral density is attenuated and osteoblast number and mineralizing surface is increased on cancellous bone.

The contradictory results reported in the human literature regarding to the relationship between the beta blockade and bone (236-238) might be explained by different doses utilized. More study is needed to determine the adequate dose of beta-blocker in various physiological conditions causing bone loss.

Importantly, in the present study, no significant differences in any outcome measures were observed with beta blockade in ad lib fed rats, suggesting that beta blockade and leptin exert their bone protective effects in the context of energy restriction and/or serum leptin deficiency, but not in the energy-balanced state. We tested the correlation between final serum leptin level and bone formation rate, which convinced the rescued serum leptin plays a role in mitigating reduction in BFR during energy restriction. Interestingly, the bone formation rates vary a great deal in very high serum leptin values (mostly seen in CONBB rats) are scattered, whereas low and mid level of serum leptin values correlate relatively well with low bone formation rate. This result suggests that there may exist a limit of level for circulating leptin exerts beneficial effect on bone.

In summary, 40% caloric restriction over 12 weeks caused bone loss at the proximal tibia and serum leptin reduction. Beta- adrenergic blockade mitigated the energy restriction induced cancellous bone loss via attenuating reduction in osteoblastic cell recruitment and activity and increase in osteoblastic cell recruitment. Reduction in serum leptin with energy restriction was mitigated by beta- adrenergic blockade, and change of serum leptin level was correlated with the bone formation rate. Reduction in leptin expression in bone marrow adipocytes observed with energy restriction was attenuated by beta-adrenergic blockade. Reduction in the number of bone lining cells and osteocytes stained positive for leptin was also attenuated by beta-blockade. We conclude that beta blockade effects on preventing the bone loss during energy restriction. Also beta blockade is associated with mitigating reduction in serum leptin, subsequently with mitigating reduction in bone mass by normalized leptin's localization to the bone cells. These results may have serious implications for those individuals who utilize energy restriction in attempts to lose weight with usage of beta blocker in order to treat hypertension or other cardiovascular disease.

CHAPTER VI

CONCLUSION AND FUTURE RESEARCH

The ultimate goals of these projects was to identify the relationship between activation of the sympathetic nervous system, altered circulating levels of leptin and decrements in markers of bone integrity during stressful condition such as mechanical unloading or energy restriction (ER). Beta adrenergic blockade was used to determine the association between mitigated reductions in serum leptin and subsequent alterations in the reductions in bone mass observed with mechanical unloading or ER. To do so, serum leptin, bone mineral density and geometry, formation and resorption parameters were documented after subjecting adult rats to hindlimg unloading and food restriction. Then, hindlimb unloaded rats or energy-restricted rats were treated with propanolol to elucidate if beta blockade attenuates bone loss during unloading or energy restriction and whether such an effect is associated with changes in serum leptin level. Leptin localization to the important cells for bone formation andresorption was also quantified.

These data document that 1) serum leptin level was reduced by unloading and by global food restriction, and was associated with the decline in bone formation rate; 2) beta-blockade mitigated unloading-induced reductions in serum leptin and was as effective as leptin administration in mitigating reduction in cancellous bone mineral density with unloading through both increased bone formation and suppressed bone resorption; and 3)

beta blockade attenuated the reduction in serum leptin, cancellous bone mineral density and bone formation rate observed with reduced energy intake and also abolished the induced increase in bone resorption; 4) the reduction in leptin expression in bone marrow adipocytes observed with ER was attenuated by beta-blockade, as was the reduction in the number of bone lining cells, osteocytes and chondrocytes which stained positive for leptin.

We conclude that bone loss during spaceflight could be aggravated by consistent reductions in food intake as is frequently observed during short-term shuttle missions. The importance of avoiding voluntary food restriction should be emphasized to astronauts or bed rest patients, because the bone loss which already occurs with disuse could be exacerbated by reduced serum leptin due to the energy restriction. We also conclude that sympathetic nervous system activation contributes to the bone loss observed during stressful condition such as spaceflight or energy restriction. Beta blockade is associated with mitigated reductions in serum leptin, bone mass, perhaps by restoring leptin's localization and signaling to important bone cell populations. These data illustrate the important role of "peripheral" leptin mechanisms in regulation of bone mass during energy restriction, as opposed to "central" pathways operating solely via the hypothalamus. Also, these data are the first to illustrate an association between beta-adrenergic signaling and leptin pathways in regulating bone cell activity in the context of energy restriction. Many adults in developed countries suffer from both cardiovascular disease and osteoporosis. The potential dual effect of beta blocker treatment on both

heart and skeletal system could be an important consideration. But recent human epidemiological studies contain contradictory results. One study showed use of beta blockers is associated with a 30% decrease in fracture risk (236), but other study showed that the use of beta blockers did not present any link to bone mineral density (238). In-depth study regarding the proper prescription of propranolol for bone treatment must be preceded.

While these results provide support for a mechanistic role of leptin in preventing bone loss during energy restriction or mechanical unloading, many questions remain. We observed that the dramatic increase of bone resorption surface during mechanical unloading or energy restriction was abolished by beta blocker treatment, but the underlying mechanism has not been demonstrated. Additional techniques could be used to investigate if regulation of osteoclast number by leptin or, more indirectly by beta blockade is mediated by the receptor activator of nuclear factor kappaB-ligand (RANKL)/osteoprotegerin (OPG) pathway. This could be accomplished by quantifying RANKL/OPG gene expression with real time PCR or RANKL/OPG expression with immuno-histostaining methods. Also, as previously discussed, the effects of altered sympathetic nervous system signaling on bone vary throughout the skeleton according to local factors. Besides limb bones that used in this study, the same investigation could be performed with the vertebral bones. These studies could provide data on whether there is a different response in axial bone and appendicular bone to the blockade of beta-

adrenergic signaling or leptin treatment during mechanical unloading or energy restriction.

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APPENDIX

IMMUNOHISTOCHEMICAL ANALYSIS OF ANTIGEN EXPRESSION USING HISTOLOGICAL SECTIONS OF PARAFFIN-EMBEDDED BONE SAMPLES

Necropsy

Immediately after tissue harvest, put bones in 4% paraformaldehyde/0.02 M sodium phosphate buffer. Store in room temperature for less than 24 hours.

Demineralization (decalcification) of bone with formic acid/sodium citrate solution.

A) Prepare a 50% formic acid/distilled water decoction:

B) Prepare a 20% sodium citrate/distilled water decoction:

C) Mix solution (A) and solution (B) together 1:1 ratio.
Put bone samples in the mixed solution and gently agitate for 2~3 days at 4ºC.
Change solution everyday.

D) Put samples in 70% ETOH and store in refrigerator before processing for embedding.

Tissue processing+ Paraffin Embedding

1. - Original fixative
2. 45 minutes 70% Alcohol 40'C
3. 45 minutes 80% Alcohol 40'C
4. 45 minutes 95% Alcohol 40'C
5. 45 minutes 100% Alcohol 40'C
6. 60 minutes 100% Alcohol 40'C
7. 60 minutes 100% Alcohol 40'C
8. 60 minutes Clearing Reagent (xylene) 40'C
9. 60 minutes Clearing Reagent (xylene) 40'C
10. 60 minutes Paraffin 1 58'C
11. 60 minutes Paraffin 2 58'C

12. 60 minutes Paraffin 3 58’

Immunohistochemistry Day 1

Melt Paraffin on Slides
Place slides in glass holders (empty bottom) and put in a 56-58 degree C. oven for 10-15 minutes.

1. *Deparaffinize slides*

Quench the slides in the following graded series of xylenes and alcohol:

1. Xylene 5 min
2. Xylene 5 min
3. 100% ethanol 3 min
4. 100% ethanol 3 min
5. 95% ethanol 3 min
6. 70% ethanol 3 min

2. Quench sections with 0.3% H2O2 in DI water for 30 min

3. Prepare an incubation box of ~ 15 cm (width) x 30 cm (length) x 10 cm (height) dimensions. Put two 25 cm round plastic tubes at the bottom approximately a slide length apart. Secure them so the slides lie flat/perpendicular to bottom of the box when you put them in.

4. Rinse with PBS using a transfer pipette: 2 times for 5 minutes each.

5. Make “wells” around each sample with Elmer’s Rubber Cement Glue around each bone slice.

6. Permeabilize with PBS/0.5% Triton for 5 minutes at room temperature.

7. Rinse with PBS 2 times for five minutes each.

8. Avidin D solution blocking step to reduce background noise: Incubate for 15 min at room temperature. Make sure that the avidin D covers the bone sample. This chemical is supplied by Vector Laboratories in a separate Avidin D/Biotin blocking kit.

> In this step, the glycoprotein avidin will bind with extremely high affinity to endogenous biotins (a vitamin) or lectins expressed in your sample. This leads to an irreversible binding of the biotin molecules that otherwise increase non-

specific binding by interfering with the biotinylated secondary antibody action or the avidin/biotinylated horseradish peroxidase macromolecular complex.

9. Rinse slightly with PBS

10. Biotin blocking step for 15 minutes at room temperature. Make sure that the biotin covers the bone sample.

Since avidin has several binding sites for biotin, the "avidin blocking step" is followed by an incubation period with biotin itself. This will irreversibly occupy *all* biotin binding sites on the avidin molecule so when the secondary biotinylated anti-body is added it will not be subjected to non-specific binding to the avidin blocking solution. In addition, the added biotins appear not to be subject to binding with the avidin/biotinylated horseradish peroxidase macromolecular complex [which is added last and should only bind to the biotinylated secondary anti body].

11. Rinse with PBS 3 times for 5 minutes each.

12. Normal goat serum (NGS) blocking step diluted in PBS/2% BSA buffer. Follow the vendor's protocol.

13. Rinse with PBS/2%BSA one time for 5 minutes.

14. Dilute the primary antibody (Ob (A20) SC 842) in buffer (PBS/2%BSA) to yield a concentration that is most effective for binding to the target antigen in your specific tissue

14. Apply primary antibody/buffer solution to each well i.e. ~ 300 µl/well. Make sure the bone sample is covered and that the well is not leaking. In order to ensure method specificity you must have a "negative control" that does not receive the primary antibody.

15. Incubate overnight at 4 ºC in a humidified chamber.

Immunohistochemistry Day 2

16. Rinse with PBS/2%BSA/0.2%Tween: 2 times 5 minutes each.

17. Rinse with PBS/2%BSA twice

18. Dilute the biotinylated secondary antibody [anti-rabbit IgG, made in goats] to 1/200 with PBS/2%BSA/~1.5-5%NGS. In order to ensure method specificity you must have a "negative control" that does not receive the secondary antibody.

19. Prepare the VECTASTAIN ABC reagent and keep in dark for 30 minutes at room temperature.

20. Rinse with PBS/2%BSA one time for 5 minutes.

21. Rinse with PBS three times for 5 minutes each.

22. Add ABC reagent and incubate in the dark for 30 minutes at room temperature. Put the lid on the box again.

23. Rinse with PBS 3 times 3 minutes each.

24. Peroxidase activity must now be revealed by adding NOVA RED
Develop with freshly prepared NOVA RED *for a minimum of 5 minutes*. The bone samples should turn pinkish/reddish soon after adding this stain.

Deionized water may inhibit peroxidase reaction! Only use distilled (DI) water.

25. Add tap water to stop developing the peroxidase activity. Rinse slides for 5 minutes using tap water [at room temperature].

26. Counterstain with hematoxylin for 1 min at room temperature.

27. Rinse slides with tap water for 15 minutes. Remove Elmer's wells with an 18-gauge needle in preparation for the last quenching step.

28. Deshydride slides with increasing grades of alcohol and two xylene (this stops potential sample shrinkage).

1. 70% ethanol 3 min
2. 95% ethanol 3 min
3. 100% ethanol 3 min
4. 100% ethanol 3 min
5. Xylene 5 min
6. Xylene 5 min

29. Coverslip one slide at a time with Eukitt glue. If the glue is too solid, soften it by adding xylenes.

VITA

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EDUCATION

Seoul National University	B.S.	Aug 2000	Food and Nutrition
Texas A&M University	Ph.D	Dec 2007	Nutrition

HONORS AND AWARDS

2001 Regents Fellowship, Texas A&M University

2003 TACSM, Student Research Presentation, Master's category, First Place,

2003 ODS Scholar Travel Award, Vitamin D & Bone Health in the 21st. Century, NIH

2004 Young Investigator Travel Award, Endocrinology & Metabolism section of American Physiology Society. (Experimental Biology meeting)

2005 Alice L. Jee Memorial Young Investigator award, 35th International Sun Valley Workshop on Skeletal Tissue Biology

2005 Plenary poster presentation, 27th American Society of Bone and Mineral Research

2007 Oral presentation at the 29th American Society of Bone and Mineral Research

PUBLICATION

Baek K, Miller S, Lemmon J, Nilsson M, and Bloomfield SA (2006) Energy and calcium deficits are major contributors to effect of global food restriction on metaphyseal bone in exercising rodents. *J Bone Miner Res.* Vol 21 (Suppl 1): M359

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