CENTRALLY ADMINISTERED GUT HORMONES AND LEPTIN ON FOOD INTAKE, BONE AND BONE MARROW ADIPOCYTES IN RATS

by

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(Under the Direction of Dr. Clifton A. Baile)

ABSTRACT

The discovery of the role of leptin on bone formation through the central nervous system marks the beginning of a new era for bone research. Several gut hormones play a role in mediating postprandial bone metabolism. Among them, GIP, GLP, and ghrelin are known to have either direct or indirect effects on bone formation. However, the idea that gut hormones binding to their receptors in the brain modulate bone metabolism has not been tested before. To investigate whether gut hormones modulate bone metabolism centrally we employed the techniques of densitometry, histomophormetry, measuring of serum bone turnover markers, as well as real time RT-PCR. The hormones we tested included leptin, GIP and ghrelin. In the first study, we found that centrally administered GIP (10 μg / inj.) decreased food intake and body weight while also affecting feeding behavior although there was no expected bone effect. Leptin treatment decreased rat tibia bone marrow size and number, and increased spinal bone mineral density. In the second study, ICV ghrelin (G20, 20 pmol / inj.) increased tibia bone mineral density. Leptin increased bone mineral density while decreasing bone marrow adipocytes size and number. The third study was designed to confirm the previous study, and to explain the underlying mechanism by measuring and comparing

the expression level of a set of pre-selected gene markers. Tibial bone mineral density was increased by leptin and G20, while spinal BMD was only increased by leptin. Gene expression data from non-adherent bone marrow cell cultures show that G20 increased gene expression of apoptosis marker, CASP2. Ghrelin also resulted in decreased adipogenesis gene expression in bone marrow. Thus, we suggest that centrally administered ghrelin resulted in increased bone mineral density by decreasing osteoclastogenesis through apoptosis, as well as by reducing bone marrow adipogenesis. In summary, gut hormones affect bone marrow adipocytes and bone metabolism when binding to receptors in the brain. Histomophormetry, densitometry, as well as measuring mRNA expression levels employing microfluidic technology provide sensitive, convenient and effective ways of measuring changes in bone marrow adipocytes and bone mass, and for developing new therapeutic strategies for treatment of osteoporosis.

INDEX WORDS: Bone, bone marrow, Real time PCR, Leptin, GIP, Ghrelin, ICV, Gene expression, Bone turnover, Histomorphometry, Densitometry

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DEDICATION

I dedicated this thesis to my beloved parents. I want you to be proud of me. Although I could not be at your side taking care of you as I would want, what I could do is to take care of myself to let you feel I am ok when away from you and feel alone in the outside world, and I will try my best to do good work that will make you feel proud. This has been and will continue to give me endless energy in my academic endeavor. I want to thank you for your love and support, without which all of my accomplishments would be impossible.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

The human bone is not an inert tissue as it may appear at first glance. Instead, an ongoing process known as bone remodeling functions in the constant renewal of bone tissue and the making of reservoirs of ions for body metabolism. Bone remodeling occurs simultaneously in all regions of the body and continuously throughout an individual's lifespan. The process involves a tightly coupled relationship between osteoblasts, bone-forming cells, and osteoclasts, bone-absorbing cells. However, as life approaches its fifth decade, an imbalance occur accompanying gonadal failure that will lead to the outpacing of osteoblasts by osteoclasts. This will result in more bone destruction and formation of less new bone. The net result is a steady loss of bone, which is called osteoporosis. (This will lead to more bone destruction and less bone formation. These changes result in further net loss of bone and osteoporosis)

The situation of osteoporosis is negatively influenced by several conditions such as stress, obesity, diabetes, alcohol and drug abuse, and smoking as well as glucocorticoids. Osteoporosis becomes increasingly prevalent in the United States today.

The discovery of the role of leptin in bone formation through the central nervous system marks the beginning of a new era of bone research (Karsenty 2001; Takeda, Elefteriou et al. 2002). In addition to regulation by the paracrine, autocrine, and endocrine mechanisms, the brain also communicates with bone. This so-called neural-osseous mechanism has become a novel regulatory mechanism for bone remodeling. Since leptin is a CNS negative regulator, it is very possible there

may also be positive regulators. New discoveries surrounding leptin and central bone regulators will provide insights for understanding the regulation of bone metabolism and opportunities for treatment of osteoporosis.

Several gut hormones are known to play a role in mediating postprandial bone metabolism. Among them, amylin, insulin, GIP, and GLP, have been demonstrated to have either direct or indirect effects on bone formation. The research on the effects of incretion culminated in the discovery of the role GIP plays in bone synthesis (Bollag, Zhong et al. 2001; Xie, Cheng et al. 2005). There is also a crosstalk between the GI tract and bone, in which the hormone acts as a messenger, indicating the availability of nutrients for bone formation. This novel "entero-osseous" route raises possibilities for developing new therapeutic strategies for osteoporosis.

Ghrelin, as a hunger hormone, is the only hormone secreted after a meal which has been shown to increase food intake either peripheral or centrally. The finding of its effect on bone cell culture (Kim, Her et al. 2005) and i.p. injections in SD and SDR rats (Fukushima, Hanada et al. 2005), as well as its opposing function to CNS leptin led to speculation that ghrelin was the second hormone after leptin that may exert an effect on bones.

Literature Review

Bone physiology

1. Bone functions and bone cells

Bone is very important connective tissue, serving protective, mechanical and metabolic functions. Its main constituents, like other connective tissue, are matrices and cells. The former is composed of mainly collagen fiber and non-collagen fiber (NCP), while the latter is composed of three types of bone cells: osteocytes, osteoblasts and osteoclasts.

Osteoblasts (OB) are the bone-lining cells responsible for bone formation. They originate from the local stromal cells under the influence of local growth factors such as FGF, PDGF and BMP, and require transcription factors such as runx2 and osterix (osx). They further differentiate into pre-OB, then mature OB, followed by a mineralization process. OB will then transform either into bone-lining cells, or will become trapped in matrix and calcify to form osteocytes.

The appearance of OB is a cluster of cuboidal cells lining the bone surface, with a round nuclear base, basophilic cytoplasm and a prominent Golgi complex. OB lines the osteoid, which is bone matrix prior to mineralization. The cell membrane of OB is rich in alkaline phosphatase (the concentration of which is an index for level of bone formation). It also has receptors for PTH, steroid-like hormones, vitamin D3, and various cytokines. It also expresses an array of cytokines such as RANKL and CSF-1, both of which have been shown to serve important functions during osteoclast (OC) differentiation (Yasuda, Shima et al. 1998). Additionally, OB expresses osteoprotegerin (OPG), a decoy receptor for RANKL, with antiosteoclastogenic effects by affecting the interaction of RANK with RANKL (Liu, Ji et al. 2003).

Osteoclasts (OC) are the bone cells responsible for bone resorption. OC are multinucleated cells, with 4-30 nuclei. They situate on the calcified bone often in a lacuna with foamy cytoplasm. The zone in direct contact with bone has the unique characteristics of a ruffled border. Calcitonin as well as RANK receptors are found in the membranes of OC (Samura, Wada et al. 2000). The OC will eventually undergo apoptosis.

Osteocytes originate from bone forming cells (osteoblasts), which become trapped in the bone matrix that they produce and later become calcified. Osteocytes are found embedded deep within the small osteocytic lacunae. However, they are not metabolically inert. Between the osteocyte plasma membrane and the bone matrix, the space is filled with extracellular fluid (ECF). Osteocytes play an important role as a mechanosensor and in local activation of bone turnover. Due to the challenges of studying this cell, the effects of osteocytes on bone metabolism remain largely unknown.

2. Bone Remodeling

Bone remodeling is involved in growth and development in young animals and in bone turnover in adult skeletons. The process does not occur randomly on bone surface; instead, it occurs either in normal growth or during bone turnover. Actually, bone formation occurs only where bone resorption has just been completed. Bone remodeling is a tightly coupled process between the two bone cells: osteoblasts and osteoclasts. It occurs at focal and discrete packets throughout the entire bone, with the whole process lasting three to four months. The sequence always starts with osteoclast activation and absorption of bone followed by osteoblast activation and repairing of the defects.

Young people experiences more bone formation than bone absorption. As a result, bone is elongated and more bone mineral is deposited. By about the fifth decade of life, the balance of bone remodeling is shifted toward more bone resorption, consequently, a net loss of bone occurs (Anderson 1997).

Bone remodeling is important not only in development, but also in the repairing process and in replacing old bone with new. In the past, the regulation of bone remodeling was thought to be a local process in which osteoclasts were directed by osteoblasts. This local mechanism does exist. Research culminated in finding of OPG and RANKL, a cytokine secreted by osteoblasts (Liu, Ji et al. 2003). OPG is a decoy receptor for RANKL with antiosteoclastogenic effect that affects the interaction of RANK with RANKL. Over-expression of OPG leads to osteopetrosis (HBM phenotype) (Nakamichi and Udagawa 2006), while deletion of OPG results in increased bone remodeling and osteoporosis (Nakamichi and Udagawa 2006). Thus, by adjusting the expression of RANKL and OPG, and the activation state of RANK on OC, the body can manage the positive and negative influence over bone absorption and bone mineral density.

However, this is not a tightly coupled process. Either bone absorption or bone formation can happen without the presence of the other (Felix, Hofstetter et al. 1996; Corral, Amling et al. 1998). This suggests that a systemic mechanism exists to regulate the process. In fact, certain hormones, such as PTH, calcitonin and sex steroids have been shown to affect bone cell activity. Local cytokines such as OPG and RANKL can also act in a systemic manner in addition to their well-known paracrine and autocrine effects.

The recent discovery of the effects of leptin on bone through the sympathetic nervous system has sparked a new wave of research concerning the regulation of bone (Ducy, Amling et al. 2000).

3. Brain and bone communication

Although no classical neural synapses are found within bone, neurotransmitters are found in close spatial proximity to bone cells, and they have receptors on bone cells. More importantly, administration of those neural ligands induces potent responses in the bone cells. The following neural ligands have been found within bone: catecholamine, CGRP, VIP, substance P, glutamine and calcitonin (Mason, Suva et al. 1997).

Leptin is a cytokine secreted from white adipose tissue (WAT) in proportion to body fat mass. Leptin plays important roles in regulating of food intake and energy expenditure within the central nervous system (Hukshorn and Saris 2004). Moreover, several clinical observations suggest that leptin is important in the modulation of bone metabolism. For example, osteoporosis is always accompanied by hypogonadoism, and obesity provides some protection against it. This indicates that body weight, reproduction and bone metabolism may be under the control of the same factor (Harvey and Ashford 2003). The best candidate for this role appears to be leptin.

Takeda et al found that the sympathetic nervous system mediates the suppressive effect of leptin on bone formation (Takeda, Elefteriou et al. 2002). Beta-2 adrenergic agonist has been known to decrease bone formation. SNS stimulation will lead to noradrenalin release from sympathetic nerve fibers in the bone tissue, the noradrenalin will then bind on the beta-2 adrenergic receptor on osteoblasts and inhibit bone formation. DBH mice are mouse model deficient in the key enzyme responsible for synthesis of noradrenalin. DBH mice seem to have a higher bone mass. Central

leptin effect on bone has been abolished in this mouse, suggesting that noradrenalin works downstream of leptin in mediating the central bone effect of leptin (Takeda, Elefteriou et al. 2002).

Recently, Baldock et al found that the activation of the NPY Y2 receptor in the hypothalamus by its ligand represses bone formation (Baldock, Sainsbury et al. 2002). These authors, for the first time, found that NPY R2 receptor-deficient mice exhibit a two-fold increase in trabecular bone volume, number and thickness. They also found that mature selective hypothalamic NPY R2 receptor knock out mice have a similar increase in those parameters within five weeks. They concluded that the NPY Y 2 receptor in the hypothalamus plays a role in bone metabolism.

Since leptin modulates the expressing of CART in the brain, it has been hypothesized that CART is also a downstream player of leptin's effect on bone, in addition to the effect of modulating the sympathetic tone. In 2005, the Karsenty group found that CART inhibits bone absorption by modulating RANKL expression (Elefteriou, Ahn et al. 2005).

Osteoporosis

1. Epidemiology, risk factor and pathogenesis

Osteoporosis, the silent factor of bone fracture, has become increasingly more prevalent in the United States and worldwide with the aging population and growing number of people diagnosed with obesity (Anderson 1997). Osteoporosis is the major cause of fractures in women over the age of 45. The main sites of fractures include the forearm, hip, humorous, tibia and vertebrae (Anderson 1997). Today, an estimated 10 million people, mainly women, are affected by osteoporosis, and as many as 18 million are diagnosed with low bone mass.

There are two types of osteoporosis: Type I and Type II (Kanis, Melton et al. 1994). Type I osteoporosis occurs in postmenopausal women with decreased trabecular bone. Type II osteoporosis occurs in both men and women, and results in age-related bone loss in the trabecular and cortical bones. Type I osteoporosis is mainly related to estrogen deficiency. Type II osteoporosis reflects composite factors involving decreased bone turnover efficiency, calcium and phosphate nutrition, intestinal and renal handling of minerals and PTH secretion (Raisz 2005). Factors such as low peak bone mass, frequent smoking, substance abuse (such as glucocorticoid) and low physical activity have been shown to increase osteoporosis (Bauer, Browner et al. 1993).

Bone turnover and remodeling occur continuously throughout life and involve a tightly coupled process by osteoblasts and osteoclasts. Bone loss occurs in postmenopausal women because the bone turnover rate is accelerated, and the bone formation rate cannot compete with the speed of bone resorption (Raisz 2005).

Sex hormone deficiency is the major factor resulting in postmenopausal bone loss. In women with healthy bone, the bone formation by osteoblasts is normally offset by PTH and local cytokine (IL-1, 6 and TNFα) induced stimulation of osteoclast activity. With sex hormone deficiency, the osteoclast activity predominates, leading to an imbalance of bone remodeling and loss of bone (Hollo 1972; Raisz 2005).

2. Current available treatments for osteoporosis

Strategies for treating osteoporosis include the use of anti-resorptive methods, bone formation agents, or a combination of the two. There is also a novel idea of targeting bone marrow adipocyte osteoporosis for treatment of osteoporosis (Hamrick 2006).

1) Anti-resorptive:

In addition to supplemental calcium and vitamin D, therapeutic protocols aiming at decreasing bone resorption include the use of calcitonin, bisphosphates, hormone replacement therapy and selective estrogen receptor modulator (SERM). One of the SERMs, raloxifene, has estrogen-like effects on bone, with anti-estrogen effects on breast and endometrium (Pfeilschifter 2001; Legrand, Hoppe et al. 2006).

2) Bone formation agents:

Novel strategies have been suggested, based on growth factor effects involved with bone development, proliferation and differentiation. Some of these, including FGF, IGF, TGF beta, GH and BMP, have been advocated as potential treatments for osteoporosis (Reginster, Halkin et al. 1999).

3) Combination of the anti-resorptive and formation agents:

The combination strategy has gained much interest. However, many issues are raised. For example, when bisphosphates are administered simultaneously with PTH, the anabolic effects of PTH are blunted by the bisphosphates via the decreasing rate of bone turnover (Lteif and Zimmerman 1998).

3. Adipogenesis and novel treatment of osteoporosis

1) Bone marrow adipogenesis is a major factor contributing to age-related bone loss: Clinically, women with osteoporosis have much more bone marrow adiposity than women with healthy bone (Odabasi, Ozata et al. 2000). The rate of bone formation rate is inversely proportional to the number of bone marrow adipocytes (Rosen and Bouxsein 2006). Bone marrow adipogenesis

is increased with conditions favoring bone loss such as estrogen depletion, disuse etc. (Cui, Wang et al. 1997; Okazaki, Inoue et al. 2002).

In addition to the clinic observation, there are also in vitro evidence showing the reciprocal relationship between bone marrow adipogenesis and osteogenesis. Mesenchymal stem cells (MSC) within bone marrow cavity can differentiate into either adipocytes or osteoblasts (Bruder, Fink et al. 1994). Conditions that favor adipogenesis have an adverse effect on osteogenesis, since more progenitor cells are directed toward adipogenesis, and vice versa. For example, TGF beta induces osteogenesis, while at the same time inhibiting adipogenesis (Richardson, Campion et al. 1989). Adipocytes secrete osteoclastogenesis cytokines, such as IL-6. In addition, adipocyte had been shown to inhibit osteoblast activity in culture (Ahdjoudj, Lasmoles et al. 2001). Adipogenesis and fat cell hypertrophy decrease the blood supply of the bone marrow compartments, which have adverse effects on bone formation (Justesen, Stenderup et al. 2001; Verma, Rajaratnam et al. 2002).

2). Targeting bone marrow adipocyte apoptosis for treatment of osteoporosis:

Leptin treatment leads to a decrease in size and number of bone marrow adipocytes in ob/ob mice, with an accompanying increase of bone mass in the hind limbs of the mice (Hamrick, Pennington et al. 2004; Hamrick, Della-Fera et al. 2005). A recent study by Dr. Mark Hamrick (Medical College of Georgia) found that VMH administration of leptin led to depleted bone marrow adipocytes in rats, as well (Hamrick, Della Fera et al. 2007). Therefore, targeting bone marrow adipocyte apoptosis research provides an innovative strategy for treatment of osteoporosis and improving bone health.

Leptin

1. Introduction

Leptin, a cytokine secreted form white adipose tissue (WAT), in proportion to body fat mass, has emerged to play an important role in modulating of bone metabolism (Coen 2004), in addition to its role in central control of food intake and energy expenditure (Hukshorn and Saris 2004). There are clinical observations that has led to people's speculation that leptin may play a role in bone metabolism. Firstly, osteoporosis is always accompanied by hypogonadoism and obesity provide some protection (Odabasi, Ozata et al. 2000). This leads people to think that the body weight, reproduction and bone metabolism may under the control of the same factor (Harvey and Ashford 2003). At the very beginning, the best candidate that fit this role appears to be leptin.

2. The effects of fat on bone loss

Although obesity is a major risk of many disease processes, and has become a heavy burden for western society, it has at least bear one virtue, that is, to prevent of osteoporosis. Studies has shown that heavier people tend to have higher bone mass, and obese menopausal women have less bone turnover rate (Friedman and Halaas 1998). Mechanical loading clearly is a factor mediating the effect of fat on bone mass (Friedman and Halaas 1998). However, the effect still exists even at non-weight bearing skeletal site, suggesting factors other than mechanical loading are also responsible for this protective effects (Thomas 2003). Recently, leptin, the 14 KD peptide secreted from fat tissue (Cinti, Frederich et al. 1997) has emerged as the best explanation for the protective effects of fat mass on bone mineral density because serum leptin levels are directly correlated with

bone mineral density (Maffei, Halaas et al. 1995; Considine, Sinha et al. 1996). Human studies have shown that leptin treatment in girls deficient in leptin significantly decreases their body weight while increasing their bone mass (LaMarca and Volpe 2004).

3. In Vitro evidences

Using human stromal cell lines, Thomas et al and other researchers found that cell lines express both short and long forms of leptin receptors (Thomas, Gori et al. 1999; Steppan, Crawford et al. 2000; Reseland, Syversen et al. 2001). However, this may depend on the stage of differentiation, since Ducy et al failed to detect leptin receptors in mature murine osteoblasts (Ducy, Amling et al. 2000).

4. Animal model

Two studies have evaluated the effect of peripheral administration of leptin in ob/ob mice. One study showed that leptin administered peripherally stimulates bone growth, with a dramatic increase in cortical bone formation as compared with the control animals (Liu C 1997).

The other study found that intraperitoneal administration of leptin to four-week-old ob/ob mice for three weeks reversed their effect on bone and osteopenia, as measured by DXA and pQCT, despite a decrease in food intake and body weight (Steppan, Crawford et al. 2000).

In contrast to the peripheral stimulatory effect, Ducy et al found that leptin exerts a negative effect on bone through the central route (Ducy, Amling et al. 2000). They found that ob/ob mice, which are deficient in leptin, and db/db mice, which have inactive leptin receptors, have high bone mineral density in their trabecular bone, with a high bone apposition rate in these sites compared with their littermates.

They further explored this phenomenon and found that leptin exerts this negative regulation on bone through a hypothalamic relay. I.C.V. injection of leptin in ob/ob mice normalized the mice's bone mass in four weeks. Takeda et al provided further evidence to show that the central antiosteogenic action of leptin is mediated via the sympathetic nervous system (Takeda, Elefteriou et al. 2002).

5. Future directions

CART deficiency produces a low bone mass phenotype due to an increase in bone absorption (Takeda, Elefteriou et al. 2002). Leptin treatment also leads to an increase in CART expression (Takeda, Elefteriou et al. 2002). Therefore, CART, in addition to effects of the SNS branch, may be a downstream result of leptin action in the central regulation of bone (Takeda, Elefteriou et al. 2002).

Our new finding with CART deficient mice (unpublished data) (CART deficiency does not affect bone mass on femur) has led us to the following speculation: circulating leptin will have a major effect on bone formation on appendicular bones such as the limb, while central leptin and the resulting CART expression work by affecting osteoclasts (bone absorption) through mediating the expression of RANKL.

Osteoblasts and adipocytes in bone marrow share MSC as common feature. Factors directing MSC to one type of osteogenesis will decrease adipogenesis, and vice-versa. The aging process is known as the infiltration of bone marrow with more fat than found in healthy young adults (Rosen and Bouxsein 2006). Fat in marrow is detrimental to bone, not only because more MSC is directed toward adipocytes, but also because it secretes an inflammatory factor which leads to osteoclastogenesis and more bone adsorption (Hamrick, Pennington et al. 2004). Therefore, certain

factors can lead to a decrease in the number of marrow adipocytes and/or promote osteogenesis and are beneficial for healthy bone. Such factors are currently undergoing extensive research.

Our findings suggest that leptin induces marrow adipocyte apoptosis through a central mechanism and further suggest that VMH (Hamrick 2006) is the area in the brain mediating those effects on bone and marrow adipocytes. This is reasonable since marrow is richly innervated with SNS fibers, providing an obvious pathway through which the central nervous system can modulate bone marrow cell populations.

SNS receptors are known to be expressed on adipocytes, especially the beat-3 receptor, and treatments with beta agonists have led to apoptosis (Hamrick, Della Fera et al. 2007). For example, one of the beta agonists, clenbuterol, has been shown to induce bone marrow adipocyte apoptosis. Therefore, leptin offers the opportunity to target bone marrow adipocyte apoptosis for treatment of osteoporosis (Nelson-Dooley, Della-Fera et al. 2005).

GIP

1. Discovery and name

GIP (Glucose-dependent insulinotropic polypeptide) is a 42 amino acid gastrointestinal hormone secreted by the intestinal K cell (Gault, O'Harte et al. 2003). GIP was first named as gastric inhibitory peptide due to its ability to inhibit the secretion of gastric acid (Brown, Pederson et al. 1969). Following the discovery of another most important function, the incretin effect, it was later re-named as glucose-dependent insulinotropic polypeptide, with the same acronym, GIP (Dupre, Greenidge et al. 1976).

2. GIP receptor and receptor distribution

The GIP receptor is a G-protein-coupled receptor belonging to the family of secretin-VIP receptors. Small intestine and salivary glands are known to secrete GIP peptide. GIP receptors have been detected in many organs, including endothelium, heart, pancreas, gut, brain, adipose tissue, pituitary and adrenal cortex (Lacroix, Bolte et al. 1992; Reznik, Allali-Zerah et al. 1992; de Herder, Hofland et al. 1996), and bone (Bollag, Zhong et al. 2000). No receptors are present on smooth muscle and liver (Usdin, Mezey et al. 1993). The wide distribution of GIP receptor mRNA suggests multiple functions in addition to incretin effect. Recently, GIP mRNA and protein had been shown to present in the brain DG area and serve important functions in neuronal cell mitogenesis (Nyberg, Anderson et al. 2005).

3. Multiple functions of GIP

A major role of GIP is its incretin effect. Abnormalities in GIP have been implicated in the pathogenesis of diabetes mellitus type II (DM type II). Nauck et al have reported that GIP-induced insulin secretion is selectively impaired in type 2 diabetes (Nauck, Stockmann et al. 1986). Miyawaki et al (Miyawaki, Yamada et al. 1999) found that early insulin secretion after oral glucose ingestion is impaired in GIPR-deficient mice in isolated pancreatic islets. In addition, a GIP receptor antibody against the GIP-R led to an impaired insulin response to an oral glucose load despite normal GLP-1 function (Lewis, Dayanandan et al. 2000).

As a principal incretin, GIP works to stimulate insulin release, and to amplify the effect of insulin on target tissues. GIP increases the affinity of the insulin receptor for insulin as well as increasing the insulin-like effects in fat cells. Incubation of isolated adipocytes with GIP was shown

to increase receptor affinity and insulin-stimulated glucose transport (Starich, Bar et al. 1985) which may serve to increase efficiency in the accumulation of triglycerides in fat cells.

GIP also works to generate excessive fat mass through direct effect on adipocytes and inhibition of lipolysis. GIP has been shown to stimulate glucose transport in isolated rat adipocytes and the incorporation of glucose into extractable lipids in one study (Hauner, Glatting et al. 1988). GIP has also been shown to stimulate the synthesis and release of LPL in cultured mouse 3T3-Ll preadipocytes (Eckel, Fujimoto et al. 1979).

GIP has been shown to inhibit the lipolytic action of glucagon in adipocytes (Dupre, Greenidge et al. 1976), which may occur as a result of competitive inhibition of glucagon binding to its receptor.

The recent demonstration that adipocytes contain functional GIP receptors (Yip, Boylan et al. 1998), is consistent with the data presented above and suggests that GIP plays a direct role in lipid metabolism. In view of the effect of GIP on fat metabolism directly and indirectly, it has been speculated for decades that GIP might have a role in etiology of diet-induced obesity (DIO) (Brown, Dryburgh et al. 1978) (Creutzfeldt, Ebert et al. 1978).

Obesity is typical associated with hyperinsulinemia, which could be attributed to excessive GIP production and/or action. In one case, ob/ob mice chronically fed a high fat diet have been shown to have increased concentrations of GIP in plasma and in the intestinal mucosa as well as increased density of GIP-secreting K-cells in the upper jejunum (Bailey, Flatt et al. 1986)

Moreover, important studies by Miyawaki on GIP receptor knockout mice (give reference for Miyawaki) provide further proof. GIPR KO mice fed a high-fat diet were clearly protected from both obesity and insulin resistance.

It is well known that changes in the nutritional state of the organism influence skeletal homeostasis. A reduction in caloric intake rapidly inhibits linear growth (Heinrichs, Colli et al. 1997). If the reduced caloric intake is accompanied by reduced calcium intake, a shift in the balance between bone formation and resorption occurs, such that bone mass decreases over time (Kuramitsu, Matsui et al. 1985).

Nutrient-induced elevations of both insulin and amylin have been implicated in normal bone metabolism (Canalis 1983; Kream, Smith et al. 1985; Cornish, Callon et al. 1998), and the ability of GIP to enhance the secretion of both hormones may contribute to an anabolic action of GIP on bone.

The "incretin" hormone, glucose-dependent insulinotropic peptide (GIP), has recently been shown to be present on osteoblasts and osteocytes, and GIP has anabolic effects on bone and bone-derived cells (Bollag, Zhong et al. 2000; Bollag, Zhong et al. 2001). Receptors for GIP present on bone cells appear to be functional: first, in that they bind GIP with affinity within the physiological range of concentrations of GIP achieved in serum postprandially; second, the functionality of GIPR in osteoblast-derived cells was demonstrated by their ability to couple to signal transduction pathways. Ligand binding can elicit cellular responses. Treatment with GIP resulted in increased collagen type I mRNA expression and ALP activity, while collagen type I message and alkaline phosphatase activity are markers for osteoblastic differentiation.

In 2003, Zhong et al. further proved that GIP dose-dependently stimulated 3H-thymidine incorporation in the osteoblastic-like cell line MG-63 (Zhong, Ding et al. 2003), which suggests that GIP can also promote bone cell proliferation.

The osteogenic effect of GIP can also be achieved indirectly: GIP increased message and secretion of transforming growth factor beta (TGF-β), an agent known to regulate osteoblastic proliferation and differentiation (Zhong, Ding et al. 2003). GIP can potentiate insulin and amylin release from the ß-cells of the pancreas (Zaidi M 1993). Insulin and amylin have been demonstrated to have anabolic effect on bone formation.

In 2001, Bollag et al. demonstrated that GIP administration can prevent the bone loss associated with ovariectomy (Bollag, Zhong et al. 2001). Based on that evidence, they proposed that GIP could serve to coordinate nutrient intake in the intestine with nutrient disposal in a variety of peripheral tissues including bone.

To provide in vivo evidence proving that GIP is involved in bone remodeling, in 2005, Xie et al. investigated the role of GIP in modulating bone turnover in GIPR KO mice, and proved the anabolic effects of GIP on bone mass (Xie, Cheng et al. 2005). Moreover, the effect of deficient GIP signaling in GIPR KO mice seems to be dependent on the site and age of bone. Over time, the decrease of bone size and mass compared with wt mice was compensated, suggesting that some hormone factor arose to compensate for the loss of GIP signaling. The site-dependent effect of GIP on bone (increased BMD in lumbar and decreased BMD in femur), the increase of leptin serum concentration in GIPR KO mice, and the well-known effect of leptin in selective effects on bone

depending on bone site--all these point to a single explanation, that is, leptin may mediate the effect of GIP on anabolic action on bone.

Recently, we found that ICV GIP administration increases expression of CART in rat brain (Duan et al). Since CART has been suggested as a downstream player of the effect of leptin on bone when administered centrally, it is possible that the compensatory bone changes in aging GIPR KO mice were mediated partly by leptin-induced changes in CART (Carlos et al., unpublished data).

Ghrelin

1. Discovery of ghrelin

In 1996, a G protein coupled receptor, which is the so-called growth hormone secretagogue receptor (GHS-R), was found mainly in the hypothalamus and the pituitary (Howard, Feighner et al. 1996). Following the discovery of this receptor, people start to look for its natural ligand in the body, unexpectedly, its endogenous ligand was found in the stomach tissue (Rosicka, Krsek et al. 2002). Ghrelin come from the Greek origin, which means “grow”, but it also share the meaning of eliciting the release of growth hormone (Rosicka, Krsek et al. 2002).

In this review, the discovery, structural characteristics, tissue distribution, and physiological function of ghrelin will be discussed, with emphasis on its effect on adipose and bone tissue.

2. Tissue distribution of ghrelin

Ghrelin is secreted mainly by the endocrine cell in the stomach and is secreted into the circulation (Date, Kojima et al. 2000). This is supported by the evidence that removing part of stomach will reduce the amount of blood ghrelin concentration by 65% (Ariyasu, Takaya et al. 2001). Ghrelin can also be produced in some other tissues, like small intestine, kidney, pancreas, testicular, etc (Kojima, Hosoda et al. 2001). In the central nervous system, the neuronal cells showing positive for ghrelin is very limited, only to the hypothermic ARC area (Kojima, Hosoda et al. 1999), which is known to be involved in the regulation o food intakes.

Ghrelin can also be synthesized in the hypothalamus (Kojima, Hosoda et al. 1999), but the number of ghrelin positive neural number of ghrelin positive neural is very few. Ghrelin can work on the pituitary via the portal vein to control the release of the growth hormone, or it may act via the neural pathway to control the feeding behavior and energy homeostasis (Kojima 2001).

3. Structure and forms of ghrelin

Ghrelin is a 28 amino acids hormone, with two forms, acyl form and des-acyl form (Hosoda, Kojima et al. 2000). In the acyl form, there is a modification in the third amino acid, the serine, by a middle chain fatty acid, octanoic acid. The Ser-3 octanoic modification is important in that it is related to ghrelin's biological activity. Upon modification, the ghrelin could bind and activate its receptor (Kojima, Hosoda et al. 1999).

The des-acyl form ghrelin lacks the receptor binding activity and GH releasing activity and it is considered as a biological inactive form (Bednarek, Feighner et al. 2000). However, it is the main form in the circulation, to our surprise.

Since the majority of ghrelin is in the form of des-acyl Ghrelin, it may not be totally inactive biologically. Recent studies (Cassoni, Papotti et al. 2001; Baldanzi, Filigheddu et al. 2002), however, indicate that des-acyl ghrelin may share with acylated ghrelin the modulation of neoplastic cell proliferation and cardiovascular cell survival *in vitro*. Moreover, one study shows that des-acyl ghrelin may offset the inhibitory effect of acylated ghrelin on insulin secretion (Broglio 2003). In addition, one study (Thompson, Gill et al. 2004) reported that acylated and des-acyl ghrelin promote adipogenesis directly *in vivo* by a mechanism independent of known GHS-Rs.

In a paper by Asakawa (Asakawa, Inui et al. 2005), des-acyl ghrelin induces a state of negative energy balance and body weight decrease by inhibiting food intake in an inverse manner to that of acylated ghrelin. Recently, Ariyasu et al.(Ariyasu, Takaya et al. 2005) found that des-acyl ghrelin overexpressing mice show thin phenotype with decreased food intake and gastric emptying rate. Therefore, the distinction between acylated ghrelin and desacyl ghrelin may call for investigation of physiological and pathological functions of ghrelin.

In view of the opposite effect of these two forms of ghrelin, it is possible to speculate that one method of regulation of energy homeostasis by the stomach is through modulation the ratio of these two forms of ghrelin.

4. GHS-R

GHS-R is a G protein coupled receptor, which exists in two forms: GHS-R 1a and 1b (Howard, Feighner et al. 1996), in which the GHS-R 1a is the functional form; while GHS-R 1b is the non-sliced, nonfunctional form.

GHS-R 1a is predominantly expressed in hypothalamus, pituitary, and in peripheral tissues such as heart, lung, intestine, pancreas, kidney etc (Guan, Yu et al. 1997). Such widespread distraction of receptor indicate that ghrelin secreted from stomach has multiple function by binding on its receptor in different tissues through endocrine, paracrine pathways (Korbonits, Goldstone et al. 2004).

In addition, a novel type of non-type 1a GHS-R was recently found to assume the activity of being bound by both the acyl form and des-acyl form ghrelin (Hosoda, Kojima et al. 2000). This novel type of receptor has been located in the endothelial and cardiomyte cells (Baldanzi, Filigheddu et al. 2002), bone marrow adipocyte (Thompson, Gill et al. 2004) as well as the adipocyte cell line 3T-3L1 cells (Choi, Roh et al. 2003).

5. Physiological functions of ghrelin

Ghrelin stimulates the growth hormone release from the anterior pituitary of mice in vitro in a dose-dependent manner (Kojima, Hosoda et al. 1999). Co-administration of ghrelin and GHRH elicits a synergistic effect in inducing the release of growth hormone (Hataya, Akamizu et al. 2001). Ghrelin may serve some additional neuroendocrine functions, such as modulation of lactotropic, corticotropic as well as gonodotropic axes (Arvat, Maccario et al. 2001).

In addition to the central role, ghrelin has been shown to have quite diverse peripheral functions. This includes modulation the secretion of gastric acid and gut motility, different kinds of cardiovascular functions, modulation of glucose metabolism and pancreatic insulin release from pancreas, as well as control the cell proliferation of several cell lines (De Ambrogi, Volpe et al. 2003; Korbonits, Goldstone et al. 2004).

6. Regulation of food intake

Ghrelin has been called "hunger hormone" (Korbonits, Goldstone et al. 2004). The feeding stimulatory effect of ghrelin is strongly accessed by its various blood concentration. First, ghrelin is released immediately before a meal, concomitantly with a decreased of blood glucose (Cummings, Purnell et al. 2001). This is the signal that triggers feeding behavior. Immediately after a meal, serum ghrelin concentration progressively decreases (Tschop, Wawarta et al. 2001). Secondly, circulating level of ghrelin is dependent on the feeding status (Asakawa, Inui et al. 2001; Toshinai, Mondal et al. 2001). That is, fasting up-regulates the level of ghrelin level, and the level of ghrelin is suppressed after re-feeding. Therefore, a role of ghrelin in the etiology of obesity has been proposed.

Ghrelin can induce weight gain and adiposity (Tschop, Smiley et al. 2000; Wren, Small et al. 2000). ICV administration of ghrelin to free-feeding rats during both light and dark phases increases food intake in a dose-dependent manner (Nakazato, Murakami et al. 2001). Neutralization of ghrelin with anti-ghrelin immunoglobulin G, also dose-dependent, suppresses starvation-induced feeding (Nakazato, Murakami et al. 2001); this suggests that endogenous ghrelin is strongly orexigenic. This orexigenic effect is independent of growth hormone because it is also observed in dwarf rats

deficient in growth hormone (Nakazato, Murakami et al. 2001). ICV administration of ghrelin was shown to induce Fos expression in 39% of NPY/AgRP-expressing neurons (Nakazato, Murakami et al. 2001) and increase both NPY and AgRP mRNA levels in the ARC (Kamegai, Tamura et al. 2000; Kamegai, Tamura et al. 2001). Its orexigenic effect is abolished by co-injection of NPY Y1 and Y5 receptor antagonists (Kojima 2001; Nakazato, Murakami et al. 2001). These results indicate that ghrelin is an upstream regulator of the orexigenic peptides NPY and AgRP.

7. Ghrelin induces adiposity

Ghrelin can induce adiposity either indirectly through stimulation of the appetite or directly through action on adipose tissue. Firstly, activation of hypothalamic feeding centers by ghrelin (Nakazato, Murakami et al. 2001; Lawrence, Snape et al. 2002) may increase substrate availability, thereby giving rise to adipogenesis.

Secondly, ghrelin may regulate adiposity via adenohypophysial hormones other than GH. For example, both PRL and ACTH secretion can be elevated by GHS-R activation (Massoud, Hindmarsh et al. 1996; Smith, Van der Ploeg et al. 1997), and both hormones potentially contribute to the regulation of adipocyte differentiation (Gregoire, Genart et al. 1991).

Hormones from peripheral endocrine glands may also contribute to the observed ghrelin-induced adipogenesis. Binding sites for the GHSs compounds are present in a range of peripheral tissues, including thyroid (Cassoni, Papotti et al. 2000),and adrenal glands (Papotti, Ghe et al. 2000). Both thyroid hormones and glucocorticoids appear to play a role in preadipocyte differentiation (Gregoire, Smas et al. 1998).

Finally, ghrelin may promote adiposity by working directly on adipose tissue. Thompson et al. recently reported that acylated ghrelin and desacyl ghrelin stimulate tibial bone marrow adipogenesis (Thompson, Gill et al. 2004). In addition, Choi (Choi, Roh et al. 2003) proved that ghrelin acts directly on adipocytes to stimulate differentiation from preadipocytes and to antagonize lipolysis. On the other hand, Zhang et al. (Zhang, Zhao et al. 2004) reported that ghrelin inhibits adipogenesis in 3T3-L1 cell lines. Therefore, the exact direct action of ghrelin on adipose tissue remains to be determined.

8. Ghrelin and bone metabolism

The possibility that ghrelin could be involved in the control of bone metabolism is speculated based on two facts: firstly, the observation that gastrectomy could induce osteopaenia (Bussabergher 1938; Ivy 1940; Rumenapf, Schwille et al. 1997). The acid procuring mucosa (where ghrelin is produced) has been shown to serve important function in bone metabolism. Loss of stomach area correlates with the concentration of ghrelin in direct proportion to bone loss.

Secondly, a number of studies showed positive effects of GHSs on bone. For example, Hexarelin has been shown to counteract the effect of bone loss following ovariectomy (Sibilia, Cocchi et al. 2002). In addition, a number of GHSs compounds had been show to increase bone turnover in human beings (Svensson, Lall et al. 2001). Therefore, ghrelin, the endogenous GHSs produced mainly in stomach, is very possibly the factor responsible for bone loss following gastrectomy.

Another question is that is the proposed protective effect of GHSs and ghrelin on bone is due to its indirect GH-releasing activity, or to its direct effects on bone cell activities. To clarify this,

Maccarinelli et al. evaluated the (Maccarinelli, Sibilia et al. 2005) effect of ghrelin on proliferation rate and on osteoblast activity in primary cultures of rat calvaria osteoblasts. They concluded that endogenous ghrelin and synthetic GHS could promote proliferation and differentiation of rat osteoblasts by acting on their specific receptors.

In 2005, a Japanese team led by Kojima proved that ghrelin directly regulates bone formation *in vitro* and *in vivo* (i.p. administration). They confirmed the expression of functional ghrelin receptors in bone cell. They further proved the direct effect of ghrelin in bone formation in SD and SDR rats, showing that bone mineral density of these rats increased following i.p injection of ghrelin (Fukushima, Hanada et al. 2005). This suggests that ghrelin still has direct effects on bone, other that those mediated by ghrelin. However, in the seeking of orthotropic hormone (referred to as gastrocalcin), Larsson et al. failed to evoke any change in calcium signal in osteoblast cell culture using ghrelin (Larsson, Norlen et al. 2002). Sun et al (Sun, Ahmed et al. 2003) reported no role for ghrelin in the maintenance of bone density since ghrelin-null mice had normal bone mineral density and normal bone mineral content. It is possible, however, that the lack of ghrelin signal triggered some other compensatory effects since multiple pathways exist to regulate these important features. Thompson et al., using a novel unilateral infusion strategy, found that ghrelin promotes bone marrow adipogenesis, which is known to be adverse for osteoblastogenesis (Thompson, Gill et al. 2004).

Until now, no report has thus far investigated the effects of brain injection of ghrelin on bone metabolism.

9. Ghrelin-leptin tango

The adipocyte hormone leptin was first discovered in 1994 (Zhang, Proenca et al. 1994). It circulates at concentrations proportional to body fat mass and inhibits food intake (Friedman and Halaas 1998). Leptin plays an important role in the homeostatic control of body fat mass.

Leptin and ghrelin have been implicated, with opposite roles, in body weight homeostasis: leptin, as a satiety factor that signals for energy abundance, and ghrelin, as an orexigenic factor that signals for energy insufficiency.

Leptin and ghrelin have opposite roles centrally, as well as peripherally. Centrally, ghrelin strongly interacts with the leptin regulatory pathway at the level of the arcuate nucleus where leptin has receptors located on the NPY neurons (Mercer, Hoggard et al. 1996). When ghrelin is co-injected with leptin, it abolishes the leptin- induced inhibition of food intake in a dose-dependent manner (Nakazato, Murakami et al. 2001; Shintani, Ogawa et al. 2001). Peripherally, ghrelin and leptin also show antagonistic activity on gastrointestinal functions (Asakawa, Inui et al. 2001). Ghrelin increases gastric acid secretion, motility and emptying (Asakawa, Inui et al. 2001),whereas leptin decreases those functions.

Recently leptin was shown to negatively regulate bone centrally via modulating sympathetic nerve activity. In view of the opposing functions of leptin and ghrelin peripherally and centrally--the peripheral *in vivo* and *in vitro* evidence of ghrelin effect on bone, the effect of ghrelin in decreasing sympathetic nerve activity and the well-known GH hormone releasing effect--it is reasonable to speculate that ghrelin may also serve an important function in modulation of bone metabolism through a neuronal pathway.

Role of adipocyte in bone marrow

1. Clinical observations

Clinical evidence shows that obese women usually have strong bone, and hence a lower osteoporosis rate (Slavkin 2000). The possible reason for this effect of higher body weight on higher bone mass may result from the increased mechanic load; furthermore, the extra estrogen secreted from fat mass also results in higher bone mass. Does that mean the more fat, the merrier? Not necessarily. It is a complex issue. Studies shows that the relationship of BM and BMI are complex , with variables dependent on age, sex and ethnic (Rosen and Bouxsein 2006).

Using MRI spectroscopy, Schellinger et al. (Schellinger, Lin et al. 2001) found increased bone marrow fat in aged women with osteoporosis. Yeung and his colleagues found postmenopausal women have nearly twice the marrow fat than premenopausal women (Yeung 2005). In addition, aged-matched study suggests that women with low BMD have significantly higher bone marrow fat compared with healthy women.

Recently, Rosen et al (Rosen, Ackert-Bicknell et al. 2004) described congenic mice that exhibited allelic suppression of skeletal and hepatic insulin-like growth factor 1 (IGF-1). These mice have significantly lower trabecular bone volume, with increased marrow fat infiltration. However, these mice are not obese. This suggests that fat redistribution, rather than generalized adiposity, might be a better indicator of impaired osteoblast genesis.

2. Leptin experiments

Ducy (Ducy, Amling et al. 2000) and his colleague Takeda (Takeda, Elefteriou et al. 2002) demonstrate that the hormone secreted from fat, leptin, modulate the bone turnover via

binding the receptor in the hypothalamus. However, these finding contradicted with several line of evidences suggesting that leptin directly promote of the osteoblast differentiations and bone resorption (Steppan, Crawford et al. 2000; Cornish, Callon et al. 2002). Probably the best explanation for this discrepancy is that leptin have parallel, effect on bone: a peripheral positive effect, and a negative central mechanism involving of activation of the sympathetic nervous systems (Elefteriou and Karsenty 2004).

A recent study by Dr. Mark Hamrick using ob/ob mice further explained the fat bone interaction. The author found leptin-deficient mice have more marrow adipocyte and less bone mass (Hamrick, Pennington et al. 2004), while treatment with leptin restores bone density, at the expense of adipocytes by apoptosis (Hamrick, Della-Fera et al. 2005). This suggests that in leptin-sensitive animals, treatment with leptin will lead to depletion of marrow adipocytes, which have a positive effect on bone formation. He further demonstrated that VMH injection of leptin in rats leads to marrow adipocyte apoptosis (Hamrick 2006). Data from this experiment suggested that the role of leptin in inducing bone marrow adipocyte apoptosis may also involve sympathetic nervous system activity. Thus, CNS leptin modulates bone metabolism, not only by acting directly on bone cell, but also by acting indirectly on the third type cell in the bone population, bone marrow adipocytes (Hamrick 2006).

3. Bone marrow adipocytes: good or bad for bone

Aside from leptin experiments, another famous experiment studied PPAR gamma. PPAR gamma is the regulator for adipogenesis (Gimble, Robinson et al. 1996). Ligand activation of PPAR gamma receptor will lead to stem cells or MSCs differentiation into adipocyte instead of bone cell

lineage (Akune, Ohba et al. 2004). Thus, it appears that there is an inverse relationship between these two cells since they share the same progenitor cells, the MSCs. Alkune et al. further demonstrated that PPAR gamma insufficiency led to increased osteoblastogenesis *in vitro* and higher trabecular bone volume *in vivo* (Rzonca, Suva et al. 2004).

With all the above evidence, it appears that bone marrow fat is bad for bone. There is, however, some opposite evidence. Evidence supporting the protective effect of fat on bone advocates that obesity increases loading on the cortical skeleton. In addition, the cytokine-like hormone, leptin, directly stimulates bone formation. Furthermore, greater aromatase activity increases estradiol, which would lead to decreased bone resorption (Gimble, Robinson et al. 1996).

VIII. Microfluidic card and bone marrow gene expression:

1. Introduction

Studying the gene expression is central in studying gene function since many cellular processes are reflected in the changes of the gene expression. There are currently four methods being used to study gene expression: RPA, northern blot, in-situ hybridization, and real-time PCR (Hod 1992), in which the last method is the most sensitive one. It is so sensitive that it can be used to quantify gene expression from a single cell. This characteristic leads to widespread application of RT-PCR in the quantification of gene expression (Rebrikov and Trofimov 2006). It also has been used to validate the result of some global scale assay, such as microarray.

In real-time PCR, several methods have arisen, in which molecular beacon, cybergreen, and Taqman real time PCR are the most important ones (Rebrikov and Trofimov 2006). The method

used in this study is Taqman real-time PCR. In the following paragraph, the principle and procedure of Taqman real-time PCR will be introduced.

2. TaqMan® real-time PCR

The method of Taqman employ two floropore, the reporter dye, which binds to the 5 prime end and the quencher dye, which binds to the 3 prime end. Before the reaction start, fluorescence of the reporter dye was quenched by the quencher dye. Once the reaction start, the Taqman polymerase starts to remove the probe from the template, separating the quencher dye from the reporter dye. Thus the fluorescence will increase in proportion to the amount of DNA synthesized (Yoshimura, Nakamura et al. 2005).

3. Normalization

RT-PCR specific error can be easily compounded with the inequality of the starting material. In order to reduce the effect of sample to sample variation, it is good idea to set a reference value to which other RNA was compared to (Romanowski, Markiewicz et al. 2007). There are three different kind of RNA that is commonly used: they are GAPDH, actin and ribosomal RNA respectively (Romanowski, Markiewicz et al. 2007).

Actin mRNA encoded for cytoskeleton protein, which is one of the earliest to be used as standard control. It is moderately expressed in most of the cell types. Till now it is still advocated as one of the most used quantitative reference for RT-PCR assay (Kreuzer, Lass et al. 1999).

GAPDH is also moderately abundant and ubiquitously expressed. It is frequently used since it is thought to be expressed constantly independent of timing and experimental manipulation.

However, nowadays, overwhelming evidence suggest it is not such a reliable reference (Edwards and Denhardt 1985).

Ribosomal RNA, which constitutes 80-90% of the total RNA, is also useful for being used as an internal control. Their level are rarely changed under normal condition which affect most RNA expression (Barbu and Dautry 1989). Moreover, it has been shown that 18S RNA is the preferred house keeping gene in bone marrow tissue gene expression (von Knoch, Jaquiery et al. 2005).

4. Quantitation of results

Two strategies have been used to quantify the result of RT-PCR, that is, the standard curve method and the comparative threshold method (Bustin, Benes et al. 2005).

In the standard curve method, first, a standard curve is built based on RNA samples of known concentration, and then the concentrations of unknown are extrapolated from the standard curve (Larionov, Krause et al. 2005).

Another quantitative method is termed as comparative Ct method, which involves compare the Ct value of samples of interest with calibrator or control (untreated sample or RNA from normal tissue). The Ct value of the unknown and calibrator was then normalized to an appropriate endogenous housing keeping gene (Wilhelm, Pingoud et al. 2003).

5. Microfluidic card and ABI PRISM® 7900HT SDS

Applied Biosystems has introduced the microfluidic card technology to study the gene expression. It is capable of high throughput of gene expression analysis, and it is quick and cost effective. The steps are very easy to carry out. For detailed information on carrying out the assay, see the following website: http://sequencingfacility.med.monash.edu.au/pdf/TLDAinformation.pdf

6. Application of microfluidic card in studying bone marrow gene expression

Since BMSCs (bone marrow stroma cells) are critically involved in maintaining the dynamic equilibrium of bone turnover, it is very important to investigate how these cells respond to anabolic treatment (Gimble, Robinson et al. 1996). The real time PCR method has been widely used for studying the effects of some anabolic bone formation agents on MSC cell differentiation, such as bisphosphonates (von Knoch, Jaquiery et al. 2005). Targeting multiple steady state mRNA levels of key genes involved in osteogenic differentiation provides a fast, reliable and cost efficient way to study anabolic agent effects on bone formation. This will have huge applications in studying bone quality and bone turnover, and in the development of new agents for prevention and treatment of osteoporosis.

CHAPTER 2

EFFECTS OF INTRCEREBROVENTRICULAR (ICV) ADMINSTRATION OF GIP AND LEPTIN ON FEEDING BEHAVIOR AND BONE MARROW IN RATS [1]

[1] Q. Li, Y.-H. Choi, D.L. Hartzell, M.A. Della-Fera, C.M. Isales, M.W. Hamrick, and C.A. Baile. To be submitted to *Physiology and Behavior*.

Abstract

Glucose-dependent insulinotropic polypeptide (GIP) is a 42-amino acid peptide synthesized and secreted from endocrine cells in the small intestine. GIP receptors have been found in the brain, and GIP mRNA and protein have recently been found present in the hippocampus. Presently, GIP serves no known function in the brain. However due to the presence of GIP receptors in the brain and the dearth of information about its central effects, the following experiment was carried out. Forty Male Sprague Dawley rats were implanted with lateral cerebroventricular (ICV) cannulas. Five treatment groups (N=8), including control (aCSF), 10 µg/d leptin, 0.1 µg/d GIP, 1 µg/d GIP, and 10 µg/d GIP, received ICV injections daily for four days. Spontaneous physical activity (SPA) and food and water intake were monitored continuously. Weight gain (WG) was decreased by both leptin (-18.2g, $p<.001$) and 10 µg/d GIP (1.5g, $p<.05$) as compared to the control (8.7g), while food intake (FI) was decreased only by leptin (34%, $p<.001$). SPA exhibited a 30% increase due to leptin, as compared to GIP treatments ($p<.05$). Fat pad weights were reduced by leptin but not by GIP. Serum leptin was decreased by both leptin and GIP (10 µg) (63.3%, $p<.001$ and 24.9%, $p<.05$, respectively). Bone marrow adipocytes size and number were significantly ($p<.05$) decreased by leptin, but not by GIP. While leptin increased spinal bone mineral density ($p<.05$), it was not affected by GIP treatment. We conclude that centrally administered GIP has a subtle effect on the control of feeding behavior and body weight, while it has no effect on bone through receptors in the central nervous system. Supported in part by the Georgia Research Alliance Eminent Scholar endowment held by Dr. Clifton A. Baile

Introduction

GIP is a 42-amino acid peptide hormone secreted by K cells of the small intestine (Yip, Boylan et al. 1998). It is one of two main incretin factors. GIP receptors are widespread throughout the body, particularly in the pancreas, adrenal cortex, brain, bone, and adipocytes (Usdin, Mezey et al. 1993). Still, the reason behind the presence of GIP receptors in the brain remains a mystery. Researchers once thought there was no endogenous GIP synthesized in the brain and that the peripheral GIP can not cross the blood-brain barrier (Woods, West et al. 1981). In fact, in the last twenty years, there has only been one study (Wood, et al, 1981) investigating the effects of GIP on the brain, which concluded that there were no effects on food intake after ICV GIP injections in rat brains (Woods, West et al. 1981). In 2005, interest in the central effects of GIP was sparked again when a study by Nyberg revealed the presence of GIP mRNA in the brain and GIP promotion of the neuronal cell proliferation and neurogenesis (Nyberg, Anderson et al. 2005).

Since the discovery of GIP receptors in adipocytes (Yip, Boylan et al. 1998), and that GIP-receptor knockout mice are resistant to diet-induced obesity (Miyawaki, Yamada et al. 2002), increasing evidence supports the notion that GIP is involved in body weight regulation (Miyawaki, Yamada et al. 2002). GIP works directly on adipocytes and via interaction with leptin and other hormones (Yip and Wolfe 2000). However, what is the role of endogenous GIP? Does peripheral GIP need to cross the blood brain barrier in order to exert its function, or is there communication between the endogenous GIP in the brain and the GIP synthesized outside the brain? Because the closely-related incretin-GLP-1-affects food intake suppression via receptor-binding in the brain (Turton, O'Shea et al. 1996), we can assume that GIP might also function in central regulation of

food intake. Thus, the purpose of this study is to investigate whether centrally administered GIP affects food intake, body weight regulation, and adipose tissue weights and apoptosis.

Additionally, several studies indicate that GIP may be involved in bone formation, linking nutrient ingestion to the formation of bone (Bollag, Zhong et al. 2001). One of the first such papers showed that GIP receptors are present and functional on the osteoblastic cells (Bollag, Zhong et al. 2000), while a second paper by the same group demonstrated that GIP can antagonize a bone loss following ovariectomy (Bollag, Zhong et al. 2001). This group also used receptor knockout mice, verifying that the mice have altered bone turnover (Xie, Cheng et al. 2005). A separate group found similar results using the GIPR knockout mice model (Tsukiyama, Yamada et al. 2006). Most recently, in 2007, a new contribution by Carlos et al. showed an increased bone mass in GIP overexpressing transgenic mice (Xie, Zhong et al. 2007). Will GIP, like leptin, exert different functions over bone peripherally versus centrally? There are no known studies on the effects of the involvement of GIP on centrally modulated bone formation.

In view of the reciprocal relationship between bone marrow adipocytes and bone cells, we expect to see changes in bone marrow adipocytes, as based upon previous studies, which indicate that GIP-receptor knockout mice have decreased fat pad weights while having increased bone marrow fatness. A second objective of this paper is to measure bone marrow adipocyte size and number following GIP treatment.

Material and methods

1. Animals and diet

Forty-four male Sprague-Dawley rats (250 – 274 g) were purchased from Harlan, Inc. (Indianapolis, IN) (two groups of 22 rats, purchased one week apart). Rats were housed in individual cages and had access to pelleted standard lab chow and water ad libitum throughout the study, unless noted. Rats were implanted with chronic lateral cerebroventricular cannulas as described below, and after recovery from surgery, rats within each block were randomly assigned to five treatment groups and moved to individual cages in the TSE Systems. Lights were on between 0900 h – 2100 h and off between 2100 – 0900 h, ambient temperature was set at 22 ± 1 º C and humidity 50 %. The Animal Care and Use Committee approved all experimental and surgical procedures in this study for The University of Georgia.

2. Peptides

All peptides were solubilized based on their net protein content. Rat leptin was purchased from R&D Systems (Minneapolis, MN). Vehicle was used to dilute the leptin and achieve a concentration of 2.0 mg/ml. Human GIP (H-5645.1000) was purchased from Bachem (San Carlos, CA).

3. Surgical procedures

Rats were anesthetized with a 3:2:1 (v/v/v) mixture (1 ml/kg ip) of ketamine HCl (Ketaset, Fort Dodge Laboratories, Fort Dodge, IA; 100 mg/ml), acepromazine maleate (PromAce, Fort Dodge; 10 mg/ml), and xylazine (Rompun, Miles, Schawnee Mission, KS; 20 mg/ml), and the hair

in the dorsum of the head as well as the area behind scapular bones was removed. Each rat was then placed in the stereotaxic instrument (Stoelting, Wood Dale, IL) and the skin disinfected with betadine solution. A 22-gauge guide cannula (C313G, Plastics One, Roanoke, VA), which was cut 13.2 mm long, was aseptically implanted into the right lateral ventricle of each rat, with the coordinates of 0.8 mm posterior to the bregma, 1.5 mm lateral to the midline, and 3.2 mm ventral to the surface of the skull based on the atlas of the rat brain (WC 1986). The cannula was held in place with four stainless steel machine screws and cranioplastic cement (Plastics One) attached to the skull. A 28-gauge stylet (C313DC, Plastics One) was installed into the guide cannula when the rat did not receive an injection. A programmable transponder (IPTT-200TM, BioMedic Data Systems, Seaford, DE) for temperature measurement was implanted under skin near the scapula of each rat using a needle-syringe type injector. To control post-surgical pain, two doses of 1.1 mg/kg banamine were give ip at 12 h intervals. Proper cannula placement was verified in two ways. First, during implantation surgery, backflow of CSF from the tip of the guide would indicate correct placement in the ventricle. Second, an ANG II drinking test was performed at the end of the recovery period, whereby a rat must consume at least 5 ml of water in 30 min following an i.c.v injection of 100 ng ANG II (100 ng/10 µl) solubilized in sterile aCSF; (Sigma, St. Louis, MO) for the cannula to be considered correctly placed. In the morning of the ANG II test, food was removed. Rats were injected with ANG II, the time recorded, in the same manner described above and the weight of each water bottle will be measured and recorded. Injection time was recorded as well. After ANG II injection, rats were returned and water bottles were provided for 30 min, followed by measurement

of the bottles. Following surgery, the rats were allowed to recover to presurgical BW, which was approximately 1 week.

4. Design and procedures

Following recovery rats were randomly assigned to the five treatments groups (including 0 (aCSF) (Control), 0.1 μg GIP (G0.1), 1.0 μg GIP (G1.0), 10 μg GIP (G10) and 10 μg rLeptin) (L10), and were transferred to the TSE behavioral monitoring system.

Treatments were administered at 24-hour intervals for four days as 10 μl injections. Each injection was administered into the lateral cerebral ventricle over 30 s, and the injector left in place for additional 30 s to allow the CSF to diffuse the drug, followed by removal of the injector and replacement of the stylet into the guide cannula.

Daily body temperature (BT), body weight (BW), food intake (FI), and water intake (WI) were recorded within 3 h after light onset. Rats were deeply anesthetized with CO_2 before decapitation on day 6 to remove the brain, blood, gastrocnemius muscle, intrascapular brown fat, retroperitoneal, epididymal, and inguinal white fat pads.

Trunk blood was collected and serum frozen at –20 ºC for measurement by Luminex of leptin, insulin and glucagon concentrations. Tissues were weighed, frozen in liquid nitrogen, and stored at –80 ºC.

5. Feeding behavior and spontaneous activity measurement

Rats were individually housed in cages equipped to automatically measure feeding and drinking behavior and spontaneous physical activity (TSE Systems, Bad Homburg, Germany). The InfraMot activity unit registers activity by sensing the body-heat image, i.e. infrared radiation, and

its spatial displacement over time. Rats were removed from the monitoring system for approximately 4 min at the same time each day for BW and BT measurements.

6. Bone marrow adipocyte size and number

The right femur of each rat was dissected free of soft tissue, fixed in 10% buffered formalin, and stored in 70% ETOH. For bone histomorphometry, the right femur was cut across in the middle of the shaft, decalcified in EDTA, embedded in paraffin, and sectioned at approximately 5um. Sections were stained with hematoxylin and eosin (H & E) to visualize adipocytes. Adipocytes were counted over the cross-sectional area of bone marrow and adipocyte density was expressed as the number of adipocyte per pixel of tissue area multiplied by 10^4. Adipocyte size was measured as the cross-sectional area of each adipocyte by digitizing the border of each cell (up to 20 cells per section) using Image-Pro® Plus image analysis software.

7. Statistics

ANOVA and LSD tests were used to determine significance of differences among treatments.

Results:

1. Food intake, body weight

Food intake (FI) and body weight (BW) of leptin groups remained significantly lower by the end of the study, FI and BW of the GIP treated rats did not change (Fig. 2.1 a, b). Leptin caused significant reductions in both cumulative apparent food intake (AFI) and cumulative net food intake (NFI) (Fig. 1 c).

At the end of the study, weight gain (WG) was decreased by both leptin (-18.15g vs 8.74g, $p<.001$) and 10 µg/d GIP (1.53g vs 8.74g, $p<.05$) compared to control (Fig. 2.1 d).

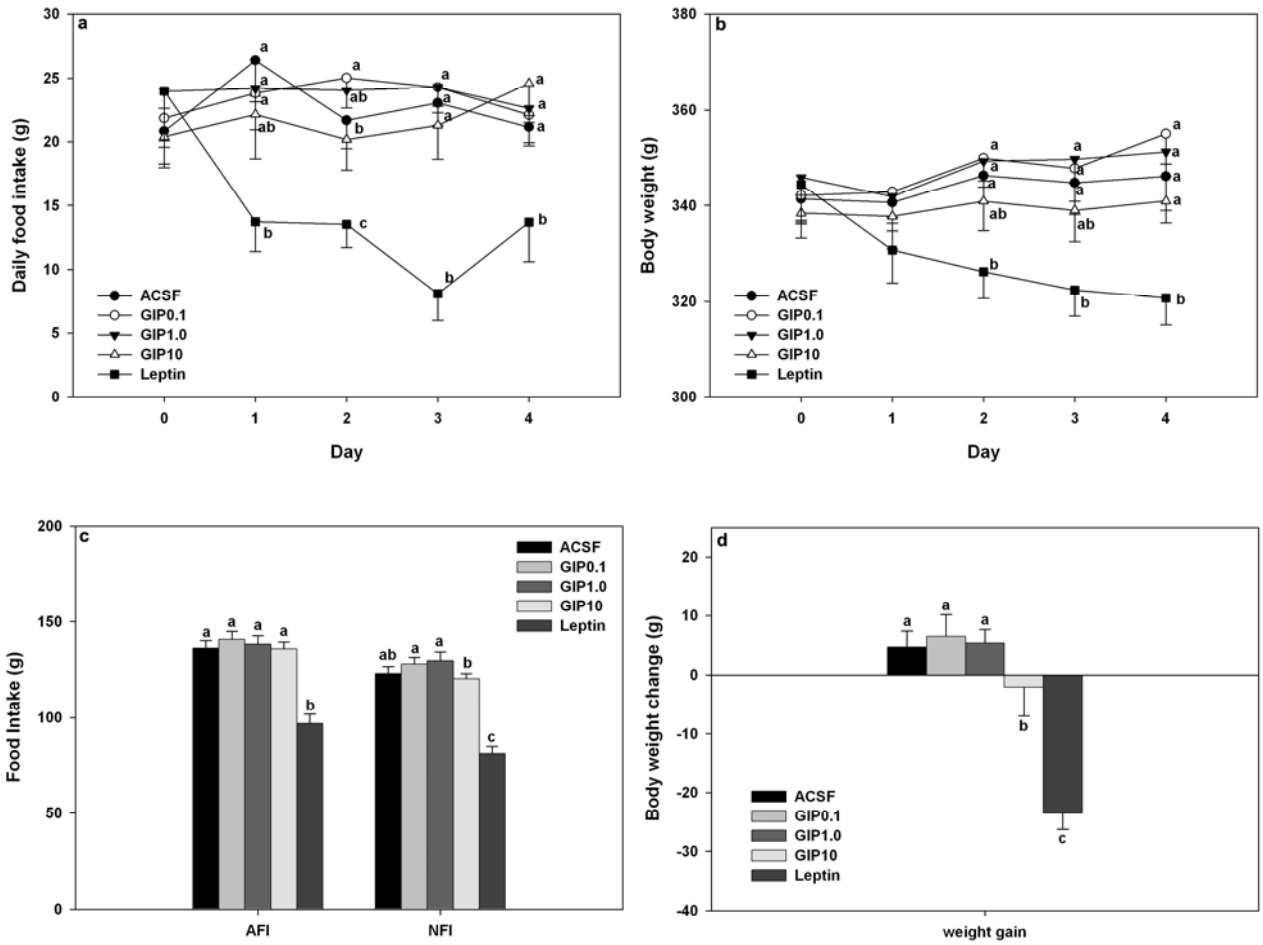

Fig.2.1 Daily food intake (a), cumulative food intake (c), body weight (b) and body weight gain (d) of rats received daily ICV injections for 4 days with aCSF (10ul), GIP 0.1 (0.1 µg/d) , GIP1.0 (1 µg/d), GIP 10 (10 µg/d), or leptin (10 µg/d). a, b: means with different letters are significantly different, p<.05. Data are mean±S.E.M. Note: AFI (apparent food intake), NFI (net food intake).

Feeding behavior

Leptin decreased meal number ($p<0.05$). G10 has a trend toward decrease in total meal number. There were no differences in individual meal size by either leptin or GIP. Eating rate was increased by Leptin and G10 ($p<0.05$); while meal durations were decreased by both leptin and G10 ($p<0.05$) (Table 2.1).

Table 2.1. Mean size, meal number and eating rate in rats injected i.c.v with 10 µl/inj. of aCSF, G0.1 (GIP 0.1 µg), G1.0 (GIP 1 µg), G10 (GIP 10 µg) or L10 (leptin 10 µg) once daily for 4 days

	aCSF	G0.1	G1.0	G10	L10
Meal size (g)	2.85±0.16	2.68±0.16	2.95±0.16	2.91±0.16	2.64±0.16
Meal number	28.6±2.3^{ab}	31.5±2.3^{a}	26.8±2.3^{ab}	24.8±2.3^{b}	14.8±2.3^{c}
Meal durations (sec)	610.9±40.7^{a}	492.4±38.8^{b}	612.8±42.4^{a}	428.7±51.8^{b}	346.4±66.8^{b}
Eating rate (g/min)	0.36±0.11^{c}	0.54±0.11^{bc}	0.44±0.12^{c}	0.84±0.14^{ab}	1.01±0.18^{a}

Data are means ± S.E.M.

^{abc}Means with different letters within each behavior parameters are different at $p<0.05$.

Note: Meal size is defined as the average quantity in gram ate per meal; meal number is defined as total number of meals ate during the whole study period; meal duration is defined as the time in minute of each meal; eating rate is defined as the size of the meal in grams divided by the duration of the meal in minutes.

Leptin increase both satiety ratio and hunger ratio ($p<0.05$) and increase inter-meal interval ($p<0.05$), while G10 only increased inter-meal interval ($p<0.05$) (Table 2.2).

Body temperature and spontaneous physical activity

Body temperature was unaffected by either GIP or leptin treatments, although there was a trend for leptin to increase body temperature (Fig.2.2 a);

Table 2.2. Satiety ratio (post-meal interval/proceeding meal size) and hunger ratio (pre-meal interval/subsequent meal size) in rats injected i.c.v with 10 µl/inj. of aCSF (Con), G0.1 (GIP 0.1 µg), G1.0 (GIP 1 µg), G10 (GIP 10 µg) or L10 (leptin, 10 µg) once daily for 4 days

	aCSF	G0.1	G1.0	G10	L10
Inter-meal interval (sec)	134.4±22.5^{c}	132.3±21.5^{c}	136.9±23.5^{bc}	209.2±28.7^{b}	318.1±37^{a}
Satiety ratio (min/g)	64.3±4.9^{b}	62.2±4.7^{b}	61.0±5.1^{b}	72.1±5.3^{b}	108.1±7.0^{a}
Hunger ratio (min/g)	68.9±5.8^{b}	72.4±5.6^{b}	72.8±6.1^{b}	79.4±6.3^{b}	108.6±8.3^{a}

Data are means ± S.E.M.

^{abc}Means with different letters within each behavior parameters are different at $p<0.05$

Note: Satiety ratio (min/g), was computed by dividing post-meal intervals in minutes with the grams of the preceding meal. Hunger ratio (min/g) is the length of the pre-meal interval (min) divided by the post-meal size (g). Intermeal interval, was the interval (sec) between individual meals.

spontaneous physical activity (SPA) was significantly increased in leptin treated rats, but not by GIP (Fig.2.2 b).

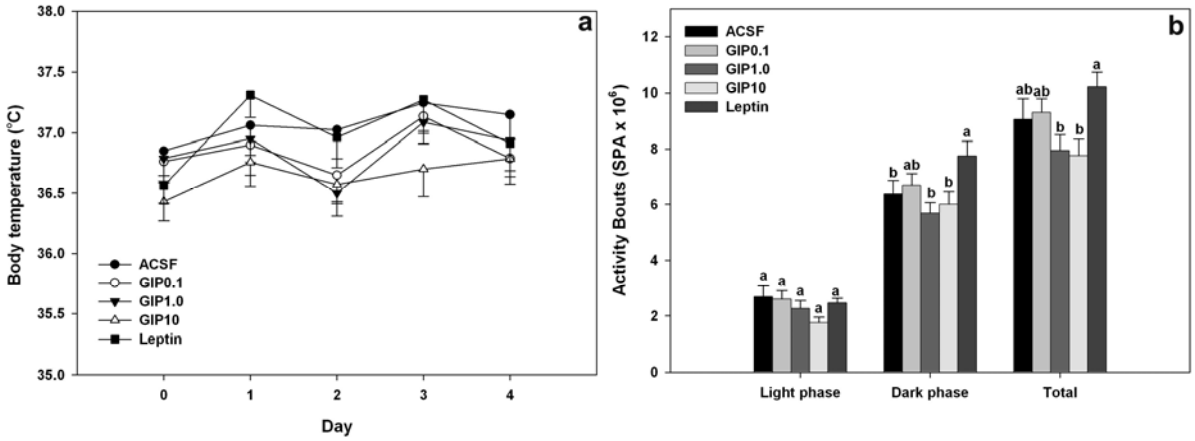

Fig.2.2. Body temperature (ºC) (a), overall, light and dark period spontaneous physical activity (SPA) (b) in rats received daily ICV injections for 4 days with aCSF (10ul), GIP 0.1(0.1 µg/d) , GIP1 (1 µg/d), GIP 10 (10 µg/d), or leptin (10 µg/d). a, b: within a day, means with different letters are significantly different, p<.05.

Tissue weights and serum glucagon, insulin and leptin concentrations

Adipose tissue mass was reduced by leptin in Epi (27.9%, p<.001), Ing (26.85%, p<.05), Rp (68.8%, p<.001), and iBAT (35%, p<.05), and was unaffected by GIP. Muscle mass was unaffected by either GIP or Leptin (Fig.2.3 a); serum leptin was decreased by leptin and GIP (10 ug) (63.3%, p<.001 & 24.9%, p<.05, respectively). Serum glucagon and insulin were unaffected by either GIP or leptin (Fig.2.3 b)

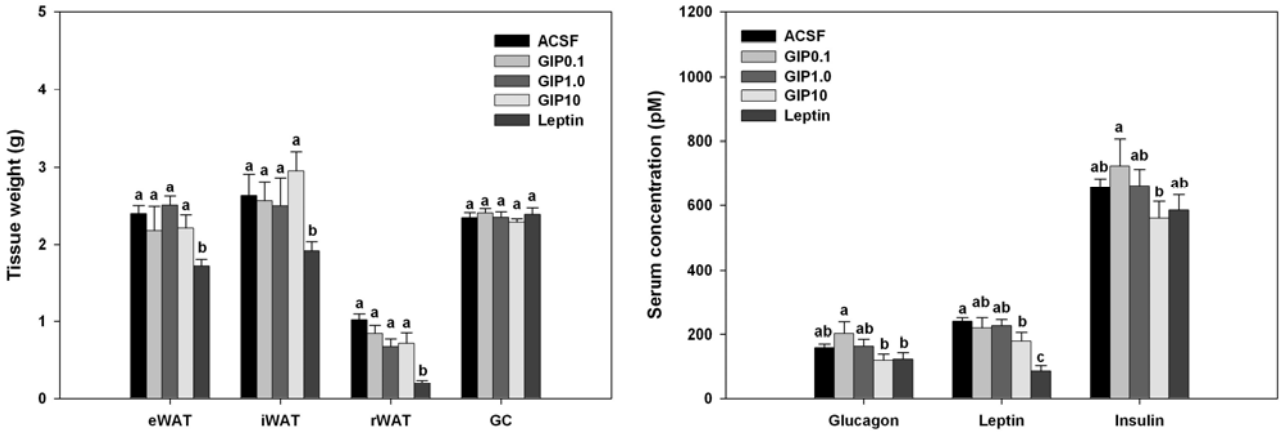

Fig.2.3. Tissue weights (g) (a) and serum concentrations of glucagon, insulin and leptin (b) in rats received daily ICV injections for 4 days with aCSF (10ul), GIP 0.1 (0.1 µg/d), GIP1 (1 µg/d), GIP 10 (10 µg/d), or leptin (10 µg/d). BAT, brown adipose tissue; eWAT, epididymal white adipose tissue(WAT); iWAT, inguinal WAT; rWAT, retroperitoneal WAT; GC, gastrocnemius muscle. Column with different letters are significantly different from each other at p<.05. a, b: within a tissue, means with different letters are significantly different, p<0.05.

Bone marrow adipocyte size, number, and spinal bone mineral density

Bone marrow adipocyte size and number were unaffected by GIP. Leptin decreased bone marrow adipocyte size and number (p<0.05) (Fig.2.4 a). Spinal bone mineral density was unaffected by GIP. Leptin increased spinal bone mineral density (p<0.05) (Fig.2.4 b).

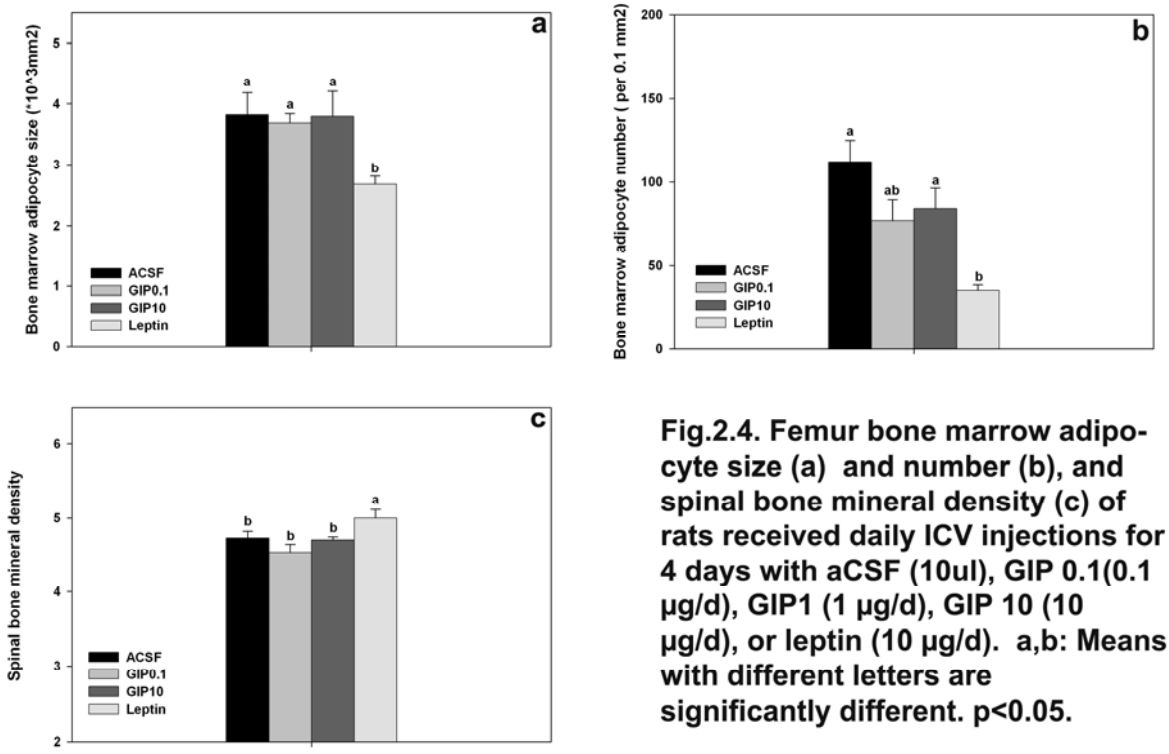

Fig.2.4. Femur bone marrow adipocyte size (a) and number (b), and spinal bone mineral density (c) of rats received daily ICV injections for 4 days with aCSF (10ul), GIP 0.1(0.1 µg/d), GIP1 (1 µg/d), GIP 10 (10 µg/d), or leptin (10 µg/d). a,b: Means with different letters are significantly different. p<0.05.

Discussion

The purpose of this study is to investigate whether centrally administered GIP affects rates of food intake, body weight regulation, adipose tissue weights and apoptosis, as well as bone marrow adipocytes. Results from this study showed that centrally administered GIP exhibited a trend of suppression of food intake, although it was not significant. This finding is in agreement with data obtained by Wood, et al (Woods, West et al. 1981).

Upon closer examination of feeding behavior data, we found that high doses of GIP have a significant effect in increasing the inter-meal interval while decreasing the meal duration. However, GIP had no effect on meal number and individual meal size. Such a finding suggests that GIP may have some slight effect on modulating feeding behavior via binding to receptors in the brain. Moreover, a study conducted by our group aiming at studying rat brain gene expression following

GIP injections, indicated that GIP injections significantly increased the gene expression of NPY - the neuropeptide that controls food intake regulation (J. Duan, University of Georgia PhD dissertation 2006, CH 7). Another study by the same person demonstrated that gene expression of selected markers of GIP receptor knockout mice showed decreased NPY expression and increased CART expression (J. Duan, University of Georgia PhD dissertation 2006, CH 6). Based on these results, we speculate that GIP has some slight effect over the rat feeding behavior either directly (by binding to receptors in the brain,) or indirectly by interaction with other signaling systems.

Although GIP had no significant effect in reducing fat pad weight, high doses of GIP decreased the serum leptin concentration. This result suggests that GIP treatment reduces lipid storage, while also indicating that GIP may exert its effect over interaction with other signaling systems, such as leptin. These findings also agree with the data of Jiuhua Duan, which showed that GIP injections induced up-regulation of vasopressin (AVP) and oxytocin (OXT), and that both were involved with central control of lipolysis through the sympathetic nervous system (J. Duan, University of Georgia PhD dissertation 2006, CH 7).

Analysis of body weight showed that high doses of GIP led to significant reduction of body weight gain in comparison to the control. Conversely, GIP seems to have no effect over spontaneous activity and body temperature. Therefore, we may conclude that weight reduction is likely due to a decrease of food intake rather than through changes in levels of activity. However, a paper by Isales, et al. has shown that GIP over-expressing transgenic mice exhibit altered behavior with increased locomotor activity (Ding, Zhong et al. 2006). They conclude that GIP receptors play a role in the modulation of spontaneous activity. In this study, we failed to detect changes in activity levels. It

could be speculated that changes in activity in their study may be due to developmental changes in this transgenic mice model, rather than due to changes in GIP level (Ding, Zhong et al. 2006).

There have been numerous studies showing that GIP may be involved in bone formation, linking nutrient ingestion to bone formation. However, no known studies have been conducted on the involvement of GIP in centrally modulated bone formation. GIP receptor knockout mice have decreased bone, while having increased bone marrow fatness (Xie, Cheng et al. 2005). Gene expression from the brain showed that CART expression was significantly up-regulated (J. Duan, University of Georgia PhD dissertation 2006, CH 7). This finding correlates with previous gene expression data from the brain of GIPR knockout mice, which showed that knockout mice have decreased expression of CART, a neuropeptide well known for its food regulating function and one of the neuropeptides having a role in modulating bone metabolism (J. Duan, University of Georgia PhD dissertation 2006, CH 6)`

In view of the reciprocal relationship between bone marrow adipocytes and bone cells, we expected to see changes in bone marrow adipocytes. We sectioned rat femur and measured bone marrow adipocyte size and number. However, we failed to detect significant changes. Reasons could be there was no effect on bone as speculated, or due to the shortness of treatment time, or because there is not such a correlation between bone marrow adipocytes and bone formation. Moreover, we did not detect changes in spinal bone mineral density following ICV GIP treatment. Although Duan found that CART expression was elevated following ICV GIP administration, we speculated that the effect of higher CART has less to do with the bone effect as the author points out, rather than related to its effect on decreased food intake.

Consistent with Hamrick (2007), this study also detected that leptin treatment decreased bone marrow adipocytes size and number. In addition, ICV leptin increased bone mineral density in spine of rats. Published data regarding the effect of leptin on bone formation is rather confusing. It seems that leptin's effect on bone depends on many factors, such as, site of bone, route and dosage of administration, as well body leptin status. Most of the data thus far were based on the leptin deficient animal, which is leptin sensitive. Information regarding the effect of leptin on leptin replete animal, however, is dearth. Hamrick et al in 2005 pointed out that effect of leptin on leptin replete animal is absent or even adverse, as compared with the beneficial effect of leptin on leptin deficient ob/ob mice. Guidobono et al (2006) found long-term infusion of leptin into the brain has no inhibitory effect on bone mineral density in spine of rats. Through this study, however, we found that ICV injection of leptin increased spinal bone mineral density in rats. The apparent disparity between this study and Guidobono et al's could be due to the length and pattern of administration of leptin. In a separate study, we found that ICV injection of leptin decreased serum concentration of IGF-1 and a trend to decrease serum osteocalcin, which is evidence that the treatment led to decreased bone turnover, which may explained the increased bone mineral density in these animal.

In summary, centrally administered leptin decreased marrow adipocytes size and number and increased spinal bone mineral density. Centrally administered GIP has a slight effect on the central control of feeding behavior and body weight. GIP also decreases serum leptin concentrations, while exhibiting no effect on bone marrow adipocytes and spinal bone mineral density.

CHAPTER 3

EFFECTS OF ICV GHRELIN AND LEPTIN ON FOOD INTAKE, SPONTANEOUS PHYSICAL ACTIVITY AND BONE IN RATS[1]

[1]Qiang Li, Yang-Ho. Choi, Diane L. Hartzell, Mary Ann Della-Fera, Jiuhua Duan, Mark W. Hamrick, and Clifton A. Baile. To be submitted to *Physiology and Behavior*.

Abstract

Ghrelin plays roles in the control food intake and body energy metabolism. Recently, ghrelin was reported to have a role in modulating bone mass peripherally. However, the hypothesis that ghrelin modulates bone metabolism by acting on its receptors in the brain has not been tested. This study, therefore, was carried out to investigate the possible effects of multiple ICV injections of ghrelin on bone. Male SD rats were implanted with lateral cerebroventricular (ICV) cannulas. Five treatment groups (N=8), including control (aCSF), 5000 ng/rat leptin, 66ng, 330ng, and 1650 ng/rat ghrelin, received twice daily ICV injections for five days. Compared with control, net food intake (NFI) and body weight gain (WG) were decreased by leptin by 38% ($p<.001$) and -6g vs -41g ($p<.001$) respectively, and were increased by 330 ng ghrelin by 8% ($p<.05$) and -6g vs 7.2g ($p=.008$) respectively. In addition, we found that 82.5% of the increase in food intake by 330 ng ghrelin resulted from additional meals during the daytime. Compared with control, leptin decreased tibial marrow adipocytes size by 46% ($p<0.01$) and number by 81% ($p<0.05$). Femoral bone mineral density (BMD) were increased by both leptin and 20pmol ghrelin ($p<0.05$), while spinal bone mineral density was significantly increased only by leptin. In conclusion, in addition to the role in regulating body energy metabolism, ghrelin may also be involved in the regulation of bone mass through a neuronal pathway. Further studies are needed to clarify the underlying mechanism of the central roles of ghrelin on bone mass.

Introduction

Ghrelin, the long sought-after endogenous ligand of the orphan GHS1a receptors, has been shown to have a variety of endocrine effects, including its role in the control of food intake and

energy metabolism (Tschop, Smiley et al. 2000; Nakazato, Murakami et al. 2001; Hosoda, Kojima et al. 2002). The speculation that ghrelin may be involved in the control of bone metabolism first came from a clinical observation that gastrectomy could induce osteopenia (Bussabergher 1938; Ivy 1940; Rumenapf, Schwille et al. 1997). A number of studies showing positive effects of GHSs (Sibilia, Cocchi et al. 1999) on bone led to the belief that the endogenous GHSs, ghrelin, may also play a part in regulating bone metabolism. The effect of ghrelin to promote proliferation and differentiation of rat osteoblasts by acting on their specific receptors were proven in2005 by two separate groups (Fukushima, Hanada et al. 2005; Maccarinelli, Sibilia et al. 2005). Furthermore, one of the teams led by Kojima proved that ghrelin directly regulates bone formation peripherally (Fukushima, Hanada et al. 2005).

The discovery of leptin's central inhibitory effect on bone (Karsenty 2001) extended the knowledge of a novel means of regulating bone mass that included a hypothalamic relay. However, leptin is not likely to be the only one. It may be just a beginning discovery in a long list of such peptides. In fact, due to its opposite roles in regulating energy balance (Kim, Namkoong et al. 2004), sympathetic nervous system activity (Matsumura, Tsuchihashi et al. 2002; Matsumura 2004), and its well-known effect on stimulation of GH secretion, ghrelin has been suggested to be involved in the regulation of bone metabolism through neuronal pathways. However, the hypothesis that ghrelin acts on its receptors in the brain to modulate bone metabolism has not been previously tested.

Because of the uncertainty about a central effect of ghrelin on osteogenesis, the present study was conducted to investigate the possible effects of multiple ICV injection of ghrelin for five days on bone marrow adipogenesis, bone formation, food intake and body weight in rats.

Materials and methods

1. Animals and diet

Forty 8-week-old male Sprague–Dawley rats (250–274 g initial BW) purchased from Harlan (Indianapolis, IN) were housed in individual cages in the TSE Systems unit. Lights were on 0900 h – 2100 h and off 2100 – 0900 h, ambient temperature was set at 22 ± 1 º C and humidity at 50 %. Rats had ad libitum access to pelleted standard lab chow (5001, PMI Nutritional International, Brentwood, MO) and water throughout the study. The Animal Care and Use Committee approved all experimental and surgical procedures in this study for The University of Georgia.

2. Peptides

Rat leptin was purchased from R & D Systems (Cat. No.: 598-LP). Vehicle (artificial cerebrospinal fluid; http://www.alzet.com/faq/proto2.htm) was used to dilute the leptin and achieved a concentration of 2.0 mg/ml. Ghrelin was purchased from Phoenix Pharmaceuticals, Inc. (Cat. No.: 031-31). All peptides were solubilized based on their net protein content.

3. Surgical procedures

Rats were anesthetized with a 3:2:1 (v/v/v) mixture (1 ml/kg i.p.) of ketamine HCl (Ketaset, Fort Dodge Laboratories, Fort Dodge, IA; 100 mg/ml), acepromazine maleate (PromAce, Fort Dodge; 10 mg/ml), and xylazine (Rompun, Miles, Shawnee Mission, KS; 20 mg/ml), and/or IsoFlo®. The hair in the dorsum of the head and in the area behind scapular bones was removed. Each rat was

then placed in the stereotaxic instrument (Stoelting, Wood Dale, IL) and the skin disinfected with betadine solution. A 22-gauge guide cannula (C313G, Plastics One, Roanoke, VA), cut 13.2 mm long, was aseptically implanted into the right lateral ventricle of each rat, with the coordinates of 0.8 mm posterior to the bregma, 1.5 mm lateral to the midline, and 3.2 mm ventral to the surface of the skull based on the atlas of the rat brain (Paxinos and Watson 1986). The cannula was held in place with four stainless steel machine screws and cranioplastic cement (Plastics One) attached to the skull. A 28-gauge stylet (C313DC, Plastics One) was inserted into the guide cannula when the rat did not receive an injection. A programmable transponder (IPTT-200TM, BioMedic Data Systems, Seaford, DE) for temperature measurement was also implanted under skin near the scapula of each rat using a needle-syringe type injector. To control post-surgical pain, two doses of 1.1 mg/kg banamine were given ip at 12 h intervals. Following surgery, the rats were allowed to recover to presurgical body weight. Proper cannula placement was verified in two ways. First, during implantation surgery, backflow of CSF from the tip of the guide indicated correct placement in the ventricle. Second, an angiotensin II (ANG II drinking test was performed at the end of the recovery period, whereby a rat must consume at least 5 ml of water in 30 min following an ICV injection of 100 ng ANG II (100 ng/10 µl) solubilized in sterile aCSF (Sigma, St. Louis, MO) for the cannula to be considered correctly placed. During the morning of the ANG II test, food was removed. Rats were injected with ANG II, in the same manner described above, the time recorded, and the weight of each water bottle measured and recorded. After ANG II injection, rats were returned to their cages and water bottles provided for 30 min, followed by measurement of the bottles. Following surgery, the rats were allowed to recover to presurgical BW for approximately one week.

4. Design and procedures

Following recovery rats were randomly assigned to the five treatments groups (including 0 (aCSF), 66 ng/ injection (G20), 330 ng/ injection (G100), 1650 ng/ injection (G500) ghrelin and 5000 ng leptin/ injection (L5), and were transferred to the TSE behavioral monitoring system.

Treatments of 10 µl injections were administered at 12-hour intervals for five days (Morning between 9:00 AM and 12:00 noon; and evening between 6:00 PM and 9:00 PM before lights were off). Each injection was administered into the lateral cerebral ventricle over 30 s, and the injector left in place for an additional 30 s to allow the CSF to diffuse the drug, followed by removal of the injector and replacement of the stylet into the guide cannula.

Daily body temperature (BT), body weight (BW), food intake (FI), and water intake (WI) were recorded within three hours after light onset. Rats were deeply anesthetized with CO_2 before decapitation on day 6 to remove the brain, blood, gastrocnemius muscle (GC), intrascapular brown fat (BAT), retroperitoneal (rWAT), epididymal (eWAT), and inguinal (iWAT) white fat pads, left and right femora and tibias, and the lumbar vertebrae L1-5. Trunk blood was collected and serum frozen at –20 ºC for measurement of glucagon, leptin, insulin, and ghrelin concentrations. Tissues were weighed, frozen in liquid nitrogen, and stored at –80 ºC until further analysis. Bones were fixed in formalin, decalcified, embedded in paraffin and sectioned. Sections were stained with H&E to visualize adipocytes.

5. Blood parameters

Leptin, insulin and glucagon concentrations were measured by Luminex. Preparation of reagents for immunoassay was based on the Lincoplex Kit, catalog # RENDO-85K-03 and Kit ID # RENDO-40836/10224 (Linco Corp., St. Charles, MO). Ghrelin and IGF-1 were assayed using RIA assays. IGF-1 RIA kit was from Diagnostic Systems Laboratories, Inc. (DSL-2900). Ghrelin RIA kit was ordered from LINCO research Inc. (Catalog # GHRT-89HK). Osteocalcin concentration was obtained using an EIA assay (Biomedical Technologies, Inc. Catalog No: BT-490).

6. Bone histomorphometry

The right femur of each rat was dissected free of soft tissue, fixed in 10% buffered formalin, and stored in 70% ETOH. For bone histomorphometry, the right femur was cut across in the middle of the shaft, decalcified in EDTA, embedded in paraffin, and sectioned at approximately 5 um. Sections were stained with hematoxylin and eosin to visualize adipocytes. Adipocytes were counted over the cross-sectional area of bone marrow and adipocyte density was expressed as the number of adipocyte per tissue area. Adipocyte size was measured as the cross-sectional area of each adipocyte by digitizing the border of each cell using Image-Pro® Plus image analysis software.

87. DEXA densitometry

DEXA densitometry (PIXImus system) was used to measure bone mass (vertebra and femur). Briefly, the excised bones were put on a 7mm Delrin tray (provided by the PIXImus Company), then densitometry of the L2-L4 vertebra and proximal portion of the right femur were obtained using similar method as used for measuring whole carcass.

8. Statistics

Data were analyzed by two-way ANOVA. Least square means were used to determine significance of differences between means, where appropriate. Data are expressed as least square means ± S.E.M., with consideration of significance at $p<0.05$.

Results

Food intake and water intake

Cumulative food intake (CFI) was increased by 330 ng ghrelin and decreased by 5000 ng leptin treatment ($p<0.05$). The 82.5% increase in food intake by the ghrelin treatment resulted from additional meals during the day, and 92.2% of the decrease in food intake by leptin resulted from reduction during night (Fig.3.1). Water intake followed a similar pattern (not shown).

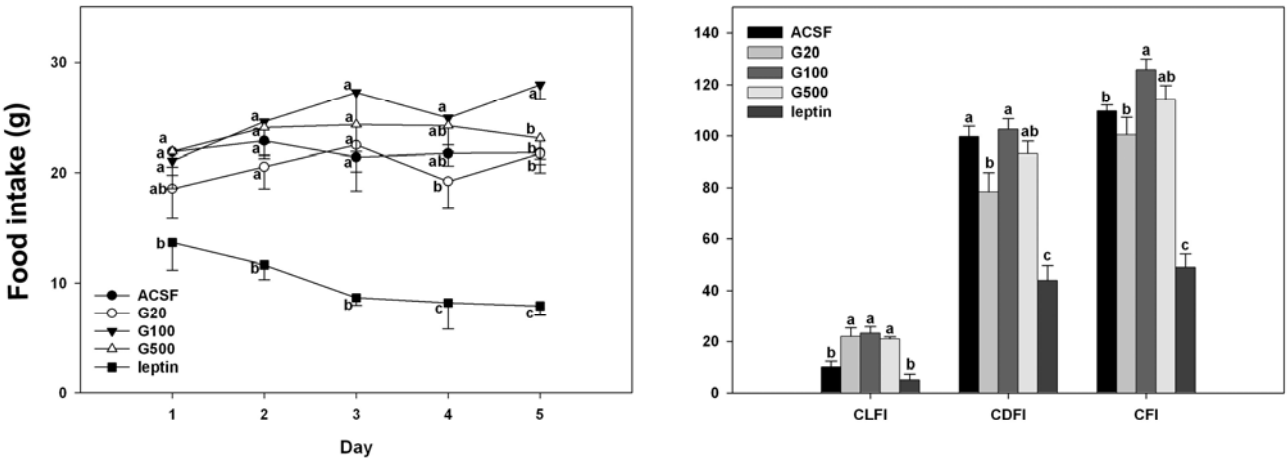

Fig.3.1. Effect of twice daily ICV injections for 5 days with aCSF (10 µl), G20 (66 ng/ injection), G100 (330 ng/ injection), G500 (1650 ng/ injection), or leptin (5000 ng/ injection) in rats on daily food intake (a) and cumulative food intake (b). a, b, c: Means with different letters are significantly different, p <0.05. Data are means ± S.E.M. Note: CLFI (light-phase food intake); DLFI (dark-phase food intake); CFI (cumulative food intake).

Taken together, the increase of food intake and water intake by ghrelin was due mainly to increases

Only leptin significantly decreased daily water intake and decreased WI-to-FI ratio (Table 3.1). during the light phase; while the decrease of food intake & water intake by leptin was due mainly to decreases during the dark phase.

Table 3.1 Average daily water intake (WI) and WI-to-FI ratio in rats receiving twice daily ICV injections for 5 days with aCSF (10 µl), G20 (66 ng/ injection), G100 (330 ng/ injection), G500 (1650 ng/ injection), or leptin (5000 ng/ injection)

Treatment	Daily WI	WI-to-FI
aCSF	31.21±1.98[a]	1.45±0.20[b]
G20	31.59±1.83[a]	1.64±0.18[b]
G100	34.03±1.71[a]	1.35±0.17[b]
G500	33.49±1.83[a]	1.50±0.18[b]
Leptin	19.98±1.71[b]	2.22±0.17[a]

Data are means ± S.E.M.
[ab]Means with different letters within a row are different, $p < 0.05$

Body weight and tissues weight:

Body weight was decreased by leptin and increased by 330 ng ghrelin (Fig 2).

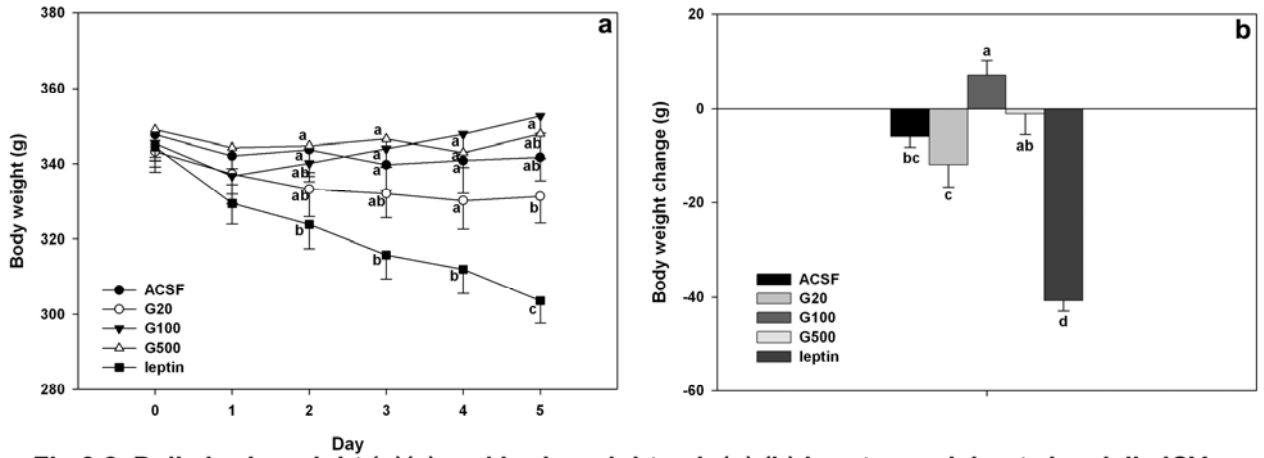

Fig.3.2. Daily body weight (g)(a) and body weight gain(g) (b) in rats receiving twice daily ICV injections for 5 days with aCSF (10 µl), G20 (66 ng/ injection), G100 (330 ng/ injection), G500 (1650 ng/ injection), or leptin (5000 ng/ injection). a, b, c: Means with different letters are significantly different, p<0.05. Data are means ± S.E.M.

Weight of BAT, iWAT, eWAT and rWAT were significantly decreased by leptin; while none were affected by ghrelin; GC weight was unaffected by either leptin or ghrelin (Fig.3).

Serum parameters

Ghrelin concentration was increased by the ICV leptin and 66 ng and 330 ng ghrelin. Leptin and insulin concentrations were significantly decreased by leptin but were unaffected by ghrelin. Glucagon level was not affected by either leptin or ghrelin (Table 3.2).

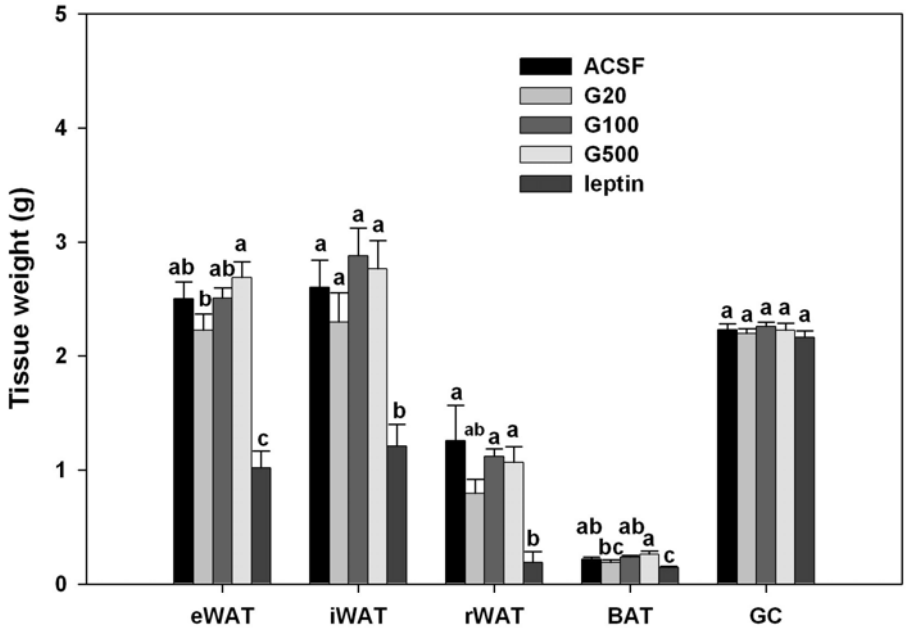

Fig.3.3. Tissues weight (g) in rats receiving twice daily ICV injections for 5 days with aCSF (10 µl), G20 (66 ng/ injection), G100 (330 ng/ injection), G500 (1650 ng/ injection), or leptin (5000 ng/ injection). BAT, brown adipose tissue; eWAT, epididymal white adipose tissue(WAT); iWAT, inguinal WAT; rWAT, retroperitoneal WAT; GC, gastrocnemius muscle. a, b, c: within a tissue, means with different letters are significantly different, p<0.05. Data are means ± S.E.M.

Bone marrow adipocyte and bone density

Adipocyte density, expressed as number of adipocytes per tissue area, and adipocyte size were significantly decreased by leptin, but not by ghrelin. Spinal BMD were increased only by leptin.

Table 3.2. Serum glucagon, insulin, leptin, and ghrelin concentrations in rats receiving twice daily ICV injections for 5 days of aCSF (10 µl), G20 (66 ng ghrelin/inj.), G100 (330 ng ghrelin/inj), G500 (1650 ng ghrelin/inj.), or leptin (5000 ng leptin/inj.).

	aCSF	G20	G100	G500	Leptin
Glucagon (pM)	36.4 ± 5.1	27.7 ± 5.3	30.6 ± 5.0	42.4 ± 5.5	29.1 ± 5.1
Insulin (pM)	310.2 ± 27.2^{a}	265.5 ± 28.4^{ab}	278.9 ± 26.3^{a}	311.9 ± 29.2^{a}	201.6 ± 27.2^{b}
Leptin (pM)	54.9 ± 6.5^{a}	54.4 ± 6.8^{a}	71.4 ± 6.3^{a}	64.5 ± 7.0^{a}	11.3 ± 6.5^{b}
Ghrelin (ng/ml)	3430.6 ± 390.7^{c}	4817.9 ± 417.7^{ab}	4621.2 ± 390.7^{b}	4421.6 ± 417.7^{bc}	5864.8 ± 390.7^{a}

Data are means ± S.E.M.
^{abc}Means with different letters within a row are different, $p < 0.05$

Table 3.3. Femoral bone marrow adipocyte size, number and bone mineral density of tibia and spine in rats receiving twice daily ICV injections for 5 days of aCSF (10 µl), G20 (66 ng ghrelin/inj.), G100 (330 ng ghrelin/inj), G500 (1650 ng ghrelin/inj.), or leptin (5000 ng leptin/inj.).

	aCSF	G20	G100	G500	Leptin
Adipocyte area (*10^{-3} mm^2)	3.44 ± 0.25^{a}	3.50 ± 0.27^{a}	3.34 ± 0.27^{a}	2.94 ± 0.27^{a}	1.80 ± 0.27^{b}
Adipocytes per 0.1mm^2	332 ± 78^{a}	265 ± 84^{ab}	287 ± 78^{ab}	254 ± 84^{ab}	83 ± 34^{b}
Tibia BMD (g/cm^2)	3.85 ± 0.078^{bc}	4.08 ± 0.085^{a}	3.78 ± 0.078^{c}	3.86 ± 0.85^{ab}	4.32 ± 0.085^{a}
Spine BMD (g/cm^2)	4.02 ± 0.12^{b}	4.28 ± 0.13^{ab}	4.18 ± 0.12^{b}	4.25 ± 0.13^{ab}	4.61 ± 0.12^{a}

Data are means ± S.E.M.
^{ab}Means with different letters within a row are different, $p < 0.05$

Femoral BMD was increased by both leptin and G20 ($p<0.05$) (Table 3.3).

Discussion

The main objective of this study was to determine if centrally administered ghrelin and/or leptin have effects on bone marrow adipocytes and bone mass. To determine whether bone density was affected, we scanned the spine and femur of rats using a PIXImus densitometer. We found that

tibia BMD was marginally increased by low-dose ghrelin. The reason could be that low-doses of ghrelin in the brain mimics the effects of endogenous ghrelin in the natural positive energy state, which will affect growth directly or indirectly through the release of growth hormone. High-dose of ghrelin in the central nervous system mimics the effect of long-term starvation, which may not be as effective in inducing body growth and development as the low dose.

Consistent with data from a published abstract by Hamrick et al (Hamrick 2006), this study also found that central leptin injections decreased marrow adipocyte size and number in rats. The depletion of adipocytes may have result from the apoptosis effect when leptin acts on its receptors in the brain.

In contrast, ICV ghrelin did not affect marrow adiposity. Ghrelin receptors are abundantly expressed in adipose tissue. A previous study (Thompson, Gill et al. 2004) showed that direct infusion of ghrelin into bone marrow increased adipogenesis. The effect of ghrelin on adipogenesis still exists even after adjusting for the factor of increased food intake (Kim, Namkoong et al. 2004). However, the effect is pattern dependent. The adipogenic effect of intermittent ghrelin exposure may be masked by the effect of enhanced GH secretion (Thompson, Gill et al. 2004). Since the twice-daily injection of ghrelin in this study was more like the intermittent administration as in a previous study, the failure to detect increased marrow adiposity could result from the effect of enhanced secretion of GH.

Consistent with previous studies on food intake (Tschop, Smiley et al. 2000; Kamegai, Tamura et al. 2001; Kojima 2001). ICV ghrelin increased food intake. The maximal food intake response resulted from the 330 ng ghrelin treatment. Because ghrelin was injected twice daily, we

compared the daytime and nighttime intakes were compared separately. The increased consumption in daytime was the primary contributor to the overall increased food intake. Nighttime intake, however, were not different from those of the control treatment.

As previously shown, serum insulin and leptin levels were positively correlated to body adiposity. Serum ghrelin levels were raised by both leptin and ghrelin treatments. The increase in ghrelin concentration associated with leptin treatments is likely to be the result of the inverse relationship of these hormones as previously reported (Kim, Namkoong et al. 2004). The plausible explanation of the increased serum ghrelin level associated with ghrelin treatments is not clear. It is unlikely that the result of the ICV injection directly influence the peripheral blood concentrations.

In conclusion, increased food intake stimulated by ghrelin ICV treatments results from additional meal intake during light phase. ICV leptin decreased marrow adipocyte size and number in appendicular bone. ICV low dose ghrelin exerted beneficial effects on bone mass as reflected in the higher PIXImus bone mineral density. Further studies are needed to clarify the underlying mechanism of the central roles of ghrelin on bone mass.

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CHAPTER 4

CNS GHRELIN AND LEPTIN HAVE OPPOSING FUNCTIONS IN THE REGULATION OF FOOD INTAKEE BUT NOT IN REGULATING OF BONE FORMATION IN RATS[1]

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Abstract

The hunger hormone ghrelin was recently found to be a direct regulator of peripheral bone formation, although its central effect on bone has not been studied. That ghrelin regulates bone formation stems from its opposing central functions to leptin. In this study, Male Sprague Dawley rats were implanted with lateral cerebroventricular (ICV) cannulas. Four treatment groups (N=6), including control (aCSF), 5 μg/rat leptin, 20 pmol (G20), and 100 pmol (G100) ghrelin/rat, received ICV injections twice daily for five consecutive days. Food intake was increased by G100 by 18% ($p<0.05$) and decreased by leptin by 51.8% ($p<0.001$). Body weight gain (WG) was decreased by leptin (-30.9g vs. -0.2g, $p<.001$), and was increased by G20 (10.6g vs. -0.2g, $p<0.05$) and G100 (15.4 g vs. -0.2g, $p<0.001$) compared with control. Leptin decreased weights of BAT by 40.4% ($p<0.05$), EP by 18.6% ($p<0.05$), and RP by 100% ($p<0.05$). G100 increased weights of EP by 24.6% ($p<0.05$) and RP by 38.5% ($p<0.05$). Serum glucagon and leptin were decreased by leptin by 67.8% ($p<0.01$) and 68.3% ($p<0.01$), respectively. Leptin decreased the serum level of insulin by 81.3% ($p<0.01$), while G100 increased the serum level of insulin by 70% ($p<0.05$). In addition, the apoptosis ratio of retroperitoneal fat tissue was increased by 125% ($p<0.05$) in leptin-administered rats. Serum IGF-1 levels were significantly decreased by leptin. Tibial bone mineral density (BMD) was elevated by both leptin and G20, while spinal BMD was significantly increased only by leptin. To investigate if centrally administered ghrelin affects the regulation of bone marrow gene expression, total RNA was extracted from both non-adherent cell and adherent bone marrow cell cultures, as well as directly from bone marrow tissue. Real-time TaqmanRT-PCR (ABI Microfluidic card) was used to quantitatively compare mRNA levels of selected osteogenesis genes. RT-PCR

results showed that G20 unregulated bone marrow mRNA expression of CASP2, MMP11 and MADH1 increased by 92.8% ($p<0.01$), 168.7% ($p<0.05$), and 114.0% ($p<0.05$), respectively, only in non-adherent cells. Since non-adherent cells are composed of osteoclasts and osteoclast progenitor cells. This may suggest ghrelin led to depletion of marrow osteoclast through apoptosis. We also compared adipogenesis gene expression from bone marrow and from white adipose tissue (EP). Comparative adipogenesis gene expression analysis showed that leptin treatment led to a decreased expression of adipogenesis markers such as Fasn, Scd1, Slc2a4 etc., while up-regulating lipolysis markers such as Mfn2 and Adrb2. Ghrelin treatment resulted in increased adipogenesis gene expression from white adipose tissue and decreased adipogenesis gene expression in bone marrow. The decreased expression of adipogenesis genes agree with the decreased marrow adipocyte size by ghrelin, which may potentially benefit for bone formation. In conclusion, centrally administered ghrelin results in increased bone mineral density. This beneficial effect of ghrelin on bone could result from decreased osteoclastogenesis as well as from reduced marrow adipogenesis. Sympathetic nervous system activity may not only mediate the action on peripheral adipocyte, but may also exert effects over bone marrow cell populations.

This study was supported in part by the Georgia Research Alliance Eminent Scholar endowment held by CAB.

Introduction

Ghrelin, the long-sought after endogenous ligand of the orphan GHS1 receptors, has been shown to have a variety of endocrine effects, including its key role in the control of food intake and energy metabolism (Tschop, Smiley et al. 2000; Nakazato, Murakami et al. 2001; Hosoda, Kojima et

al. 2002). The speculation that ghrelin may be involved in the control of bone metabolism first came from a clinical observation that gastrectomy could induce osteopenia (Bussabergher 1938; Ivy 1940; Rumenapf, Schwille et al. 1997). A number of studies showing positive effects of GHSs (Sibilia, Cocchi et al. 1999) on bone indicated that the endogenous GHSs, ghrelin, may also play a part in regulating bone metabolism.

In 2005, the effect of ghrelin in promoting proliferation and differentiation of rat osteoblasts by acting on their specific receptors was proven by two separate groups (Fukushima, Hanada et al. 2005; Maccarinelli, Sibilia et al. 2005). Furthermore, one of the teams led by Kojima proved that ghrelin directly regulates peripheral bone formation (Fukushima, Hanada et al. 2005).

The discovery of leptin's central inhibitory effect on bone (Karsenty 2001) is now known to involve a hypothalamic relay. However, leptin is likely not the only brain peptide to have a role on bone physiology. In fact, due to its opposite roles in regulating the body's energy system (Kim, Namkoong et al. 2004) and sympathetic nervous system (Matsumura, Tsuchihashi et al. 2002; Matsumura 2004), as well as its well-known effect on stimulation of GH secretion, ghrelin's regulation of bone metabolism occurs through neuronal pathways. However, the hypothesis that ghrelin acts on its receptors in the brain to modulate bone metabolism has not been tested until our lab investigated the possible effects of multiple ICV injections of ghrelin on food intake, body weight, bone marrow adipogenesis and bone formation in rats. Results from this study were consistent with previous reports on the effects of leptin and ghrelin on food intake, water intake, body weight, body temperature, tissue weights, and a series of blood parameters. In addition, we found that central leptin decreased marrow adipocyte size and number in appendicular bone while

increasing bone mineral density in axial and appendicular bone. These findings were consistent with a previous study by this lab, which showed that VMH leptin decreases marrow adipocyte size and number through apoptosis in normal rats (Hamrick, Della Fera et al. 2007).In contrast, these findings were opposite the current view of leptin's inhibitory effect on axial bone via the hypothalamus relay (Karsenty 2001).

Moreover, our previous study found that low dosages of ghrelin (20pmol) exerted beneficial effects on bone by increasing tibia bone mineral density. This study, therefore, was designed to confirm the results of the previous study. In addition, we employed histomophormetry, densitometry, and measurement of serum bone markers, as well as microfluidic card technology to map the gene expression profile of bone marrow cell populations to describe the mechanism of the central effects of ghrelin on bone.

Three main cell types found in bone marrow are osteoclasts, osteoblasts and adipocytes. Osteoblasts and adipocytes share a common mesenchymal stem cell precursor (Pittenger, Mackay et al. 1999); a shift in differentiation, survival or elimination rates from one lineage to another could lead to an altered ratio of fat to bone cells. A decrease in bone volume is often accompanied by an increase in bone marrow (BM) fat. Our previous studies showed that central leptin injection induced bone marrow adipocytes to undergo apoptosis (Hamrick, Della-Fera et al. 2005), while central ghrelin administration increased bone density and induced a trend toward decreased marrow adipocyte size and number. It is therefore reasonable to speculate that either central leptin or ghrelin administration can modulate the ratio of cell populations in bone marrow.

Materials and methods

1. Animals and diet

Twenty-four 8-week-old male Sprague–Dawley rats (250–274 g initial BW) purchased from Harlan (Indianapolis, IN) were housed in individual cages in the TSE Systems unit. Lights were on between 0900 h – 2100 h and off between 2100 – 0900 h, ambient temperature was set at 22 ± 1 º C and humidity at 50 %. Rats had ad libitum access to pelleted standard lab chow (5001, PMI Nutritional International, Brentwood, MO) and water throughout the study. The Animal Care and Use Committee approved all experimental and surgical procedures in this study for The University of Georgia.

2. Peptides

Dr. Arieh Gertler (The Hebrew University, Israel) generously provided rat leptin. Vehicle (artificial cerebrospinal fluid; http://www.alzet.com/faq/proto2.htm) was used to dilute the leptin and achieved a concentration of 2.0 mg/ml. Ghrelin was purchased from Phoenix Pharmaceuticals, Inc. (Cat. No.: 031-31). All peptides were solubilized based on their net protein content.

3. Surgical procedures

Rats were anesthetized with a 3:2:1 (v/v/v) mixture (1 ml/kg i.p.) of ketamine HCl (Ketaset, Fort Dodge Laboratories, Fort Dodge, IA; 100 mg/ml), acepromazine maleate (PromAce, Fort Dodge; 10 mg/ml), and xylazine (Rompun, Miles, Schawnee Mission, KS; 20 mg/ml), and/or IsoFlo®, and the hair in the dorsum of the head as well as the area behind scapular bones was removed. Each rat was then placed in the stereotaxic instrument (Stoelting, Wood Dale, IL) and the skin disinfected with

betadine solution. A 22-gauge guide cannula (C313G, Plastics One, Roanoke, VA), cut 13.2 mm long, was aseptically implanted into the right lateral ventricle of each rat with the coordinates of 0.8 mm posterior to the bregma, 1.5 mm lateral to the midline and 3.2 mm ventral to the surface of the skull, based on the atlas of the rat brain (Paxinos and Watson 1986). The cannula was held in place with four stainless steel machine screws and cranioplastic cement (Plastics One) attached to the skull. A 28-gauge stylet (C313DC, Plastics One) was inserted into the guide cannula when the rat did not receive an injection. A programmable transponder (IPTT-200TM, BioMedic Data Systems, Seaford, DE) for temperature measurement was also implanted under the skin near the scapula of each rat using a needle-syringe type injector. To control post-surgical pain, two doses of 1.1 mg/kg banamine were given ip at 12 h intervals. Following surgery, the rats were allowed to recover to presurgical body weight. Proper cannula placement was verified in two ways. First, during implantation surgery, backflow of CSF from the tip of the guide indicated correct placement in the ventricle. Second, an angiotensin II (ANG II drinking test) was performed at the end of the recovery period, whereby a rat must consume at least 5 ml of water in 30 min following an ICV injection of 100 ng ANG II (100 ng/10 μl) solubilized in sterile aCSF; (Sigma, St. Louis, MO) for the cannula to be considered correctly placed. During the morning of the ANG II test, food was removed. Rats were injected with ANG II, in the same manner described above, the time recorded, and the weight of each water bottle measured and recorded. After ANG II injection, rats were returned and water bottles provided for 30 min, followed by measurement of the bottles. Following surgery, rats were allowed to recover to presurgical BW for approximately one week.

4. Design and procedures

Following recovery, rats were randomly assigned to the four treatments groups (including 0 (aCSF), 66 ng/ injection (G20), 330 ng/ injection (G100) and 5000 ng leptin/ injection (L5)), and were transferred to the TSE behavioral monitoring system.

Treatments of 10 µl injections were administered at 12-hour intervals for five days (morning between 9:00 AM and 12:00 AM; and evening between 6:00 PM and 9:00 PM before lights were turned off). Each injection was administered into the lateral cerebral ventricle over 30 s, and the injector left in place for an additional 30 s to allow the CSF to diffuse the drug, followed by removal of the injector and replacement of the stylet into the guide cannula.

Daily body temperature (BT), body weight (BW), food intake (FI) and water intake (WI) were recorded within 3 h after light onset. Rats were deeply anesthetized with CO_2 before decapitation on day six to remove the brain, blood, gastrocnemius muscle (GC), intrascapular brown fat (BAT), retroperitoneal (rWAT), epididymal (eWAT) and inguinal (iWAT) white fat pads. Tibias were used for measuring bone mineral density and for visualization and quantification of marrow adipocytes. Femurs and front legs were used for extracting bone marrow RNA. Trunk blood was collected and serum frozen at –20 ºC for measurement of glucagon, leptin, insulin, IGF-I, ghrelin and osteocalcin concentrations. Tissues were weighed, frozen in liquid nitrogen and stored at –80 ºC until further analysis. Bones were fixed in formalin, decalcified, embedded in paraffin and sectioned. Sections were stained with H&E to visualize adipocytes.

5. Feeding behavior and spontaneous activity measurement

Rats were individually housed in cages equipped to automatically measure feeding and drinking behavior and spontaneous physical activity (TSE Systems, Bad Homburg, Germany). The InfraMot activity unit registers activity by sensing the body-heat image, i.e. infrared radiation and its spatial displacement over time. Rats were removed from the monitoring system for approximately four minutes at the same time each day for BW and BT measurements.

6. Blood parameters

Leptin, insulin and glucagon concentrations were measured by Luminex. Preparation of reagents for immunoassay was based on the Lincoplex kit (Catalog # RENDO-85K-03, and Kit ID # RENDO-40836/10224, Linco Corp., St. Charles, MO). Ghrelin and IGF-1 were assayed using RIA assays. IGF-1 RIA kit was from Diagnostic Systems Laboratories, Inc. (DSL-2900). Ghrelin RIA kit was ordered from LINCO Research, Inc. (Catalog # GHRT-89HK). Osteocalcin concentration was obtained using an EIA assay (Biomedical Technologies, Inc., Catalog No: BT-490).

7. Bone DEXA densitometry and histomophormetry

The right and left tibia of each rat was dissected free of soft tissue. Length and weight of each bone was measured. PIXImus system was used to measure bone mineral density. Briefly, the excised bones were put on a 7mm Delrin tray (provided by the PIXImus Company); measurement of BMD of tibias was obtained following the method for measuring whole carcass.

Bones were fixed in 10% buffered formalin (neutral buffered formalin, Fisher SF 93-4) at room temperature for 48 hours before transfer and stored in 70% ETOH at 4 ºC. For bone

histomorphometry, the tibia was cut across in the proximal middle third of the shaft, decalcified in EDTA, embedded in paraffin, and sectioned at approximately 5 μm. Sections were stained with hematoxylin and eosin to visualize adipocytes. Adipocytes were counted over the cross-sectional area of bone marrow, with adipocyte density expressed as the number of adipocyte per tissue area. Adipocyte size was measured as the cross-section area of each adipocyte by digitizing the border of each cell using Image-Pro® Plus image analysis software.

8. RNA extractions and microfluidic card gene expression

Bone marrow RNA was extracted using two independent routes, cell culture system and RNA direct extraction from marrow tissue.

The left femur ends of rat were removed and put in minicentrifuger (4 ºC). Immediately after bone marrow was centrifuged out of cavity, 1 ml of pre-warmed DMEM (with 10% FBS) was added. After quantification of the cell number, cells were seeded in 100 mm tissue culture dish at density of 5x107 per 10 ml of media. 2 hours after plating, non-adherent cells were transferred into a new plate, and the original plate was washed with 5 ml of medium before transferring to the new plate; another 5 ml of medium was added to the original plate; 24 hours after initial plating, both original (adherent cell) and the new plates (non-adherent cell) were washed with 1X PBS twice to remove the floating red blood cells; followed by RNA extraction according to the kit specifications (QIAGEN Catalog #74104 RNeasy Mini Kit) (Appendix 1). After reverse transcription, real-time TaqmanRT-PCR (ABI Microfluidic cards) was used to quantitatively compare mRNA levels of selected genes in the bone marrow cell cultures.

Marrow from the right femur and front legs were pooled together following centrifuge. After that, 1 ml of stabilization reagent (Roche Cat.No.1-934-317) was immediately added. Samples were stored at -20 ºC before carrying out the RNA extraction procedure with Trizol.

RNA extraction with Trizol follows the procedure as described by the product insert with some modification. For the remainder of the steps, see detailed description of the Trizol method in Appendix 2.

The integrity of the RNA produced from all samples used was verified and quantified using an RNA 6000 Nano Assay and the Agilent 2100 Bioanalyzer (Agilent Technologies).

One hundred ng of total RNA in a 20-µl reaction was reverse-transcribed using the cDNA Archive Kit (Applied Biosystems, Inc., part #4322171) according to the manufacturer's protocol using MultiScribeTM Reverse Transcriptase. Reactions were incubated initially at 25 ºC for ten minutes and subsequently at37 ºC for 120 minutes. Quantitative PCR (TaqmanTM) assays were chosen for the transcripts to be evaluated, from Assays-On-DemandTM (ABI), a pre-validated library of QPCR assays incorporated into 384-well MicroFluidic cardsTM. All of the oligonucleotide primer and fluorogenic probe sets for TaqmanTM real time PCR were from ABI. Two µl of the cDNA samples, along with 50 µl of 2× PCR master mixes were loaded into respective channels on the microfluidic card followed by a brief centrifugation (3000 rpm for three minutes). The card was then sealed and real-time PCR and relative quantification was carried out on the ABI PRISM 7900 Sequence Detection System. The cycle conditions were: 94.5 ºC for 15 minutes, followed by 40 cycles of 97 ºC for 30 s and 59.7 ºC for one minute. Data were analyzed using sequence detection systems software, and the relative quantification, which presents the fold difference of mRNA level in treatment groups relative

to the aCSF control group. mRNA expression was normalized by using 18S RNA as an endogenous control to normalize the differences in the amount of total RNA added to each reaction. The ΔCT values were first calculated by using CT for a specific gene mRNA minus CT for 18S RNA mRNA in the sample. Then the mean mRNA expressions from the treatment groups were compared with the aCSF control group using the formula: relative quantification = 2-ΔΔCT (ΔΔCT is the average aCSF control group ΔCT values minus the average experimental group ΔCT values, and ΔΔCT of one equates to a twofold difference in cDNA added into the PCR reaction).

The following 23 genes were selected based on their ability to be detected in a mixed cell environment, and to express genes specific to one type of cell, e.g., osteoblasts, adipocytes, etc. Also included were genes regulating stromal cell fate, such as the well-established markers of bone marrow adipogenesis (PPAR gamma), osteogenesis (ANXA5, BGLAP, BGN), and apoptosis (BBC3 and CASP2):

Pre- adipocyte: GATA-3 (Tong, Dalgin et al. 2000)and DLK1 (Lee, Villena et al. 2003))

Adipocyte: LEP (Machinal-Quelin, Dieudonne et al. 2002) , RTN, FABP4 (Baxa, Sha et al. 1989), PPARG (Tontonoz, Hu et al. 1995)

Apoptosis: BBC3 (Chipuk, Bouchier-Hayes et al. 2005) and CASP2 (Bergeron, Perez et al. 1998)

Osteogenesis: ANXA5 (Rodriguez-Garcia, Kozak et al. 1996), BGLAP (Ducy and Karsenty 1995), BGN (Xu, Bianco et al. 1998), CD36 (Coburn, Knapp et al. 2000), SPARC (Termine, Kleinman et al. 1981), MMP11 (Nagase, Barrett et al. 1992), CTSK (Saftig, Hunziker et al. 1998), FN1, SERPINH1 (Clarke, Cates et al. 1991)

Growth Factor: EGF (Carpenter and Cohen 1979), FGF14 (Smallwood, Munoz-Sanjuan et al. 1996), MADH1 (Riggins, Thiagalingam et al. 1996), SRA1 (Caretti, Schiltz et al. 2006), TNF (Broudy, Kaushansky et al. 1986), VEGF (Fukumura, Xavier et al. 1998)

Control: 18S RNA

In order to measure the adipogenesis-related gene markers in marrow, and compare with the expression profiles from white adipose tissue, a different card was designed. This card incorporated key osteogenesis-related genes, which were not included in the previously mentioned cards. The following 23 genes were grouped into clusters based on their functions.

Thermogenesis: UCP1 (Lowell, V et al. 1993), UCP2 (Arsenijevic, Onuma et al. 2000), UCP3 (Argyropoulos, Brown et al. 1998), MFN2 (Bach, Pich et al. 2003), LEPR (Cohen, Zhao et al. 2001), LEP.

Apoptosis: TNF

Adipogenesis: SLC2A4 (Birnbaum 1989), CEBPA (Cao, Umek et al. 1991), GPD1 (Menaya, Gonzalez-Manchon et al. 1995), SCD1 (Cohen, Miyazaki et al. 2002), FASN (Casado, Vallet et al. 1999)

Lipolysis: ADRB3 (Clement, Vaisse et al. 1995), LIPE (Holst, Langin et al. 1996), LPL (Etienne and Brault 1992)

Adipocyte differentiation: PPARG, PPARA (Kersten, Desvergne et al. 2000)

Bone-related parameters: ADRB2 (Elefteriou, Ahn et al. 2005), CBFA1 (Ducy, Starbuck et al. 1999), ALPL (Moore, Curry et al. 1999), BMP2 (Cheng, Jiang et al. 2003), COL1A1 (Aitchison, Ogilvie et al. 1988)

Endogenous control: GAPDH, 18S RNA

9. Statistics

Data were analyzed by two-way ANOVA. Least square means were used to determine significance of differences between means, where appropriate. Data are expressed as least square means ± S.E.M., with consideration of significance at $p<0.05$.

Results

Part I General physiological data

Food intake and body weight

Cumulative food intake was increased by G100 and decreased by leptin treatment ($p<0.05$) (Fig.4.1.). Water intake followed a similar pattern, but only leptin decreased water intake significantly (data not shown). Leptin decreased body weight gain significantly ($p<0.05$), and low and high dose ghrelin increased body weight gain significantly ($p<0.05$). There is no difference between low and high dose ghrelin treatment (Fig.4.1.).

Feeding Behavior

While leptin decreased meal number, increased meal interval, and increased satiety ratio, ghrelin affected only inter-meal interval to a significant level. In addition, there was no difference in the other parameters, and there were no night and day time related differences (Table 4.1, Table 4.2).

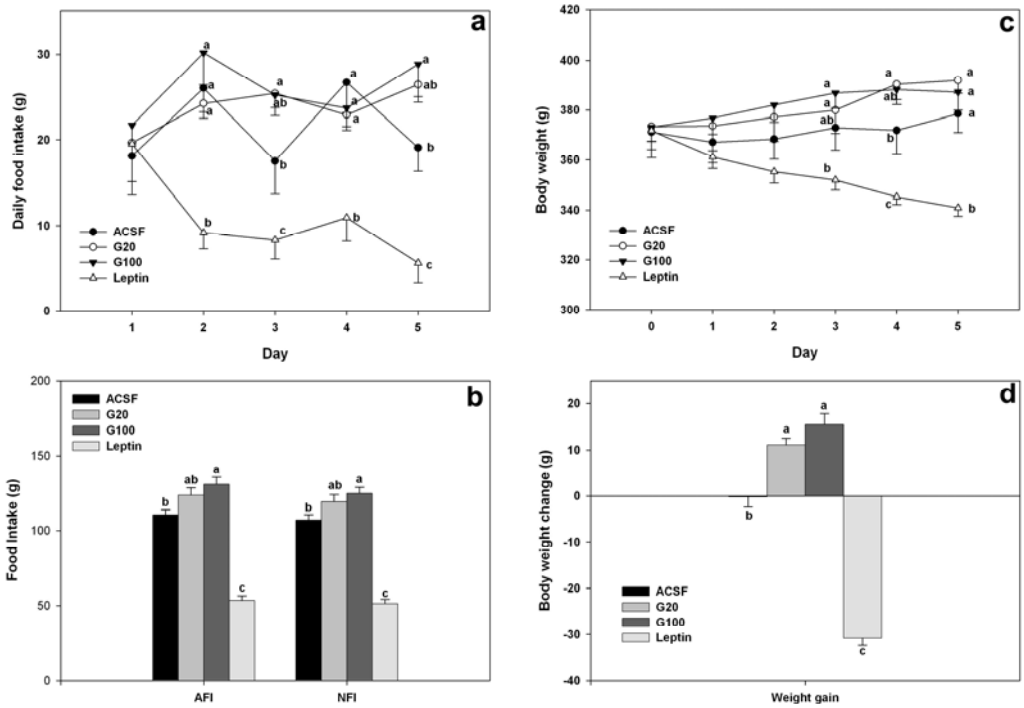

Fig.4.1. Effect of twice daily ICV injections for 5 days with aCSF (10 µl), G20 (20 pmol ghrelin/inj.), G100 (100 pmol ghrelin /inj.), or leptin (5 ug leptin/injection) in rats on daily food intake (a), cumulative food intake (b), daily body weight (c), and body weight gain (d). a, b, c: Means with different letters are significantly different, p <0.05. Data are means ± S.E.M. Note: AFI (apparent food intake); NFI (net food intake).

Tissues weight

Leptin treatment decreased BAT, EP and RP weights ($p<0.05$); 100 pmol ghrelin increased EP and RP weights ($p<0.05$); GC and soleus weights were unaffected by either leptin or ghrelin. (Fig.4.2)

Table 4.1. Mean size, meal number and eating rate in rats injected i.c.v with 10 µl/inj. of aCSF (Con), G 20 (Ghrelin 20 pmol), G100 (Ghrelin 100 pmol), L5 (leptin, 5 µg) twice daily for 5 days

		aCSF	G 20	G100	Leptin
Meal size (g)	24 h mean	2.97±0.34	3.66±0.37	3.08±0.36	3.13±0.44
	Light	3.04±0.34	3.4±0.37	3.0±0.34	4.3±0.45
	Dark	2.9±0.34	3.9±0.34	3.2±0.36	1.96±0.46
Meal number	24 h total	19.3±3.2[a]	18.7±3.2[a]	20.7±3.2[a]	8.5±3.6[b]
	Light	3.54±0.27	3.47±0.29	3.1±0.27	1.75±0.37
	Dark	3.1±0.28	3.17±0.27	4.0±0.29	1.36±0.51
Meal durations (sec)	24 h mean	408.0±67.5	453.5±67.3	533.8±56.6	139.8±135.9
	Light	334.5±34.3	445.9±37.1	475.7±33.9	111.6±70.4
	Dark	481.4±35.2	461.0±32.0	591.9±28.4	167.9±71.2
Eating rate (g/min)	24 h mean	0.92±0.21	0.96±0.22	0.46±0.20	1.62±0.36
	Light	1.08±0.13	1.08±0.15	0.47±0.14	2.1±0.23
	Dark	0.76±0.14	0.84±0.14	0.45±0.13	1.12±0.25

Data are means ± S.E.M.
[abc]Means not denoted with a common letter are different, *p<0.05*

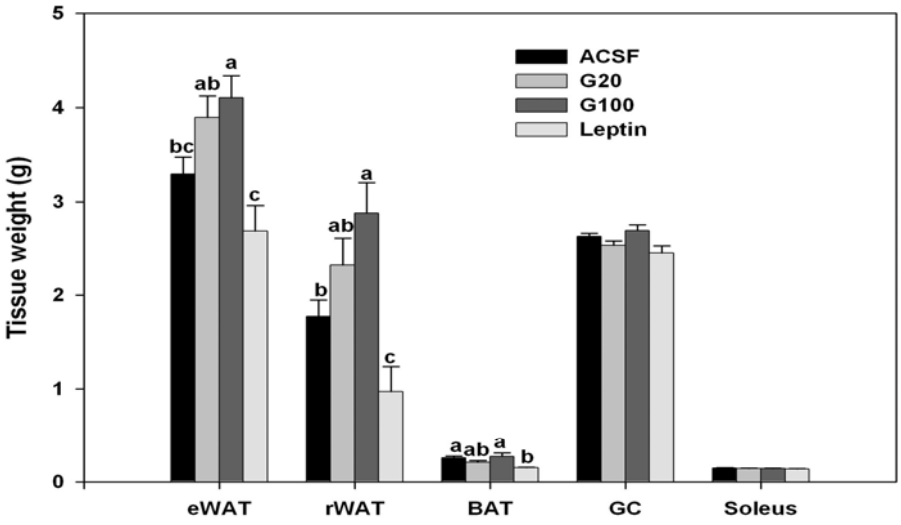

Fig.4.2. Tissues weight (g) in rats receiving twice daily ICV injections for 5 days with aCSF (10 µl), G20 (20 pmol ghrelin/ inj.), G100 (100 mol ghrelin/ inj.), or leptin (5 ug/ inj.). BAT, brown adipose tissue; eWAT, epididymal white adipose tissue(WAT); iWAT, inguinal WAT; rWAT, retroperitoneal WAT; GC, gastrocnemius muscle. a, b, c: within a tissue, means with different letters are significantly different, p<0.05. Data are means ± S.E.M.

Table.4.2. Satiety ratio (post-meal interval/proceeding meal size) and hunger ratio (pre-meal interval/subsequent meal size) in rats injected i.c.v with 10 µl/inj. of aCSF (Con), G 20 (Ghrelin 20 pmol), G100 (Ghrelin 100 pmol), L5 (leptin, 5 µg) twice daily for 5 days

		aCSF	G 20	G100	Leptin
Inter-meal interval (sec)	24 h mean	191.2±35.4^{b}	165.6±37.1^{b}	128.6±34.2^{b}	480.3±60.5^{a}
	Light	223.0±24.7^{b}	179.3±27.4^{bc}	133.5±26.2^{c}	695.1±43.4^{a}
	Dark	159.4±25.9^{b}	152.0±25.9^{b}	123.7±23.6^{b}	265.4±45.4^{a}
Satiety ratio (min/g)	24 h total	59.9±5.9^{b}	59.6±6.0^{b}	61.1±5.6^{b}	145.8±14.0^{a}
	Light	75.6±9.4	70.0±10.3	68.1±9.9	180.4±18.2
	Dark	44.1±10.1	49.2±9.8	54.1±8.9	111.2±28.3
Hunger ratio (min/g)	24 h mean	80.1±6.5^{b}	63.7±6.6^{b}	62.1±6.1^{b}	108.5±15.2^{a}
	Light	75.5±11.6	68.1±12.7	62.0±12.3	145.0±22.4
	Dark	84.6±12.4	59.4±12.1	62.3±11.0	71.9±34.8

Data are means ± S.E.M.
^{abc}Means not denoted with a common letter are different, $p<0.05$

Spontaneous physical activity and body temperature

Daytime activity was increased by both leptin and 100pmol ghrelin ($p<0.05$); leptin also increased total activity level ($p<0.05$); body temperature was unaffected by either leptin or ghrelin (Fig.4.3)

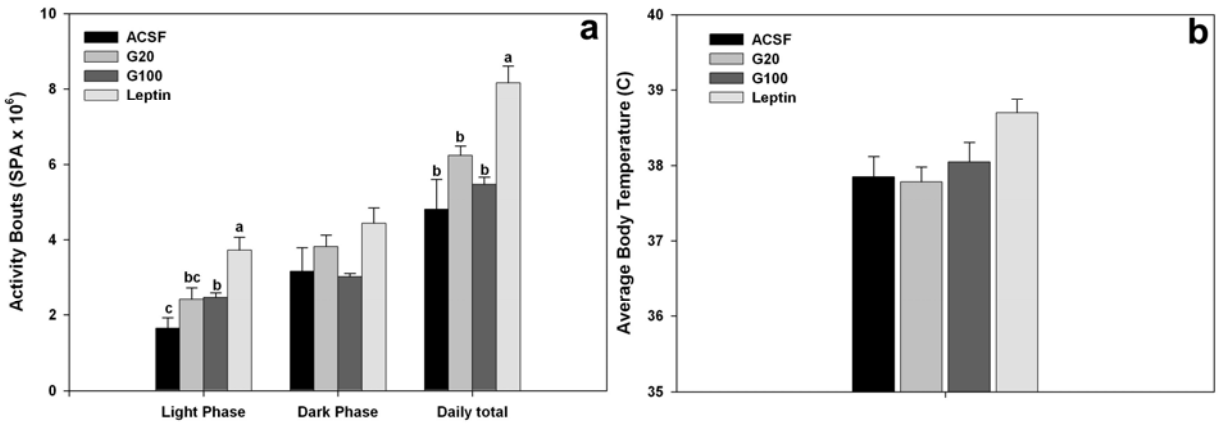

Fig.4.3. Activity (a) and body temperature (b) in rats receiving twice daily ICV injections for 5 days with aCSF (10 µl), G20 (20 pmol ghrelin/ inj.), G100 (100 mol ghrelin/ inj.), or leptin (5 ug/ inj.). a, b, c: Means with different letters are significantly different, p<0.05. Data are means ± S.E.M.

Serum parameters and adipose tissue apoptosis

Serum glucagon, leptin and insulin levels were decreased by leptin ($p<0.05$), and 100 pmol ghrelin increased serum insulin level ($p<0.05$). Serum IGF-1 level was significantly decreased by leptin ($p<0.05$), while serum osteocalcin level showed a trend toward a decrease by both leptin and low dose ghrelin (Table 4.3).

Table 4.3. Serum glucagon, insulin, leptin, IGF-1, and osteocalcin concentrations in rats receiving twice daily ICV injections for five days with aCSF (10 µl), G20 (20 pmol ghrelin/inj.), G100 (100 pmol ghrelin/inj), or leptin (5 ug leptin/inj).

	aCSF	G20	G100	Leptin
Glucagon (pM)	24.3 ± 3.7^{a}	21.6 ± 3.7^{a}	18.4 ± 3.7^{ab}	7.8 ± 3.7^{b}
Insulin (pM)	68.6 ± 13.2^{b}	75 ± 13.2^{b}	116.6 ± 13.2^{a}	12.9 ± 13.2^{c}
Leptin (pM)	109 ± 15.9^{a}	134.9 ± 15.9^{a}	136.9 ± 15.9^{a}	34.5 ± 15.9^{b}
IGF-1 (ng/ml)	2060.6 ± 193.1^{a}	2174.0 ± 200.9^{a}	2252.1 ± 193.1^{a}	1316.1 ± 193.1^{b}
Osteocalcin (ng/ml)	36.1 ± 4.4	23.6 ± 4.6	33.2 ± 4.4	26.7 ± 4.4

Data are means ± S.E.M.

^{abc}Means with different letters within a row are different, $p < 0.05$

ICV leptin treated rats had significantly higher numbers of apoptotic nuclei than control as shown by the TUNEL assay ($p<0.05$) (Figure 4.4)

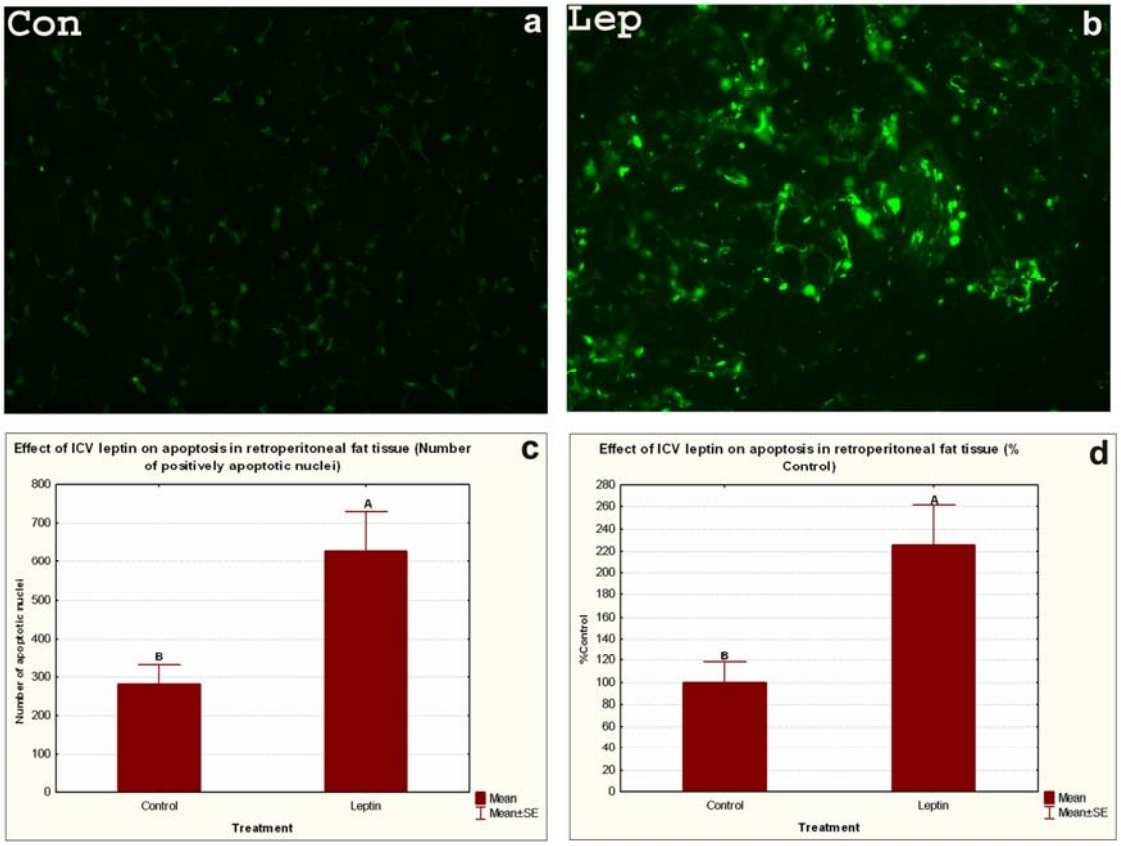

Fig.4.4. Adipose tissue (RP) apoptosis by leptin. (a) control (b) leptin TUNEL assay staining; (c) number of apoptotic nuclei, (d) ratio of apoptotic nuclei. a, b: Means with different letters are significantly different, p<0.05. Data are means ± S.E.M.

Tibial bone marrow adipocyte and bone mineral density

Adipocyte number and size were significantly decreased by leptin ($p<0.05$), adipocyte size was significantly decreased by G20 ($p<0.05$). Analysis of bone mineral density data showed that tibial bone mineral density was elevated by both leptin and G20 ($p<0.05$) (Fig.4.5.).

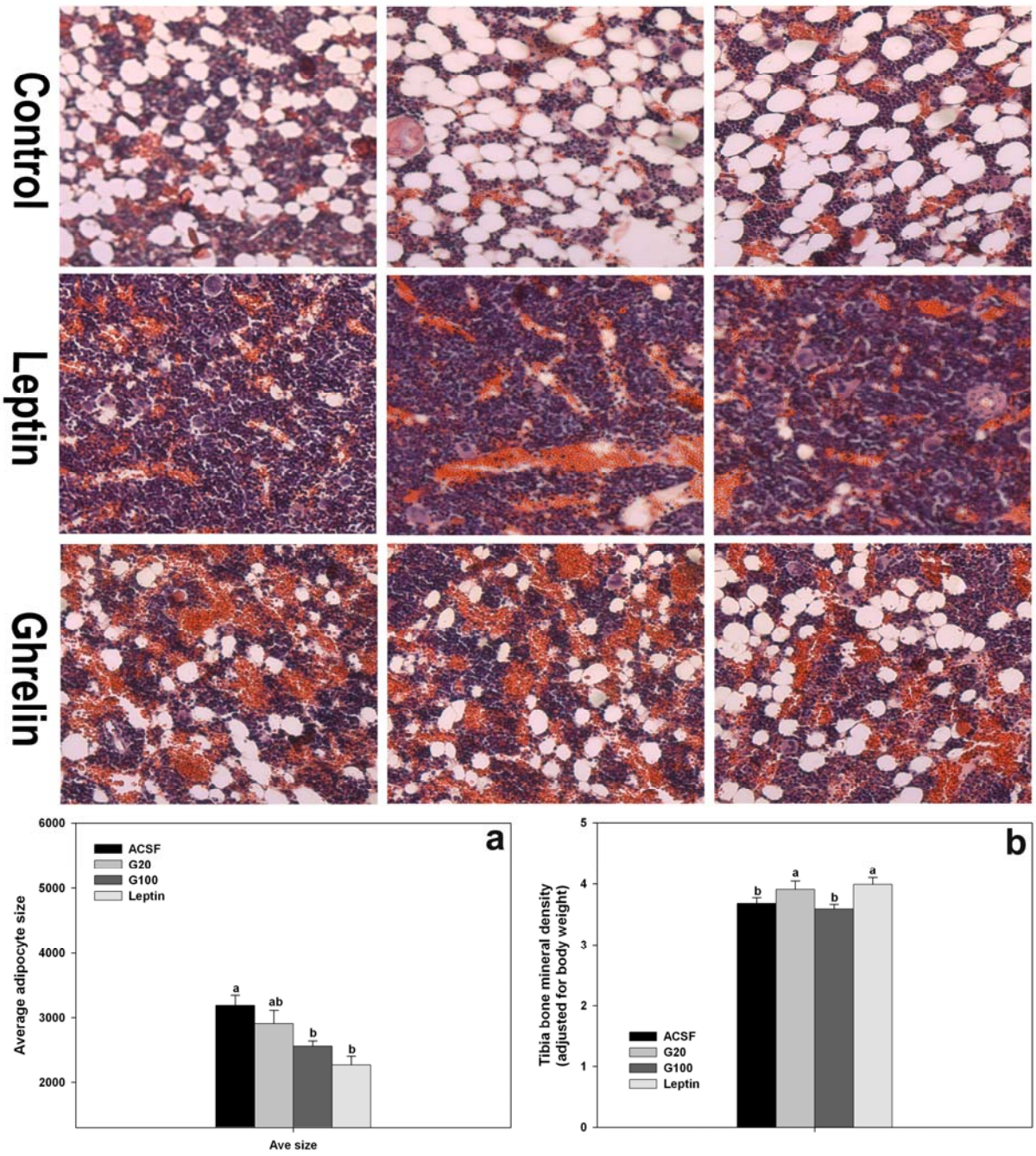

Fig. 4.5. HE stain of transverse section of tibia. Comparison of tibial bone marrow adipocyte size (a), and tibial bone mineral density (b) in rats receiving twice daily ICV injections for 5 days of aCSF (10 µl), G20 (20 pmol ghrelin/inj.), G100 (100 pmol ghrelin/inj.), or leptin (5µg leptin/inj.). Data are means ± S.E.M. a,b: Means with different letters are different, $p<0.05$.

Part II Comparisons of osteogenesis gene expression from bone marrow

RT-PCR results showed G20 unregulated bone marrow mRNA expression of CASP2, MMP11, MADH1 by 92.8% ($p<0.01$), 168.7% ($p<0.05$), and 114.0% ($p<0.05$) respectively in non-adherent cells (Fig.4.6). None of these gene expression changes was found in adherent cells or in bone marrow directly (data not shown). Leptin did not cause significant gene expression changes (data not shown).

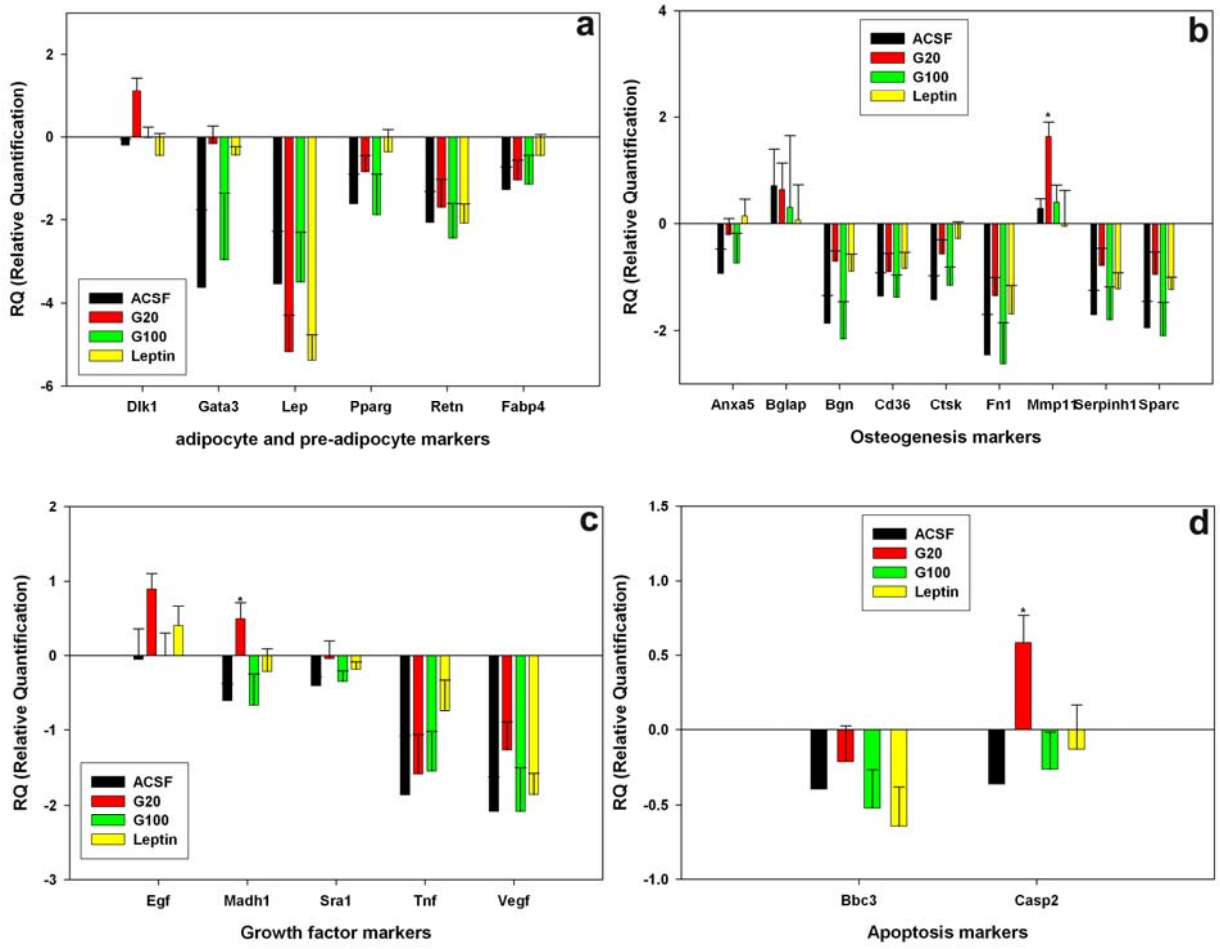

Fig 4.6. Effect of twice daily ICV injections for 5 days with aCSF (10 µl), G20 (20 pmol ghrelin/injection), G100 (100 pmol ghrelin/injection), or leptin (5 µg leptin/ injection) in rats on gene expression changes in non-adherent bone marrow cell culture. Genes are groups based on their function on adipogenesis (a) osteogenesis (b) growth factor effect (c) and apoptosis (d). Note: *, p <0.05. Data are means ± S.E.M.

Part III Comparative study of gene expression profile of adipocyte in bone marrow and epididymal fat tissue

Comparison of expression of thermogenesis & lipolysis and adipogenesis gene markers from RNA extracted from epididymal fat and from bone marrow

For thermogenesis and lipolysis markers, in epididymal fat tissue (Epi), leptin treatment up regulated lipolysis marker ADRB-2 and thermogenesis marker Mfn2, while down regulated leptin expression. None of those markers seems being significantly affected by ghrelin treatment (Fig4.6.) (a). in bone marrow, those set of marks were unaffected by either ghrelin or leptin (Fig4.6.) (b);

For adipogenesis markers, in epididymal fat tissue, leptin treatment highly significantly ($p<0.001$) down-regulate of expression of FASN and SLC2A4, and significantly ($p<0.05$) down-regulate expression of SCD1; while the expression levels of Fasn and SCD1 were significantly up regulated by G100 (Fig4.6.) (c); while in bone marrow tissue, we saw an opposite effect of ghrelin compared with that in EP tissue, we saw a that some adipogenesis-related markers, such as Cebpa, Fasn, and Slc2a4 were down-regulated by ghrelin; while leptin treatment appeared to take the same action as it did in adipose tissue, two adipogenesis markers, FASN ($p<0.05$) and Scd1 ($p<0.001$), were down-regulated by leptin (Fig4.6.) (d).

Discussion

Most studies examining bone density following ghrelin treatments showed beneficial effects (Bohan 2003; Fukushima, Hanada et al. 2005); yet only peripheral ghrelin treatments were reported. The effects of injecting ghrelin into the cerebral ventricles on bone metabolism had not been studied previously. One report documented the lack of changes in bone mass in mice measured by DEXA after subcutaneous once-daily injections of approximately 400pmol ghrelin for two weeks

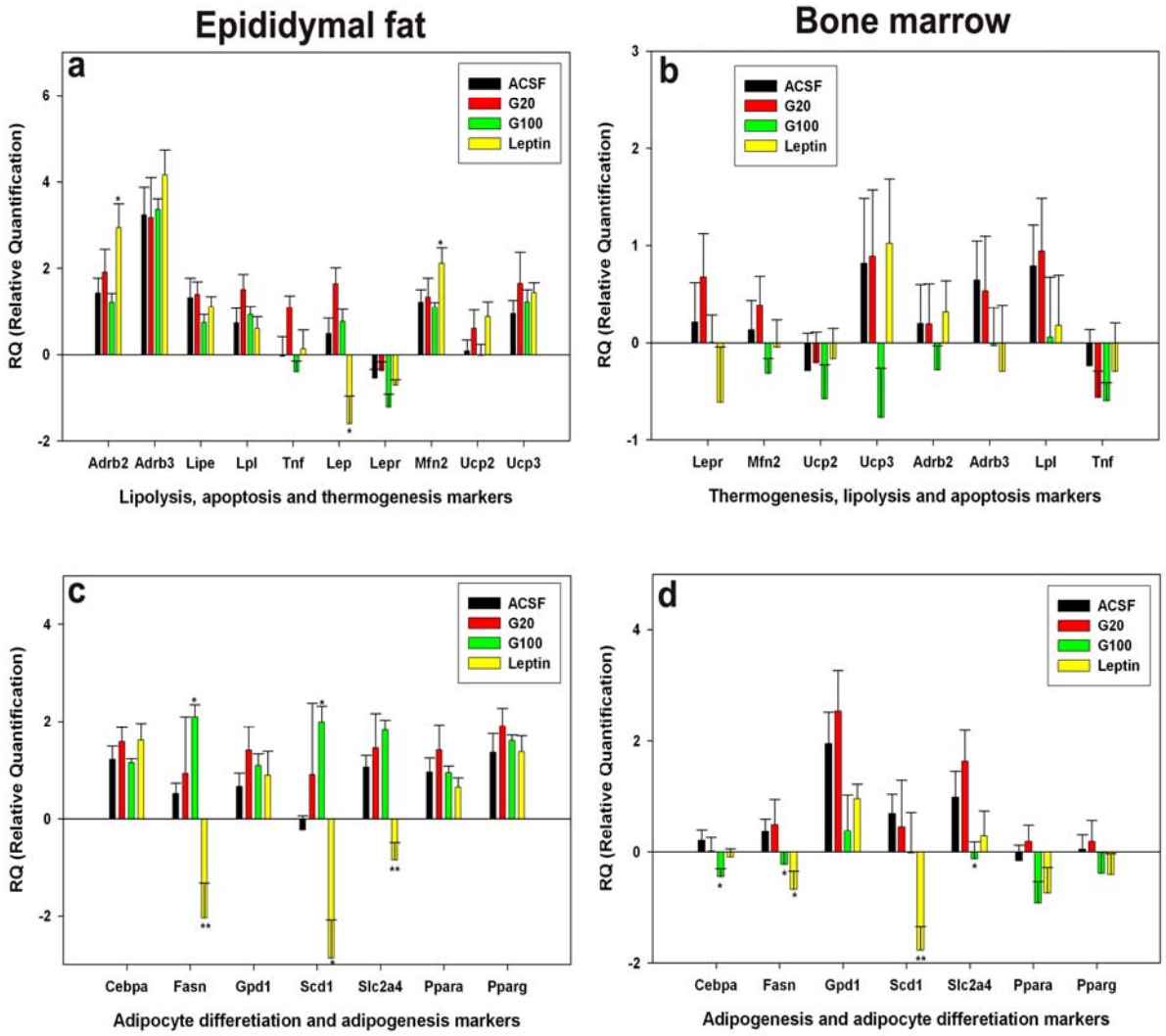

Fig 4.7. Comparative study of twice daily ICV injections for 5 days with aCSF (10 µl), G20 (20 pmol ghrelin/injection), G100 (100 pmol ghrelin/injection), or leptin (5 µg leptin/ injection) in rats on gene expression in epididymal fat pad (a) (c) and in bone marrow cell culture (b) (d). Genes are groups based on their function on either thermogenesis & lipolysis or osteogenesis. Note: **p<0.001,*p <0.05. Data are means ± S.E.M.

(Tschop, Smiley et al. 2000). Because of its effect on energy metabolism, GH secretion, sympathetic nerve activity, and various in vitro evidence and clinical observations, ghrelin has been suggested to be involved in the regulation of bone metabolism through a neuronal pathway. Therefore, the main objective of this study was to determine the effects of ghrelin and/or leptin ICV-administered to bone marrow adipocytes and bone.

In agreement with our previous study on ghrelin, as well as with results of other groups, we found in this new study that high dose and low dose ghrelin (20pmol (G20), 100pmol (G100), injected twice daily at 12-hour intervals for five days in 10 μl injections) increased body weight gain compared with the control dose, while leptin deceased body weight.

The effect of ghrelin on body weight is apparently related to its effect on stimulating food intake as well as to the increased efficiency of adipogenesis(Wren, Small et al. 2001). Increased body weight was reflected in the increase of adipose tissue weight (Epi and Rpi). The increase of daytime activity by 100 pmol of ghrelin appears due to the increase of the frequency of feeding during daytime. The reduction of body weight by leptin is due to its effect on increasing activity, as well as a decrease of food intake. Tissue weights were also reduced by leptin treatment (Epi, Rpi and BAT).

Tissue weight data agrees with serum concentration of leptin data, as well as with insulin and glucagon. Leptin and insulin are makers of body fuel gauge levels (Diamond and Eichler 2002): the greater the body's adipose deposit, the higher the serum leptin and insulin level. Therefore, it is reasonable to see a corresponding decrease of leptin and insulin serum concentration when leptin treatment decreases adipose tissue weight.

A TUNEL apoptosis assay of adipose tissue was carried out using 4% paraformaldehyde fixed adipose tissue. It was found that the apoptotic cell count in RP fat tissue of leptin-administered rats is significantly higher than in the control animals, which means that the decrease of adipocytes by central leptin occurs via the induction of apoptosis.

Also observed was an increase in white adipose tissue weight (Epi and Rpi) and a corresponding increase of serum insulin level by the 100 pmol ghrelin treatment. This apparently is due to the effect of ghrelin on increasing food intake, as well as increased efficiency of adipogenesis(Wren, Small et al. 2001).

Tibia bone mineral density increased because of both ghrelin and leptin treatments. These results coincide with our previous ghrelin study.

The effects of leptin on bone density were not unexpected, since Hamrick's results previously demonstrated that central leptin treatment decreases marrow adipocyte size and number (Hamrick, Della-Fera et al. 2005). The decrease of adiposity in marrow could be a factor in the increase of bone mass.

Consistent with data of Hamrick et al (Hamrick 2006), this study found that central leptin injections decreased marrow adipocyte size and number in rats. The depletion of adipocytes may have resulted from the apoptotic effect of leptin acting on its receptors in the brain.

Because ghrelin administration affects bone mass growth and development by acting on the GH/IGF axis, an increase in IGF-1 by both leptin and ghrelin is expected. Surprisingly, however, in this study the serum concentration of IGF was unaffected by ghrelin, while being decreased by leptin. This result proves consistent with one independent studies (Kim, Namkoong et al. 2004). This

above reference also suggested that the effects of leptin and ghrelin on bone are independent of IGF-1 levels (one study showed that a low dose of leptin i.p. injection slowed bone loss while having no effect on serum IGF-1 concentration).

In another study, continuous intracerebroventricular infusion of leptin activated bone remodeling with a negative balance (Guidobono, Pagani et al. 2006). Urine deoxypyrydinoline and serum osteocalcin remained more elevated in the leptin-treated group when compared with controls. This study found that four days of leptin injections increased bone mass in axial as well as appendicular mass. The IGF-1 level was decreased, with an accompanying decrease in secretion of osteocalcin. Such findings serve as evidence for the slowing down of the bone remodeling process. We speculate the increased bone mass is a result of decreased osteoclatogenesis rather than increased bone formation through osteoblastogenesis. A loss of marrow adipocytes also supports the idea of decreased osteoclastogenesis, since adipocyte promote osteoclastogenesis (Fried, Bunkin et al. 1998; Maurin, Chavassieux et al. 2000).

The original study was planned to extract bone marrow RNA and to use marrow tissue directly to compare the gene expression of selected markers. However, it became apparent that in viewing multiple cell populations inside the marrow, it would be hard to interpret gene expression results. The experimental design was therefore altered to include a bone marrow cell culture. The resultant adherent cells would mostly be stromal cells, which are important progenitor cells for osteoblasts (Jilka 2002). Concern that a cell culture could remove the effects of the original treatment led to a change in the experimental design in two ways: first by parallel RNA extraction and gene expression from both tissues and cell cultures; second, RNA extraction from the cell

cultures performed within 24 hrs after plating, to reduce the effects of culturing. Since two independent publications (Wlodarski, Galus et al. 2004) advocated the idea that non-adherent bone marrow cells are a rich source of cells forming bone *in vivo*, the design also included non-adherent cells in the RNA extraction material (procedure described by Gimble et al) (Gimble, Robinson et al. 1996).

A series of pilot studies were run to determine the optimal conditions for assay of good quality RNA extraction. An RNeasy kit (Qiagen) was utilized to extract the RNA from the cell culture because it is a sensitive method especially useful when the cell number is limited (The culture was limited to one day, and the cell count was small before division). Adding a stabilization regent immediately after collection of bone marrow was critically important for the preservation of RNA integrity. Additionally, a pre-treatment with NH_4CL lysis buffer (which removes most of the red blood cells) before RNA extraction using Trizol was equally important in to maintain the integrity of RNA.

It appears unlikely that non-adherent, instead of adherent cells, showed gene expression changes, despite the fact that adherent stromal cells are progenitors for adipocytes and osteoblasts (Jilka 2002). Because the tibial BMD was increased by leptin and low dose ghrelin, expectations were to see gene expression changes in the adherent stromal cells or in the gene expression from RNA extracted directly from bone marrow tissue. However, only a few marker changes in non-adherent cells were noted. Further examination of the composition of the non-adherent cell population gave some clues. Since the non-adherent population is composed mostly of hematopoietic (blood cell lineage), those hematopoietic, non-adherent cells could be osteoclast

progenitors. However, there are many other hematopoietic cell types, such as granulocytes, macrophages, myeloid-derived dendritic cells, etc. An increase of bone mineral density within five days, as well as an increase of CASP2 gene expression in those non-adherent cells, led to speculation that this increase of bone mineral density is the result of apoptosis of osteoclasts.

The matrix metalloproteinases (MMPs) comprise a family of more than 20 zinc-dependent endopeptidases. The role of matrix metalloproteinases in osteoclast biology is poorly defined, but in other tissues they have been linked with tumor-promoting activities such as the activation of growth factors (Coussens, Fingleton et al. 2002). MMPs are required for tumor metastasis and angiogenesis. MADH1 is involved in regulation of osteogenesis is mediated by TNF β pathway (Kulterer, Friedl et al. 2007). Therefore, the up-regulation of MMP11 and the growth factor MADH1 may result from increased hemopoitic activity. Bone marrow up-regulates hematopoietic function and therefore contributes to increased osteogenesis activity (Ilizarov, Palienko et al. 1984).

To investigate the effects of the treatment on the third population of bone marrow--the bone marrow adipocytes -a set of adipogenesis-related markers was expressed, using cDNA samples from the bone marrow and those gene expression changes were compared with those from epididymal tissue. Genes were clustered into three groups based on their functions: adipogenesis markers, lipolysis markers, and osteogenesis markers. Most of the interesting findings were from the adipogenesis markers.

In epididymal tissue, leptin treatment increased lipolysis expression and thermogenesis gene markers while down-regulating adipogenesis markers such as FASN, SLC2A4 and SCD1. These

findings coincide with the decreased epididymal tissue weight data obtained via leptin treatment. Such findings were also demonstrated in 2000 by Soukas et al (Soukas, Cohen et al. 2000).

Ghrelin treatment up regulated the gene expression of Fasn and Scd1 from epididymal tissue, which is consistent with the increased epididymal weight in ghrelin-treated rats. These results are supported by a study by Theander-Carrillo et al (Theander-Carrillo, Wiedmer et al. 2006), in which storage-promoting enzymes in white adipose tissue were markedly increased, while thermogenesis-related mitochondrial uncoupling proteins 1 and 3 were down-regulated in brown adipose tissue. Since this dose-dependent effect was independent of the hyperphagia effect by ghrelin, the study concluded that this effect might be mediated by the sympathetic nervous system (Theander-Carrillo, Wiedmer et al. 2006)

In bone marrow, leptin treatment led to a decreased expression of adipogenesis markers, which is consistent with its action on epididymal tissue. However, the action of ghrelin on bone marrow adipocyte was opposite to its effects on epididymal tissue. A decrease in bone marrow adipogenesis gene markers was observed in ghrelin-treated animals. This decreased adipogenesis is consistent with the decreased adipocyte size observed in treatment with ghrelin.

Since this study also showed a trend toward increased serum IGF-1 level caused by ghrelin, this decreased bone marrow adiposity may be mediated by the lipolysis action of GH and IGF-1; in fact, several studies have demonstrated the lipolysis action of GH on visceral adipocyte (Lucidi, Parlanti et al. 2002), 3T3-L1 adipocyte culture (Asada, Takahashi et al. 2000), and bone marrow adipocyte (Gevers, Loveridge et al. 2002).

We conclude that the beneficial effect of ghrelin on bone likely results from decreased osteoclastogenesis and reduced marrow adipogenesis. The sympathetic nervous system does not only mediate the action on peripheral adipocytes, but also mediates it effects on bone marrow cell populations.

Leptin induced loss of bone marrow adipocyte, which in turn led to increased bone mass. However, no major changes in osteogenesis gene expression were detected. Due to the fast action of leptin, at the point of tissue collection changes in gene expression may had already diminished, although the there were accumulative bone effects as reflected by increased bone mineral density. Further studies in which bone marrow is sampled at different time points may help to better define the effects of leptin on bone.

The hypothesis that ghrelin affects bone through the inhibition of osteoclastogenesis requires further testing.

CHAPTER 5

CONCLUSION

This aim of these studies was to test the hypothesis that centrally administered gut peptides acting on their receptors in the brain modulate bone formation. The two peptides tested were GIP and ghrelin. Leptin was used as a positive control. Several studies previously conducted in this lab studied the effects of centrally administered leptin on bone.

For the GIP study, although we failed to define a central mediated effect of GIP on bone, we did find that when GIP is administered centrally, it could induce changes in feeding behavior. This study, to our knowledge, is the second in the past 20 years to investigate the central effects of GIP on food intake regulation. The first study, conducted in 1981 by Woods, found that GIP played no role in regulating food intake when given centrally. Similarly, our study did not find effects of a lower dose of GIP on food intake. However, we did find that a higher dose of GIP decreased body weight gain, although the decrease in food intake was not significant. We analyzed feeding behavior in an effort to detect changes in some parameters of feeding behavior, and found that the high dose of GIP led to changes in feeding time and inter-meal intervals. Thus, we conclude that the centrally administered GIP induced some subtle changes in feeding behavior.

The effects of GIP on bone, when administered peripherally, in studies of cell culture systems, and in genetically modified receptor knockout or GIP over-expressed mice have been studied extensively (Bollag, Zhong et al. 2000; Bollag, Zhong et al. 2001; Xie, Cheng et al. 2005; Xie, Zhong et al. 2007). However, the effects of centrally administered GIP on bone remain little

studied. In our study, GIP failed to induce changes in bone mineral density like leptin. We also failed to detect changes in bone marrow adipocytes. Although Jiuhua Duan et al. (J. Duan, University of Georgia PhD dissertation 2006, CH 7) found that CART expression was unregulated by GIP in the same study in the rat brain, we speculate that changes in CART had more to do with the GIP effect on food intake repression rather than its effect on bone metabolism.

Two ghrelin studies were completed. In the first study, we investigated whether ghrelin had the expected effect on bone when administered centrally. The second study investigated the mechanisms underlying the effects on bone. We learned from previous studies that, like GIP, the effects of ghrelin on bone metabolism have been shown both *in vitro* in cell culture systems, as well as *in vivo when delivered* peripherally in rats. Moreover, ghrelin has opposing biological functions to leptin both inside and outside the brain (Brzozowski, Konturek et al. 2001). Thus, it is possible that ghrelin will also have an opposing function on bone through the central nervous system. Upon completion of our initial study, we found that although a 100 pmol ghrelin appears more effective in inducing changes in food intake and body weight gain, 20 pmol ghrelin seems to have a more potent effect on bone. We also found that low dose ghrelin treatment increased bone mineral density in rat tibia, as measured by PIXImus.

The second study employed RT-PCR combined with microfluidic technology to define the mechanism of ghrelin on bone by checking the expression level of mRNA in a set of pre-selected gene markers, which were grouped into different biological functions such as osteogenesis, adipogenesis, apoptosis and growth factors. Since bone marrow adipocytes and osteoblasts share the same progenitor cells, and since there is a reverse relationship between adipocytes and osteoblasts,

we also employed bone histomophormetry to examine changes in bone marrow adipocytes. We measured serum IGF-1 and serum osteocalcin levels, which are well-known bone turnover markers (Thiede, Smock et al. 1994).

We confirmed our results of low dose ghrelin on bone through densitometry and histomophormetry. It was found that low dose ghrelin up-regulated mRNA expression of a few gene markers responsible for osteogenesis in primary, non-adherent bone marrow cell culture systems. At first, it seemed unlikely that non-adherent cells--as opposed to adherent cells--showed gene expression changes, despite the fact that adherent stromal cells are progenitors for adipocytes and osteoblasts. However, looking at the composition of the non-adherent cell population revealed that this is true because the non-adherent population is composed mostly of hematopoietic (blood cell lineage). Those hematopoietic, non-adherent cells could be osteoclast progenitors. Since we saw an increase of bone mineral density within five days and an increase of CASP2 gene expression in those non-adherent cells, we speculate that this increase of bone mineral density in such a short period may result from apoptosis of osteoclasts, thus shifting the balance of the bone remodeling toward bone formation. This decreased osteoclatogenesis was corroborated by the associated decreased serum osteocalcin.

Our results indicate that leptin-induced depletion of marrow adipocytes was accompanied by an increase in bone mineral density. These findings are consistent with the work of others. However, we failed to detect bone marrow gene expression changes by leptin. It is possible, however, that we did not include other important gene markers of osteogenesis, or that because of

the rapidity of the action of leptin to induce gene expression, the changes in gene expression were already diminished prior to the time of enthanization of the animals.

To investigate the effects of the treatments on the third population of bone marrow--the marrow adipocytes--we expressed a set of adipogenesis-related markers using cDNA samples from the bone marrow and compared those gene expression changes to those in epididymal tissue. Results indicated that leptin treatment led to a decreased expression of adipogenesis markers such as FASN, SCD1, SLC2A4 etc. in both adipose tissue and bone marrow, while up-regulating lipolysis markers such as MFN2 and ADRB2, only in adipose tissue. Ghrelin treatment resulted in increased adipogenesis gene expression from adipose tissue and decreased adipogenesis gene expression in bone marrow. These decreased adipogenesis related markers by leptin and ghrelin are consistent with the histomophormetry data, which showed leptin, and ghrelin decreased bone marrow adipocyte size. Therefore, we conclude that the beneficial effect of ghrelin on bone could also result from a reduced marrow adipogenesis.

Contrary to our expectations, we found that leptin significantly decreased serum IGF levels. Although we found two other independent studies which showed that ICV leptin decreased serum IGF-1 and had no changes induced by ghrelin (Kim, Namkoong et al. 2004), our findings could possibly be due to the assay itself. The effect of IGFBP on the assay may also play a role in such findings (Kim, Namkoong et al. 2004). Furthermore, we found that in addition to its effect on the long bone, leptin also increased BMD in the spine of rats both in this study and in the previous GIP study. This is a novel addition to the well-known effect of leptin on appendicular bone. Another reason may be attributed to changes in serum concentration of leptin following central leptin

administration. There is an abrupt reduction of serum leptin concentration, which follows central leptin administration, which is different from when leptin is given peripherally. This may in part explain leptin's effects on bone metabolism, both directly and indirectly, by its interaction with vast networks of the cytokine and endocrine systems to exert effects on bone (Coen 2004).

In summary, we conducted two gut peptide experiments and defined the role of leptin and its effects on bone. Moreover, we demonstrated that ghrelin also affects bone marrow population and bone metabolism through the central nervous system, and that the beneficial effects of ghrelin on bone could result from decreased osteoclastogenesis, as well as from reduced marrow adipogenesis. Sympathetic nervous system activity may not only mediate the action of ghrelin on peripheral adipocytes, but may also mediate its effects on bone marrow cell populations.

We studied bone by histomophormetry, densitometry, and bone marrow RNA expression level using microfluidic technology. This technology is known to be fast, reliable and sensitive in assessing effects on bone and adipocytes, as well as in interpretation of underlying mechanisms.

As osteoporosis becomes increasingly more prominent both in the US and worldwide, scientists continue to investigate methods of cures and therapeutics. The traditional approach of slowing down bone resorption is progressively being replaced by agents of bone formation. The strategy of targeting adipocyte apoptosis will provide a promising new hope for the prevention and treatment of osteoporosis.

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APPENDICIES

I Bone Marrow Extraction and Plating of Bone Marrow Stromal Cells

Supplies:

- Microcentrifuge capable of spinning 13,000 rpm
- Microcentrifuge tube (2 ml, RNase-free) autoclaved
- Biosafety cabinet
- Water bath heated to 37°C
- Steri 250 Glass bead sterilizer (used to re-sterilize instruments between animals)
- Sterile surgery kit (composed of two pairs of forceps and small scissors)
- P-200 pipette and sterile 200 µl pipette tips
- DMEM (4.5 mg/ml glucose) supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml Amphotericin B.
- 70% ETOH (used to wipe down surfaces of biosafety cabinets and water baths)

Bone marrow extraction and handling

- Dissect the fore and hind limbs from the body, taking care not to break the bones.

(The following is performed aseptically in biosafety cabinet -- note-wear gloves and a mask)

- Remove the skin and carefully separate the femur and tibia, removing as much muscle as possible from each bone. Note: Bone marrow WILL NOT be extracted from the tibias. The right and left tibias will be placed in 10% buffered formalin and 70% ETOH respectively.
- Clip off the proximal and distal ends of each bone with sterile scissors leaving the bone shafts intact.
- Transfer the bones into autoclaved RNase-free microcentrifuge tubes appropriately labeled to maintain identification of the animal, bone, and study number. Centrifuge the samples at 13,000 rpm for 1 minute.
- Discard the bone shaft and freeze the marrow collected in the bottom of the 2.0 ml centrifuge directly in liquid nitrogen for Beadlyte analysis (right femur), or add 1 ml of stabilization reagent to marrow collected from both front legs, or add 200 µl of pre-warmed media to the bone marrow extract from left femur.
- Transfer resuspended bone marrow extracts into a 15 ml conical tube. Add 3 ml of media previously prewarmed maintain the samples at room temperature until the dissections are completed then continue with the following steps as noted below.
- add stabilization reagent immediately after spinning down the bone marrow after (1ml, aspirate up and down for a few times and make 2 aliquots and then put in -20 C for temporarily storage before extraction)

Cell Separation

(Performed aseptically in biosafety cabinet -- note-wear gloves)

- Disrupt the stromal cells by sequential aspiration through 18 and 20 gauge needles fixed to a 3 ml syringe.
- Centrifuge the disrupted cells in the same 15 ml centrifuge tube at 1200 rpm for 10 minutes, discard the supernatant and resuspend in 2 ml of fresh media.
- Mix thoroughly and remove a small aliquot (~40 µl) for cell counting.

Cell Counting

(conducted on bench top)

Same as in Appendix 1

The hardest part is being able to distinguish RBC from the bone marrow cells however it can be done by knowing the following characteristics of each cell type. The bone marrow cells are larger and nucleated, whereas the RBCs are round, transparent, and shiny without nucleus and appear pale pink in color as compared to bone marrow cells.

Cell Plating

(Performed aseptically in biosafety cabinet -- note-wear gloves)

- Seed cells at a density of 5x107 per 10 ml of media in a 100mm tissue culture dish.
- After two-hour incubation at 37°C and 5% CO2 remove the non-adherent cells by changing media.
- Return to the incubator and the following day extract the RNA

II Trizol RNA Extraction Procedure

RNA extraction with Trizol involves some modification with the addition of a NH_4CL (DEPC water based solution) and PBS (DEPC water based solution) treatment. Marrows were taken from the freezer to allow the stabilization reagent to thaw; this was followed by centrifuging for five minutes at room temperature. The stabilization reagent was discarded and the pellet saved,. Then 1 ml of pre-cooled NH_4Cl buffer was added and the tubes left on ice for 15 minutes. Samples were centrifuged for five minutes at 4 C to remove the NH_4Cl; the pellet was then washed once with 1XPBS. To allow formation of good pellet, the centrifuge was extended to 10 minutes at 4 C. For the remainder of the steps, see detailed description of the Trizol method.

1. Transfer sample to 1ml flat bottom tube with 4 beca beads (1.0 mm Zirconia beca beads, Biospec Product, Inc.)
2. Place sample in a 2x24 Mixer Mill adaptor set (Cat. # 69998, Qiagen) and secure into the Retsch Mixer Mill (MM300) to homogenize at a frequency of 30 shakes per second for 7min at room temperature
3. Remove sample from Mixer Mill and store at room temperature for 10 min
4. Add 200 µl of chloroform, mix gently by inverting the sample 5 times and then allow to sit at room temperature for 2 min
5. Centrifuge tube at 12,000 rpm for 15 min at 4°C (Sorvall Fresco)
6. Place sample on ice and remove the upper layer, which contains the RNA of the 3-layer suspension and place in a 2 ml RNase free microcentrifuge tube, which has been appropriate, labeled.
7. Add 500 µl of isopropanol, mix gently by inverting 5 times and hold at RT for 10 minutes
8. Centrifuge at 4°C for 10 min at 12,000 rpm
9. Dump off the supernatant assuring that the pellet remains in the bottom of the tube
10. Add 1 ml of newly prepared 75% ETOH and vigorously hand shake the tube to wash the pellet
11. Centrifuge at 4°C for 8,000 rpm for 8 min
12. Carefully pipette off the ETOH
13. Allow the pellet to partially dry in the fume hood by keeping the lid off the tube and checking frequently (no more than 5 min)
14. Re-suspend the pellet in 15 µl of DEPC-treated water and pipette the solution up and down within the tip to obtain an even mix, until the pellet is no longer visible.
15. Remove 2 µl of the RNA solution and place in a newly labeled RNase-free microcentrifuge and give to Roger Nilsen for RNA quantification using an Agilent 2100 Bioanalyzer. The remaining sample (~13 µl) will be divided into (3) RNase-free microcentrifuge tubes 1 sample for cDNA and 2 backup samples. Keep samples on ice or store at -80°C.

Materials:
A. Chemicals
1) 75% ETOH

2) Chloroform
3) Isopropanol
4) DEPC-treated water
5) NH4Cl
6) NaHCO3
7) EDTA
8) NaCl
9) KCl
10) KH2PO4
11) NaHPO4 7H2O

Chemical name	Vendor	Cat #	Lot#
Ethyl Alcohol	EM science	EX 0289-4	05C15GA
Chloroform	ACROS	61028-1000	B00F2919
Isopropanol	Sigma	190764	06539HC
DEPC treated wa (0.1%)	Biosource	395000	311085
Trizol Reagent	Invitrogen	15596-018	1301182
NH4Cl	Fisher	130900	053006
NaHCO3	J.B.Baker	3506-01	J46740
EDTA	J.B.Baker	8993-01	A43644
NaCl	Fisher	S271-500	960598
KCl	J.B.Baker	3040-01	L41595
KH2PO4	Sigma	P5379	074K0160
Na2HPO4 7H2O	EM Science	SX0715-1	41289215

B. Equipment
12) Microcentrifuge (RT & 4°C)
13) Retsche Mixer Mill, 2X24 adaptor set, 1.0mm beca beads)
14) Agilent 2100 Bioanalyzer
15) -80°C freezer
16) Chemical fume hood
17) Biological safety cabinet
18) Surgical Equipment (treated with RNase-zap, followed by autoclaving)
    a. Joyce Chen Poultry Scissors
    b. forceps (Fisherbrand Cat. # 138046)
    c. glass bead sterilizer
    d. Nolvasan solution

C. Supplies
19) Pipettes (200 µl and 1000 µl)
20) 2.0 ml autoclaved RNAse-free microcentrifuge tubes

21) 1.5 ml autoclaved RNAse-free microcentrifuge tubes (for extracting marrow from front legs)
22) 1.0 ml autoclaved RNAse-free flat-bottom microcentrifuge tubes
23) Ice bucket & ice

Item name	Vendor	Cat #	Lot#
Tips (Rnase-free), 200 µ	Ambion	12650	1026
Tips (Rnase-free), 1000	Ambion	12660	776

Solution Preparation:

1. 20 ml NH_4Cl lysis buffer:
0.16 g of NH_4Cl
0.0168 g of NaHCO3
0.0074 g of EDTA
Dissolved in 20 ml of DEPC water

2. 50 ml of 1X PBS:
0.4 g of NaCl
0.01 g of KCl
0.01 g of KH2PO4
0.0575 g of Na2HPO4 7H2O
Dissolved in 50 ml of DEPC water

3. 75% ETOH (for 10 ml sample)
7.5 ml of 100% ETOH
2.5 ml DEPC treated water

III Immunohistochemical Assay for Apoptotic Cells in White Adipose Tissue

Quantitative analysis on adipose tissue apoptosis provides a better understanding of the effect of treatment of obesity. One easily measured feature of apoptotic cells is the break-up of the genomic DNA which results in the appearance of "DNA laddering" in agarose gel electrophoresis while normal cells the genomic DNA remains largely intact and does not display this "laddering". One method we have employed is using fragmental DNA quantification by separating small apoptotic induced fragmental DNA from large intact genomic DNA. The idea is excellent but not without technical limitation, large variations, and at times it masks the fact of apoptosis. Thus, a relative more constant quantification method is needed to support our findings. We will also compare this method with the TUNEL (terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling) method to determine which best fits our purpose on detection and quantification of adipose tissue apoptosis.

TUNEL staining on adipose tissue

(Based on Molecular Probes TUNEL cell kit, with modification). Fragmental DNA in apoptotic cells results in multiple 3'-hydroxyl ends in the DNA, thus by labeling the 3'-hydroxyl ends of the DNA with bromolated deoxyuridine triphosphate nucleotides (Br-dUTP) followed with antibody linked with fluorescent signal, apoptotic cells could be identified. The enzyme terminal deoxynucleotidyl transferase (TdT) catalyzes this template independent addition of deoxyribonucleotide triphosphates to the 3'-hydroxyl ends of double- or single-stranded DNA with either blunt, recessed or overhanging ends non-apoptotic cells do not incorporate significant amounts of the Br-dUTP owing to the lack of exposed 3'-hydroxyl DNA ends. Thus, apoptotic cells could be identified and analyzed.

1. Tissue fixed in 4% paraformaldehyde (Shibuya M 1992 Biotech Histochem).
2. Standard paraffin embedding process including dehydration of the fixed tissue through series of alcohol and xylene using automatic TissueTek process machine.
3. Embed sample in paraffin and cool down in stainless steel cassette, properly labeled.
4. Cut 5 μM thick section using microtone and transfer on glass slide.
5. Keep slide on slide heater at 56ºC for 1 hour. Cool down in room temperature.
6. Immerse slides in xylene (or xylene substitute) for 5 minutes at room temperature. Repeat using fresh xylene for second 5-minute incubation.
7. Immerse slides in 100%ethanol for 5 minutes at room temperature. Repeat with fresh 100% ethanol for additional 5-minute incubation.
8. Immerse slides in 95% ethanol for 3 minutes at room temperature.
9. Immerse slides in 80% ethanol for 3 minutes at room temperature.
10. Immerse slides in 75% ethanol for 3 minutes at room temperature.
11. Immerse slides in 50% ethanol for 3 minutes at room temperature.
12. Immerse slides in dH2O for 3 minutes at room temperature.

13. Immerse slides in 1 × PBS for 3 minutes at room temperature.
14. Immerse slides in 10 mM Tris (20 ug/µl Proteinase-K added @ 5ul/ 100 ml Tris) for 30 minutes at 37 ºC.
15. Immerse slides briefly in 1 × PBS and carefully dry the glass slide around the section.
16. Prepare a DNA-labeling solution, 100 µl per sample: Mix 20 µl of reaction buffer, 1.5 µL of TdT enzyme (yellow cap), 16.0 µl of Br-dUTP and 62.5 µl of dH2O.
17. Incubate slide at 37ºC in a humid box (could be home made by putting wet paper towel into a large tip box with a small tip loader, or commercial available one) for 1 hr.
18. Briefly wash with 1.0 ml of rinse buffer, repeat once.
19. Prepare 100 µl of antibody staining solution per sample by mixing 5.0 µl of the Alexa Fluor 488 dye–labeled anti-BrdU antibody with 95 µl of Rinse Buffer. Incubate 30 minutes at room temperature in humid box, avoid light.
20. Add 0.5 ml of the Propidium Iodide/RNase A staining buffer to each sample. Incubate the cells for an additional 30 minutes at room temperature. Protect the samples from light during the incubation.
21. Add one drop of anti-fade solution (prolong, VectorLab) and covered with glass cover.
22. Observe with fluorescent microscopy; take 9 photos from randomized field views per sample (200~400x magnification folds).
23. Analyze using ImagePro software
24. Statistical Analysis

Note: slides could be saved by wrapped with aluminum foil in -20 ºC freezer for couple of months without fluorescent bleach out if handling properly.