

Central leptin versus ghrelin: effects on bone marrow adiposity and gene expression

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Received: 29 July 2009 / Accepted: 12 October 2009 / Published online: 30 October 2009
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Abstract This study compared the central effects of ghrelin and leptin on body and bone marrow adiposity and gene expression in adipose tissue and bone marrow. Male Sprague–Dawley rats were injected intracerebroventricular (ICV) twice daily with control, 66 ng ghrelin (G66), 330 ng ghrelin (G330), or 5 µg leptin (L5) for 5 days. Food intake (FI) and body weight (BW) were measured daily. Gene expression in adipose tissue and bone marrow was assessed using RT–PCR. Leptin reduced FI ($P < 0.05$) and BW ($P < 0.05$), whereas ghrelin increased BW ($P < 0.05$) without affecting FI. Leptin decreased fat pad weights, whereas ghrelin (G330) increased fat pad weights ($P < 0.05$). In epididymal adipose tissue, leptin increased expression of lipolysis marker ADRB2 and thermogenesis marker MFN2 and decreased expression of adipogenic markers, FASN, SLC2A4, and SCD1, whereas ghrelin increased expression of FASN and SCD1. Leptin decreased bone marrow adipocyte size and number; however, ghrelin had no effect on these parameters. In whole bone marrow, leptin decreased expression of FASN and SCD1 and increased expression of DLK1, whereas ghrelin (G330) decreased expression of COL1A1. Thus, leptin induces similar changes in bone marrow and adipose tissue gene

expression, reflecting the decreased adiposity in both compartments.

Keywords Food intake · Body weight · Bone marrow · Gene expression

Abbreviations

ADRB2	Adrenergic, beta-2 receptor
ALPL	Alkaline phosphatase
ANXA5	Annexin A5
BBC3	Bcl-2 binding component 3
BGLAP	Bone gamma-carboxy glutamate (gla) protein
BGN	Biglycan
BMP2	Bone morphogenetic protein 2
CASP2	Caspase 2
CD36	CD36 molecule (thrombospondin receptor)
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha
COL1A1	collagen type I alpha 1
CTSK	Cathepsin K
DLK1	Delta-like 1 homolog
EGF	Epidermal growth factor
FABP4	Fatty acid binding protein 4
FASN	Fatty acid synthase
FGF14	Fibroblast growth factor 14
FN1	Fibronectin 1
GAPDH	Glyceraldehyde-3-phosphate
GATA-3	GATA binding protein 3
GPD1	Glycerol-3-phosphate dehydrogenase 1
LEP	Leptin
LEPR	Leptin receptor
LIPE	Lipase, hormone-sensitive
LPL	Lipoprotein lipase

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SMAD1	SMAD family member 1
MFN 2	Mitofusin 2
MMP11	Matrix metalloproteinase 11
PPAR α	Peroxisome-proliferator-activated receptor alpha
PPAR γ	Peroxisome-proliferator-activated receptor gamma
RTN	RTN hypothetical protein
SCD1	Stearoyl-CoA desaturase 1
SERPINH1	Serine (or cysteine) peptidase inhibitor, clade H member 1
SLC2A4	Solute carrier family 2 (facilitated glucose transporter), member 4
SPARC	Secreted protein, acidic cysteine-rich (osteonectin)
SRA1	Steroid receptor RNA activator 1
TNF	Tumor necrosis factor
UCP1	Uncoupling protein 1
UCP3	Uncoupling protein 2
UCP3	Uncoupling protein 3
VEGF	Vascular endothelial growth factor precursor
EP	Epididymal fat pad
RP	Retroperitoneal fat pad
ING	Inguinal fat pad
GC	Gastrocnemius muscle

Introduction

Leptin's role in the regulation of adiposity is widely acknowledged. Numerous studies have demonstrated that through hypothalamic relays, leptin alters eating behavior, physical activity levels, lipid mobilization, and adipose tissue apoptosis [1–3]. More recently, leptin has also been identified as a candidate hormone linking adiposity to changes in bone turnover [4–7]. Leptin not only has direct effects on osteoblasts, osteoclasts, and bone marrow stromal cells [8–10], but also can indirectly affect bone turnover by stimulating growth hormone secretion and altering sympathetic output to bone via the hypothalamus [4, 7, 11]. Leptin's centrally mediated effects on bone also include a reduction in bone marrow adiposity, at least in part by triggering apoptosis of marrow adipocytes [11, 12].

Ghrelin, originally identified as a growth hormone secretagogue, has recently been shown to increase adiposity [13, 14]. Like leptin, ghrelin acts directly on osteoblasts to stimulate bone growth [15]. In contrast to leptin, ghrelin has been shown to act centrally to reduce sympathetic output [16]; however, the central effects of ghrelin on bone turnover and bone marrow adiposity have not been investigated. Our study was designed to investigate the short-term effects of centrally administered leptin and

ghrelin on bone marrow and body adiposity and to compare changes in gene expression following central leptin or ghrelin treatment in adipose tissue, whole bone marrow, and bone marrow stromal cells.

Results

Body weight, food intake, tissue weights, and serum hormone levels

Rats treated with leptin ate less and lost weight during the 5-day period (Fig. 1a, b), whereas rats treated with the high dose of ghrelin (G330) gained weight ($P < 0.05$) (Fig. 1a), but did not eat more than either control rats or rats treated with G66 (Fig. 1b).

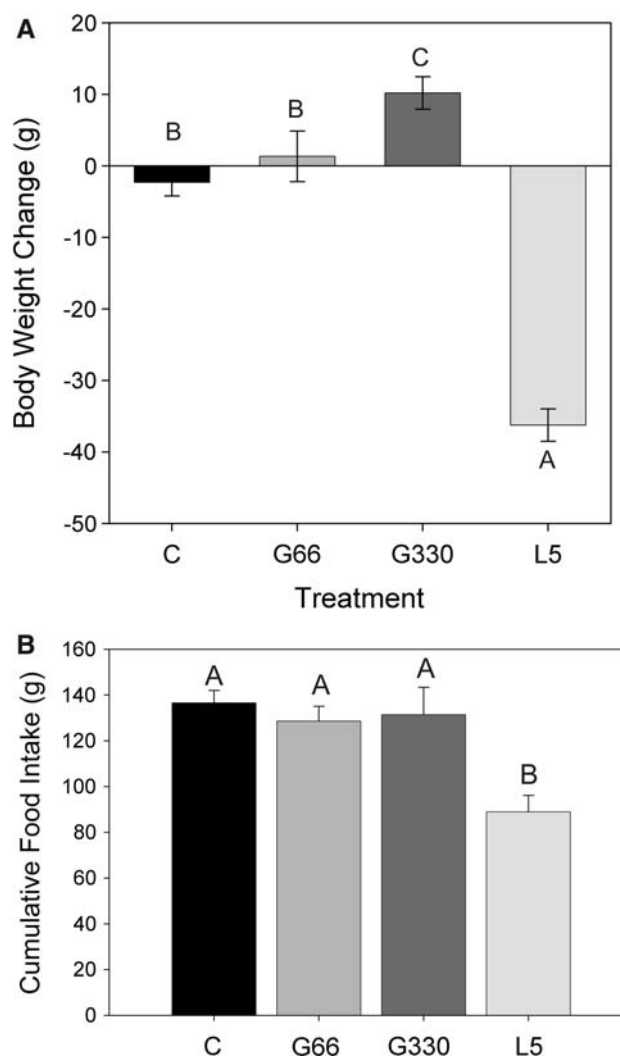


Fig. 1 Cumulative change in BW (a) and cumulative 5 day FI (means \pm SEM) in rats injected twice daily ICV with 10 μ l/injection of aCSF (Control), G66 (Ghrelin 66 ng/injection), G330 (Ghrelin 330 ng/injection), L5 (leptin, 5 μ g/injection) for 5 days (b). Means not designated with a common letter are different, $P < 0.05$

Leptin treatment decreased brown fat (BAT), epididymal (EP), retroperitoneal (RP), and inguinal (ING) fat pad weights ($P < 0.05$); G330 increased EP and RP weights ($P < 0.05$) (Fig. 2). Gastrocnemius muscle (GC) and soleus muscle weights were unaffected by either leptin or ghrelin (data not shown).

Serum leptin and insulin levels (Fig. 3) were decreased by leptin treatment ($P < 0.05$), whereas leptin levels were increased ($P < 0.05$) by G330. Osteocalcin levels were unaffected by leptin or G330.

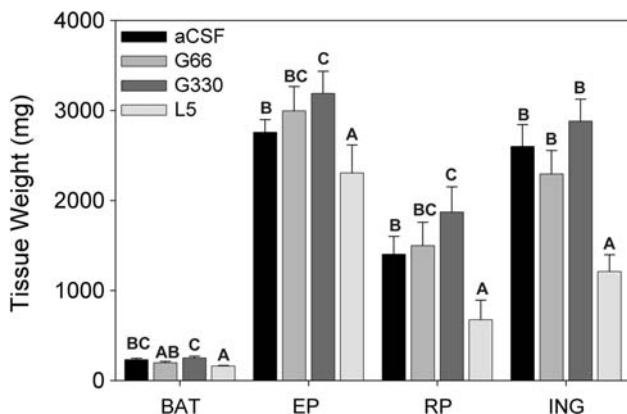


Fig. 2 Weights of EP, RP, ING fat pads, and BAT from rats injected twice daily ICV with 10 μ l/injection of aCSF (Control), G66 (Ghrelin 66 ng/injection), G330 (Ghrelin 330 ng/injection), L5 (leptin, 5 μ g/injection) for 5 days. Data shown are means \pm SEM. Means not designated with a common letter are different, $P < 0.05$

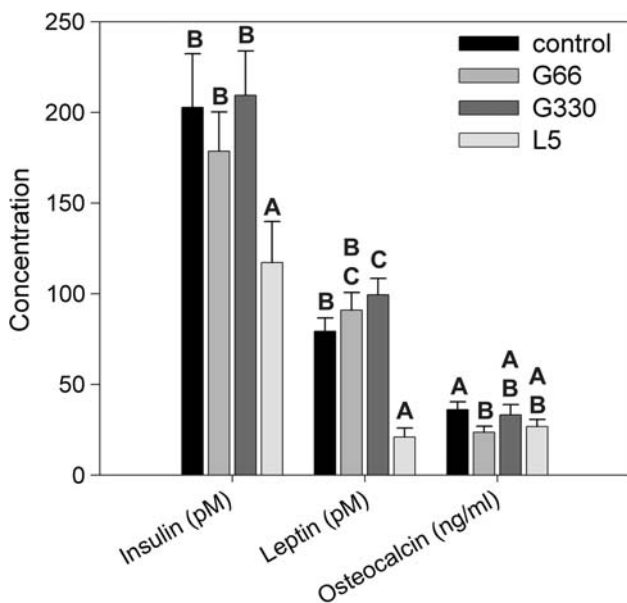


Fig. 3 Serum concentrations of insulin, leptin, and osteocalcin (mean \pm SEM) in rats injected twice daily ICV with 10 μ l/injection of aCSF (Control), G66 (Ghrelin 66 ng/injection), G330 (Ghrelin 330 ng/injection), L5 (leptin, 5 μ g/injection) for 5 days. Means not designated with a common letter are different, $P < 0.05$

Bone marrow adiposity and gene expression in whole bone marrow and isolated bone marrow stem cells

Leptin decreased bone marrow adipocyte density and adipocyte size, whereas ghrelin had no effect (Table 1).

In bone marrow stem cells (Fig. 4a), leptin treatment decreased expression of DLK1 ($P < 0.05$), whereas ghrelin had no effect on gene expression. In whole bone marrow (Fig. 4b) from rats treated with G330, expression of COL1A1 was decreased ($P < 0.05$) while leptin treatment

Table 1 Bone marrow adipocyte density and number (mean \pm SEM) in rats injected ICV with 10 μ l/injection of aCSF (Control), G66 (Ghrelin 66 ng), G330 (Ghrelin 330 ng), L5 (leptin, 5 μ g) twice daily for 5 days

Treatment	Adipocyte density (cells/mm ² $\times 10^{-3}$)	Adipocyte number/0.1 mm ²
aCSF	3.44 \pm 0.27 ^a	332.1 \pm 90.9 ^a
G66	3.50 \pm 0.36 ^a	265.2 \pm 104.7 ^a
G330	3.34 \pm 0.14 ^a	323.1 \pm 71.0 ^a
L5	1.80 \pm 0.21 ^b	83.5 \pm 25.6 ^b

a,b,c Means not designated with a common letter are different, $P < 0.05$

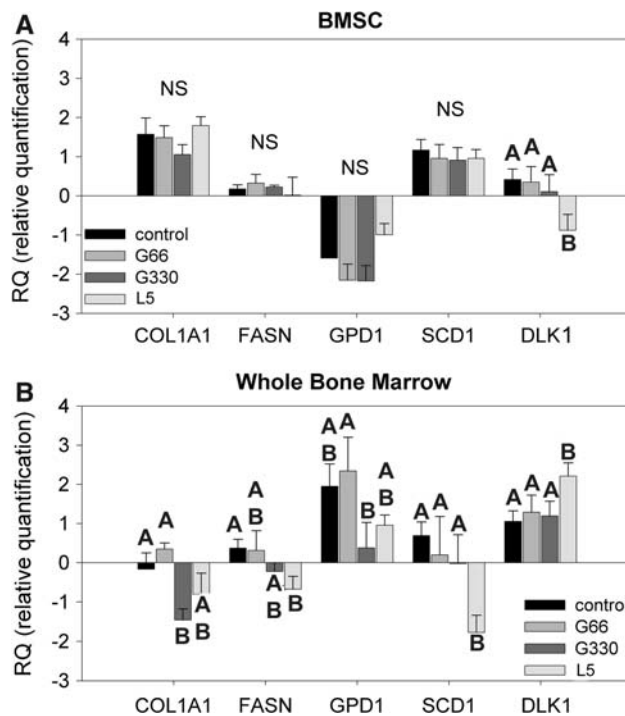


Fig. 4 mRNA expression of selected genes in bone marrow stem cells (a) and whole bone marrow (b) of rats given twice daily ICV injections of aCSF (Control), G66 (Ghrelin 66 ng/injection), G330 (Ghrelin 330 ng/injection), or L5 (Leptin 5 μ g/injection). Data are expressed as means \pm SEM for each group normalized to the endogenous control. Within each gene, means not designated with a common letter are different, $P < 0.05$

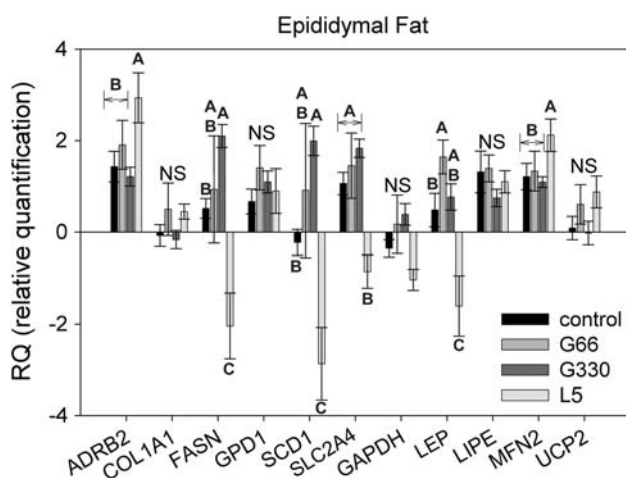


Fig. 5 mRNA expression of thermogenesis- and lipolysis-related genes in epididymal fat from rats given twice daily ICV injections of α CSF (Control), G66 (Ghrelin 66 ng/injection), G330 (Ghrelin 330 ng/injection), or L5 (leptin 5 μ g/injection). Data are expressed as means \pm SEM for each group normalized to the endogenous control. Within each gene, means not designated with a common letter are different, $P < 0.05$

decreased expression of FASN and SCD1 ($P < 0.05$) and increased expression of DLK1 ($P < 0.05$).

In EP fat (Fig. 5) from rats treated with leptin, expression of ADRB2 and MFN2 was increased ($P < 0.05$), while expression of FASN, SCD1, SLC2A4/GLUT4, and LEP was decreased ($P < 0.05$). In contrast, G330 increased expression of FASN and SCD1 ($P < 0.05$), and LEP expression was increased ($P < 0.05$) by G66 treatment.

Discussion

As we and others have previously shown, rats treated with leptin consumed significantly less food, lost weight, and body fat and had reduced serum leptin and insulin concentrations during the treatment period. In EP fat pads, leptin treatments up-regulated the expression of genes involved in both lipolysis (ADRB2) and thermogenesis (MFN2) while down-regulating the adipogenesis-related genes FASN, SCL2A4/GLUT4, and SCD1. These findings are in agreement with the decrease in EP fat pad weights resulting from leptin treatment. Gallardo et al. [17] also found a decrease in FASN expression in epididymal adipose tissue after a 7-day infusion of only 0.2 μ g/day leptin ICV in rats, and Zhang et al. [18] found decreased expression of FASN and SCD1 after a 14-day peripheral administration of leptin in ob/ob mice. In contrast, rats treated with the high dose of ghrelin had increased body weight (BW) and adiposity and increased serum leptin levels even though food intake (FI) was not increased.

Furthermore, adipose tissue gene expression of FASN, SCD1, and leptin was increased. The finding of increased adiposity without increased FI supports findings of a previous study indicating that central ghrelin can alter adipocyte metabolism independently of an effect on FI. Other studies have shown that ghrelin stimulates adipogenesis [19], alters energy partitioning into fat [20], and decreases sympathetic activity and metabolic rate [21], also supporting a FI-independent effect of ghrelin on adipose tissue metabolism. In our study, the lack of effect on FI was likely due to the much lower doses that we tested, compared to other studies showing both increased adiposity and increased FI with ICV or peripheral ghrelin treatment (see, for example, [14, 22]).

Leptin plays a significant role in bone metabolism and can stimulate osteogenesis through direct effects on osteoblasts, in addition to promoting both bone resorption and bone growth via CNS pathways [4, 7, 23]. We have previously shown that leptin administered either peripherally in ob/ob mice or directly into the ventromedial hypothalamus of Sprague–Dawley rats caused a marked reduction in bone marrow adipocyte size and density, which was associated with increased levels of active caspase-3, a critical protease involved in apoptosis [11, 12]. The results of this study confirmed those findings: rats treated with leptin ICV had decreased adipocyte density and size in tibial bone marrow. Furthermore, whole bone marrow from leptin-treated rats had decreased expression of FASN and SCD1, although we did not find increased expression of genes associated with apoptosis. Leptin-treated rats also had increased expression of DLK1 in whole bone marrow. This is an interesting finding because the protein encoded by this gene has been shown to inhibit maturation and lipid synthesis in preadipocytes [24]; thus, the increased expression of DLK1 is consistent with decreased bone marrow adiposity. Because the protein product of DLK1 has also been shown to inhibit osteoblastic differentiation of bone marrow stem cells [25]; the decreased expression of DLK1 in tibial BMSC from leptin-treated rats is also consistent with the promotion of osteogenesis by leptin, although the treatment period in this study was too short to demonstrate an osteogenic effect.

Recently, ghrelin has been found to influence bone turnover through direct stimulation of osteoblast proliferation [15]; however, no previous studies have investigated a centrally mediated effect of ghrelin on bone. In our study, ghrelin-administered ICV had no effect on bone marrow adiposity, and of the genes we investigated, only expression of COL1A1 in whole bone marrow was affected by ghrelin treatment. Thus, at least at the doses tested in this study, ghrelin did not appear to alter tibial bone marrow adiposity.

Conclusions

Central administration of leptin for a 5-day period reduced bone marrow adiposity and decreased expression of adipogenesis-related genes and increased expression of lipolysis-related genes in bone marrow. Central administration of ghrelin increased adiposity in spite of the lack of increase in FI, but had no effect on bone marrow adiposity. Further studies with a dose range of ghrelin that increases both FI and adiposity should provide an interesting comparison for the effects on both adipose tissue and bone marrow gene expression.

Materials and methods

Two experiments were carried out. In both experiments, FI, BW, adipose tissue weights, bone marrow adipocyte size and density, and serum leptin, insulin, and osteocalcin levels were measured. In addition, in experiment 2, expression of specific genes in epididymal adipose tissue and bone marrow was measured.

Animals and diet

Eight-week-old male Sprague–Dawley rats (250–274 g initial BW; eight per treatment group in experiment 1, six per treatment group in experiment 2) purchased from Harlan (Indianapolis, IN, USA) were housed in individual cages in the TSE Systems unit (Bad Homburg, Germany). Lights were on 0900–2100 hours and off 2100–0900 hours, ambient temperature was set at $22 \pm 1^\circ\text{C}$ and humidity at 50%. Rats had ad libitum access to pelleted standard lab chow (5001, PMI Nutritional International, Brentwood, MO, USA) and water throughout the study. The Animal Care and Use Committee approved all experimental and surgical procedures in this study.

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 to a concentration of 2.0 mg/ml. Ghrelin was purchased from Phoenix Pharmaceuticals, Inc. (Belmont, CA, USA, Cat. No.: 031-31). All peptides were solubilized based on their net protein content.

Surgical procedures

Rats were surgically prepared with cannulas directed toward the right lateral cerebral ventricle [26]. Following surgery, the rats were allowed to recover to presurgical BW prior to

administration of treatments. Proper cannula placement was verified by the backflow of CSF from the tip of the guide as well as by an angiotensin II challenge test. Rats that drank ≥ 5 ml of water in 30 min in response to ICV injection of 100 ng/10 μl angiotensin (Sigma, St. Louis, MO, USA) were considered to have the cannula correctly placed [26].

Design and procedures

Following recovery, rats were randomly assigned to treatment groups, including control (*a*CSF), 66 ng ghrelin/injection (G66), 330 ng ghrelin/injection (G330), and 5 μg leptin/injection (L5), and were transferred to the TSE behavioral monitoring system. Injections (10 μl) were administered at 12-h intervals for 5 days. The total amount of ghrelin administered per day for the high-dose treatment was similar to that previously shown to stimulate feeding and increase hypothalamic NPY expression in Sprague–Dawley rats [27].

In both experiments, rats were removed from their cages each day 3 h after light onset for measurement of body temperature, BW, and FI. On day 6, rats were deeply anesthetized with CO_2 before decapitation to remove the brain, blood, GC, soleus muscle, intrascapular BAT, RP, EP, and ING white fat pads, and left and right femora, tibiae and humeri. Trunk blood was collected and the serum was frozen at -20°C for measurement of leptin, insulin, and osteocalcin concentrations. Adipose tissues were weighed, frozen in liquid nitrogen, and stored at -80°C until further analysis. In experiment 2, the EP samples were subsequently used for measuring gene expression. In both experiments, tibias were used for visualization and quantification of marrow adipocytes. In experiment 2, femora and humeri were used for extracting bone marrow RNA.

Bone histomorphometry

The right tibia of each rat was dissected free of soft tissue, fixed in 10% buffered formalin, and stored in 70% ethanol. For bone histomorphometry, the tibia was cut across the middle 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, and adipocyte density was expressed as the number of adipocytes 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.

Blood parameters

Leptin concentrations were measured by the Luminex 100 IS system (Luminex, Inc., Austin, TX, USA). Preparation

of reagents for immunoassay was based on the Lincoplex Kit, catalog # RENDO-85K-03 and Kit ID # RENDO-40836/10224 (Linco Research, St. Charles, MO, USA). Osteocalcin concentration was obtained using an EIA assay (Biomedical Technologies, Inc., Stroughton, MA, USA, Catalog No.: BT-490).

RNA extractions and microfluidic card gene expression

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

Cell culture system

The proximal and distal ends of one femur from each rat were removed and the femur was put in a centrifuge tube and centrifuged at $13000\times g$ for 1 min at 4°C in order to extract the marrow. After centrifugation, the bone was removed and 1 ml of pre-warmed DMEM (with 10% FBS) was added to the marrow. After quantification of the cell number, cells were seeded in 100 mm tissue culture dishes at a density of 5×10^7 per 10 ml of media. Two hours after plating, non-adherent cells were removed by washing once with 5 ml of medium. Twenty-four hours after initial plating, the adherent bone marrow stem cells, which can differentiate into either osteoblasts or adipocytes, were washed twice with $1\times$ phosphate buffered saline (PBS) in order to remove the floating red blood cells; followed by RNA extraction using the RNeasy Mini kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's instructions. After reverse transcription, real-time Taqman RT-PCR (ABI Microfluidic cards) was used to quantitatively measure mRNA levels of selected genes in the bone marrow stem cells.

Whole bone marrow and adipose tissue extraction

Marrow from the second femur and both humeri from each rat were extracted by centrifugation as described above. Samples were pooled by rat following centrifugation. One milliliter of stabilization reagent (Roche Cat. No.: 1-934-317) was immediately added. Samples were stored at -20°C until RNA was extracted. Stabilization reagent was removed by washing samples with NH_4Cl followed by PBS. RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Samples were homogenized in 500 μl Trizol reagent, then centrifuged at $12000\times g$ for 15 min at 4°C , and the aqueous phase was removed and mixed with 0.5 ml of isopropyl alcohol. After centrifugation at $12000\times g$ for 10 min at 4°C , the pellet was washed with 1 ml of 75%

ethanol and then dissolved in 12 μl RNase-free water. One microliter of the 12 μl sample was used as an integrity check and for quantification by the Agilent 2100 bioanalyzer and RNA 6000 Nano Assay (Agilent Technologies, Foster City, CA, USA).

Total RNA was isolated from EP, using Trizol reagent in accordance with the manufacturer's protocol. One hundred milligrams of frozen adipose tissue from each sample was homogenized by adding 1 ml of Trizol reagent. The samples were centrifuged at $12000\times g$ for 15 min at 4°C and the aqueous phase was removed and mixed with 0.5 ml of isopropyl alcohol. After centrifugation at $12000\times g$ for 10 min at 4°C , the pellet was washed with 1 ml of 75% ethanol and then dissolved in 12 μl RNase-free water. One microliter of the 12 μl sample was used as an integrity check and for quantification by the Agilent 2100 bioanalyzer and RNA 6000 Nano Assay (Agilent Technologies).

One hundred nanograms of total RNA in a 20- μl reaction was reverse-transcribed using the cDNA Archive Kit (Applied Biosystems, Inc., Foster City, CA, USA) according to the manufacturer's protocol using MultiScribeTM Reverse Transcriptase. Reactions were incubated initially at 25°C for 10 min and subsequently at 37°C for 120 min. 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 cards. All of the oligonucleotide primer and fluorogenic probe sets for TaqmanTM real-time PCR were from ABI. Two microliters of the cDNA samples, along with 50 μl of $2\times$ PCR master mixes, were loaded into respective channels on the microfluidic card followed by a brief centrifugation ($311\times g$ for 2 min). The card was then sealed and real-time PCR and relative quantification (RQ) was carried out on the ABI PRISM 7900 Sequence Detection System. The cycle conditions were 94.5°C for 15 min, followed by 40 cycles of 97°C for 30 s and 59.7°C for 1 min. Data were expressed as RQ, which presents the fold difference of mRNA level in treatment groups relative to the *a*CSF control group, and were analyzed using sequence detection systems software. mRNA expression was normalized by using 18S RNA as an endogenous control. The ΔCT values were first calculated by using CT for a specific gene mRNA minus CT for 18S RNA mRNA in the sample. Then the mean mRNA expression from the treatment groups was compared with the *a*CSF control group using the formula: $\text{RQ} = 2^{-\Delta\Delta\text{CT}}$ ($\Delta\Delta\text{CT}$ is the average *a*CSF control group ΔCT values minus the average experimental group ΔCT values, and $\Delta\Delta\text{CT}$ of one equates to a twofold difference in cDNA added into the PCR reaction). Twenty-three genes were selected based on their ability to be detected in a mixed cell environment, and to express genes specific to osteoblasts or adipocytes (Table 2).

Table 2 Probes used in real time-PCR (microfluidic card #1, markers specific to osteoblasts or adipocytes)

Function or tissue		Gene	Gene ID
Tissue-specific gene expression	GATA binding protein 3	GATA3	Rn00484683_m1
	Delta-like 1 homolog	DLK1	Rn00587011_m1
Adipocyte	Leptin	LEP	Rn00565158_m1
Lipogenesis	Fatty acid binding protein 4	FABP4	Rn00670361_m1
	CD36 molecule	CD36	Rn00580728_m1
Adipogenesis	Peroxisome proliferator activated receptor γ	PPARG	Rn00440945_m1
Apoptosis	Bcl-2 binding component 3	BBC3	Rn00597992_m1
	Caspase-2	CASP2	Rn00574684_m1
Osteogenesis	Annexin A5	ANXA5	Rn00565571_m1
	Bone gamma-carboxy glutamate protein	BGLAP	Rn00566386_g1
	Biglycan	BGN	Rn00567229_m1
	Secreted protein, acidic cysteine-rich (osteonectin)	SPARC	Rn00561955_m1
	Matrix metalloproteinase 11	MMP11	Rn00564319_m1
	Cathepsin K	CTSK	Rn00580723_m1
	Fibronectin 1	FN1, SERPINH1	Rn00569575_m1
	Epidermal growth factor	EGF	Rn00563336_m1
	Fibroblast growth factor 14	FGF14	Rn00438914_m1
	SMAD family member 1	SMAD1	Rn00565555_m1
Apoptosis, insulin sensitivity	Tumor necrosis factor α	TNF	Rn99999017_m1
Angiogenesis	Vascular endothelial growth factor	VEGF	Rn00582935_m1
Endogenous control	18S	18S	Hs99999901_s1

Table 3 Probes used in real time-PCR (microfluidic card #2, specific to osteogenesis and adipogenesis)

Function		Gene	Gene ID
Energy expenditure	Uncoupling protein 1	UCP1	Rn00562126_m1
	Uncoupling protein 2	UCP2	Rn01754856_m1
	Uncoupling protein 3	UCP3	Rn00565874_m1
	Adrenergic receptor, beta 2	ADRB2	Rn00560650_s1
	Mitofusin 2	MFN2	Rn00500120_m1
	Leptin receptor	LEPR	Rn00561465_m1
	leptin	LEP	Rn00565158_m1
Apoptosis	Tumor necrosis factor α	TNF	Rn99999017_m1
Adipogenesis, lipogenesis	solute carrier family 2 (facilitated glucose transporter), member 4	SLC2A4/GLUT4	Rn00562597_m1
	CCAAT/enhancer binding protein alpha	CEBPA	Rn00560963_s1
	Glycerol-3-phosphate dehydrogenase 1	GPD1	Rn00573596_m1
	Stearoyl-coenzyme A desaturase 1	SCD1	Rn00594894_g1
	Fatty acid synthase	FASN	Rn00569117_m1
Lipolysis	Lipase, hormone sensitive	LIPE/HSL	Rn00563444_m1
	Lipoprotein lipase	LPL	Rn00561482_m1
Adipocyte differentiation	Peroxisome proliferator-activated receptor γ	PPAR γ	Rn00440945_m1
	Peroxisome proliferator-activated receptor α	PPAR α	Rn00566193_m1
Osteogenesis	Alkaline phosphatase	ALPL	Rn00564931_m1
	Bone morphogenic protein 2	BMP2	Rn00567818_m1
	Collagen type I alpha 1	COL1A1	Rn00801649_g1
Endogenous control	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	Rn99999916_s1
	18S	18S	Hs99999901_s1

In order to measure the adipogenesis-related gene markers in bone marrow, and to compare with the expression profiles from white adipose tissue, a different card was designed (Table 3). This card also incorporated key osteogenesis-related genes that were not included in the previously described cards.

Statistical analysis

Data for daily FI, BW, and weight gain, adipose tissue weights, bone marrow adipocyte size and density, and serum leptin, insulin, and osteocalcin levels from the two experiments were combined and analyzed using a 4-way (experiment, block, day, and treatment) ANOVA. Fisher's LSD test was used to determine significance of differences among means. Data are expressed as mean \pm SEM, with consideration of significance at $P \leq 0.05$.

For the gene expression data, two-way ANOVA (treatment and block) was used to determine significance of treatment effects for RQ values within each tissue/cell type; Fisher's LSD test was used to determine significance of differences among treatment means. Data are expressed as means \pm SEM, with consideration of significance at $P < 0.05$.

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

References

1. Y.-H. Choi, C.L. Li, D. Hartzell, D.E. Little, M.A. Della-Fera, C.A. Baile, ICV leptin effects on spontaneous physical activity and feeding behavior in rats. *Behav. Brain Res.* **188**, 100–108 (2008)
2. M.A. Della-Fera, H. Qian, C.A. Baile, Adipocyte apoptosis in the regulation of body fat mass by leptin. *Diabetes Obes. Metab.* **3**, 299–310 (2001)
3. G. Fruhbeck, M. Aguado, J. Gomez-Ambrosi, J.A. Martinez, Lipolytic effect of in vivo leptin administration on adipocytes of lean and ob/ob mice, but not db/db mice. *Biochem. Biophys. Res. Commun.* **250**, 99–102 (1998)
4. P. Ducy, M. Amling, S. Takeda, M. Priemel, A.F. Schilling, F.T. Beil, J. Shen, C. Vinson, J.M. Rueger, G. Karsenty, Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass. *Cell* **100**, 197–207 (2000)
5. F. Eleftheriou, S. Takeda, K. Ebihara, J. Magre, N. Patano, C.A. Kim, Y. Ogawa, X. Liu, S.M. Ware, W.J. Craigen, J.J. Robert, C. Vinson, K. Nakao, J. Capeau, G. Karsenty, Serum leptin level is a regulator of bone mass. *Proc. Natl Acad. Sci. USA* **101**, 3258–3263 (2004)
6. T. Thomas, The complex effects of leptin on bone metabolism through multiple pathways. *Curr. Opin. Pharmacol.* **4**, 295–300 (2004)
7. M.W. Hamrick, S.L. Ferrari, Leptin and the sympathetic connection of fat to bone. *Osteoporos. Int.* **19**, 905–912 (2008)
8. W.R. Holloway, F.M. Collier, C.J. Aitken, D.E. Myers, J.M. Hodge, M. Malakellis, T.J. Gough, G.R. Collier, G.C. Nicholson, Leptin inhibits osteoclast generation. *J. Bone Miner. Res.* **17**, 200–209 (2002)
9. J.O. Gordeladze, C.A. Drevon, U. Syversen, J.E. Reseland, Leptin stimulates human osteoblastic cell proliferation, de novo collagen synthesis, and mineralization: impact on differentiation markers, apoptosis, and osteoclastic signaling. *J. Cell. Biochem.* **85**, 825–836 (2002)
10. T. Thomas, F. Gori, S. Khosla, M.D. Jensen, B. Burguera, B.L. Riggs, Leptin acts on human marrow stromal cells to enhance differentiation to osteoblasts and to inhibit differentiation to adipocytes. *Endocrinology* **140**, 1630–1638 (1999)
11. M.W. Hamrick, M.A. Della-Fera, Y.H. Choi, D. Hartzell, C. Pennington, C.A. Baile, Injections of leptin into rat ventromedial hypothalamus increase adipocyte apoptosis in peripheral fat and in bone marrow. *Cell Tissue Res.* **327**, 133–141 (2007)
12. M.W. Hamrick, M.A. Della-Fera, Y.H. Choi, C. Pennington, D. Hartzell, C.A. Baile, Leptin treatment induces loss of bone marrow adipocytes and increases bone formation in leptin-deficient ob/ob mice. *J. Bone Miner. Res.* **20**, 994–1001 (2005)
13. M. Tschop, D.L. Smiley, M.L. Heiman, Ghrelin induces adiposity in rodents. *Nature* **407**, 908–913 (2000)
14. C. Theander-Carrillo, P. Wiedmer, P. Cettour-Rose, R. Nogueiras, D. Perez-Tilve, P. Pfluger, T.R. Castaneda, P. Muzzin, A. Schurmann, I. Szanto, M.H. Tschop, F. Rohner-Jeanrenaud, Ghrelin action in the brain controls adipocyte metabolism. *J. Clin. Invest.* **116**, 1983–1993 (2006)
15. N. Fukushima, R. Hanada, H. Teranishi, Y. Fukue, T. Tachibana, H. Ishikawa, S. Takeda, Y. Takeuchi, S. Fukumoto, K. Kangawa, K. Nagata, M. Kojima, Ghrelin directly regulates bone formation. *J. Bone Miner. Res.* **20**, 790–798 (2005)
16. K. Matsumura, T. Tsuchihashi, K. Fujii, I. Abe, M. Iida, Central ghrelin modulates sympathetic activity in conscious rabbits. *Hypertension* **40**, 694–699 (2002)
17. N. Gallardo, E. Bonzon-Kulichenko, T. Fernandez-Agullo, E. Molto, S. Gomez-Alonso, P. Blanco, J.M. Carrascosa, M. Ros, A. Andres, Tissue-specific effects of central leptin on the expression of genes involved in lipid metabolism in liver and white adipose tissue. *Endocrinology* **148**, 5604–5610 (2007)
18. W. Zhang, M.A. Della-Fera, D.L. Hartzell, D. Hausman, C.A. Baile, Adipose tissue gene expression profiles in ob/ob mice treated with leptin. *Life Sci.* **83**, 35–42 (2008)
19. A.M. Wren, C.J. Small, C.R. Abbott, W.S. Dhillo, L.J. Seal, M.A. Cohen, R.L. Batterham, S. Taheri, S.A. Stanley, M.A. Ghatei, S.R. Bloom, Ghrelin causes hyperphagia and obesity in rats. *Diabetes* **50**, 2540–2547 (2001)
20. R. Nogueiras, M.H. Tschop, J.M. Zigman, Central nervous system regulation of energy metabolism: ghrelin versus leptin. *Ann. N. Y. Acad. Sci.* **1126**, 14–19 (2008)
21. M. Yukawa, D.S. Weigle, C.D. Davis, B.T. Marck, T. Wolden-Hanson, Peripheral ghrelin treatment stabilizes body weights of senescent male Brown Norway rats at baseline and after surgery. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **294**, R1453–R1460 (2008)
22. S. Strassburg, S.D. Anker, T.R. Castaneda, L. Burget, D. Perez-Tilve, P.T. Pfluger, R. Nogueiras, H. Halem, J.Z. Dong, M.D. Culler, R. Datta, M.H. Tschop, Long-term effects of ghrelin and ghrelin receptor agonists on energy balance in rats. *Am. J. Physiol. Endocrinol. Metab.* **295**, E78–E84 (2008)
23. V. Cirmanova, M. Bayer, L. Starka, K. Zajickova, The effect of leptin on bone: an evolving concept of action. *Physiol. Res.* **57**(Suppl 1), S143–S151 (2008)
24. C. Garces, M.J. Ruiz-Hidalgo, E. Bonvini, J. Goldstein, J. Laborda, Adipocyte differentiation is modulated by secreted delta-like (dlk) variants and requires the expression of membrane-associated dlk. *Differentiation* **64**, 103–114 (1999)

25. B.M. Abdallah, P. Boissy, Q. Tan, J. Dahlgaard, G.A. Traustadottir, K. Kupisiewicz, J. Laborda, J.M. Delaisse, M. Kassem, *dlk1/FA1* regulates the function of human bone marrow mesenchymal stem cells by modulating gene expression of pro-inflammatory cytokines and immune response-related factors. *J. Biol. Chem.* **282**, 7339–7351 (2007)
26. Y.H. Choi, C. Li, K. Page, A. Westby, M.A. Della-Fera, J. Lin, D.L. Hartzell, C.A. Baile, Melanocortin receptors mediate leptin effects on feeding and body weight but not adipose apoptosis. *Physiol. Behav.* **79**, 795–801 (2003)
27. M. Shintani, Y. Ogawa, K. Ebihara, M. Aizawa-Abe, F. Miyanaga, K. Takaya, T. Hayashi, G. Inoue, K. Hosoda, M. Kojima, K. Kangawa, K. Nakao, Ghrelin, an endogenous growth hormone secretagogue, is a novel orexigenic peptide that antagonizes leptin action through the activation of hypothalamic neuropeptide Y/Y1 receptor pathway. *Diabetes* **50**, 227–232 (2001)