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Leptin Regulates Bone Formation via the Sympathetic Nervous System

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Summary

We previously showed that leptin inhibits bone formation by an undefined mechanism. Here, we show that hypothalamic leptin-dependent antiosteogenic and anorexigenic networks differ, and that the peripheral mediators of leptin antiosteogenic function appear to be neuronal. Neuropeptides mediating leptin anorexigenic function do not affect bone formation. Leptin deficiency results in low sympathetic tone, and genetic or pharmacological ablation of adrenergic signaling leads to a leptin-resistant high bone mass. β-adrenergic receptors on osteoblasts regulate their proliferation, and a β-adrenergic agonist decreases bone mass in leptin-deficient and wild-type mice while a β-adrenergic antagonist increases bone mass in wild-type and ovariectomized mice. None of these manipulations affects body weight. This study demonstrates a leptin-dependent neuronal regulation of bone formation with potential therapeutic implications for osteoporosis.

Introduction

In vertebrates, bone mass is maintained constant through the interplay of two functions: bone resorption by osteoclasts and bone formation by osteoblasts. The concerted action of these two cell types defines bone remodeling. The fact that osteoporosis, the most frequent bone remodeling disease, is also the most frequent degenerative disease in developed countries (Cooper and Melton, 1996) explains why identifying molecular regulators of bone remodeling is such an important question of bone biology.

The precision of the recovery following inducible osteoblast ablation in adult mice led us to postulate the existence of a systemic control of bone formation (Corral et al., 1998). The high incidence of osteoporosis following gonadal failure (Riggs et al., 1998) and its low incidence in obese people (Felson et al., 1993; Tremollieres et al., 1993) suggested a hypothesis whereby bone mass, body weight, and reproduction would be controlled by the same hormone(s). Testing this hypothesis revealed that leptin, a hormone regulating body weight and gonadal function, is also a powerful inhibitor of bone formation, i.e., an antiosteogenic factor (Ducy et al., 2000). Leptin-deficient (*ob/ob*), leptin receptor-deficient, and lipodystrophic mice that have in common decreased leptin signaling have the same high bone mass (HBM) phenotype. That these three mutant mouse strains, characterized by a great disparity in body weight, displayed the same bone phenotype demonstrated that it is leptin signaling, not body weight, that controls bone mass. Infusion of leptin into the third ventricle (ICV) of *ob/ob* or wild-type (wt) mice decreased bone mass and bone formation parameters, establishing the existence of a central component in the control of bone formation (Ducy et al., 2000). These results are in agreement with the initial hypothesis since obese people that are protected from osteoporosis are resistant to leptin central action (Ahima and Flier, 2000). The importance of leptin antiosteogenic function is underscored by the fact that leptin deficiency is the only known condition resulting in the coexistence of HBM and hypogonadism, a condition that otherwise favors bone loss.

Much progress has been made in identifying mechanisms whereby leptin exerts its anorexigenic function. Chemical lesioning, molecular elucidation of mouse mutant strains, and generation of neuropeptide-deficient mice have identified hypothalamic neurons synthesizing orexigenic or anorexigenic molecules that are targets of leptin anorexigenic action (Elias et al., 1999; Cowley et al., 2001; DeFalco et al., 2001). In contrast, the cellular and molecular bases of leptin antiosteogenic function remain unknown. We do not know which hypothalamic areas and networks are targeted by this function of leptin, or if the same neuropeptides mediate anorexigenic and antiosteogenic functions of leptin. We further do not know either the nature (i.e., humoral or neuronal) or the identity of the mediator(s) emanating from the brain and controlling bone formation. These questions are of paramount importance from a biomedical viewpoint as their answers may lead to the generation of drugs enhancing bone formation, possibly without affecting body weight. Such drugs are in great demand to treat osteoporosis.

To decipher the bases of leptin antiosteogenic function, we used chemical lesioning, genetic, physiological, and molecular analyses. Our studies suggest that the anorexigenic and antiosteogenic modes of leptin action are distinct, identify a neuronal regulation of bone formation, and point toward a therapeutically useful way of manipulating this pathway.

Results

Identification of Hypothalamic Antiosteogenic Areas

The mediobasal hypothalamus has previously been shown to be a critical target of leptin action (Tartaglia et

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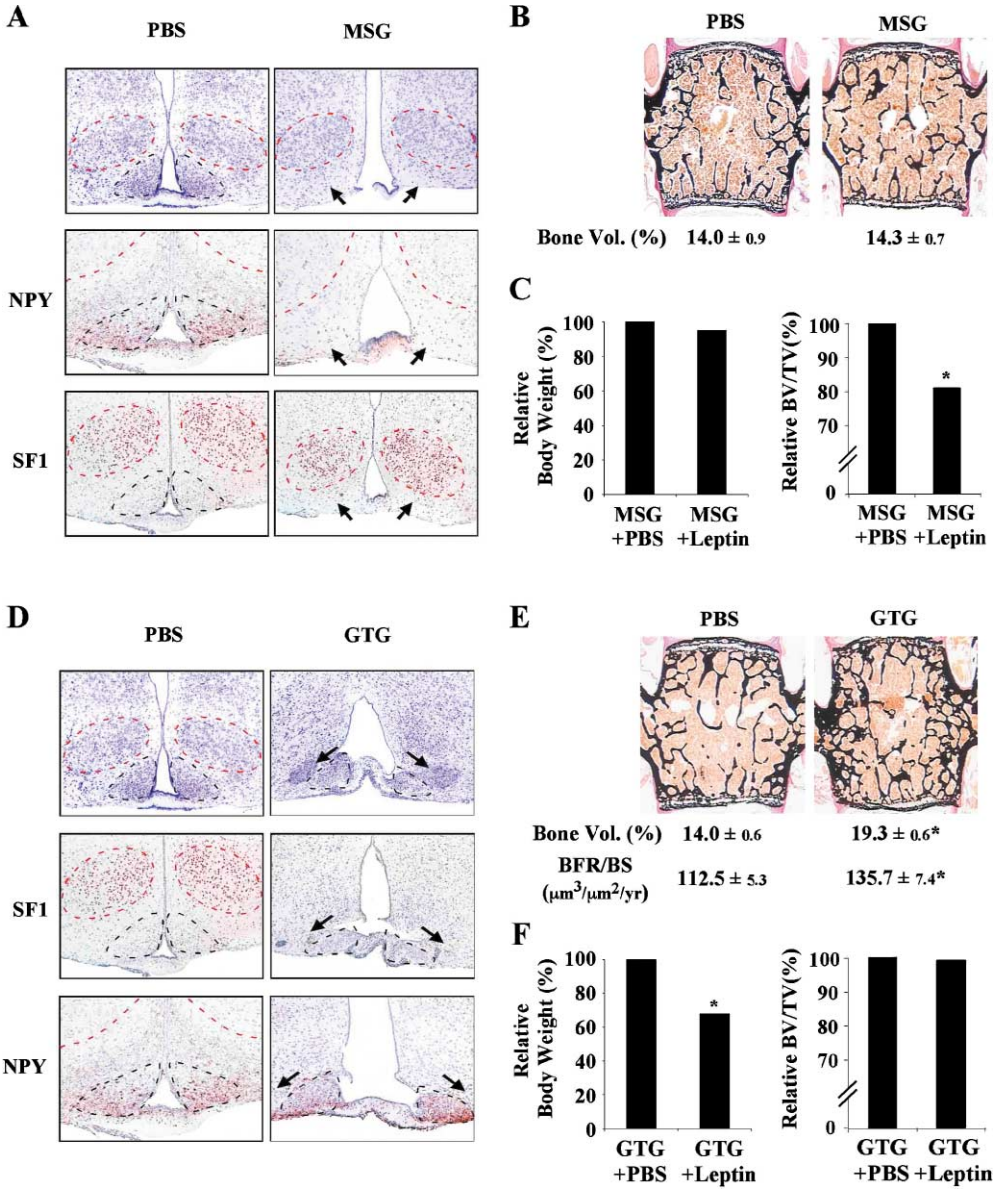

Figure 1. Dissociation of Leptin Anorexigenic and Antiosteogenic Actions in the Hypothalamus

(A–C) MSG lesion.

(A) Effect on hypothalamic structure. Cresyl violet staining (top) immunohistochemistry for NPY (middle) and SF1 (bottom). The arcuate nuclei (ARC) and the VMH are encircled by black and red dotted lines, respectively. The planes of brain sections are located −1.7 from bregma according to Paxinos' reference atlas (Franklin and Paxinos, 1997). MSG treatment markedly affected ARC structure and NPY expression (arrows), while VMH structure and SF1-expressing neurons were preserved.

(B) Histological analysis of vertebrae of MSG-lesioned mice; bone volume (% bone vol./tissue volume) is not affected.

(C) Leptin ICV infusion does not affect body weight (left) but decreases bone volume (right) in MSG-lesioned *ob/ob* mice (MSG+leptin).

(D–F) GTG lesion.

(D) Effect on hypothalamic structure. Cresyl violet staining (top) immunohistochemistry for SF1 (middle) and NPY (bottom). GTG treatment affected the VMH, resulting in a dense scar and markedly altering the distribution of SF-1-expressiong neurons while preserving ARC structure and NPY expression.

(E) Histological analysis of vertebrae of GTG-lesioned mice; bone volume and bone formation rate (BFR/BS) are increased.

(F) Leptin ICV infusion decreases body weight (left) but does not affect bone volume (right) in GTG-treated animals (GTG+leptin). Asterisks indicate statistically significant differences between experimental and control groups ($p < 0.01$).

al., 1995; Fei et al., 1997). Two nuclei in this hypothalamic region, the ventromedial hypothalamic nucleus (VMH) and arcuate nucleus (ARC), have the highest density of neurons expressing *ObRb*, the signal-transducing form of the leptin receptor, and both nuclei play a critical role in mediating leptin anorexigenic function. The role of neurons of these nuclei in mediating leptin antiosteogenic function was first assessed by chemical lesioning followed by leptin ICV infusion.

We first treated newborn pups with monosodium glu-

tamate (MSG), which damages circumventricular neurons expressing the glutamate receptor (Olney, 1969). As shown by histology, MSG treatment predominantly affected ARC structures (Figure 1A and see Supplemental Figures S1 and S2 at http://www.cell.com/cgi/content/full/111/3/305/DC1). This was illustrated by the near absence of neurons synthesizing neuropeptide Y (NPY) (Figure 1A). In contrast, immunohistochemical studies using an antibody against steroidogenic factor 1 (SF1), a marker of VMH neurons (Ikeda et al., 1995; Dellovade et al., 2000), indicated that the majority of these neurons were unaffected by MSG treatment. No lesions outside the hypothalamus could be observed in any of the animals treated. 12-week-old MSG-treated mice had a normal bone mass as determined histologically by measurement of their bone volume (Figure 1B). To further evaluate the role of MSG-sensitive neurons in leptin antiosteogenic function, we performed leptin ICV infusion in *ob/ob* mice treated with MSG. MSG treatment blocked the ability of leptin ICV infusion to decrease body weight but not to decrease bone mass (Figure 1C). The normal bone mass of MSG-treated mice along with the decrease in bone mass following leptin ICV infusion despite MSG-induced lesions in *ob/ob* mice indicates that although leptin anorexigenic function required their presence, its antiosteogenic action can take place when MSG-sensitive neurons are absent.

Next we treated 4-week-old wt mice with gold thioglucose (GTG), a compound that destroys glucose-sensitive neurons (Debons et al., 1962). In each mouse analyzed, the deleterious effect of GTG treatment was revealed by a dense scar distorting the anatomy of the ventral hypothalamus, resulting in a collapse of the third ventricle (Figure 1D and see Supplemental Figures S3 and S4 at http://www.cell.com/cgi/content/full/111/3/305/DC1). Immunohistochemical studies and cell counting in GTG-treated mice showed a marked decrease of neurons expressing SF1, whereas NPY-expressing neurons were largely but not entirely spared (Figure 1D). Bone histological analysis revealed that 12-week-old GTG-treated mice displayed a HBM phenotype whose severity was nearly identical to that of *ob/ob* mice (Figure 1E). As with *ob/ob* mice, this HBM was due to an increase in bone formation defined by an increase in the bone formation rate (Figure 1E). Urinary elimination of deoxypyridinoline (dpd), a collagen breakdown product indicative of osteoclast activity, and osteoclast numbers were normal, indicating that bone resorption was not overtly affected by GTG treatment (data not shown). These findings established that neurons sensitive to GTG are involved in the control of bone formation.

To determine whether GTG-sensitive neurons were implicated in leptin antiosteogenic function, we performed leptin ICV infusion in GTG-treated *ob/ob* mice. Leptin ICV infusion decreased the body weight of these animals, indicating that it was effective (Figure 1F). However and despite the extreme sensitivity of *ob/ob* mice to leptin, its ICV infusion failed to decrease their bone mass (Figure 1F). These data indicate that GTG-sensitive neuronal networks are necessary for leptin antiosteogenic function. The observation that GTG or MSG treatment affected differentially leptin antiosteogenic and anorexigenic functions suggests that these two functions are executed, at least partly, by distinct neuronal pathways without, however, defining precisely their neuroanatomical locations.

Leptin Antiosteogenic Function Does Not Require Known Anorexigenic Neuropeptides

To refine our results from chemical lesions, we used a genetic approach. It has been demonstrated that binding of α-melanocyte-stimulating hormone (αMSH), produced by ARC neurons, to neurons expressing melanocortin 4 receptor (MC4-R) and melanocortin 3 receptor (MC3-R) is required for leptin anorexigenic function (Huszar et al., 1997; Vaisse et al., 1998; Yeo et al., 1998; Cowley et al., 2001). To assess the role of melanocortin signaling in leptin antiosteogenic function, we analyzed mutant mouse strains with disrupted melanocortin signaling and *ob/ob* mice treated with a melanocortin receptor agonist.

A^y/a mice have decreased melanocortin signaling due to the binding to melanocortin receptors of the agouti protein, a competitive antagonist of melanocortin receptor signaling with a high affinity for MC4-R (Miller et al., 1993; Lu et al., 1994; Fan et al., 1997). As a result, *A^y/a* mice develop a late-onset obesity accompanied by a central resistance to leptin anorexigenic function (Halaas et al., 1997) that resembles the phenotype observed in *Mc4-r*-deficient mice (Huszar et al., 1997). In contrast, multiple lines of evidence indicate that leptin antiosteogenic function does take place when melanocortin signaling is disrupted. First, *A^y/a* mice have a normal bone mass (Ducy et al., 2000). Second, *A^y/a* mice are not resistant to leptin antiosteogenic function, as long-term ICV leptin infusion decreased bone volume comparably in *A^y/a* mice and wt mice, secondary to a decrease in the bone formation rate (Figure 2A). Third, *Mc4-r*-deficient mice have a normal bone mass (Figure 2B). Fourth, ICV infusion of MTII, a MC4-R/MC3-R agonist (Fan et al., 1997) in *ob/ob* mice, did not affect their bone mass while it significantly decreased their body weight (Figures 2C and 2D).

Another anorexigenic polypeptide whose expression is regulated by leptin is cocaine amphetamine related transcript (CART) (Kristensen et al., 1998). Although CART regulates body weight in mice (Asnicar et al., 2001), *Cart*-deficient mice did not display HBM (data not shown). Taken together, these experiments indicate that melanocortin- and CART-signaling pathways, which are critical for leptin anorexigenic action, are not required for its antiosteogenic function. These results are consistent with the observation that MSG-sensitive neurons, which are the main hypothalamic source of αMSH, are dispensable for leptin antiosteogenic action.

The analysis of *Mc4-r*-deficient mice addressed another concern that could not be studied in mice deficient in leptin signaling. The HBM observed in absence of leptin signaling raised the possibility that it could be secondary to coexisting hyperinsulinism. The normal bone mass in *Mc4-r*-deficient mice despite their elevated plasma insulin levels argues that hyperinsulinism does not lead to HBM (Table 1). Three other lines of evidence presented below further dissociate plasma insulin levels and bone mass regulation.

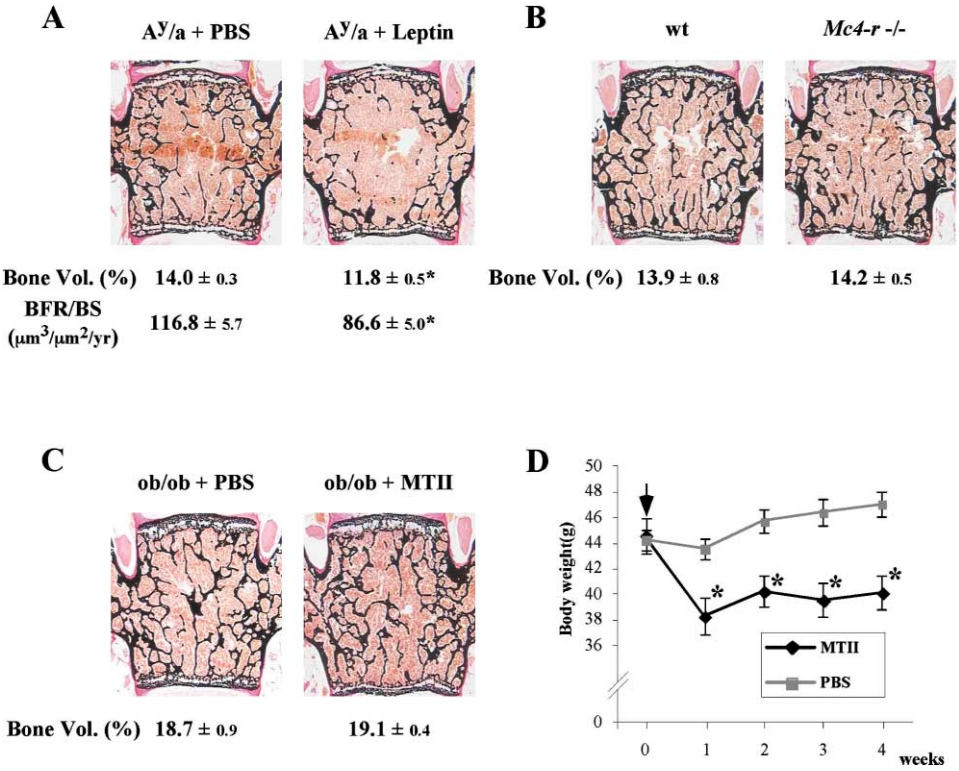

Figure 2. Anorexigenic Neuropeptides Do Not Control Bone Formation

(A) Histological analysis of vertebrae of 4-month-old agouti yellow mutant mice (A^y/a) infused ICV with PBS or leptin. ICV leptin infusion causes a decrease in bone volume (Bone vol. %) and bone formation rate (BFR/BS).

(B) Histological analysis of vertebrae of 3-month-old *Mc4-R*-deficient mice (*MC4-R*$^{-/-}$); bone volume is normal.

(C and D) Histological analysis of vertebrae of *ob/ob* mice infused ICV with PBS or MTII; bone volume is not affected (C), whereas body weight is decreased (D). Asterisks indicate statistically significant differences between experimental and control groups ($p < 0.01$). Error bars represent SEM.

Peripheral Mediation of Leptin Antiosteogenic Function

We next asked whether the signal(s) emanating from the hypothalamic antiosteogenic network was/were of humoral or of neuronal nature. To that end we relied on crosscirculation (parabiosis) experiments between *ob/ob* animals (that have no circulating leptin). Parabiosis experiments were performed as described in Experimental Procedures and dye injection confirmed effective crosscirculation with >95% equilibrium between the parabiotic animals after 2 hr. Two weeks after parabiosis, we implanted in one mouse of each parabiosed pair a pump infusing leptin ICV. The absence of measurable leptin in serum of all the animals analyzed ruled out a leakage of leptin into the general circulation (data not shown). Four weeks later, the animals were sacrificed and analyzed. As expected, bone mass dropped significantly in the *ob/ob* mouse receiving leptin ICV; in contrast, we never observed any modification of bone mass in the contralateral mouse (Figure 3A). Although it does not rule out the existence of a short-lived humoral mediator, this experiment raised the possibility of a neuronal mediation of leptin antiosteogenic function.

Table 1. Serum Insulin Levels and Bone Mass

	Insulin (ng/ml)	Bone Volume
Wild-type	0.8 ± 0.3	Normal
MC4-R$^{-/-}$	23.0 ± 6.0[a]	Normal
Dbh$^{-/-}$	1.0 ± 0.4	High
ob/ob	19.6 ± 0.8	High
ob/ob+isoproterenol	4.4 ± 0.4	Low
Wild-type+propranolol	0.8 ± 0.1	High

[a] Huszar et al., 1997

None of the experiments presented above excluded the possibility that leptin could also affect osteoblast function by acting locally. To test this hypothesis, two transgenic mouse lines were generated that use the osteoblast-specific fragment of the α*1(I) collagen* promoter (Rossert et al., 1995) to drive *Leptin* expression in osteoblasts [α*1(I)–leptin*] (Figure 3B). Northern blot analysis and immunocytochemistry demonstrated a high level of leptin synthesis by osteoblasts (Figures 3B and 3C) that resulted in a slight increase in plasma leptin level although it remained within the normal range (wt, 3.2 ± 0.4 ng/ml versus α*1(I)* leptin, 5.6 ± 0.8 ng/ml, n = 7 per genotype). The bioactivity of leptin transcribed by this transgene was established by showing that following DNA transfection leptin increased the activity of a Stat3-dependent luciferase reporter construct in 293 cells expressing *ObRb* (Figure 3D). Despite this high local level of bioactive leptin, the bone mass of the transgenic mice was indistinguishable from that of wt

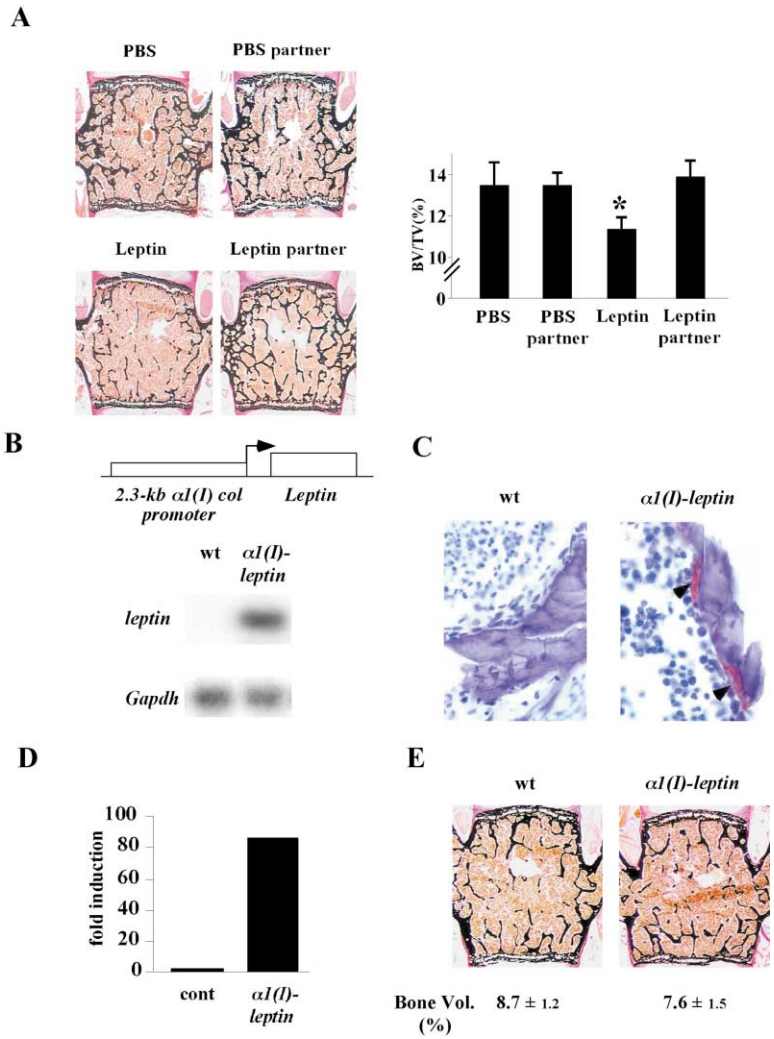

Figure 3. Peripheral Mediation of Leptin Antiosteogenic Function

(A) Histological analysis of vertebrae of parabiosed *ob/ob* mice subsequently infused with PBS or leptin. Leptin ICV infusion decreases the bone volume (Bone vol. %) of the ipsilateral mouse (Leptin), but not in the contralateral mouse (Leptin partner). No effect was observed with PBS (PBS and PBS partner).

(B) Generation of osteoblast-specific leptin (*α1(I)-leptin*) transgenic mice. Top: schematic representation of the construct. The mouse *leptin* cDNA is under the control of the 2.3 kb osteoblast-specific fragment of the *α1(I) collagen* promoter. Bottom: Northern blot analysis of the transgene expression in bone.

(C) Leptin immunoreactivity is detectable in bones of *α1(I)-leptin* mice but not of wt mice.

(D) Bioactivity analysis of the *α1-leptin* transgene performed in 293 cells expressing *ObRb* cotransfected with the *α1(I)-leptin* transgene and a Stat3-dependent promoter-luc construct.

(E) Histological analysis of vertebrae of 12-month-old *α1(I)-leptin* transgenic mice. Leptin expression in osteoblasts does not affect bone volume. Asterisks indicate statistically significant differences between experimental and control groups ($p < 0.01$). Error bars represent SEM.

mice at any age including 1 year (Figure 3E). This result indicates that the primary basis of leptin antiosteogenic function is not a direct action on osteoblasts.

Sympathetic Regulation of Bone Formation

A well-characterized consequence of leptin deficiency is a reduced activity of the sympathetic nervous system (SNS) (Bray and York, 1998). Moreover, it has been proposed that the VMH mediates leptin-induced increase in catecholamine secretion (Ruffin and Nicolaidis, 1999; Satoh et al., 1999). These observations as well as our own findings led us to explore the role of the SNS in the control of bone formation.

As SNS function is mediated through adrenergic receptors, we studied mutant mice deficient in dopamine β-hydroxylase (DBH), an enzyme necessary to produce norepinephrine and epinephrine, the catecholamine ligands for adrenergic receptors. Histological examination revealed the existence of a HBM in *Dbh*-deficient mice, albeit less severe than the one observed in *ob/ob* mice (Figure 4A). This finding was significant since *Dbh*-deficient mice have an increase in serum corticosterone and in dopamine levels (Alaniz et al., 1999), two conditions favoring low bone mass (Adachi et al., 1993; Alaniz et al., 1999; Bliziotes et al., 2000). This HBM was secondary to an increase in the bone formation rate and in the number of osteoblasts while markers of bone resorption were normal (Figures 4A–4D and data not shown). The HBM observed in *Dbh*-deficient mice was not associated with hyperinsulinism or with other hormonal perturbations besides a high corticosterone level (Table 1 and data not shown). Catecholamines are released from two main sources: the sympathetic nerves and the adrenal glands. To determine whether the adrenal production of catecholamines is involved in the regulation of bone mass, we analyzed wt mice in which the adrenal medulla, the main source of circulating epinephrine (Young and Landsberg, 1998), had been surgically removed 4 weeks previously. Histological analysis showed that removal of the adrenal medulla did not affect bone mass (Figure 4E). These results establish the existence of a neuronal regulation of bone formation.

We next asked whether there was a direct link between leptin central antiosteogenic function and the SNS regulation of bone formation. To that end we performed leptin ICV infusion in *Dbh*-deficient mice. This infusion led to a near disappearance of the gonadal fat pad in all *Dbh*-deficient mice treated, indicating that

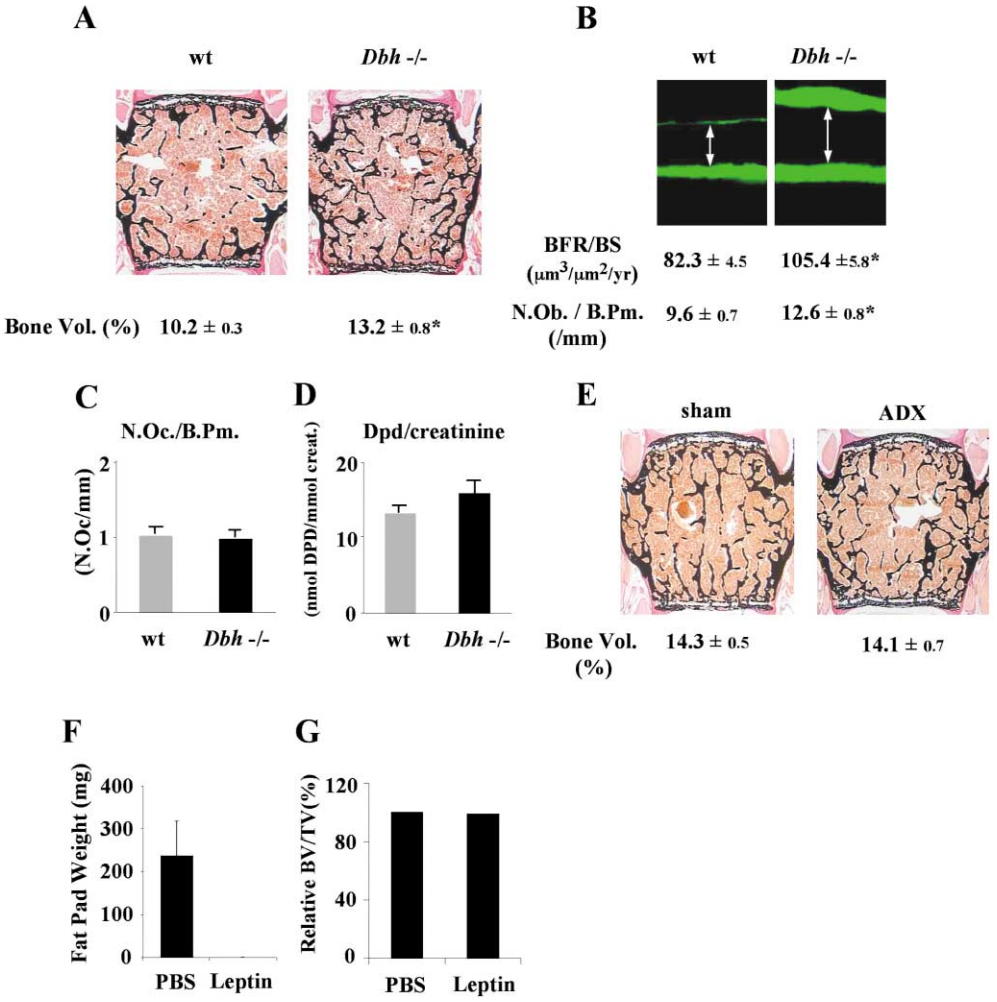

Figure 4. Sympathetic Control of Bone Formation

(A) Histological analysis of vertebrae of 12-month-old *Dbh*-deficient mice ($Dbh^{-/-}$). Bone volume (Bone vol. %) is significantly increased in $Dbh^{-/-}$ mice.

(B) Calcein double labeling, bone formation rate (BFR/BS, white arrows), and number of osteoblasts (N.Ob/B.Pm) are increased in $Dbh^{-/-}$ mice.

(C and D) Normal osteoclast number (N.Oc/B.Pm) and deoxypyridinoline crosslinks elimination in *Dbh*-deficient mice.

(E) Histological analysis of vertebrae of adrenal medullectomized mice (ADX); bone volume is not affected.

(F and G) Leptin icv infusion in $Dbh^{-/-}$ mice. Fat pad weight is decreased (F) while bone volume is not affected (G) by the treatment. Asterisks indicate statistically significant differences between experimental and control groups ($p < 0.01$). Error bars represent SEM.

centrally delivered leptin can affect body weight regulation in the absence of norepinephrine and epinephrine (Figure 4F). Strikingly, leptin failed to decrease the bone mass of *Dbh*-deficient mice (Figure 4G), demonstrating that leptin antiosteogenic function requires a functional SNS.

Functional Adrenergic Receptors on Osteoblasts

For a sympathetic regulation of bone formation to exist, several requirements have to be fulfilled. The first one is that functional adrenergic receptors must be present on osteoblasts. Gene expression analysis by RT-PCR and Northern blot showed the presence of β_2-adrenergic receptor transcripts, but of no other transcripts for adrenergic receptors, in primary mouse osteoblast cultures (Figure 5A). Immunohistochemical analysis of long bones from transgenic mice expressing *LacZ* under the control of the osteoblast-specific fragment of the α*1(I) collagen* promoter verified the presence of β2-adrenergic receptors on osteoblasts (Figure 5B). No other adrenergic receptor subtype could be detected. Moreover, axons immunoreactive with anti-neurofilament and anti-tyrosine hydroxylase antibodies were observed in the vicinity of osteoblasts (Figures 5C and 5D). Electron micrographs confirmed the presence of unmyelinated peripheral nerve axons coursing through the marrow adjacent to bone trabeculae and to osteoblasts (Figure 5E). β-adrenergic receptors are G-coupled receptors that signal through the cAMP pathway (Benovic et al., 1988). Thus, to assess the biological relevance of the presence of β_2-adrenergic receptors on osteoblasts, we

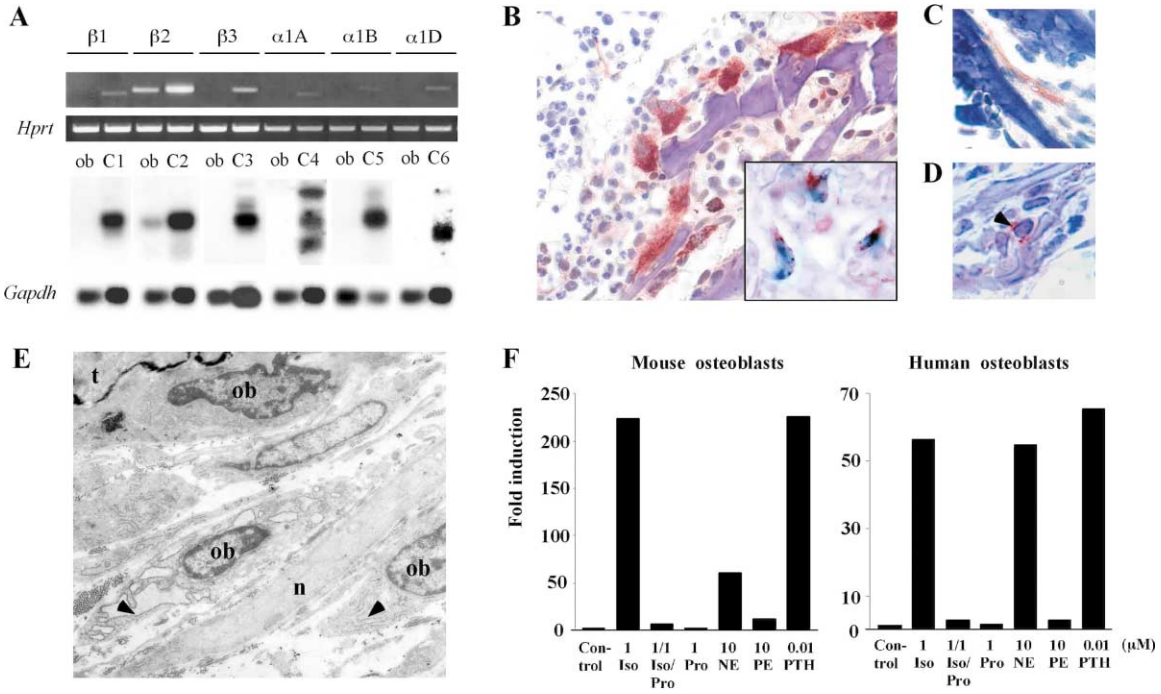

Figure 5. Presence of Functional Adrenergic Receptors on Osteoblasts

(A) RT-PCR (top) and Northern blot analyses (bottom) of α- and β-adrenergic receptor expression. Only β2-adrenergic receptor expression can be detected in osteoblasts. Samples were from primary osteoblasts (ob), heart (C1, C2, C4, and C6), brown adipose tissue (C3), and liver (C5). *Hprt* amplification and *Gapdh* expression were used as controls for loading and RNA integrity.

(B) Immunolocalization of β2-adrenergic receptors in bones of wt mice and transgenic mice overexpressing LacZ in osteoblasts (inset). Mononucleated cells express β2-adrenergic receptors and those cells are X-gal positive, i.e., osteoblasts.

(C and D) Immunolocalization of neurofilament (C) and tyrosine hydroxylase (D, arrow) adjacent to osteoblasts.

(E) Electron micrographs of bone sections of 2-day-old mice. A nerve (n) is located in close vicinity to osteoblasts (ob) and bone trabeculae (t).

(F) In murine primary osteoblasts (left) and human SaOS-2 osteoblastic cells (right), cAMP production is induced by β-adrenergic receptor agonists (isoproterenol, iso; norepinephrine, NE) and inhibited by the addition of β-adrenergic antagonist (propranolol, Pro). PTH was used as a positive control.

treated these cells with isoproterenol, a β-adrenergic agonist, alone or in the presence of propranolol, a β-adrenergic receptor antagonist; with norepinephrine, the natural ligand of β-adrenergic receptors; with phenylephrine, an α-adrenergic agonist; or with vehicle and measured cAMP production. As a positive control we used parathyroid hormone (PTH), which binds to another G-coupled receptor present in osteoblasts (Gardella and Juppner, 2001). Isoproterenol or norepinephrine but not phenylephrine treatment increased cAMP production to a similar extent as PTH. These effects were abolished by propranolol treatment. The same results were obtained using mouse and human osteoblasts (Figure 5F).

Decreased Bone Mass but Persistent Obesity in Sympathomimetic-Treated *ob*/*ob* Mice

A second requirement is that treatment of *ob*/*ob* mice with sympathomimetic agents should decrease their bone mass. To address this point, 1-month-old *ob*/*ob* mice were treated for 6 weeks with the β-adrenergic agonist isoproterenol or vehicle. As shown in Figure 6A, long-term isoproterenol treatment (3 mg/kg/day) resulted in a massive bone loss in the vertebrae and long bones of *ob*/*ob* mice. This was secondary to a marked decrease in the bone formation rate and in the number of osteoblasts per bone perimeter (Figure 6B) while bone resorption parameters were unaffected (data not shown). Identical results were obtained when using 10 mg/kg/day of isoproterenol (data not shown). Two aspects of this experiment are of particular importance. First, this low bone mass developed while *ob*/*ob* mice remained hyperinsulinemic, further dissociating hyperinsulinism and bone mass regulation (Table 1). Second, at these two doses, isoproterenol decreased bone mass without affecting body weight, demonstrating the existence of a range of doses in which isoproterenol affects selectively bone mass (Figures 6A and 6C). To determine the role of the SNS in animals that have none of the metabolic and neurological abnormalities caused by leptin deficiency, we repeated this experiment in wt mice. Isoproterenol again significantly decreased bone mass, bone formation rate, and osteoblast number without affecting their body weight (Figures 6D–6F). The increased expression of *uncoupling protein 1* (*Ucp1*) in brown adipose tissue indicated that isoproterenol mimicked an increase in sympathetic activity in both *ob*/*ob* and wt mice (Figure 6G; Scarpace and Matheny, 1998). Taken together, these data identify the SNS, acting through β_2 adrenergic receptors, as a regulator of bone formation independent of the effect it may have on body weight.

To elucidate the bases of the antiosteogenic effect of isoproterenol in vivo, we studied osteoblast prolif-

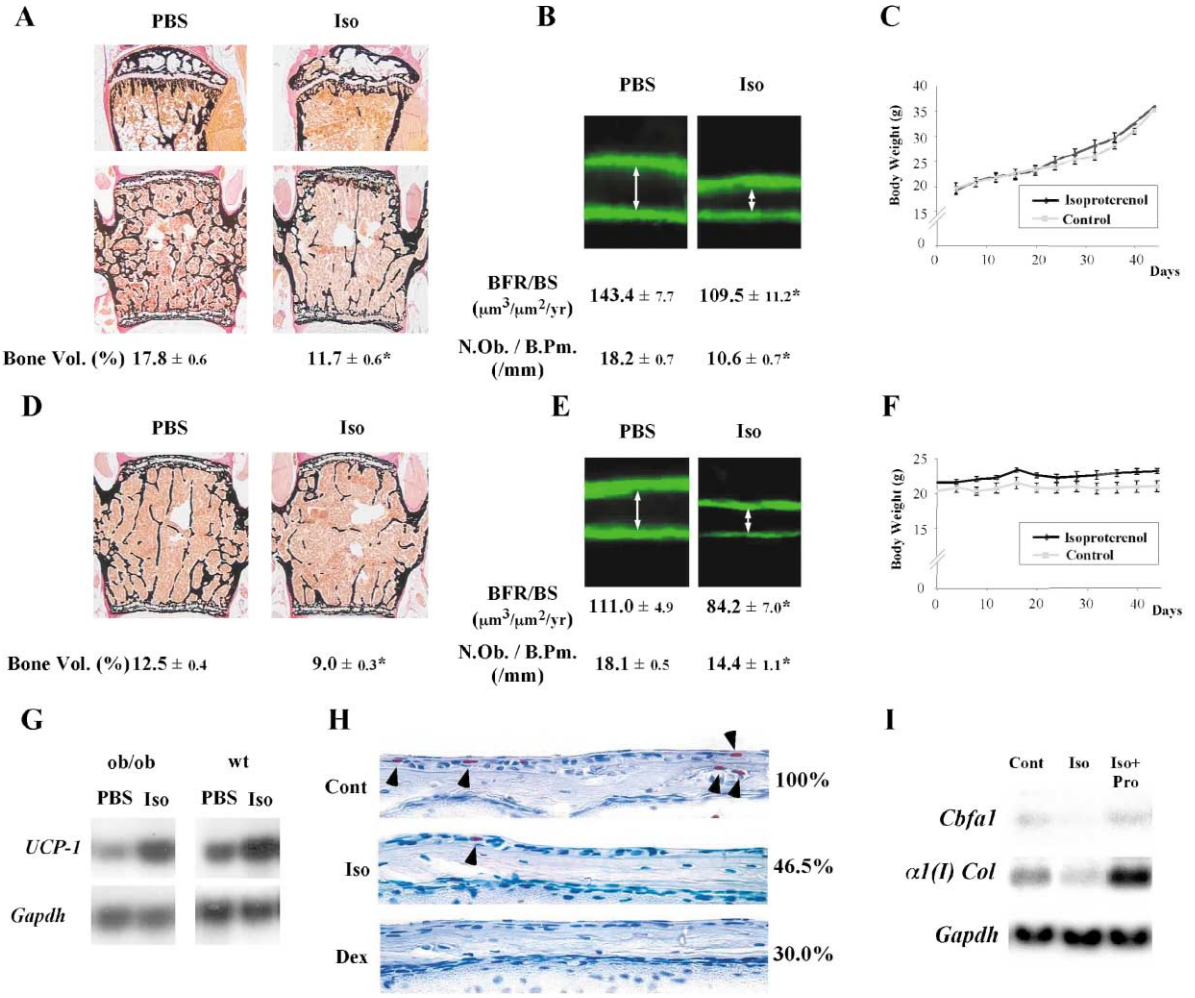

Figure 6. A β-Adrenergic Agonist Inhibits Bone Formation

Isoproterenol (Iso) treatment of *ob/ob* (A–C) and wt (D–F) mice. (A and D) Histological analysis of vertebrae and tibia (A). Bone volume (Bone vol. %) is decreased by Iso treatment. (B and E) Calcein double labeling (top), bone formation rate (BFR/BS, white arrows), and osteoblast number (N.Ob/B.Pm) are decreased by Iso treatment. (C and F) Body weight analysis. These doses of Iso do not affect body weight.

(G) Northern blot analysis. Iso increases *UCP-1* expression in brown adipose tissues (top).

(H) Osteoblast proliferation. Immunolocalization of BrdU incorporation (red staining, arrows) in calvariae of Iso, dexamethasone (Dex), or vehicle (Cont)-treated mice. Percentages of BrdU-positive cells relative to control are indicated on the right.

(I) Northern blot analysis of *Cbfa1* and *α1(I) collagen* expression in primary osteoblasts treated by Iso, Iso and propranolol (Iso+Pro), or vehicle (Cont). *Gapdh* expression was used as an internal control for all Northern blots. Asterisks indicate statistically significant differences between experimental and control groups ($p < 0.01$).

eration, gene expression, and apoptosis. Following bromodeoxyuridine (BrdU) labeling in vivo, calvariae of isoproterenol-treated wt mice showed a nearly 2-fold reduction in the number of positive cells compared to calvariae of control mice, indicating that isoproterenol reduces osteoblast proliferation (Figure 6H). As a control we used dexamethasone, which severely reduced osteoblast proliferation. Osteoblast gene expression studies revealed that isoproterenol treatment decreased the expression of *Cbfa1*, a transcription factor controlling osteoblast function (Ducy et al., 1999), and *α1(I) collagen*, a gene encoding the main component of the bone extracellular matrix (Figure 6I). Treatment with propranolol, a β-adrenergic antagonist, reversed the inhibitory effect of isoproterenol on gene expression (Figure 6I). To study apoptosis, we looked at *Caspase 3* expression and Annexin-V staining levels and did not observe any change in isoproterenol-treated versus control osteoblasts (data not shown). These results indicate that the antiosteogenic function of the SNS is secondary to an inhibition of osteoblast proliferation and function.

A β-Adrenergic Antagonist Increases Bone Mass in Wild-Type and Ovariectomized Mice

The most important biomedical requirement for regulation of bone formation by the SNS is that β-adrenergic antagonists (β blockers) should increase bone mass by increasing bone formation. To address this point, wt mice were treated for 5 weeks with propranolol (0.4 mg/day). This treatment resulted in a significant increase in bone mass in vertebrae and long bones (Figure 7A). In some propranolol-treated mice, the bone volume

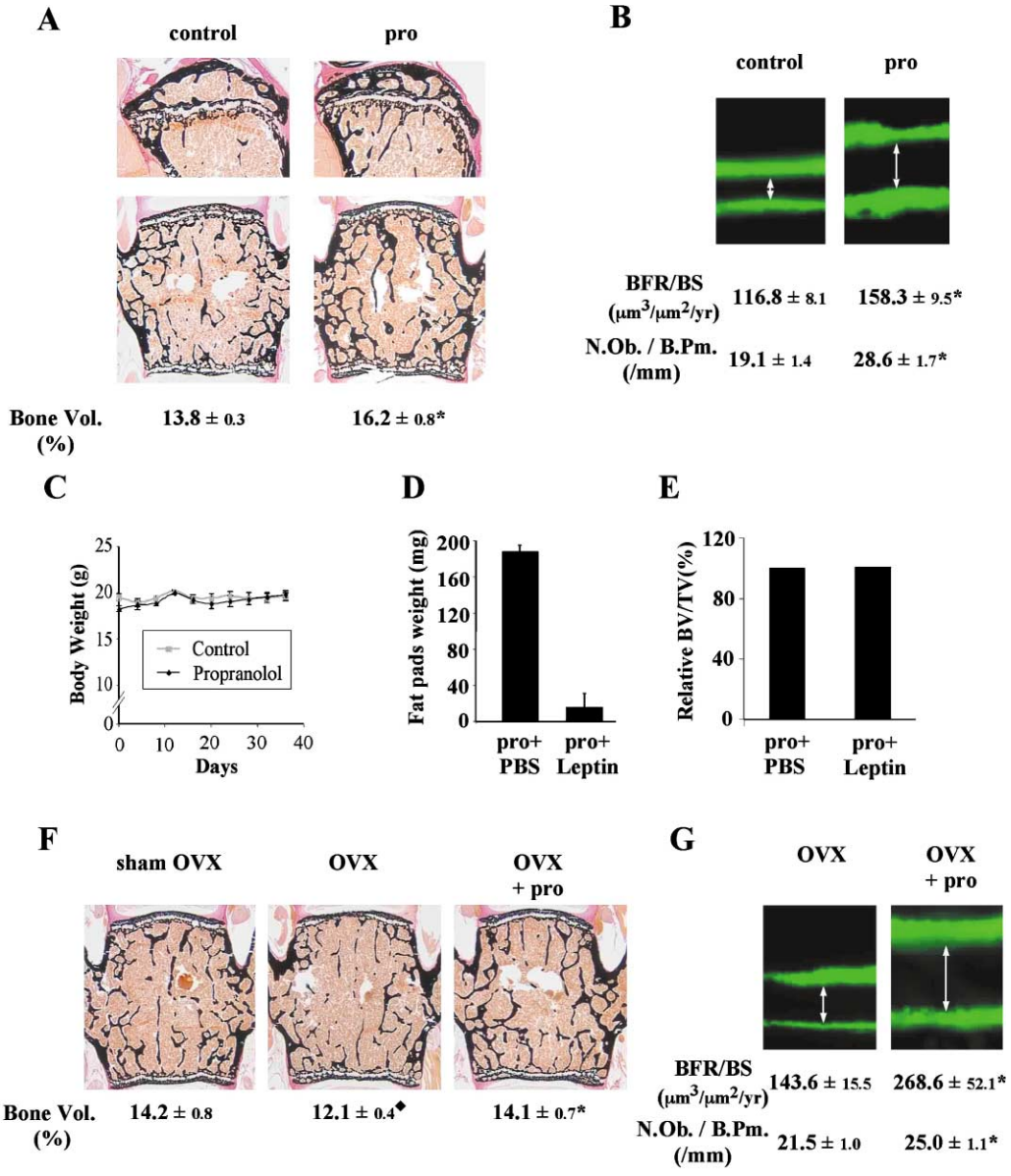

Figure 7. A β-Adrenergic Antagonist Increases Bone Mass

Propranolol (Pro) treatment of wt (A, C), wt receiving leptin ICV infusion (D, E), ovariectomized (OVX), and sham-operated (Sham OVX) (F and G) mice. Histological analysis of vertebrae (A, B, E–G) and tibiae (A). Bone volume (Bone vol. %) is increased by Pro treatment (A and F). Calcein double labeling (top), bone formation rates (BFR/BS, white arrows), and osteoblast numbers (N.Ob/B.Pm) are increased in propranolol-treated mice (B and G). Body weight (C) is not affected. Leptin ICV infusion decreases fat pad weight but not bone mass in pro-treated wt mice (D and E). Pro treatment prevents bone loss by increasing bone formation parameters in OVX mice (F and G). Asterisks indicate statistically significant differences between experimental and control groups ($p < 0.01$). Diamond indicates statistically significant differences between OVX and sham-operated mice ($p < 0.01$). Error bars represent SEM.

matched what is observed in *ob/ob* mice. This increase in bone mass was secondary to an increase of both the bone formation rate and the number of osteoblasts (Figure 7B). The change in bone mass induced by propranolol occurred while the body weight and fat pad weight of the animals remained normal (Figure 7C and data not shown). Propranolol did not cause hyperinsulinism or other hormonal modifications (Table 1 and data not shown). Next, we asked if there was a link between leptin antiosteogenic function and the effect of β blockade on bone mass. To that end we infused leptin ICV in propranolol-treated mice. Centrally delivered leptin led to the disappearance of fat pads in propranolol-treated mice but did not affect their bone mass (Figures 7D and 7E). This experiment is a second argument supporting the hypothesis that leptin antiosteogenic function requires sympathetic activity.

This effect of propranolol on bone formation observed in wt mice suggested that it might mitigate the osteoporosis observed following estrogen depletion. To test if it was the case, we performed ovariectomy in 6-week-old wt mice and treated them for 7 weeks with proprano-

lol or vehicle. Estrogen depletion by ovariectomy decreased bone mass in vehicle-treated mice. In contrast, propranolol-treated ovariectomized mice had a normal bone mass (Figure 7F). This preventative effect of propranolol treatment was due to a striking increase in the bone formation rate and in the osteoblast number that were both significantly higher than in control ovariectomized mice (Figure 7G). This latter result underscores the physiological and therapeutic importance of the sympathetic regulation of bone formation.

Discussion

We have identified neuronal networks required for leptin antiosteogenic function and demonstrated that the SNS is a negative regulator of bone formation. Remarkably, this inhibitory role of the SNS on bone formation could be modulated without affecting body weight. Besides providing a molecular basis for leptin antiosteogenic function, these findings establish a biochemical basis for the neuronal regulation of bone remodeling. That a β-adrenergic antagonist could overcome the deleterious effect of ovariectomy on bone without affecting body weight has major implications for treatment of osteoporosis.

Specificity of Leptin Antiosteogenic Function

Our analysis provides additional evidence in favor of a critical role played by the hypothalamus in the mediation of leptin antiosteogenic function. It also implies, with all the inherent limitations of chemical lesion studies, that GTG-sensitive neuronal networks are a target of leptin antiosteogenic action while MSG-sensitive neurons may be dispensable for this action. We wish to stress, however, that chemical lesioning studies have limitations and cannot identify precisely the location of subgroups of neurons in the hypothalamus required for leptin antiosteogenic function. These limitations are inherent to the technique and will only be overcome by further studies using a series of neuronal subpopulation-specific inactivations of the leptin receptor in mice. Likewise, chemical lesions do not rule out that other neuronal populations located elsewhere in the brain may be involved in leptin antiosteogenic function provided, however, that they express *ObRb*, the gene encoding the signal transducing form of the leptin receptor.

We also used genetically modified mouse strains to determine whether leptin antiosteogenic and anorexigenic functions were using identical neuronal relays. One neuropeptide implicated in leptin anorexigenic function is αMSH, which is produced by ARC neurons and that binds to the melanocortin receptors. This signaling pathway is disrupted in A^y/a and *Mc4-r*-deficient mice that do not display any bone mass abnormality. Moreover, A^y/a mice are not resistant to leptin antiosteogenic function, whereas treatment of *ob/ob* mice with a melanocortin receptor agonist decreased their body weight without affecting their bone mass. These results, indicating that melanocortin agonists are not major regulators of bone formation, are consistent with the absence of an overt effect of MSG-induced damage on leptin antiosteogenic function. CART is another anorexigenic neuropeptide whose expression in the hypothalamus is regulated by leptin (Kristensen et al., 1998). *Cart*-deficient mice develop, on a high fat diet, a mild obesity (Asnicar et al., 2001), yet they do not have HBM. The ability to differentially modulate the anorexigenic and the antiosteogenic effect of leptin as observed in MSG- and GTG-treated mice and in mutant mouse strains suggests that these two functions are executed at least in part by distinct neuronal networks. We remain aware, however, that a direct demonstration will have to await a genetic identification of leptin antiosteogenic neurons using cell-specific inactivation of the leptin receptor in the nervous system.

Sympathetic Activity and Regulation of Bone Formation

While we were unable to provide evidence showing that leptin acts locally on osteoblasts or that hyperinsulinism explains leptin antiosteogenic function, several lines of evidence establish that the SNS regulates bone formation in vivo. These include results of parabiosis experiments, analysis of *Dbh*-deficient mice, and treatment of *ob/ob*, wt, or wt ovariectomized mice with sympathetic agonists or antagonists. In our experiments, the effects of the SNS on bone mass are independent of changes in body or fat mass, a dissociation that has major therapeutic implications. Also of considerable importance is that the failure of leptin ICV infusion to decrease bone mass in *Dbh*-deficient and in propranolol-treated mice establishes a direct link between leptin antiosteogenic function and sympathetic activity.

Other observations in mice and rats support the existence of a sympathetic regulation of bone formation. For instance, Dopamine transporter-deficient mice, in which rapid uptake of dopamine into presynaptic terminals does not occur, are osteopenic (Bliziotes et al., 2000). This latter observation suggests that the increased level of circulating dopamine observed in *Dbh*-deficient mice (Alaniz et al., 1999) may have limited rather than created or amplified their bone phenotype. Likewise, the anabolic effect of β blockers on bone formation could account for their beneficial effects in bone fracture in rats (Minkowitz et al., 1991). Although cross-circulation experiments in parabiosed mice do not exclude the possibility of a short-lived humoral mediator of leptin antiosteogenic function, the result of the adrenalectomy experiment would argue against such a mechanism. Furthermore, the low bone mass of panhypopituitarism patients does not support this notion (Kaufman et al., 1992).

Leptin as a Master Hormone

The pleiotropic functions of leptin that include control of body weight, reproduction, and bone formation are reminiscent of the many functions of hormones such as cortisol, estrogen, thyroid hormone, and insulin. By analogy with the notion that master genes orchestrate cell differentiation programs during development, we propose that leptin along with these other hormones defines a group of "master hormones" involved in the control of several important homeostatic functions. In particular, the existence of HBM despite an increase in bone resorption in the absence of leptin signaling indicates that control of bone formation is one of the

primary functions of leptin. Master hormones may make use of different modes of action to achieve their various functions. Analysis of glucocorticoid receptor mutants has revealed that cortisol, the hormone binding to this receptor, uses different pathways to achieve different functions (Reichardt et al., 1998; Karst et al., 2000). The evidence presented here distinguishing its anorexigenic and antiosteogenic mode of action suggests that the same may be true in the case of leptin. The ability of master hormones to use different pathways to fulfill multiple functions in vertebrates may have been one way to integrate the increasing complexity of large animal homeostasis during evolution.

Biomedical Implications

Does this regulatory pathway operate in humans as well as in mice? To date only correlative lines of evidence can be gathered. One such line of evidence indicates that a sympathetic regulation of bone mass does exist in human and plays an important role. Reflex sympathetic dystrophy is a human disease characterized by manifestations of hyperadrenergic activity including osteoporosis. Remarkably, treatment of patients affected by this disease with β blockers corrects most manifestations including the osteoporosis (Schwartzman, 2000). The existence of an osteoporosis in a human disorder characterized by excessive sympathetic activity and the disappearance of the osteoporosis with β blockers treatment is entirely consistent with our finding in mice and suggests that additional studies should be performed in other forms of osteoporosis. Indeed, although many drugs can effectively stop bone destruction in this disease, there is a need for drugs that enhance bone formation. The observation that propranolol, a widely used drug with no major deleterious effects, can significantly increase bone formation and bone mass without affecting body weight provides novel opportunities to design efficient bone-forming drugs.

Experimental Procedures

Animals, Treatments, and Surgical Procedures

Wild-type (C57BL/6J) and mutant (C57BL/6J A^y/a, C57BL/6J *ob*/*ob*) mice were obtained from the Jackson laboratory and adrenal medullectomized mice from Harlan Tecklad laboratory. $Dbh^{-/-}$ mice were rescued as previously described (Thomas et al., 1998). Isoproterenol (Sigma) was injected intraperitoneally (ip) once daily for 6 weeks at 30 mg/kg (wt) or 3 mg/kg (*ob*/*ob*). Propranolol (Sigma) was added to the drinking water at a concentration of 0.5 g/l. For ICV infusion, a 28-gauge cannula (Brain infusion kit II, Alza) was implanted into the third ventricle as previously described infusing human leptin (Sigma) at 8 ng/hr or MT-II at 125 ng/hr (Phoenix Pharmaceuticals) for 28 days (Ducy et al., 2000). The cannula was connected to an osmotic pump (Alza) placed in the dorsal subcutaneous space of the animal. For parabiosis, longitudinal incisions were made in the skin and in the peritoneal cavity along one side on each mouse. Edges of the incisions were connected by suture to induce coelioanastomosis. Crosscirculation was quantified by injecting 1 ml/kg 0.25% Evans blue into the tail vein of one mouse of the parabiosed pair. After 30 min blood was collected from both mice by retroorbital bleeding and the blood exchange rate was calculated according to standard techniques (Harris and Martin, 1984). All parabiosed pairs had an hourly exchange rate above 2%. Two weeks after parabiosis, a pump delivering leptin ICV in one animal of each pair was implanted. For GTG lesioning, 4-week-old C57BL/6J mice were given a single ip injection of either PBS or GTG (0.5 mg/g). For MSG lesioning, 2-day-old C57BL/6J pups were injected daily subcutaneously with either PBS or MSG (2 mg/g) for 10 days.

Generation of Transgenic Mice and Molecular Studies

The *α1(I) leptin* transgene was generated by cloning the mouse leptin cDNA downstream of the 2.3 kb osteoblast-specific fragment of the *α1(I)collagen* promoter. Transgenic founders were generated by standard techniques (Ausubel, 1995). Genotypes were determined by PCR. Progenies of two lines expressing the transgene were analyzed. Northern blot analyses were performed using total RNA or poly(A)$^+$ RNA according to standard protocols. RT-PCR analysis of adrenergic receptor expression was performed on random-primed cDNA for 27 cycles. All primer sequences are available upon request.

Histological Procedures, Immunocytochemistry, and Proliferation

Specimens were embedded in paraffin and sectioned at 6 μm. Brains were stained with 0.1% cresyl violet using standard procedures. Immunohistochemistry was performed according to standard protocols (Ausubel, 1995). In vivo osteoblast proliferation assays were performed on newborn mice treated daily for 5 days with 40 μg isoproterenol, 4 μg dexamethasone, or vehicle. BrdU (0.4 mg) was injected ip at day 5, and mice were sacrificed 2 hr later. BrdU incorporation was detected by immunohistochemistry using Zymed BrdU staining kit (Zymed Laboratories, Inc.). Four pups per treatment were analyzed and 10 calvariae sections were counted per animal. NovaRED was used as a chromogenic peroxidase substrate. Sections were counterstained with hematoxylin.

Histological analyses were performed on undecalcified sections stained by von Kossa and counterstained by von Gieson (Ducy et al., 2000). Static and dynamic histomorphometric analyses were performed according to standard protocols (Parfitt et al., 1987) using the Osteomeasure Analysis System (Osteometrics, Atlanta). Six to twelve animals were analyzed for each group. Statistical significance was assessed by Student's t test.

Cell Cultures and Bioassays

Primary osteoblast cultures were established as previously described (Ducy et al., 2000) and maintained in αMEM/0.1 mg/ml ascorbic acid supplemented with 10% FBS. SaOS-2 were grown in MEM/10% FBS. For cAMP assay, confluent cultures were incubated with serum-free medium containing 100 μM IBMX (3-isobutyl-1-methylxanthine, Sigma) for 8 min before PBS, PTH(1-34) (Bachem), isoproterenol, norepinephrine, phenylephrine (Sigma), or propranolol were added for 5 min. Intracellular cAMP concentration was measured by immunoassay (R&D Systems). For gene expression analyses, primary osteoblasts were treated for 72 hr with appropriate drugs in 0.1% FBS. Hormone serum levels were quantified using immunoassay kits from Peninsula Laboratories (Insulin) or Alpco Diagnostics (leptin). Deoxypyridinoline crosslinks and Creatinine were measured in morning urines using the Quidel kits. α1(I) leptin bioactivity was verified by cotransfection of 293 cells expressing *ObRb* with a STAT3-responsive-luc reporter construct, pSVβgal plasmid, and α1(I) leptin expression vector or mock. Twenty-four hours later, luciferase and β-galactosidase activities were measured. Data represents ratios of luciferase/β-galactosidase activities, and values are mean of six independent transfection experiments.

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