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Original Article

Hylonome

CART deficiency increases body weight but does not alter bone strength

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Abstract

The regulation of bone metabolism mediated by leptin is a complex process that is not clearly understood. Recent studies suggest that CART (cocaine-amphetamine related transcript) is a significant neuronal co-factor when combined with leptin. CART deficiency is thought to result in low trabecular bone mass, but since leptin exerts contrasting effects on trabecular and cortical bone it is possible that cortical bone may not respond to the absence of CART signaling in the same manner as trabecular bone. We tested the hypothesis that CART deficiency decreases cortical bone mass, density, and strength by examining femora of adult wild-type mice ($CART^{+/+}$) and CART-deficient mice ($CART^{-/-}$). DEXA densitometry (PIXImus system) was used to measure whole-bone mineral content (BMC) and mineral density (BMD) from right femora, and pQCT used to calculate densitometric and geometric parameters from the femur midshaft. Femora were also tested in three-point bending, and sections of the tibia analyzed histologically to determine bone marrow adipocyte density (N.At./M.Ar) and endocortical osteoclast number (N.Oc/B.Pm). The control mice weighed less than the mice lacking CART ($P<0.001$), but mechanical testing data showed no differences ($p>0.05$) in ultimate force, energy to fracture, stiffness, or intrinsic properties such as ultimate stress, ultimate strain, or modulus. CART-deficient mice did not differ from normal controls in whole-femur BMC ($p=0.09$), BMD ($p=0.19$), midshaft cortical bone thickness ($p=0.67$), midshaft cortical bone area ($p=0.59$) or N.Oc/B.Pm ($p=0.94$), although CART deficiency was associated with a three-fold increase in bone marrow adipocyte density ($p<0.001$). Our data suggest that while the central, neuroendocrine regulation of bone mass via CART signaling may have effects on trabecular mass, absence of CART expression does not significantly alter cortical bone geometry, density, or strength.

Keywords: Leptin, Adipocyte, Bone Mass, Cortical Bone, Body Weight

Introduction

The regulation of bone metabolism mediated by leptin is a complex process that is not clearly understood. The findings of increased trabecular bone formation and high bone mass within the proximal tibia and lumbar vertebrae of ob/ob mice[1], with similar observations in the db/db mouse, led to the conclusion that leptin signals through its known receptor to control bone mass. Intracerebroventricular (ICV) infusion of leptin into the third ventricle resulted in bone loss in both ob/ob and wild-type mice, suggesting that leptin acts via the central nervous system to inhibit bone formation[1]. The absence of detectable leptin in the serum of ICV-administered ob/ob mice also supports a central mode of leptin action. A parabiosis model, in which only one ob/ob mouse of a parabiosed pair received ICV leptin, was used to confirm the key role hypothalamic leptin plays in regulating bone formation[2]. Correction of the ob/ob skeletal phenotype by loss of bone mass was only achieved in the leptin recipient and not in the contralateral mouse[2], indicating that similar to its control of energy homeostasis by central mediation, the action of leptin on bone can be regulated through a similar hypothalamic relay.

There are, however, contradictory findings when assessing leptin treatment on bone mass in rodents. Some studies have

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described ob/ob mice (leptin-deficient) that were administered leptin as having a "high bone mass"[1,3,4], while Steppan et al.[5] observed that leptin-deficient mice had lower bone mass than normal mice. One explanation of the discrepancies of results reported may be due to mode of leptin administration. Peripheral (SQ) administration of leptin could increase bone growth and indices of bone formation[5-7] whereas central (ICV) infusion of leptin might lead to bone loss[1], revealing that leptin can regulate bone mass through alternative pathways: one involving a direct stimulatory effect on bone growth when administered peripherally and another that is indirect, involving a hypothalamic relay that suppresses bone formation when administered centrally[8]. However, in the recent study by Iwaniec et al.[9] in which ob/ob mice were injected in the hypothalamus with a recombinant adenovirus expressing leptin, femoral bone mass and length increased while cancellous bone volume in both the femur and spine declined. Together these studies indicate that, regardless of whether leptin is administered centrally or peripherally, leptin decreases cancellous bone volume while at the same time increasing cortical bone mass.

Because of the complexity of understanding leptin's role in the regulation of bone, several recent studies have been conducted with neuronal co-factors such as beta-adrenergic receptors (β-AR), neuropeptide Y (NPY), and CART that are regulated by leptin expression in the hypothalamus. Sympathetic tone is reduced in leptin-deficient mice[10], and studies involving β-AR agonists and antagonists demonstrate that the central actions of leptin are mediated via the sympathetic nervous system (SNS). Resembling the sympathetic hypotonic ob/ob mouse are mice lacking both β1 and β2-adrenergic receptors, which demonstrate increased trabecular number but decreased cortical bone[11]. Administration of β-AR antagonists increased bone mass in vertebrae and long bones of ob/ob and wild-type mice[2]. Conversely, administration of β-AR agonists restored sympathetic activity in ob/ob mice, and decreased bone mass in both the ob/ob and wild-type mice without affecting body weight[2,12], demonstrating that the modulation of SNS activity can affect bone remodeling. It should, however, be noted here that neonatal sympathectomy has no demonstrable effects on cortical bone[13]. Another neuronal co-factor that interacts with leptin is NPY, a target of leptin signaling within the hypothalamus that regulates energy balance, modulating feeding behavior, and bone metabolism through at least five receptors (Y1, Y2, Y4, Y5, and Y6). The finding that trabecular and cortical bone formation and volume are increased in the absence of NPY signaling in mice[14-18] strongly supports a role for NPY receptor signaling in the regulation of bone formation. Thus, Y2 receptor signaling along with leptin regulates bone formation via a hypothalamic relay in both trabecular and cortical bone.

Through recent studies, CART (cocaine-amphetamine related transcript) has been revealed to be an important neuropeptide regulated by leptin. CART is expressed at high levels in the mouse hypothalamus[19], and CART expression is upregulated with injection of psychostimulants, suggesting that CART may antagonize the effects of these drugs[20]. CART has so far been detected in the posterior pituitary, the adrenal medulla, and endocrine cells in the gut[19]. CART peptide also decreases food intake after intracerebroventricular injection in rats, implicating CART as a potential regulator of feeding, satiety, and body weight[21]. CART expression in the hypothalamus is also upregulated by leptin[22]. Recently, it was found that CART-deficient mice have low bone mass in their spine, suggesting that the effects of leptin on bone mass in the axial skeleton may be mediated at least in part by CART signaling, acting through a central, neuroendocrine signaling pathway[12]. It has been proposed that while leptin may increase bone resorption by stimulating RANK-ligand (RANKL) expression in osteoblasts, leptin can also inhibit osteoclast activity by increasing CART expression, which decreases RANKL expression by osteoblasts[12].

The effects of CART deficiency on bone mass have so far only been examined in trabecular bone of the vertebrae. As noted above, CART deficiency is thought to result in low trabecular bone mass, but since leptin exerts different effects on cortical and trabecular bone it is possible that cortical bone may not respond to the absence of CART signaling in the same manner as trabecular bone. Periosteum is richly innervated and is supplied by a dense network of sensory and sympathetic fibers[23], but it is unknown how altering CART affects periosteal and endosteal apposition during growth and aging. This is an important consideration, because periosteal expansion and cortical bone cross-sectional geometry are key determinants of hip fracture risk[24]. We tested the hypothesis that CART deficiency decreases cortical bone mass, density, and strength by examining femora of adult CART-deficient mice. It was predicted that bone mineral content, density, femoral fracture strength, and toughness would be lower in CART-deficient mice than normal mice, due to an increase in bone resorption. The long-term objective of this study is to better understand the role of central, neuroendocrine signaling pathways in regulating bone metabolism and bone strength.

Methods

Sample

Male mice, 6 months of age were used for this study. The sample size includes 18 CART-deficient ($CART^{-/-}$) mice on a C57BL6 background and 18 wild-type ($CART^{+/+}$) C57BL6 mice[25]. Mice were obtained from the $CART^{-/-}$ breeding colony at Eli Lilly and shipped live to MJK at the Yerkes Primate Facility. Mice were euthanized by CO_2, weighed, and the left hindlimb dissected free and stored at -20C for mechanical testing. The right hindlimb was fixed for 24 hours in neutral buffered formalin and then stored in 70% ETOH prior to densitometry.

Densitometry

Dual-energy X-ray absorptiometry (DEXA) densitometry (PIXImus system) was used to measure whole-bone mineral content (BMC) and mineral density (BMD) from right femora. pQCT densitometry, a reliable approach for measuring trabecular and cortical bone area, mineral content, mineral density, and cross-sectional geometry in small rodent bones[26-28], was then used to calculate BMC, BMD, cortical area, cortical thickness, periosteal circumference, and endosteal circumference from a single cross-section, 1 mm thick, at the femur midshaft. Sections were scanned at 4 mm/sec with a voxel size of .070 mm and a threshold value of 524.0 mg/cm^3 using a Norland Stratec XCT-Research peripheral quantitative computed tomography (pQCT) machine housed in the Department of Biochemistry and Molecular Pathology at the Northeast Ohio Universities College of Medicine (NEOUCOM).

Mechanical testing

Left femora were dissected free, thawed in cold water at room temperature for 1 hour, and then prepared for mechanical testing in 3-point anteroposterior bending using a Vitrodyne V1000 Materials Testing system (Liveco Inc., Burlington, VT, USA). Femora were mounted on stainless steel fixtures spaced 5 mm apart and 2.5 mm either side of center. Testing was linear displacement control with a displacement rate of 0.10 mm/sec using a Transducer Techniques 5 kg load cell. Femora were loaded to failure with data points recorded every .01 sec. Structural, or extrinsic properties, such as ultimate force (Fu; height of curve), stiffness (S; slope of curve), ultimate displacement (du; width of curve), and energy to fracture (U; area under curve) were calculated from load-displacement curves[29,30]. The cross-sectional moment of inertia (CSMI) was calculated for each specimen using the external diameter and cortical thickness, measured from histological sections (described below). $CSMI=\pi*(2R-t)^3*t/8$, where 2R equals the external diameter and t equals the cortical thickness. Ultimate stress (σ_u) was calculated as $F_u\ Lb/8I$, ultimate strain (ε_u) as $d_u*(6b/L^2)$, and Young's modulus (E) as $S*(L^3/48I)$ where L equals length of the test specimen (5 mm), b equals anteroposterior diameter of the specimen, and I is the cross-sectional moment of inertia (CSMI).

Histology and histomorphometry

The right tibia and L4 vertebra were decalcified in 4% EDTA for approximately 1 week and then embedded in paraffin following standard procedures. Transverse cross-sections were cut at 5 μm and stained with hematoxylin and eosin (H&E) to yield tibal sections used for measuring total section area (T.Ar), marrow area (M.Ar), cortical area (Ct.Ar), cortical thickness (Ct.Wi), and adipocyte density (N.At./M.Ar). Adipocytes were counted over a 0.50 mm^2 area. Trabecular volume and total bone volume were measured from H&E stained sections of the vertebrae to determine relative bone volume fraction (BV/TV). Sections from the tibia and L4 vertebra were also stained for tartrate-resistant acid phosphatase (TRAP) as a marker of active osteoclasts. Osteoclasts were counted on the tibial endosteal surface and expressed as a number of osteoclasts per bone perimeter (N.Oc/B.Pm).

Statistical analysis

Single-factor ANOVA with genotype as the factor was used to test for between-group differences in the densitometric and biomechanical variables examined, and ANCOVA was used to adjust variables for differences in body weight employing body mass as a covariate. Significance is established at $p<0.05$.

Results

The wild-type mice weighed significantly less than the CART$^{-/-}$ mice ($p<0.001$), but CART$^{-/-}$ mice did not differ from wild-type mice in whole-femur BMC ($p=0.09$), BMD ($p=0.19$), midshaft cortical bone thickness ($p=0.67$), or midshaft cortical bone area ($p=0.59$; Table 1, Figure 1a). ANCOVA results indicate that femur BMC is lower in CART$^{-/-}$ than in wild-type mice when the data are adjusted for body weight (Table 1), however no other parameters differ between groups in ANCOVA analyses (Tables 1, 2). Mechanical testing data (Table 2) showed no differences ($p>0.05$) in ultimate force, energy to fracture, stiffness, or intrinsic properties such as ultimate stress, ultimate strain, or modulus. Histological sections stained for osteoclast activity showed few osteoclasts along the endosteal surface of the proximal tibia in wild-type mice and CART$^{-/-}$ mice (Figure 1c), and no significant differences in osteoclasts per bone perimeter (N.Oc/B.Pm) between the groups ($p=0.94$; Figure 2a). H&E staining for adipocyte number show more adipocytes in the bone marrow of the CART$^{-/-}$ mice (Figure 1b) and greater bone marrow adipocyte density (N.At/M.Ar) than the wild-type mice ($p<0.001$; Figure 2b). Data from the L4 vertebrae demonstrate that bone volume fraction and osteoclasts per bone perimeter are similar between the two groups of mice (Figure 3).

Discussion

Evidence from the leptin-deficient ob/ob mouse, and from mice lacking beta-adrenergic receptors, suggest that cortical and trabecular bone are regulated through different leptin-mediated pathways[31]. Leptin suppresses NPY and stimulates beta-adrenergic receptors, both of which increase cortical bone formation, whereas activation of the beta-adrenergic pathway appears to inhibit trabecular bone formation and may increase trabecular resorption. Likewise, Elefteriou et

Parameter	CART$^{+/+}$ (n=18)		CART$^{-/-}$ (n=18)		P-value (ANOVA)	P-value (ANCOVA)*
	MEAN	SD	MEAN	SD		
Body mass (g)	31.3	3.0	35.5	2.6	$p<.001$	- - -
Femur BMC (g)	0.023	0.004	0.021	0.001	ns	$p<.05$
Femur BMD (g/cm^2)	0.051	0.005	0.050	0.002	ns	ns
Midshaft BMC (mg/mm)	0.73	0.16	0.72	0.14	ns	ns
Midshaft BMD (mg/cm^3)	515.9	57.5	519.6	50.3	ns	ns
Cortical area (mm^2)	0.88	0.10	0.86	0.04	ns	ns
Cortical thickness (mm)	0.19	0.05	0.19	0.02	ns	ns
Periosteal circumference (mm)	4.4	0.40	4.3	0.40	ns	ns
Endosteal circumference (mm)	3.0	0.80	3.1	0.21	ns	ns

*ANCOVA results refer to analyses using body mass as a covariate.

Table 1. Body mass and densitometric properties of the femur from wild-type (CART$^{+/+}$) and CART-deficient (CART$^{-/-}$) mice.

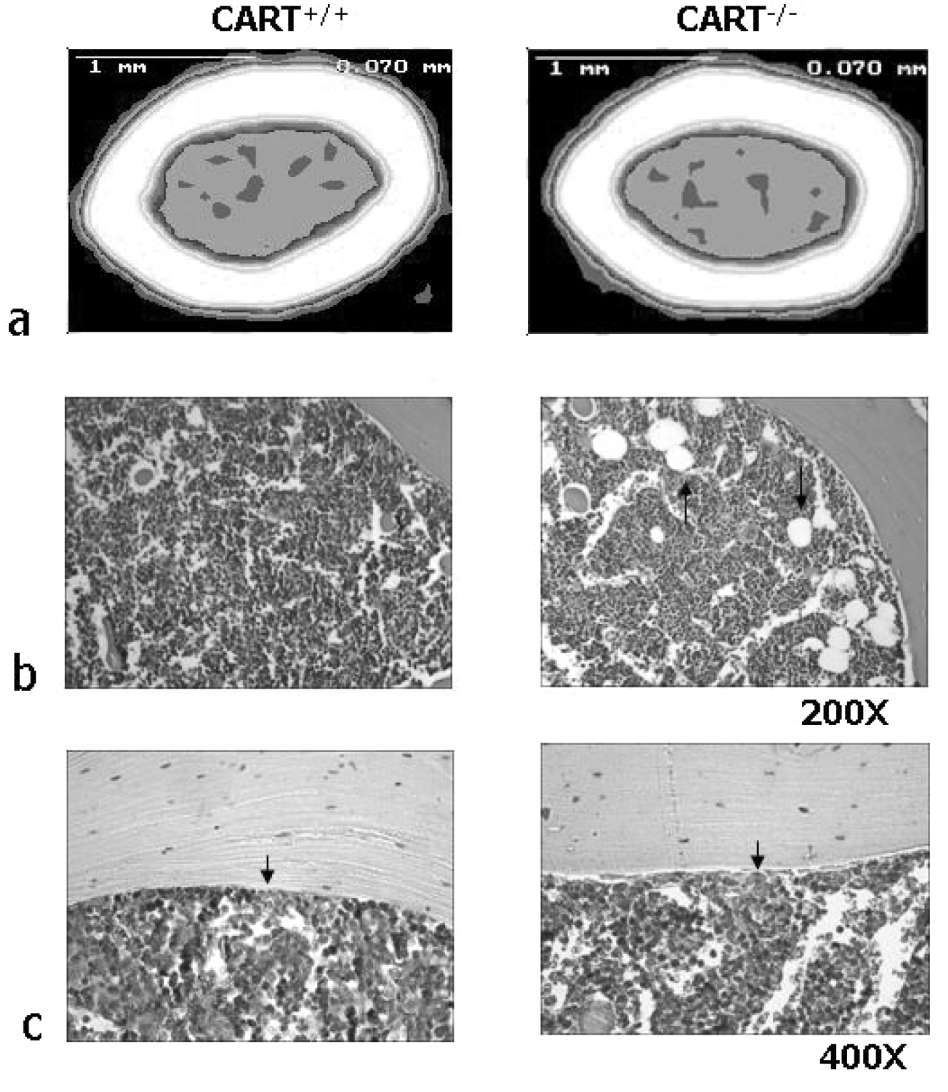

Figure 1. pQCT cross-sections of the femur (**a**), H&E stained tibial cross-sections (**b**), and TRAP stained tibial sections (**c**) from CART$^{-/-}$ (left column) and CART$^{+/+}$ (right column) mice. Note that the two strains of mice do not differ in cortical bone structure (top), bone marrow adipocyte number (middle), and osteoclast number (bottom).

Parameter	$CART^{+/+}$ (n=18)		$CART^{-/-}$ (n=18)		*p*-value (ANOVA)	*p*-value (ANCOVA)*
	MEAN	SD	MEAN	SD		
Fu (N)	21.7	2.8	21.3	1.6	ns	ns
Du (mm)	0.61	0.21	0.51	0.16	ns	ns
S (N/mm)	65.5	12.9	67.9	13.1	ns	ns
U (N-mm)	8.1	2.3	7.1	1.9	ns	ns
CSMI (mm^4)	0.083	0.031	0.080	0.01	ns	ns
Ultimate stress (σ_u; N/mm^2)	198.4	30.9	204.1	29.9	ns	ns
Ultimate strain (ε_u)	0.17	0.06	0.15	0.05	ns	ns
Young's modulus (*E*; N/mm^2)	14.2	6.2	14.1	3.4	ns	ns

*ANCOVA results refer to analyses using body mass as a covariate.

Table 2. Mechanical testing data of the femur from wild-type ($CART^{+/+}$) and CART-deficient ($CART^{-/-}$) mice.

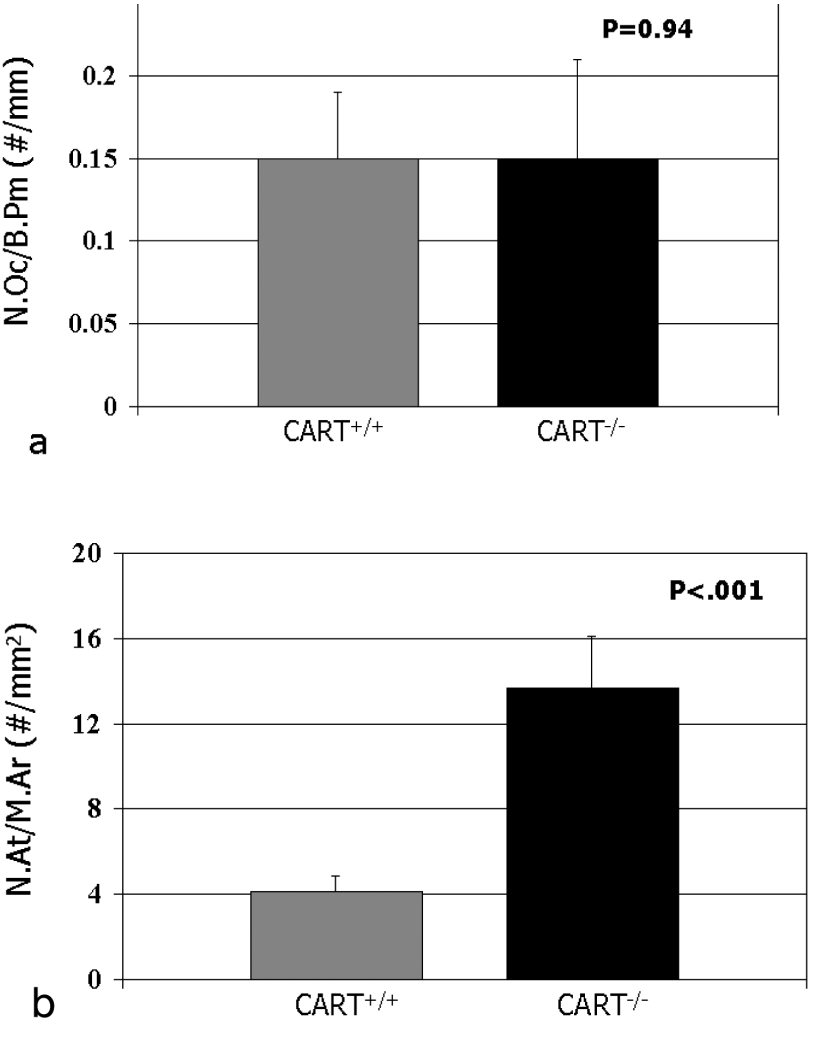

Figure 2. Histograms showing tibial osteoclast per surface (**a**) and bone marrow adipocyte number (**b**).

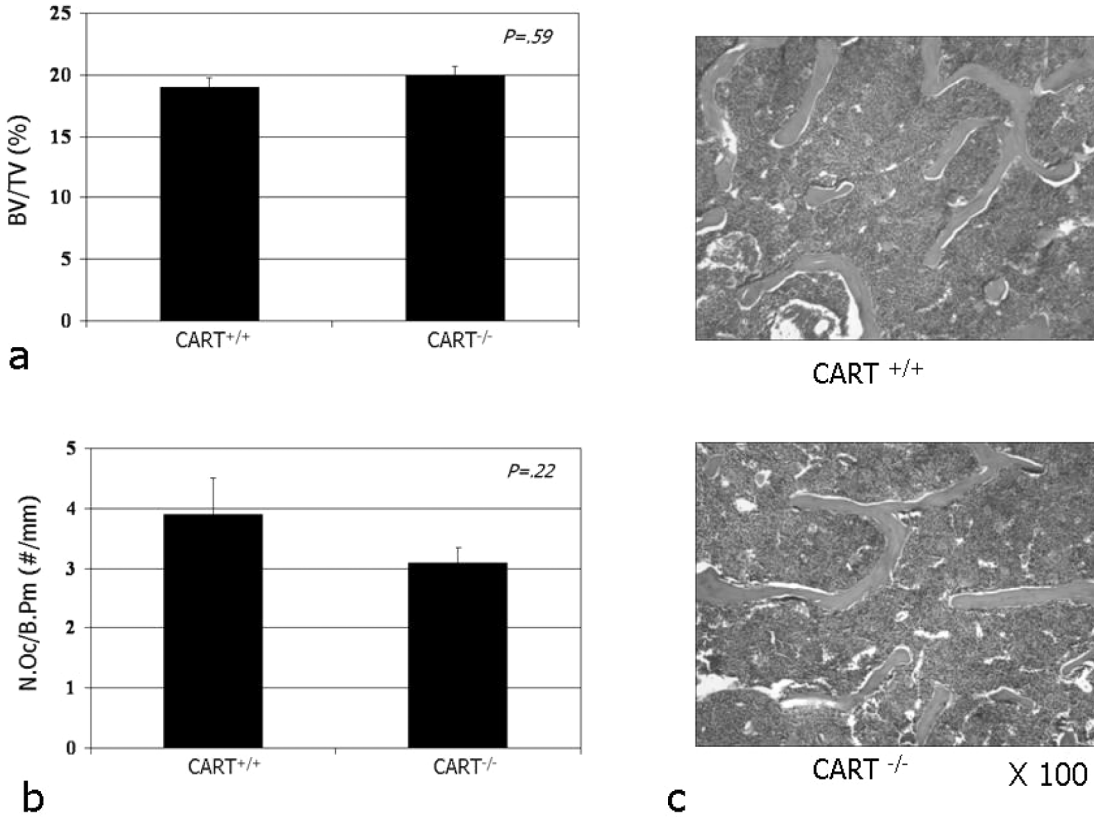

Figure 3. (**a**) Trabecular bone volume fraction (BV/TV), (**b**) osteoclasts per trabecular bone perimeter, and (**c**) sections of the vertebral body from L4 vertebrae of wild-type ($CART^{+/+}$) and CART-deficient ($CART^{-/-}$) mice.

al.[12] found that the absence of CART leads to a decrease in trabecular bone volume because of an isolated increase in osteoclast activity following RANKL overexpression by osteoblasts. Our findings indicate that the deletion of CART yields no detectable increase in the number of endocortical osteoclasts or vertebral osteoclasts, and no decrease in cortical thickness, cortical area, or trabecular bone volume fraction. Thus, leptin signaling appears to be anabolic for cortical bone, and hence the body fat-bone axis links cortical bone with weight support[31]. It is unclear why our results for trabecular bone volume fraction and osteoclast number differ from those of Elefteriou et al.[12], since the mice were of the same age, although the animals in our study were male and the mice in their study were female. It is also possible that feeding behavior and body composition (and hence bone metabolism) may differ between knockout mice from different colonies. For example, food intake and fat pad weights were slightly lower in $CART^{-/-}$ mice compared to wild-type mice in the study by Elefteriou et al.[12], whereas fad pad weights were similar and food intake increased in the $CART^{-/-}$ studied by Zhang et al.[32].

Leptin is a factor that plays a major role in regulating appetite, energy expenditure, and body weight, and leptin treatment can induce apoptosis of fat cells (adipocytes) in peripheral fat pads as well as in bone marrow[33-35]. Leptin can also act directly on bone marrow cells to enhance their differentiation to osteoblasts and inhibit their differentiation to adipocytes[36]. Age plays a role in adipocyte accumulation, both in bone marrow and in peripheral fat depots. Marrow tissue in the limb bones is known to accumulate adipocytes with age, and this may contribute directly to bone loss and increased risk of fracture[37,38]. The increase in bone marrow adipocyte density observed among the CART-deficient mice in this study suggests that CART may mediate leptin's effect on adipogenesis in bone and lipid mobilization. This is further indicated by the fact that central (icv) infusions of CART peptide reduce fat pad weights in rodents[19]; however, it should be noted that the increase in marrow adipogenesis observed among the CART knockout mice is much less than that observed among leptin-deficient ob/ob mice. For example, we found that the adipocyte density in the $CART^{-/-}$ mice was approximately 14 adipocytes/mm sq, whereas ob/ob mice were shown to have an adipocyte density of approximately 47 adipocytes/mm sq[32].

Although CART may play a role in mediating leptin's effects on bone marrow adipogenesis and adipocyte apoptosis, it does not appear to be involved in linking body weight with bone mass, especially cortical bone thickness and area.

CART-deficient mice weigh significantly more than wild-type mice, but show no differences in femur BMD, periosteal circumference, area moment of inertia, or strength and toughness with wild-type mice. Our data therefore suggest that while the central, neuroendocrine regulation of bone mass via CART signaling may have effects on trabecular mass, absence of CART expression does not significantly alter cortical bone geometry, density, or strength. While central leptin signaling and hypothalamic CART expression are known to regulate body weight, food intake, and satiety, it is likely that the beta-adrenergic and NPY pathways play a greater role in mediating the fat-bone axis downstream of leptin.

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