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Palmitic Acid and DGAT1 Deficiency Enhance Osteoclastogenesis, while Oleic Acid-Induced Triglyceride Formation Prevents It

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Abstract

Both obesity and diabetes mellitus are associated with alterations in lipid metabolism as well as a change in bone homeostasis and osteoclastogenesis. We hypothesized that increased fatty acid levels affect bone health by altering precursor cell differentiation and osteoclast activation. Here we show that palmitic acid (PA, 16:0) enhances receptor activator of NF- κ B ligand (RANKL)-stimulated osteoclastogenesis and is sufficient to induce osteoclast differentiation even in the absence of RANKL. TNF α expression is crucial for PA-induced osteoclastogenesis, as shown by increased TNF α mRNA levels in PA-treated cells and abrogation of PA-stimulated osteoclastogenesis by TNF α neutralizing antibodies. In contrast, oleic acid (OA, 18:1) does not enhance osteoclast differentiation, leads to increased intracellular triglyceride accumulation, and inhibits PA-induced osteoclastogenesis. Adenovirus-mediated expression of diacylglycerol acyl transferase 1 (DGAT1), a gene involved in triglyceride synthesis, also inhibits PA-induced osteoclastogenesis, suggesting a protective role of DGAT1 for bone health. Accordingly, Dgat1 knockout mice have larger bone marrow-derived osteoclasts and decreased bone mass indices. In line with these findings, mice on a high-fat PA-enriched diet have a greater reduction in bone mass and structure than mice on a high-fat OA-enriched diet. Thus, we propose that TNF α mediates saturated fatty acid-induced osteoclastogenesis that can be prevented by DGAT activation or supplementation with OA.

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Additional Supporting Information may be found in the online version of this article.

Disclosures

All authors state that they have no conflicts of interest.

Keywords

OSTEOCLASTOGENESIS; PALMITIC ACID; TNFA; DGAT1; TRIGLYCERIDES

Introduction

Bone undergoes constant remodeling, balancing osteoclast-mediated bone resorption, and osteoblast-regulated bone formation. Under normal conditions, these processes secure the stability of bone mass. Systemic or local abnormalities, such as hormonal deregulation⁽¹⁾ or inflammation,⁽²⁾ compromise bone homeostasis and may enhance bone resorption by osteoclasts, resulting in reduced bone mass.

Osteoclasts are multinucleated cells that derive from hematopoietic monocyte/macrophage precursor cells.⁽²⁾ Osteoclastogenesis mostly relies on activation of precursor cells by the receptor activator of NF- κ B ligand (RANKL). RANKL is produced by osteoblasts, osteocytes,^(3,4) stromal cells, T and B lymphocytes, and endothelial cells⁽⁵⁾ and binds to the specific receptor activator of NF- κ B (RANK). Differentiation of macrophages into osteoclasts is accompanied by fusion with adjacent macrophages, resulting in formation of big multinucleated cells. Osteoclastogenesis can be assessed by size and multinuclearity, as well as by gene expression levels of tartrate-resistant acid phosphatase (TRAP). Tumor necrosis factor α (TNF α) can also induce osteoclastogenesis through a RANKL/RANK-independent mechanism.^(6–10) Induction of pro-inflammatory cytokines, such as TNF α and IL-6, has been implicated in bone loss that occurs in chronic inflammatory diseases such as rheumatoid arthritis and periodontitis,^(11,12) as well as in diabetes.^(12,13)

Systemic metabolic derangements in obesity, diabetes mellitus, and the metabolic syndrome are linked to abnormal bone homeostasis. Although obesity itself has traditionally been associated with increased bone mass,⁽¹⁴⁾ mainly attributed to enhanced bone formation in response to weight-bearing mechanical stimulation,⁽¹⁵⁾ increased fat mass and hyperlipidemia have been shown to decrease bone mineral density and increase fracture risk in young^(16–18) and adult individuals.^(19–25) Previous studies on the impact of hyperlipidemia and increased circulating fatty acid (FA) levels on osteoclastogenesis have been inconclusive.^(26,27) We hypothesize that saturation of FA is an important variable promoting osteoclastogenesis and osteolysis. Our study aimed to investigate this hypothesis and identify mechanisms that mediate these actions.

Materials and Methods

RAW 264.7 cell cultures

RAW 264.7 cells were plated into 6-well plates at a density of 150,000 cells/well in 10% fetal bovine serum (FBS) (ATCC, Manassas, VA, USA) plus 1% penicillin/streptomycin (ATCC), DMEM (4500 mg/L glucose) (ATCC) supplemented with 20 ng/mL RANKL (PeproTech, Rocky Hill, NJ, USA). In the fatty acid treatments, 1% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) was added to the medium along with 0.2 mM palmitic acid (PA) or 0.2 mM oleic acid (OA) or 0.2 mM PA + 0.2 mM OA diluted in

methanol. Control cells were treated with equivalent amount of methanol and BSA. Anti-TNF α antibody (10 μ g/mL, Millipore, Billerica, MA, USA) was added to the culture medium. Cells were cultured for 5 days, and the medium was replenished on day 2 and day 4.

Animals

All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Columbia University. Mice were maintained under appropriate barrier conditions in a 12-hour light-dark cycle and received food and water *ad libitum*. Mice were anesthetized by isoflurane inhalation.

Bone marrow cell cultures

Bone marrow cells (BMC) were extracted from mouse femurs that were aseptically removed, and adherent soft tissue was dissected away. Bone ends were cut off with razor blades, and the marrow cavity was flushed with α -MEM. The BMC were collected and plated in 6-well plates at a density of 4×10^6 cells/well in 10% FBS plus 1% penicillin/streptomycin α -MEM (ATCC) supplemented with 50 ng/mL macrophage colony-stimulating factor (MCSF) (PeproTech) and 20 ng/mL RANKL. Cells were cultured for 5 days, and the medium was replenished on day 2 and day 4. On day 5, cells were fixed and stained for TRAP.

Animals' fatty-acid (FA)-enriched diet to induce weight gain

Four-week old male C57BL/6 mice were randomly divided into groups ($n = 9$ /group) and put on either PA- or OA-enriched diet (Research Diets Inc., New Brunswick, NJ, USA) or normal chow diet for 3 months. The high caloric FA-enriched diets contained an increased amount of FA (20% of calories from fatty acids, whereas chow contains a total of 10%) to induce weight gain (Supplemental Table S1). Animal weight was monitored once a week.

Analysis of osteoclast morphological and area of osteoclast resorption

Osteoclast cell area and resorption area were measured using ImageJ software (NIH, Bethesda, MD, USA). All TRAP + cells with at least 3 nuclei were considered osteoclasts.

RNA purification and gene expression analysis

Total RNA purification, cDNA generation, and quantitative real-time PCR were performed as previously described.⁽²⁸⁾ Incorporation of SYBR Green dye into the PCR products was monitored with an iCycler BioRad (Hercules, CA, USA) sequence detection system. Samples were normalized against 18 S. The sequences of the primers can be provided upon request.

Fatty acid and glucose oxidation assays

FA and glucose oxidation were measured in osteoclasts derived from RAW 264.7 cells after 5 days of treatment with RANKL and undifferentiated (day 1 post-RANKL stimulation) RAW 264.7 cells. Cells were cultured as described above. On day 1 and day 5, cells were harvested in PBS, incubated at 37°C for 2 hours in modified Krebs-Ringer buffer (MKR)

(115 mm NaCl, 2.6 mm KCl, 1.2 mm KH₂PO₄, 10 mm NaHCO₃, 10 mm HEPES [pH 7.4]) that contained 2% BSA, 0.2 mmol/mL palmitate, and 10 µCi/mL [9,10-³H]palmitate (PerkinElmer, Waltham, MA, USA) for assessment of FA oxidation or MKR that contained 0.2 mmol/mL glucose and 10 µCi/ml D-[3-³H]glucose (PerkinElmer) for assessment of glucose oxidation and were gassed with 95% O₂ and 5% CO₂. Then, water was extracted with chloroform: methanol (2:1) extraction. Palmitate or glucose oxidation was determined by measuring the amount of ³H₂O in the aqueous phase.

Analysis of osteoclast resorptive activity

Cells were cultured in the presence of 0.2 mM PA or 0.2 mM OA or 0.2 mM PA + 0.2 mM OA on dentine discs (Immunodiagnostic Systems Inc., Scottsdale, AZ, USA) for 5 days. Resorption lacunae were scanned using electron microscopy.

Cellular neutral lipid staining

Intracellular neutral lipids were stained with Oil Red O, as described previously.⁽²⁹⁾

Construction of recombinant adenovirus expressing human DGAT1

The plasmid that contained the cDNA of human DGAT1 (pOTB7-hDgat1, accession number BC023565) was purchased by Open Biosystems, Inc. (Pittsburgh, PA, USA). The hDGAT1 cDNA was extracted by partial digestion with XhoI and EcoRI restriction enzymes and subcloned into the same restriction sites of the pcDNA3.1 vector. The hDGAT1 cDNA was subsequently excised from the pcDNA 3.1 vector with HindIII and XbaI digestion and cloned in corresponding sites of the pAdTrackCMV vector. The recombinant adenoviruses were constructed as previously described⁽³⁰⁾ using the Ad-Easy-1 system. Usually, titers of 0.5 to 2 × 10¹⁰ plaque-forming units (pfu)/mL were obtained.

Infection of cells with recombinant adenoviruses

RAW 264.7 cells were grown in DMEM containing 10% FBS and 1% penicillin/streptomycin for 2 days. Cells were infected with Ad-DGAT1 or control adenovirus-expressing GFP (Ad-GFP) at a 250 multiplicity of infection (MOI) in DMEM supplemented with 2% heat-inactivated horse serum and 1% penicillin/streptomycin. Eight hours post-infection, the medium was removed and fresh 10% FBS-containing medium was added.

ELISA

Serum levels of proteins were determined using ELISA, according to the manufacturers' recommendations ($n = 5$ to 7/group; R&D Systems [Minneapolis, MN, USA], Abcam [Cambridge, UK], MyBioSource [San Diego, CA, USA]).

Bone densitometry

BMD was measured using dual-energy X-ray absorptiometry (DXA) on a Lunar PIXImus (GE Lunar, Madison, WI, USA) X-ray densitometer for small animals using Lunar PIXIMUS2 version 2.10 software. The mice ($n = 7$ to 8) were scanned at 10 mm/s with a resolution of 0.5 mm × 0.5 mm. BMD was determined in a window that excluded the head area.

Micro-CT analysis

Scans were performed starting from the fused growth plate with a scan length of 3 mm back to the diaphysis direction in the distal and the midshaft of femurs ($n = 6$ to 8/group) with a scanning length of 1 mm using a micro-computed tomography system (vivaCT 40, Scanco Medical, Bassersdorf, Switzerland). The scans were performed using 21- μ m resolution at 55 KV exciting voltage and 200 ms integration time. The total scan time for each sample was 15 minutes for distal femur scan and 5 minutes for midshaft scan. With a manually defined volume of interest (VOI), the following three-dimensional structural parameters were analyzed with the analyses script built in micro-CT machine: bone volume fraction (BV/TV, %), trabecular number (Tb.N, 1/mm), trabecular thickness (Tb.Th, mm), trabecular separation (Tb.Sp, mm), bone surface to bone volume ratio (BS/BV, 1/mm), and bone mineral density (BMD, mg HA/ccm).

Histomorphometric analysis

Femurs ($n = 5$ /group) were isolated and fixed in 4% paraformal-dehyde at 4°C. Afterward, they were decalcified in 15% EDTA and dehydrated in progressive concentrations of ethanol, cleared in xylene, and embedded in paraffin. Five-micromolar sections were cut and stained for TRAP and counterstained with hematoxylin. Measurements were taken 150 μ m distal from the growth plate and 150 μ m away from cortical bone using Bioquant Osteo 2012 (Nashville, TN, USA). Measurements included percent trabecular bone volume (BV/TV), number of osteoclasts per bone surface (N.OC/BS), osteoblast surface (ObS), and percent osteoclast surface (OcS/BS).⁽³¹⁾

Statistical analysis

Comparisons between groups were performed using GraphPad (La Jolla, CA, USA) InStat 3 statistical software. Data from more than two groups were analyzed by one-way ANOVA. Comparisons between two groups were performed using unpaired two-tailed Student's *t* tests. All values are presented as means $\pm$ SE. Differences between groups were considered statistically significant at $p < 0.05$.

Results

Osteoclastogenesis is associated with increased fatty acid oxidation

To investigate the impact of FAs on osteoclast differentiation, we used macrophage-derived RAW 264.7 cells, which we differentiated into mature osteoclasts by stimulating them with RANKL, as described previously.^(2,32) Treatment of RAW 264.7 cells with RANKL for 5 days converted macrophages from small, round, mononuclear cells into large multinucleated osteoclasts that stain positive for TRAP (Fig. 1A). Palmitate oxidation was increased in osteoclasts (day 5 post-stimulation) by 45% compared with undifferentiated cells (day 1 post-stimulation) (Fig. 1B). Accordingly, expression of FA catabolism genes was increased in osteoclasts. Particularly, we found a fivefold increase in peroxisome proliferator-activated receptor γ coactivator (PGC) 1 β expression (Fig. 1C), which regulates mitochondrial functional capacity and cellular energy metabolism,^(33,34) whereas PGC1 α mRNA was not detected in this system. The mRNA levels of carnitine palmitoyl-transferase (CPT) 1 α , an

enzyme that mediates a rate limiting step in β -oxidation,^(35–37) was increased threefold in osteoclasts (Fig. 1C). Furthermore, we found a threefold increase in the expression of CD36 (Fig. 1C), which is a scavenger receptor that mediates the uptake of long-chain FAs (LCFA) and oxidized low-density lipoproteins.⁽³⁸⁾ Moreover, there was a 3.9-fold increase in glucose oxidation of RANKL-stimulated osteoclasts compared with undifferentiated cells (Supplemental Fig. S1), as shown previously.^(39–41) Accordingly, differentiation of primary BMC to osteoclasts with RANKL/MCSF also increased fatty acid oxidation markers, such as fatty acyl-CoA oxidase (AOX, fivefold), Cpt-1 α (40%), and PGC-1 β (15-fold) (Supplemental Fig. S2A). Thus, RANKL-mediated osteoclastogenesis is associated with increased FA and glucose utilization.

Treatment of cells with PA but not OA enhances osteoclastogenesis

To further investigate the role of FAs in osteoclastogenesis, we differentiated RAW 264.7 cells into osteoclasts using RANKL in the presence of either 0.2 mM PA, or 0.2 mM OA, the two most abundant dietary FAs in the Western diet and in serum.⁽⁴²⁾ Combined treatment with RANKL and PA led to the formation of large multinucleated osteoclasts with intense TRAP staining, whereas treatment with RANKL and OA resulted in smaller osteoclasts similar in size to the control cells treated with RANKL only (Fig. 2Aa, 2Ab, and Supplemental Fig. S3). Moreover, PA-treated cultures had more osteoclastic resorption compared with control cells (Fig. 2Ba, 2Bb). Our morphological findings were consistent with TRAP mRNA levels, which increased ~sevenfold in the PA-treated cells compared with control cells that were treated with RANKL only, whereas they remained unchanged in the OA-treated cells (Fig. 2C). PA induced a more profound increase (52-fold) in the gene expression levels of the dendritic cell-specific transmembrane protein (DC-STAMP) (Fig. 2C), an osteoclast precursor fusion marker, the osteoclast stimulatory transmembrane protein (OC-STAMP; 21-fold) (Fig. 2C), an osteoclast differentiation marker, and a vacuolar (V-) ATPase proton pump (Atp6v0d2; 14-fold) (Fig. 2C), which carries out an extracellular acidification function that is crucial for bone resorption. PA also increased gene expression levels of FA utilization markers, such as PGC1 β (eightfold) (Fig. 2C) and CPT1 α (50-fold) (Fig. 2D) compared with cells stimulated with RANKL only. Treatment of primary BMC with RANKL/MCSF and PA reproduced the increase in PGC1 β expression levels, although to a less pronounced extent (40%), compared with cells treated with RANKL/MCSF only (Supplemental Fig. S2B). CPT1 α was not further increased by PA treatment, but the increased levels of AOX (twofold) indicate increased fatty acid oxidation levels compared with cells treated with RANKL/MCSF only (Supplemental Fig. S2B). OA treatment increased CPT1 α expression (20-fold) but did not affect PGC1 β compared with cells stimulated with RANKL only (Fig. 2C). Thus, PA, but not OA, leads to enhanced osteoclastogenesis.

PA enhances osteoclastogenesis by upregulating TNF α and RANK expression, whereas OA increases triglyceride formation in osteoclasts

To investigate the mechanisms by which PA enhances osteoclastogenesis, we analyzed gene expression of markers for endoplasmic reticulum (ER) stress,⁽⁴³⁾ apoptosis,⁽⁴⁴⁾ and inflammation⁽⁴⁵⁾ that are linked with PA-mediated lipotoxicity. Osteoclast precursor cells were cultured in the presence of RANKL and either 0.2 mM PA or 0.2 mM OA. PA did not

change the expression of CHOP, an ER stress marker, or caspase 9, an apoptosis marker (Fig. 2C). However, TNF α mRNA increased eightfold in PA-treated cultures but not in OA-treated cells compared with cells stimulated with RANKL only (Fig. 2C). OA led to a threefold increase in CHOP expression (Fig. 2C) compared with cells stimulated with RANKL only.

We next investigated whether increased osteoclastogenesis in PA-treated RAW 264.7 cultures could be the result of a change in RANKL/RANK expression. RANK expression was increased (fivefold) in PA-treated cultures but not in OA-treated cells (Fig. 2C). This suggests that PA-treated osteoclasts may be more susceptible to exogenous RANKL stimulation. Accordingly, primary BMC that were treated with RANKL/MCSF and PA had increased RANK expression levels (60%) compared with cells treated with RANKL/MCSF only (Supplemental Fig. S2B). As anticipated, RANKL mRNA was not detected in control as well as in PA and OA cultures of RAW 264.7 cells. Thus, PA increased TNF α and RANK expression, which both can enhance the differentiation of precursor cells into osteoclasts.

OA increases triglyceride formation in various cell culture models.^(28,46,47) As shown by Oil-Red-O staining, triglyceride levels were increased in osteoclasts treated with RANKL and OA, which did not occur in the cells treated with RANKL and PA or in control RANKL-treated cells (Fig. 2D). Accordingly, gene expression of DGAT1, which catalyzes triglyceride synthesis,⁽⁴⁸⁾ was increased 3.7-fold in the OA-stimulated cells. PA treatment also increased DGAT1 mRNA levels, but this increase was lower (twofold) (Fig. 2C). Taken together, PA increases osteoclastogenesis and upregulates TNF α , which do not occur in OA-treated cultures that have increased triglyceride formation.

Combined PA and OA treatment attenuates PA-induced osteoclastogenesis

Combined treatment with PA and OA alleviates lipotoxicity of PA in various cell culture models.^(49–51) Accordingly, we observed that combined PA and OA treatment in the presence of RANKL ameliorated the impact of PA and produced osteoclasts that were similar in number and size to RANKL only-treated control cells (Fig. 3A, B, and Supplemental Fig. S4). Additionally, combined PA and OA treatment reduced TRAP (Fig. 3C), PGC1 β (Fig. 3C), TNF α (Fig. 3C), and RANK (Fig. 3C) mRNA levels compared with PA-treated cells. Furthermore, analysis of BMC isolated from C57BL/6 mice and cultured with RANKL/MCSF and combination of PA and OA showed a trend toward lower osteoclastic activity compared with PA-treated cells (Supplemental Fig. S5).

PA induces osteoclastogenesis in the absence of RANKL

We then investigated whether PA can stimulate osteoclast differentiation even in the absence of RANKL because TNF α has been shown to induce osteoclastogenesis independent of the RANKL pathway.^(4–8) Stimulation of RAW 264.7 cells with PA alone for 5 days in the absence of RANKL resulted in formation of large osteoclasts (Fig. 4Aa, b). However, cells in the control and OA cultures did not differentiate well (Fig. 4Aa, b, and Supplemental Fig. S6). Accordingly, PA increased TRAP (twofold) and TNF α (2.5-fold) mRNA levels (Fig. 4B). Of note, the degree of PA-mediated osteoclastogenesis in the absence of RANKL was lower compared with RANKL-stimulated osteoclast formation. Osteoclasts that formed in

PA-treated cultures in the absence of RANKL were smaller in size and number with a less profound increase in TRAP expression during differentiation compared with osteoclasts that formed upon RANKL stimulation. Next, we observed that addition of OA to the PA cultures, which is known to attenuate the impact of PA in other cell culture models,^(49–51) also blocked PA-induced enlargement of osteoclasts (Fig. 4Aa, b), TRAP, and TNF α gene expression levels (Fig. 4B).

TNF α is the major mediator of PA-induced osteoclastogenesis

To address whether TNF α mediates PA-induced osteoclastogenesis, we treated RAW 264.7 cells with PA and a neutralizing anti-TNF α antibody (10 μ g/mL) for 5 days. Anti-TNF α antibody inhibited PA-induced osteoclast formation (Fig. 4C) and prevented the increase in TRAP mRNA, but it did not affect TNF α gene expression levels (Fig. 4D).

PA-induced osteoclastogenesis is inhibited by adenovirus-mediated expression of DGAT1

OA increased DGAT1 expression and led to triglyceride accumulation in osteoclasts (Fig. 2C). This finding, combined with the fact that OA did not enhance osteoclastogenesis (Fig. 2A, B) and inhibited PA-induced formation of osteoclasts (Fig. 3A–C, Fig. 4A, B), prompted us to investigate whether triglyceride formation may protect from increased osteoclastogenesis. To address this hypothesis, we generated a recombinant adenovirus that contains a single plasmid that expresses independently both GFP and DGAT1, which catalyzes TG formation⁽⁵²⁾ (Fig. 5A) driven by separate CMV promoters (Ad-DGAT1). RAW 264.7 cells were infected with the Ad-DGAT1 (MOI: 250) or control Ad-GFP and were treated with PA to induce osteoclastogenesis. DGAT1 overexpression enhanced triglyceride formation in both control and PA-treated cells, as shown by Oil-Red-O staining (Fig. 5B), inhibited PA-induced osteoclastogenesis (Fig. 5B, C), and decreased TRAP and TNF α mRNA compared with cells treated with Ad-GFP and PA (Fig. 5D). RAW 264.7 cells exhibit relatively low infectivity by adenoviruses. Therefore, a number of cells remained uninfected by the Ad-DGAT1, as shown by lack of GFP expression. Notably, osteoclasts, which formed in cultures treated with Ad-DGAT1 and PA, were consistently non-GFP-expressing cells (Supplemental Fig. S7), ie, cells that did not overexpress DGAT1. This emphasizes the importance of DGAT1 expression in the prevention of PA-mediated differentiation of cells into osteoclasts. In contrast, cultures treated with PA and control Ad-GFP had fully mature GFP-expressing osteoclasts (Fig. 5C), confirming that GFP overexpression did not inhibit cell fusion and osteoclast differentiation. Taken together, adenovirus-mediated DGAT1 expression inhibits PA-induced formation of osteoclasts.

DGAT1 gene deletion enhances osteoclastogenesis

Because DGAT1 inhibits PA-induced osteoclastogenesis, we hypothesized that DGAT1 deficiency would lead to increased osteoclastogenesis. To address this hypothesis, we isolated bone marrow cells from DGAT1 knockout mice (*Dgat1*^{−/−}) and WT controls and stimulated them with MCSF and RANKL. BMC from *Dgat1*^{−/−} mice that were stimulated with MCSF and RANKL produced larger multinucleated osteoclasts in comparison to osteoclasts from DGAT1-expressing mice (Fig. 6Aa, b, and Supplemental Fig. S8). Accordingly, TRAP and TNF α mRNA levels were increased by 30% and 50%, respectively,

in *Dgat1*^{-/-} derived osteoclasts (Fig. 6Ac). OC-STAMP mRNA levels of RANKL-treated *Dgat1*^{-/-} cells were increased by 30% compared with WT control cells (CTRL: 1 ± 0.08 , *Dgat1*^{-/-}: 1.3 ± 0.1 ; $p < 0.05$). DC-STAMP (CTRL: 1 ± 0.12 , *Dgat1*^{-/-}: 1.06 ± 0.12) and *Atp6v0d2* (CTRL: 1 ± 0.2 , *Dgat1*^{-/-}: 0.6 ± 0.1) mRNA levels were not increased in *Dgat1*^{-/-} osteoclasts. To address whether these changes in osteoclastogenesis are the result of systemic changes of TNF α or RANKL, we measured these cytokines in serum of *Dgat1*^{-/-} and WT mice. We detected lower serum levels of TNF α and RANKL in *Dgat1*^{-/-} compared with WT controls (ELISA: *Dgat1*^{-/-} versus WT: TNF α pg/mL 4.7 ± 0.3 versus $8.1 \pm 0.3^*$, RANKL pg/mL: 594 ± 0.65 versus $732 \pm 2.1^*$, $*p < 0.0001$, $n = 6$ to 7), indicating that the observed phenotype was not because of systemic inflammatory changes.

Bone quality is compromised in *Dgat1*-deficient mice

Using micro-CT and DXA, we analyzed bone mineral density (BMD) of *Dgat1*^{-/-} mice and littermate controls (Fig. 6Ba, b). BMD measured in femur trabecular bone was significantly lower in *Dgat1*^{-/-} mice, as shown in Fig. 6Bb. *Dgat1*^{-/-} mice had a trend toward lower whole body BMD compared with WT controls. Three-dimensional reconstruction of trabecular bone with CT revealed reduced bone microarchitecture in the femurs of *Dgat1*^{-/-} mice compared with littermate controls (Fig. 6C). The calculated morphometric indices of bone volume fraction (BV/TV), trabecular number (Tb.N), and trabecular thickness (Tb.Th) were reduced by 36% (Fig. 6Cb), 23% (Fig. 6Cc), and 10% (Fig. 6Cd), respectively, whereas trabecular spacing (Tb.Sp) was increased by 30% (Fig. 6Ce). Histomorphometric analysis showed that BV/TV (Fig. 6Db) was decreased (24%) and osteoclast number (OcN) (Fig. 6Df) was increased by 65% in *Dgat1*^{-/-} mice compared with controls. In addition, osteoclast surface (OcS) (Fig. 6De), osteoclast surface per bone surface (OcS/BS) (Fig. 6Dc), and osteoclast number per bone surface (OcN/BS) (Fig. 6Dd) were marginally increased. However, these were only trends that did not reach statistical significance. Furthermore, bone formation marker serum type 1 procollagen N-terminal (PINP) was lower in *Dgat1*^{-/-} mice compared with WT controls (ELISA PINP ng/ μ L: 228.41 ± 2.32 versus 482.71 ± 2.41 , $*p < 0.0001$, $n = 5$ /group) and osteoblast surface ObS was slightly, however, nonsignificantly decreased (Fig. 6Dg). Taken together, our experiments demonstrate that *Dgat1*-deficient mice have abnormal bone architecture and increased levels of osteoclast differentiation.

PA-enriched high-fat diet results in increased reduction of bone mass and loss of bone microarchitecture in overweight mice

We then investigated whether the FA profile of a high-fat diet can modulate bone microarchitecture during weight gain. C57BL/6 mice were fed isocaloric high-fat diets enriched in either OA or PA. After 12 weeks, all animals on FA-enriched diets weighed significantly more compared with animals on a normal caloric standard chow diet (31.7 ± 1.5 versus $41 \pm 1.2^*$, $39.3 \pm 1.0^*$, $*p < 0.05$) and lost bone over time (Fig. 7). However, the amount of bone loss was significantly greater in animals that were on a PA-enriched diet versus OA-enriched diet. PA-fed animals had lower BV/TV (Fig. 7Ba), BMD (Fig. 7Bb), and TbN (Fig. 7Bd) and higher TbSp (Fig. 7Bc) compared with OA-fed animals. These findings indicate that the FA profile of the diet rather than weight gain and obesity alone compromises bone density.

Discussion

Bone health may be compromised by metabolic abnormalities, such as increased fat mass,^(19,22–24) elevated blood glucose,⁽⁵³⁾ and lipid levels.^(20,54) Our study focused on the impact of PA and OA, the most abundant saturated and monounsaturated FAs in the Western diet and in serum,⁽⁴²⁾ on osteoclastogenesis. The FA concentrations used for this study correspond to a molecular ratio of PA/OA:BSA = 1.32, which is a typical ratio found in obesity and hypertriglyceridemia.^(49–52,55–57) We propose that PA induces a proinflammatory response via TNF α that increases osteoclastogenesis. Excess OA, on the other hand, inhibits a proinflammatory response by fostering neutral triglyceride formation. Many studies have demonstrated that PA promotes inflammation in several organs, as opposed to OA, which has anti-inflammatory properties.^(58–61) Given the contribution of inflammation to bone degradation^(6,7,9,11) and impaired bone formation,^(62,63) the pro-inflammatory nature of PA may account for its aggravating impact on bone health by either promoting osteoclast or inhibiting osteoblast formation. PA increased expression of RANK, which may make cells more prone to RANKL stimulation. We also show that lack of DGAT1, which catalyzes the formation of TG, reduces bone mass indices and enhances formation of osteoclasts. Furthermore, we found significantly lower bone structure indices in mice that were on a PA-enriched diet compared with mice on an OA-enriched diet. Previous studies had shown an inverse correlation between weight gain-initiated fat mass accumulation and bone mass, mainly attributed to an increase in osteoclastogenesis and osteoclastic activity.^(64,65) Thus, we propose that saturated FAs mediate a lipotoxic effect on the skeleton, via enhancement of osteoclast formation.

Several studies have shown that PA is more toxic than OA^(28,46,47,66–68) because of the accumulation of toxic lipids, such as ceramide and diacylglycerol, and the induction of ER stress,⁽⁴³⁾ apoptosis,⁽⁴⁴⁾ or inflammation.⁽⁴⁵⁾ However, treatment of RAW 264.7 cells with ceramide,⁽⁶⁹⁾ as well as treatment of rat osteoclasts with diacylglycerol,⁽⁷⁰⁾ inhibits RANKL-mediated osteoclastogenesis and bone resorption. We show that PA directly increased TNF α mRNA but not markers of ER stress or apoptosis in osteoclasts. Earlier studies showed that TNF α suffices to induce osteoclastogenesis independently of the RANKL-RANK interaction.^(6–10) In our study, administration of a neutralizing anti-TNF α antibody to PA-treated cells blocked PA-driven osteoclast formation. A previous study had suggested that combined treatment of RAW 264.7 cells with saturated FAs and differentiation factors inhibits osteoclastogenesis.⁽²⁶⁾ However, the authors had measured only absolute number of osteoclasts per culture regardless of size and multinuclearity of the cells. Consistent with this study,⁽²⁶⁾ our data show that because osteoclasts in cultures treated with PA and RANKL are bigger and occupy a larger space; actual cell number is smaller for the given area (Supplemental Figs. S3, S4, and S6). Similarly, another study showed that treatment of BMCs with saturated FAs, such as lauric acid or PA, enhanced osteoclast survival.⁽²⁷⁾

OA is more efficiently converted to TG and does not cause a pro-inflammatory response the way PA does.^(28,46,47) Consistently, treatment of RANKL-stimulated cells with OA increased triglyceride (TG) formation and did not enhance osteoclastogenesis, nor did it modulate RANK and TNF α gene expression. On the other hand, OA increased CHOP gene

expression, an ER stress marker. This finding is consistent with previous studies showing association of prolonged OA treatment with ER stress⁽⁷¹⁾ and indicates that ER stress may not enhance osteoclast formation. The benefit of OA in preventing lipotoxicity has been demonstrated with combined PA and OA cell treatment.^(28,46,47) Similarly, combined treatment of RANKL-stimulated RAW 264.7 cells with PA and OA inhibited PA-induced enhancement of osteoclastogenesis and reduced RANK and TNF α gene expression. Thus, OA may prevent PA-induced osteoclastogenesis.

We also demonstrated that prevention of PA-induced osteoclastogenesis by OA may be attributed to the enhanced formation of TG as shown by adenovirus-mediated expression of DGAT1, the enzyme that catalyzes TG formation,⁽⁴⁸⁾ in PA-treated cells. This treatment increased TG, reduced TNF α mRNA, and inhibited PA-induced osteoclastogenesis. Consistent with the beneficial effect of DGAT1 in the inhibition of osteoclastogenesis, *Dgat1*^{-/-} mice have decreased BMD and bone mass and increased RANKL-mediated differentiation of BMC to osteoclasts. Thus, DGAT1 may protect from osteoclastogenesis.

The association of saturated FAs with increased osteoclastogenesis links metabolic abnormalities, such as increased fat mass, diabetes, and dyslipidemia, with compromised bone health. Currently the relationship between obesity and bone mass is controversial. Although epidemiologic data show protection from bone loss in obesity,⁽⁷²⁻⁷⁶⁾ patients with metabolic syndrome and diabetes mellitus have increased bone loss and fracture risk,⁽⁷⁷⁻⁷⁹⁾ as well as reduced fracture healing because of increased osteoclastogenesis.^(80,81) Increased bone mass in individuals with greater body mass may represent an indirect adaptive mechanism for greater load.⁽⁸²⁾ However, although increased body weight appears to prevent bone loss, increase in total body fat may be a risk factor for bone health.⁽⁸³⁾ Thus, the positive effects of increased body mass should be separated from effects of increased fat mass and the coexistence of metabolic derangements affecting bone turnover. Animal and human data for young^(16-18,84) and adult individuals⁽¹⁹⁻²⁵⁾ reveal poor bone health and increased fracture risk with increased fat mass. Our data from high-fat-fed mice demonstrate that fat source determines the amount of bone loss in mice. However, obesity is a complex metabolic abnormality that includes multiple deregulations such as modulations in hormones and adipokines that may compromise bone remodeling. Therefore, additional effects of obesity on bone health may not be excluded. Future studies might also investigate whether the detected impact of excess PA and OA levels is specific to the complexity of a high-fat diet or can also be found in a normal caloric diet. Furthermore, several methodological limitations required that some of our in vitro experiments were performed only in RAW264.7-derived cultures. These results will ultimately need to be repeated in a primary cell model.

In conclusion, we show that saturated FAs can enhance RANKL-mediated osteoclast differentiation (Fig. 7) via upregulation of TNF α and RANK. Indirect effects of saturated FAs on bone formation may also not be excluded. Increased TG formation by OA supplementation or DGAT1 activation prevents PA-driven osteoclastogenesis, indicating potential therapeutic targets for increasing bone health.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Osteoclasts are associated with increased levels of fatty acid oxidation. (A) TRAP staining of RAW 246.7 cells 1 day (macrophages) and 5 days (osteoclasts indicated by arrows) post-stimulation with RANKL. (B) [^3H]-palmitate oxidation in osteoclasts compared with undifferentiated cells; $*p < 0.05$, $n = 4$ to 5. (C) RT-PCR analysis for TRAP, PGC1 β , CPT1 α , and CD36 mRNAs; $*p < 0.05$, $**p < 0.01$, $n = 5$ to 8.

Fig. 2.

PA enhances osteoclastogenesis. (A) a) TRAP staining in osteoclasts (5 days post-stimulation with RANKL) treated with PA or OA. b) Average size of osteoclasts treated with PA or OA. Average size was calculated from measurement of 233 osteoclasts in control treatment, 202 osteoclasts in PA-treated cultures, and 160 osteoclasts in OA-treated cultures; *** $p < 0.001$. (B) a) Analysis of osteoclastic resorption using scanning electron microscopy. Cells were cultured in the presence of PA or OA on dentin discs. b) Resorbed area in cultures treated with PA or OA versus CTRL; PA versus CTRL; * $p < 0.05$, $n = 7$ to 10. (C) RT-PCR for TRAP, DC-STAMP, OC-STAMP, Atp6v0d2, PGC1β, CPT1, TNFα, CHOP, Caspase9, RANK, and DGAT1 mRNAs; * $p < 0.05$, *** $p < 0.001$, $n = 4$ to 6. D. Neutral lipid staining with Oil-Red-O in osteoclasts treated with PA or OA.

Fig. 3.

Combined treatment with PA and OA inhibits PA-enhanced osteoclastogenesis. (A) TRAP staining in osteoclasts (5 days post-stimulation with RANKL) treated with PA, OA, or PA and OA. (B) Average size of osteoclasts treated with PA, OA, or PA and OA. Average size was calculated from measurement of 173 osteoclasts in control treatment, 108 osteoclasts in PA-treated cultures, 109 osteoclasts in OA-treated cultures, and 133 osteoclasts in cultures treated with combination of PA and OA; * $p < 0.05$. (C) RT-PCR analysis for *TRAP*, *PGC1 β* , *TNF α* , and *RANK* mRNAs; * $p < 0.05$, *** $p < 0.001$, $n = 4$ to 9.

Fig. 4.

PA induces osteoclastogenesis in the absence of RANKL via increased $TNF\alpha$ expression, whereas combined treatment with PA and OA inhibits this effect. (A) a) TRAP staining for osteoclasts in RAW 264.7 cells after 5 days of treatment with PA, OA, or PA and OA. b) Average size of osteoclasts treated with PA, OA, or PA and OA. Average size was calculated from measurement of 173 osteoclasts in control treatment, 108 osteoclasts in PA-treated cultures, 109 osteoclasts in OA-treated cultures, and 133 osteoclasts in cultures treated with combination of PA and OA. (B) RT-PCR analysis for *TRAP* and *TNFα* mRNAs; ** $p < 0.01$, *** $p < 0.001$, $n = 4$ to 9. (C) TRAP staining for osteoclasts in RAW 264.7 cells treated for 5 days with PA, anti- $TNF\alpha$ antibody, or PA and anti- $TNF\alpha$ antibody. (D) RT-PCR analysis for *TRAP* and *TNFα* mRNAs. * $p < 0.05$, *** $p < 0.001$, $n = 3-9$.

Fig. 5. DGAT1 inhibits PA-induced osteoclastogenesis. (A) RT-PCR analysis for *DGAT1* mRNA; *** $p < 0.001$, $n = 6$. (B, C) Neutral lipid staining with Oil-Red-O (B) and GFP fluorescence (C) in RAW 264.7 cells after 5 days of treatment with PA and Ad-GFP, or Ad-DGAT1 (MOI: 250). (D) RT-PCR analysis for *TRAP* and *TNFα* mRNAs; * $p < 0.05$, *** $p < 0.001$, $n = 4$ to 6.

Fig. 6.

Osteoclastogenesis is increased and femoral bone volume is decreased in *Dgat1*^{-/-} mice. (A) a) TRAP staining; b) average size of osteoclasts (**p* < 0.05; average osteoclast size was calculated from measurement of 311 WT osteoclasts and 267 *Dgat1*^{-/-} osteoclasts); and c) RT-PCR analysis for *TRAP*, *TNF α* , and *DGAT1* mRNAs derived from bone marrow cells from 20-week-old *Dgat1*^{-/-} mice and WT mice; **p* < 0.05, ***p* < 0.01, *n* = 6 to 9. (B) a) DXA scans of 12-week-old *Dgat1*^{-/-} and WT mice. b, c) BMD of *Dgat1*^{-/-} and WT mice measured from DXA scan (b, *n* = 7 to 8) and micro-CT (c, femur trabecular bone, *n* = 6 to 7,

* $p < 0.01$). (C) a) 3D reconstruction of femoral trabecular bone in 12-week-old *Dgat1*^{-/-} and WT mice. b–e) Morphometric indices calculated from micro-CT analysis of femurs in 12-week-old *Dgat1*^{-/-} and WT mice, BV/TV (b), Tb.N (c), Tb.Th (d), and Tb.Sp (e). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 6$ to 8. (D) Histomorphometric analysis. a) Longitudinal histological sections of femurs of *Dgat1*^{-/-} and WT mice. b–g) Morphometric indices calculated from histomorphometric analysis of femurs derived from 12-week-old *Dgat1*^{-/-} and wild-type mice. BV/TV (b), OcS/BS (c), OcN/BS (d), OCS (e), OCN (f), ObS (g). * $p < 0.05$, $n = 5$.

Fig. 7. Mice on a PA-enriched high-fat diet lose more bone than mice on an OA-enriched diet. (A) Micro-CT analysis: 3D reconstruction of femoral trabecular bone from mice after being on a high-fat diet for 12 weeks. (B) a–d) Morphometric indices calculated from micro-CT analysis of femurs BV/TV (a), BMD (b), Tb.Sp (c), and Tb.Th (d); $n = 9$ (PA-, OA-HFD), $n = 5$ (NFD), $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. (C) Proposed pathway of how excess levels of PA and OA influence osteoclastogenesis. PA increases TNF α and RANK gene expression. Increased RANK renders cells more susceptible to exogenous RANKL

stimulation and subsequent osteoclast differentiation. Increase in TNF α ignites osteoclastogenesis even by itself. OA supplementation or DGAT1 overexpression attenuates PA-mediated osteoclastogenesis by promoting triglyceride formation, which may also have an indirect effect on osteoblastogenesis.

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