Adiponectin Inhibits Osteoclastogenesis and Bone Resorption via APPL1-mediated Suppression of Akt1*

Qisheng Tu‡, Jin Zhang§, Lily Q. Dong‡, Eileen Saunders‡, En Luo‡, Jean Tang‡, and Jake Chen‡†

From the ‡Division of Oral Biology, Department of General Dentistry, Tufts University School of Dental Medicine, Boston, Massachusetts 02111, the §School of Dentistry, Shandong University, Jinan, Shandong 250012, China, and the ¶Department of Pharmacology, Cellular, and Structural Biology and the Barshop Center for Longevity and Aging Studies, University of Texas Health Science Center, San Antonio, Texas 78229

Adiponectin is an adipokine playing an important role in regulating energy homeostasis and insulin sensitivity. However, the effect of adiponectin on bone metabolism shows contradictory results according to different research studies. In this study femurs were isolated from genetically double-knockout mice and were transplanted into adiponectin knock-out mice or wild type mice to investigate the effect of temporary exposure to adiponectin deficiency on bone growth and metabolism. We found that the growth of bone explants in adiponectin knock-out mice was significantly retarded. Histological analysis, microcomputed tomography analysis, and tartrate-resistant acid phosphatase staining revealed reduced trabecular bone volume, decreased cortical bone, and increased osteoclast number in bone explants in adiponectin knock-out mice. We then found that adiponectin inhibits RANKL-induced osteoclastogenesis from RAW264.7 cells and down-regulates RANKL-enhanced expressions of osteoclastogenic regulators including NFAT2, TRAF6, cathepsin K, and tartrate-resistant acid phosphatase. Adiponectin also increases osteoclast apoptosis and decreases survival/proliferation of osteoclast precursor cells. Using siRNA specifically targeting APPL1, the first identified adaptor protein of adiponectin signaling, we found that the inhibitory effect of adiponectin on osteoclasts was induced by APPL1-mediated down-regulation of Akt1 activity. In addition, overexpression of Akt1 successfully reversed adiponectin-induced inhibition in RANKL-stimulated osteoclast differentiation. In conclusion, adiponectin is important in maintaining the balance of energy metabolism, inflammatory responses, and bone formation.

Adipose tissue is not just an inert organ for energy storage. It also secretes proinflammatory cytokines and synthesizes a wide range of biologically active molecules known as adipokines (1, 2). Adiponectin, a 30-kDa protein containing a collagen-repeat domain at the N terminus and a globular domain at the C terminus, is among these adipokines (3). It has been reported that adiponectin plays an important role in regulating energy homeostasis and insulin sensitivity, and plasma adiponectin levels correlate positively with insulin sensitivity (4, 5). APPL1 (adapter protein containing pleckstrin homology domain, phosphotyrosine domain, and leucine zipper motif), is the first identified protein interacting with adiponectin receptors and is suggested to be an adaptor protein responsible for the mediation of adiponectin signal transduction (6). Knockdown of APPL1 expression resulted in a significant reduction in insulin-stimulated Akt phosphorylation (6). In addition to its insulin-sensitizing effect, adiponectin has also been reported to have potent anti-inflammatory properties by suppressing the expressions of inflammatory cytokines while inducing production of anti-inflammatory cytokines (7–9). However, unlike other adipose tissue-derived molecules, adiponectin mRNA and plasma protein levels were shown to decrease in obesity and type 2 diabetes mellitus (T2DM) patients (10, 11). In 3T3-L1 adipocyte, TNF-α was shown to suppress the transcription of the adiponectin gene, which might explain the lower adiponectin mRNA levels in obesity associated adipose tissue, where TNF-α production was increased (12).

Ample clinical research studies have demonstrated the association between adiponectin and bone metabolism in various patient populations; however, with conflicting results. Several studies reported a significant inverse relationship between serum adiponectin level and bone mineral density (BMD) (13, 14), whereas other studies showed that serum adiponectin level was positively correlated with BMD (15) and other studies failed to find any associations between adiponectin level and BMD (16, 17). Although most researchers found that adiponectin stimulates osteoblast proliferation and differentiation (18, 19), the contradictory results remain inconclusive for the effect of adiponectin on osteoclast activity. For example, some researchers found that adiponectin not only inhibits macrophage colony-stimulating factor- and RANKL-induced osteoclast differentiation but also suppresses bone-resorption activity of osteoclasts (20–22). In contrast, Luo et al. (18) showed that adiponectin indirectly promotes osteoclastogenesis via enhancing RANKL expression and suppresses osteoprotegerin expression in human osteoblasts but has no direct effect on the differentiation of human osteoclast precursor

The abbreviations used are: T2DM, type 2 diabetes mellitus; BMD, bone mineral density; BSP, bone sialoprotein; GFP, green fluorescent protein; EGF, enhanced GFP; TRACP, tartrate-resistant acid phosphatase; Oxs, osterix; MNC, multinucleated cells; TRAF6, TNF receptor-associated factor 6; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

* This work was supported, in whole or in part, by National Institutes of Health Grant Grants DE14537 and DE16710 (to J. C.)
1 To whom correspondence should be addressed: One Kneeland St., Boston, MA 02111. Tel.: 617-636-2729; Fax: 617-636-0878; E-mail: jk.chen@tufts.edu.
Adiponectin Suppresses Bone Resorption

With this approach we can exclude the effects of long-term adaptation and compensation and eliminate any possible effects of mechanical loading on bone metabolism. We also performed in vitro studies to investigate the roles of adiponectin in osteoclastogenesis and the underlying molecular mechanisms.

**EXPERIMENTAL PROCEDURES**

**Bone Explantation**—Intramuscular bone explantation was performed as described previously with minor modifications (36). Briefly, bone donors were 3-day-old mBSP9.0Luc/β-actin–EGFP transgenic mice double-labeled with EGFP driven by β-actin promoter and luciferase driven by bone sialoprotein (BSP) promoter (37). After euthanasia, femurs from both sides of the donor mice were isolated and explanted into the back muscles of 8–10-week-old wild-type or adiponectin knock-out mice (stock no. 008195, The Jackson Laboratory, Bar Harbor, ME). Both the mBSP9.0Luc/β-actin–EGFP transgenic mice and the adiponectin knock-out mice have been backcrossed to C57BL/6J for at least eight generations before the bone explantation study; therefore, the donor mice and the recipient mice were both in the C57BL/6J genetic background. Femurs were explanted from the donor mouse. One femur was transplanted into an adiponectin knock-out mouse and the other into a wild type (WT) mouse; the mice were littermates generated by crossing a pair of heterozygous adiponectin+/− mice. At weeks 2 and 4 postoperatively, luciferase and EGFP expressions were measured using an IVIS Imaging System 200 Series in the live animals (Xenogen Corp., Alameda, CA) as described previously (37, 38). Four weeks after explantation, the bone explants were harvested to measure bone length and ash/dry weight (39). X-ray (radiography) was performed using a radiographic inspection unit (Faxitron X-ray Corp., Wheeling, IL). High-resolution microcomputed tomography (CT40; Scanco Medical, Basserdorf, Switzerland) was used to scan and evaluate bone volume fraction and BMD in the femurs as previously described (40). The three-dimensional structure was constructed by three-dimensional morphometric analysis with built-in microcomputed tomography system software. Histological analysis and tartrate-resistant acid phosphatase (TRACP) staining (using a leukocyte acid phosphatase staining kit with a tartrate concentration of 6.7 mM (38)) were also performed to evaluate the bone remodeling processes in these bone explants. The photos were taken and analyzed using a Nikon Eclipse E600 microscope and Spot Advanced software (Diagnostic Instruments, Inc., Sterling Heights, MI) as described previously (38).

Mice were maintained and used in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals prepared by the Institute on Laboratory Animal Resources, National Research Council (Department of Health and Human Services Publication NIH 86–23, 1985) and by guidelines established by the Institutional Animal Care and Use Committee of the Tufts-New England Medical Center (Boston, MA).

**Plasmids and Purification of Recombinant Adiponectin Protein**—pET15b bacterial expression vector encoding the C-terminal part of human adiponectin (amino acids 106–244) was used to purify globular adiponectin as a His-tagged protein.

...
Adiponectin Suppresses Bone Resorption

Adiponectin Deficiency Results in Retarded Growth in Bone Explants—IVIS Imaging demonstrated that both luciferase and EGFP expressions could be detected in the explantation regions of the live mice 2 weeks after surgery (Fig. 1, A and B). EGFP signals in the bone explants were slightly higher in WT mice than in adiponectin knock-out mice, whereas luciferase activity of bone explants was much stronger in WT mice than in adiponectin null mice. Four weeks after explantation, the intensities of EGFP signals and luciferase activity in the bone explants both decreased in WT and adiponectin knock-out mice, with their signals continuing to be weaker in adiponectin knock-out mice than in WT mice (Fig. 1, C and D).

Four weeks after bone explantation, the bone explants were isolated and subjected to further analyses. We found that bone explants in both mice groups remained viable and increased in size (Fig. 2A). However, the bone explants in adiponectin knock-out mice demonstrated a slower growth rate than in the WT mice (Table 1). X-ray, histological analysis, and microcomputed tomography showed that bone explants in adiponectin knock-out mice displayed reduced trabecular bone volume and decreased cortical bone (Fig. 2, A–C, and Table 2). TRACP staining further indicated that more TRACP stained cells with three or more nuclei.

Four weeks after bone explantation, the bone explants were isolated and subjected to further analyses. We found that bone explants in both mice groups remained viable and increased in size (Fig. 2A). However, the bone explants in adiponectin knock-out mice demonstrated a slower growth rate than in the WT mice (Table 1). X-ray, histological analysis, and microcomputed tomography showed that bone explants in adiponectin knock-out mice displayed reduced trabecular bone volume and decreased cortical bone (Fig. 2, A–C, and Table 2). TRACP staining further indicated that more TRACP stained cells with three or more nuclei.

Adiponectin Inhibits RANKL-induced Osteoclast Formation from RAW264.7 Cells—In vitro osteoclastogenesis was performed to investigate whether adiponectin could inhibit osteoclastogenesis from RAW264.7 cells stimulated by RANKL. Our results showed that the cells efficiently differentiated into
TRACP-positive multinucleated cells (MNCs) in the presence of RANKL stimulation, whereas globular adiponectin strongly inhibited RANKL-stimulated formation of TRACP-positive MNCs (Fig. 3, A and B). We also counted the number of MNC TRACP+ cells presented in these RAW264.7 cell cultures. We found that the number of MNC TRACP+ cells treated with RANKL and adiponectin was lower than that of cells treated with RANKL alone (Fig. 3C).

**Adiponectin Inhibits RANKL-induced Activation of Osteoclastogenic Regulators**—Calcineurin-dependent activation of NFAT2 is sufficient to induce osteoclast differentiation and is the master regulator of osteoclastogenesis (44). Upon the activation of calcineurin by extracellular stimuli and after Ca\(^{2+}\)-mediated signaling, NFAT2 forms a complex with calcineurin and is then dephosphorylated and translocated into the nuclei as an active form (45). TNF receptor-associated factor 6 (TRAF6) also plays an essential role in RANKL-induced activation of NF-κB in osteoclasts. We, thus, monitored mRNA levels of NFAT2 and TRAF6 as well as mRNA levels of another two osteoclastic differentiation markers, TRACP and cathepsin K, in RAW264.7 cells after being treated with adiponectin (1 μg/ml) and/or RANKL (50 ng/ml) for 72 h. Western blot analysis showed that adiponectin treatment caused an inhibition in the RANKL-triggered up-regulation and nuclear translocation of NFAT2 (Fig. 4B). These results clearly indicated that the negative effects of adiponectin on RANKL-induced osteoclast differentiation and activity are mediated by the inhibition of TRAF6 and NFAT2.

**Adiponectin Strongly Inhibits Cathepsin K Promoter Activity**—RAW264.7 cells were stably transfected with pGL3-CtpsK-luciferase and treated with globular adiponectin (0–2.0 μg/ml) with the presence of RANKL (50 ng/ml) for 72 h. RAW264.7 cells stably transfected with pGL3-CtpsK-luciferase were also treated with 1 μg/ml globular adiponectin and 50 ng/ml RANKL for 0, 24, 48, and 72 h. We found that adiponectin strongly inhibited cathepsin K promoter activity in a dose- and time-dependent manner (Fig. 5, A and B).

APPL1 was reported to be a key adaptor protein mediating adiponectin signaling and coordinating different signaling pathways (6). To investigate whether the inhibitory effect of adiponectin on cathepsin K promoter is mediated by APPL1,
Adiponectin Suppresses Bone Resorption

RAW264.7 cells stably expressing pGL3-CtpsK-luciferase were transfected with siRNA construct specifically targeting APPL1 and treated with globular adiponectin (1.0 μg/ml) and RANKL (50 ng/ml) for 72 h. Cells transfected with scrambled siRNA served as a control. As shown in Fig. 5C, transfection with active siRNA led to an 89% reduction in APPL1 protein levels compared with scrambled siRNA controls. Luciferase assays showed that the inhibitory effect of adiponectin on cathepsin K was antagonized by the siRNA specifically targeting APPL1 (Fig. 5D). These results indicated that the inhibitory effects of adiponectin on osteoclast differentiation and activity are mediated by APPL1.

Adiponectin Decreases Osteoclast Survival/Proliferation, Up-regulates DNA Condensation, and Increases Caspase-dependent Apoptosis in RAW264.7 Cells—RAW264.7 cells were treated with RANKL (50 ng/ml) and/or globular adiponectin (1 μg/ml) for 3 and 5 days. Osteoclast survival/proliferation was monitored using an MTT assay (Invitrogen), and we found that survival/proliferation of RAW264.7 cells was decreased by globular adiponectin treatment. Cells treated with both globular adiponectin and RANKL exhibited even lower MTT readings (Fig. 6A).

The effects of adiponectin on DNA condensation were also determined using a single-stranded DNA apoptosis ELISA kit (Chemicon International; Temecula, CA), which is the most specific and definite hallmark of caspase-dependent and -independent apoptosis. Cells used for this study were RAW264.7 cells treated with RANKL (50 ng/ml) and/or globular adiponectin (1 μg/ml) for 3 and 5 days. We found that DNA condensation was up-regulated by either RANKL or globular adiponectin treatment. However, the most prominent increase in DNA condensation was observed in cells treated with both RANKL and globular adiponectin (Fig. 6B).
To indirectly evaluate which type of apoptosis adiponectin could be enhancing (i.e. caspase-8-dependent death receptor pathway or caspase-9-dependent stress pathway), RAW264.7 cells were treated with RANKL (50 ng/ml) and/or globular adiponectin (1 μg/ml) for 3 and 5 days. The activity levels of executioner caspase-3 and initiator caspases-8 and -9 were determined using Caspase-Glo® Assay (Promega). Our results showed that the activity levels of the three caspases were all higher in adiponectin-treated RAW264.7 cell cultures than in those treated with RANKL alone. The increased activity levels of caspases-3, -8, and -9 in RAW264.7 cells treated with both RANKL and adiponectin were shown to be even more prominent (Fig. 6, C–E).

Adiponectin Shows Discrepant Effects on Different Akt Isoforms, Which Is Also Mediated by APPL1—Akt activity has been reported to be associated with osteoclast survival (46) and differentiation (47). To investigate whether the effect of adiponectin on osteoclast apoptosis is mediated by Akt activity, RAW264.7 cells overexpressing APPL1 were treated with RANKL (50 ng/ml) for 3 days. In addition, RAW264.7 cells with a normal APPL1 level were treated with RANKL (50 ng/ml) and/or adiponectin (1 μg/ml) for 3 days. Whole protein lysates were prepared and subjected to a fluorescence peptide substrate-based assay (Invitrogen) to determine Akt activity. We found that in RAW264.7 cells treated with adiponectin with or without RANKL, Akt activity was significantly decreased. APPL1 overexpression also decreased Akt activity to a similar level as that observed in the adiponectin-
Adiponectin Suppresses Bone Resorption

Adiponectin Suppresses Bone Resorption—To test whether adiponectin regulates transcriptional activities of Osx and BSP promoters, full-length Osx promoter (−11002/−13) or full-length BSP promoter (−9256/−30) construct, installed in pGL3-basic vector, was stably transfected into MC3T3-E1 cells. The cells were then treated with 1 μg/ml globular adiponectin and/or 50 μg/ml ascorbic acid for 7 days. Luciferase assay indicated that adiponectin alone up-regulated the transcription activities of Osx and BSP promoters to similar levels as those stimulated by ascorbic acid when compared with the untreated cells. Cells treated with both ascorbic acid and adiponectin demonstrated a more prominent increase in luciferase levels (Fig. 8).

FIGURE 5. Adiponectin inhibition of cathepsin K promoter activity mediated by APPL1. RAW264.7 cells were stably transfected with pGL3-CtpsK-luciferase construct in which osteoclast-specific expression of luciferase was driven by cathepsin K promoter and treated with globular adiponectin (0–2.0 μg/ml) in the presence of RANKL (50 ng/ml) for 72 h. Luciferase assays were performed using Lumat LB 9501 (EG&G Berthold). A and B, adiponectin strongly inhibits cathepsin K promoter activity in a dose- and time-dependent manner. a, p < 0.05, versus untreated cells; b, p < 0.01, versus untreated cells. C, APPL1 siRNA is shown. The sense and antisense sequences of RNAi were chemically synthesized and ligated into the pSIREN-DNR vector (BD Biosciences BDTM Knock-out RNAi system). For generation of the APPL1 siRNA stable cell lines, RAW264.7 cells were transfected with the APPL1 RNAi construct or the scrambled control and selected with 5 μg/ml puromycin. The effect of RNAi on APPL1 expression was tested by Western blot (top panel). Equal loading of protein in cell lysates was determined by Western blot using an anti-β-Actin antibody (bottom panel). D, the effects of APPL1 siRNA on adiponectin-induced inhibition of cathepsin K expression are shown. RAW264.7 cells stably expressing pGL3-CtpsK-luciferase were transfected with a siRNA construct specifically targeting APPL1 and treated with RANKL (50 ng/ml) and/or globular adiponectin (1.0 μg/ml) for 72 h. Cells transfected with scrambled siRNA served as controls. a, p < 0.05 adiponectin treated cells versus corresponding untreated cells; b, p < 0.05 APPL1 siRNA versus scrambled control in adiponectin-treated cells.

Adiponectin Strongly Enhances Osterix and BSP Promoter Activity in MC3T3-E1 Osteoblasts—To test whether adiponectin regulates transcriptional activities of Osx and BSP promoters, full-length Osx promoter, full-length Osterix promoter (−2020/+13) or full-length BSP promoter (−9256/+30) construct, installed in pGL3-basic vector, was stably transfected into MC3T3-E1 cells. The cells were then treated with 1 μg/ml globular adiponectin and/or 50 μg/ml ascorbic acid for 7 days. Luciferase assay indicated that adiponectin alone up-regulated the transcription activities of Osx and BSP promoters to similar levels as those stimulated by ascorbic acid when compared with the untreated cells. Cells treated with both ascorbic acid and adiponectin demonstrated a more prominent increase in luciferase levels (Fig. 8).
Adiponectin Suppresses Bone Resorption

Adiponectin is the most abundant adipokine derived from adipose tissue that displays insulin-sensitizing effects, and a reduced level of adiponectin closely relates to the pathophysiology of insulin resistance and T2DM (49). As mentioned previously, adiponectin was also found to be associated with bone metabolism; however, the previous studies have had inconsistent results as indicated by different studies, including clinical association, animal, and in vitro studies. To find out the real, direct role of adiponectin in bone metabolism, we established a bone explantation model to exclude the effects of long term adaptation and pathological compensation so that these adiponectin knock-out mice may not display any obvious bone phenotypes. In contrast, the short term exposure of bone explants in an adiponectin-deficient environment, which breaks the original balance of bone remodeling, displays noticeable effects on bone metabolism.

Our results, derived from a loss-of-function animal model, were consistent with a previous study using a gain-of-function animal model in which adiponectin was temporarily overexpressed via an adiponectin–adenovirus treatment; the researchers observed that trabecular bone mass increased, number of osteoclasts was reduced, and levels of plasma NTx, a bone-resorption marker, decreased (20). In addition, the use of a spontaneously diabetic animal model, WBN/Kob rats, also showed that low serum adiponectin levels resulted from hyperglycemia and obesity are partly associated with low bone mineral density, which is considered to be the cause of diabetes-related bone fragility (15, 50). All of these findings indicated that short term physiological or pathological changes in the serum adiponectin level have a significant impact on bone metabolism. In addition, the direct effect of adiponectin is to promote bone formation while inhibit bone resorption.

In fact, the adiponectin level of human beings keeps fluctuating with changes in other physiological or pathological factors such as fat mass or diabetes and would never be the same as that of a gene knock-out animal model. Therefore, as long as the effect of other confounding factors such as mechanical loading is removed, the role of adiponectin level on bone metabolism

FIGURE 6. Adiponectin effects in the survival/proliferation and apoptosis of RAW264.7. The cells were cultured on 96-well plates treated with RANKL (50 ng/ml) and/or globular adiponectin (gADIPONECTIN, 1 μg/ml) for 3 and 5 days. A, adiponectin mediates inhibitory effects in osteoclasts, as determined by the MTT assay. MTT results of three different experiments are presented as the mean ± S.E. a, p < 0.05 versus untreated cells; b, p < 0.05 versus cells treated with RANKL alone. B, globular adiponectin and RANKL-mediated up-regulation in DNA condensation in RAW264.7 cells is shown. Cells were differentiated as in A, and their DNA condensation was measured at 5, 7, and 9 days as described previously (43). Results are presented as the mean ± S.E. a, p < 0.05 versus untreated cells; b, p < 0.05 versus cells treated with RANKL alone. C, D, and E, adiponectin increases caspase activities in RAW264.7 cells. RAW264.7 cells were treated with RANKL (50 ng/ml) and/or globular adiponectin (1 μg/ml) for 3 and 5 days. The activity levels of executioner caspase-3 and initiator caspases-8 and -9 were determined using a Caspase-Glo® assay (Promega). Results are presented as the mean ± S.E. a, p < 0.05 versus untreated cells; b, p < 0.05 versus RANKL-treated cells.

DISCUSSION

Although no obvious bone phenotypes have been reported in adiponectin knock-out mice, we found that the short term exposure to an adiponectin-deficient environment has a negative effect on bone mineral density, which was indicated by retarded bone growth, decreased trabecular bone, impaired cortical bone structure, and more active bone resorption activity due to an increased number of osteoclasts in the bone explants embedded in adiponectin null mice. In the adiponectin knock-out mice, adiponectin is completely removed from the very beginning of, and throughout the life process. Therefore, a better balance could be established in these animals between bone formation and bone resorption via physiological adaptation and pathological compensation so that these adiponectin knock-out mice may not display any obvious bone phenotypes.
Adiponectin inhibits Akt activation in RAW264.7 cells. A, RAW264.7 cells with a normal APPL1 level were treated with RANKL (50 ng/ml) and/or adiponectin (1 μg/ml) for 3 days. RAW264.7 cells overexpressing APPL1 were treated with RANKL (50 ng/ml) alone for 3 days. Whole protein lysates were prepared and subjected to fluorescence peptide substrate-based assay to determine Akt activity (Omnia lysate assay kit). a, p < 0.05 versus untreated cells; b, p < 0.05 versus RANKL-treated cells. B, adiponectin exhibits discrepant effects on different Akt isoforms. RAW264.7 cells were treated with globular adiponectin (gAd, 1 μg/ml) and/or RANKL (50 ng/ml) for 72 h, and quantitative RT-PCR was performed to determine the changes in expression levels of different Akt isoforms. Results are presented as the mean ± S.E. a, p < 0.05 versus untreated cells; b, p < 0.05 versus RANKL-treated cells. C and D, effects of adiponectin on Akt phosphorylation are shown. Whole protein lysates were subjected to Western blotting to determine phosphorylation and the total levels of Akt, Akt1, and Akt2, and the detection of β-actin was used to normalize the band intensities. Normalized levels of Akt are shown in D. a, p < 0.05 versus untreated cells; b, p < 0.05 versus RANKL-treated cells. E and F, RAW264.7 cells were transfected with plasmids encoding Akt1 or Akt2 and were treated with RANKL (50 ng/ml) and/or adiponectin (0.5 μg/ml) for 3 days. pGL3-CtpsK-luciferase was co-transfected. E, luciferase assays showed that Akt1 reversed adiponectin-induced inhibition in RANKL-enhanced luciferase level, whereas Akt2 failed to show such an effect. Results are presented as the mean ± S.E. a, p < 0.05 versus untreated cells; b, p < 0.05 versus RANKL-treated cells. F, a Western blot shows the nuclear translocation of NFAT2 after Akt1 or Akt2 transfection. Results are presented as the mean ± S.E. a, p < 0.05 versus untreated cells; b, p < 0.05 versus RANKL-treated cells.
could easily be observed. Indeed, in a recently published clinical study, the researchers found a positive relationship between the total serum adiponectin level and BMD in patients with T2DM after adjustment for body weight and waist circumference (15).

Although one group of researchers reported that adiponectin has no direct effect on osteoclasts (18), most other in vitro studies have found that adiponectin significantly inhibits macrophage colony-stimulating factor- and RANKL-induced osteoclast differentiation (20–22). Yet, the molecular mechanism underlying the inhibitory effect of adiponectin on osteoclasts was not fully elucidated. In this study we found that adiponectin suppresses RANKL-induced osteoclastogenesis through decreasing expression levels of osteoclastogenic regulators, NFAT2 and TRAF6, as well as another two osteoclastic differentiation markers, TRACP and cathepsin K. Moreover, adiponectin indirectly suppresses osteoclast function through decreasing the osteoclast survival/proliferation rate and increasing osteoclast apoptosis. Using a siRNA construct specifically targeting APPL1, we found that the inhibitory effects of adiponectin on osteoclasts are mediated by APPL1, the first identified adaptor protein in adiponectin signaling.

To further investigate the underlying molecular mechanisms, we monitored the total and phosphorylation levels of Akt. Akt, also known as PKB, is a serine/threonine protein kinase and is a downstream target of phosphatidylinositol 3-kinase. Ample studies have demonstrated that enhanced Akt activity is critical in maintaining osteoclast survival (46) and enhancing osteoclast differentiation (47, 51). In this study we observed that adiponectin treatment significantly induced APPL1-mediated down-regulation of Akt activity in osteoclast precursor cells, which strongly indicated that the suppressive effect of adiponectin on osteoclasts results from the decreased Akt level.

However, in regard to adiponectin-mediated effects on the Akt pathway, various studies have previously demonstrated inconsistent results in different cells. It was reported that in C2C12 myoblasts, adiponectin displays a synergistic effect on Akt activation with insulin (6), whereas another study indicated that adiponectin suppresses hepatic stellate cell proliferation through, at least partly, inhibition of the Akt pathway (48). There are three Akt family members, Akt1/PKBa, Akt2/PKBb, and Akt3/PKBc, with Akt1 and Akt2, but not Akt3, being ubiquitously expressed in various tissues (52, 53). Akt2 is expressed more predominantly in insulin target tissues such as fat, liver, and muscle (52, 53). Accordingly, although Akt1−/− mice and Akt2−/− mice showed similar phenotypes, only Akt2−/− mice exhibited severe diabetes (53–55). These gene knock-out studies revealed non-redundant functions of different Akt isoforms, which indicated that different Akt isoforms may display unique functions. Briefly, Akt1 is essential in cell proliferation and organism growth, whereas Akt2 is an important regulator of metabolic regulation (54, 55). To clarify whether the inhibitory effects of adiponectin on osteoclast differentiation and survival are mainly mediated by Akt1, we performed real time RT-PCR and a Western blot to determine expression changes in each individual Akt isofrom. We found that adiponectin treatment mainly decreased the total and phosphorylated levels of Akt1 but not those of Akt2. Moreover, Akt1 overexpression in RAW264.7 cells reversed adiponectin-induced inhibition in RANKL-enhanced nuclear translocation of NFAT2 and cathepsin K expression, whereas Akt2 overexpression had no significant effect on adiponectin-induced biological changes in differentiating RAW264.7 cells. Previous findings have shown that phosphorylation of NFAT2 by GSK-3β prevents nuclear translocation of NFAT2 (56), whereas Akt signaling inhibits GSK-3β activity via phosphorylation of Ser-21 and Ser-9 residues (57, 58). Together with these previous findings, our results indicated that while during osteoclast differentiation, Akt1 removes NFAT2-targeted genes. Our results provided strong evidence that the reduction of Akt1 activity is the main reason for adiponectin-mediated inhibition in osteoclast formation. Using siRNA specifically targeting APPL1, we found that the effect of adiponectin on Akt activity is also mediated by APPL1. Based on these findings, we have described a model of the cross-talk between the adiponectin and RANKL/RANK signaling pathways (Fig. 9).

In addition to the findings showing that adiponectin inhibits osteoclastogenesis, we found that adiponectin promotes osteogenic differentiation, as indicated by increased promoter activities of Oxs and BSP after adiponectin treatment. Oxs is an
Adiponectin Suppresses Bone Resorption

Essential osteogenic transcription factor, and BSP is an important extracellular bone matrix protein that has long been used as a bone-specific marker. These results suggest that adiponectin enhances osteoblast differentiation and new bone formation through up-regulating expression levels of osteogenic transcription factors and bone matrix proteins.

As an insulin-sensitizing hormone, adiponectin plays an important role in regulating energy homeostasis and insulin sensitivity (4, 5). In addition, adiponectin has potent anti-inflammatory properties by suppressing inflammatory cytokines while activating anti-inflammatory cytokines (7–9). Showing inflammatory properties by suppressing inflammatory cytokines with preparation of this manuscript.

Acknowledgment—We thank Dana Murray for secretarial assistance with preparation of this manuscript.

REFERENCES

1. Spiegelman, B. M., and Flier, J. S. (2001) Cell 104, 531–543
2. Friedman, J. M. (2000) Nature 404, 632–634
3. Shapiro, L., and Scherer, P. E. (1998) Curr. Biol. 8, 335–338
4. Weyer, C., Funahashi, T., Tanaka, S., Hotta, K., Matsuzawa, Y., Pratley, R. E., and Tataranni, P. A. (2001) J. Clin. Endocrinol. Metab. 86, 1930–1935
5. Hotta, K., Funahashi, T., Bodkin, N. L., Ortmeyer, H. K., Arita, Y., Hansen, B. I., Langefeld, C. D., Carr, J. J., and Bowden, D. W. (2003) J. Bone Miner. Res. 18, 116–125
6. Zillikens, M. C., Uitterlinden, A. G., van Leeuwen, J. P., Berends, A. L., Pols, H. A., van Dijk, K. W., Oostra, B. A., van Duijn, C. M., and Rivadeneira, F. (2010) Calcif. Tissue Int. 86, 116–125
7. Vondracek, S. F., Voelkel, N. F., McDermott, M. T., and Valdez, C. (2009) Int. J. Chron. Obstruct. Pulmon. Dis. 4, 267–277
8. Luo, X. H., Guo, L. J., Xie, H., Yuan, L. Q., Wu, X. P., Zhou, H. D., and Liao, E. Y. (2006) J. Bone Miner. Res. 21, 1648–1656
9. Lee, H. W., Kim, S. Y., Kim, A. Y., Lee, E. J., Choi, J. Y., and Kim, J. B. (2009) Stem Cells 27, 2254–2262
10. Oshima, K., Nampei, A., Matsuda, M., Iwaki, M., Fukuhara, A., Hashimoto, J., Yoshikawa, H., and Shimomura, I. (2005) Biochem. Biophys. Res. Commun. 331, 520–526
11. Yamaguchi, N., Kuikita, T., Li, Y. J., Martinez Argueta, J. G., Saito, T., Hanazawa, S., and Yamashita, Y. (2007) FEBS Immunol. Med. Microbiol.
Adiponectin Suppresses Bone Resorption

22. Yamaguchi, N., Kukita, T., Li Y. J., Kamio, N., Fukumoto, S., Nonaka, K., Ninomiya, Y., Hanazawa, S., and Yamashita, Y. (2008) FEBS Lett. 582, 451–456

23. Shinoda, Y., Yamaguchi, M., Ogata, N., Akune, T., Kubota, N., Yamauchi, T., Terauchi, Y., Kadowaki, T., Takeuchi, Y., Fukumoto, S., Ikeda, T., Hoshi, K., Chung, U. I., Nakanura, K., and Kawaguchi, H. (2006) J. Cell. Biochem. 99, 196–208

24. Williams, G. A., Wang, Y., Callon, K. E., Watson, M., Lin, J. M., Lam, J. B., Costa, J. L., Orpe, A., Broom, N., Naot, D., Reid, I. R., and Cornish, J. (2009) Endocrinology 150, 3603–3610

25. Goulding, A., Grant, A. M., and Williams, S. M. (2005) J. Bone Miner. Res. 20, 2090–2096

26. Hsu, Y. H., Venners, S. A., Terwedow, H. A., Feng, Y., Niu, T., Li, Z., Laird, H., Richards, W. G., Bannon, T. W., Noda, M., Clement, K., Vaisse, C., and Christiansen, C. (1999) Bone 25, 1622–1627

27. Zhao, L. J., Liu, Y. J., Liu, P. Y., Hamilton, J., Recker, R. R., and Deng, H. W. (2006) J. Bone Miner. Res. 21, 1775–1788

28. Ealey, K. N., Kaludjerovic, J., Archer, M. C., and Ward, W. E. (2008) Exp. Biol. Med. (Maywood) 233, 1546–1553

29. Tanaka, H., and Seino, Y. (2004) J. Steroid Biochem. Mol. Biol. 89–90, 343–345

30. Li, S., Tu, Q., Zhang, J., Stein, G., Lian, J., Yang, P. S., and Chen, J. (2008) J. Cell. Physiol. 215, 204–209

31. Ravn, P., Cizza, G., Bjarnason, N. H., Thompson, D., Daley, M., Wasnich, R. D., McClung, M., Hosking, D., Yates, A. J., and Christiansen, C. (1999) J. Bone Miner. Res. 14, 1622–1627

32. Preshaw, P. M., Foster, N., and Taylor, J. J. (2007) Periodontol. 2000 45, 138–157

33. Stolk, R. P., Van Daele, P. L., Pols, H. A., Burger, H., Hofman, A., Birkenhager, J. C., Lamberts, S. W., and Grobbee, D. E. (1996) Bone 18, 545–549

34. Daccquin, R., Davey, R. A., Laplace, C., Levasseur, R., Morris, H. A., Goldberg, S. R., Gebre-Medhin, S., Galson, D. L., Zajac, J. D., and Karsenty, G. (2004) J. Cell Biol. 164, 509–514

35. Ealey, K. N., Kaludjerovic, J., Archer, M. C., and Ward, W. E. (2008) Exp. Biol. Med. (Maywood) 233, 1546–1553

36. Hildebrandt, T., Laib, A., Müller, R., Dequeker, J., and Rüegsegger, P. (1999) J. Bone Miner. Res. 14, 1167–1174

37. Tu, Q., Yamauchi, M., Pageau, S. C., and Chen, J. (2004) Biochem. Biophys. Res. Commun. 316, 461–467

38. Tu, Q., Zhang, J., Fix, A., Wang, Y., Zhang, Z. Y., and Chen, J. (2008) J. Cell. Physiol. 217, 40–47

39. Valverde, P., Tu, Q., and Chen, J. (2005) J. Bone Miner. Res. 20, 1669–1679

40. Hirotani, H., Tsuboy, N. A., Woo, J. T., Stern, P. H., and Clipstone, N. A. (2004) J. Biol. Chem. 279, 13984–13992

41. Cyert, M. S. (2001) J. Biol. Chem. 276, 20805–20808

42. Shishodia, S., and Aggarwal, B. B. (2006) Oncogene 25, 1463–1473

43. Yano, A., Tsutsumi, S., Soga, S., Lee, M. J., Trepel, J., Osada, H., and Neckers, L. (2008) Proc. Natl. Acad. Sci. U.S.A. 105, 15541–15546

44. Adachi, A., and Tashjian, A. H. (2008) Hepatology 47, 677–685

45. Maeda, N., Shimomura, I., Kishida, K., Nishizawa, H., Matsuda, M., Nagaretani, H., Furuyama, N., Kondo, H., Takahashi, M., Arita, Y., Komuro, R., Ouchi, N., Kihara, S., Tochino, Y., Okutomi, K., Horie, M., Takeda, S., Aoyama, T., Funahashi, T., and Matsuzawa, Y. (2002) Nat. Med. 8, 731–737

46. Nelson, R. G., Shlossman, M., Budding, L. M., Pettitt, D. J., Saad, M. F., and Christiansen, C. (1999) Bone 23, 89–90, 1546–1553

47. Yano, A., Tsutsumi, S., Soga, S., Lee, M. J., Trepel, J., Osada, H., and Neckers, L. (2008) Proc. Natl. Acad. Sci. U.S.A. 105, 15541–15546

48. Manning, B. D., and Cantley, L. C. (2007) Cell 131–138

49. Sugatani, T., and Hruska, K. A. (2005) J. Biol. Chem. 280, 3583–3589

50. Hanada, M., Feng, J., and Hemmings, B. A. (2004) Biochim. Biophys. Acta 1697, 3–16

51. Garofalo, R. S., Orena, S. J., Rafidi, K., Torchia, A. J., Stock, J. L., Hildebrandt, A. L., Coskran, T., Black, S. C., Brees, D. J., Wicks, J. R., McNeish, J. D., and Coleman, K. G. (2003) J. Clin. Invest. 112, 197–208

52. Cho, H., Thorvaldsen, J. L., Chu, Q., Feng, F., and Birnbaum, M. J. (2001) J. Biol. Chem. 276, 38349–38352

53. Beals, C. R., Sheridan, C. M., Turck, C. W., Gardner, P., and Crabtree, G. R. (1997) Science 275, 1930–1934

54. Doble, B. W., and Woodgett, J. R. (2003) Cell Sci. 116, 1175–1186

55. Manning, B. D., and Cantley, L. C. (2007) Cell 129, 1261–1274

56. Xu, H., Barnes, G. T., Yang, Q., Tan, G., Yang, D., Chou, J. C., Sole, J., Nichols, A., Ross, J. S., Tartaglia, L. A., and Chen, H. (2003) J. Clin. Invest. 112, 1821–1830

57. Weisberg, S. P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R. L., and Ferrante, A. W., Jr. (2003) J. Clin. Invest. 112, 1796–1808

58. Saito, T., Shimazaki, Y., and Sakamoto, M. (1998) N. Engl. J. Med. 339, 482–483

59. Nelson, R. G., Shlossman, M., Budding, L. M., Pettit, D. J., Saad, M. F., Genco, R. J., and Knowler, W. C. (1990) Diabetes Care 13, 836–840

60. Pischon, T., Heng, N., Bernimoulin, J. P., Kleber, B. M., Willrich, S. N., and Pischon, T. (2007) J. Dent. Res. 86, 400–409

61. Fantuzzi, G. (2005) J. Allergy Clin. Immunol. 115, 911–919; quiz 920