The bone anabolic carotenoid *p*-hydroxycinnamic acid promotes osteoblast mineralization and suppresses osteoclast differentiation by antagonizing NF-κB activation

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Received March 15, 2012; Accepted May 25, 2012

DOI: 10.3892/ijmm.2012.1043

Abstract. Numerous plant derived nutritional factors including *p*-hydroxycinnamic acid (HCA), a member of the carotenoid family, have long been held to possess bone protective properties. Studies in animals have provided a mechanistic basis for these observations by demonstrating the capacity of HCA to promote bone formation and suppress bone resorption *in vivo*. However, the molecular mechanism by which HCA achieves these effects remains unclear. We have demonstrated that a centralized mechanism by which several other nutritional factors achieve similar effects is through modulation of the nuclear factor-κB (NF-κB) signal transduction pathway. NF-κB activation is essential for osteoclast formation and resorption but potently antagonizes osteoblast differentiation and mineralization. In this study we demonstrate that HCA does indeed antagonize the activation of NF-κB by the key osteoclastogenic cytokine receptor activator of NF-κB (RANKL) in RAW264.7 osteoclast precursors, suppressing their differentiation into osteoclasts. Furthermore, HCA augmented the *in vitro* differentiation of MC3T3 preosteoblastic cells into mineralizing osteoblasts and relieved the inhibitory action of tumor necrosis factor-α (TNF-α)-induced NF-κB signaling on transforming growth factor-β (TGF-β)- or bone morphogenetic protein-2 (BMP-2)-induced Smad activation, an important pathway in osteoblast commitment and differentiation. Our data provide a mechanism to explain the dual pro-anabolic and anti-catabolic activities of HCA.

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Key words: *p*-hydroxycinnamic acid, nuclear factor-κB, osteoclast, osteoblast, osteoporosis

Introduction

A number of natural factors and nutritional supplements have long been believed to promote skeletal health. However, it is only relatively recently that these effects have been ratified in animal models and *in vitro* systems and in a few instances such as vitamin K_2, in clinical trials (1). However, their mechanisms of action remain poorly defined. We have previously investigated the mode of action of a number of micronutrients and nutritional factors on bone cells including quercetin, zinc, vitamin K_2, honokiol and the carotenoid β-cryptoxanthin (2-7). Interestingly, we reported that all of these agents appear to utilize a common centralized mechanism for rebalancing bone turnover by differentially regulating bone formation and resorption through suppression of the nuclear factor-κB (NF-κB) signal transduction pathway (2-7).

Indeed NF-κB is established as a critical component of osteoclast differentiation and activity and is an essential signal mediated through association of receptor activator of NF-κB (RANKL) with its receptor RANK (8,9). NF-κB antagonists have been demonstrated to ameliorate bone loss associated with estrogen deficiency in mice, a model of postmenopausal osteoporosis (10), in animal models of rheumatoid arthritis (11) and in bone loss associated with multiple myeloma (12).

More recently the role of NF-κB signaling in osteoblast differentiation has been investigated and found to be potently inhibitory. This finding was consistent with the well-established inhibitory action on osteoblastic differentiation and mineralization of the inflammatory cytokine tumor necrosis factor-α (TNF-α), a potent activator of NF-κB (13). In fact, we have demonstrated *in vivo* that even physiological concentrations of TNF-α achieve a magnitude sufficient to significantly impede bone formation and reduce maximum achievable peak bone mineral density (BMD) in mice *in vivo* (14). Other studies have reported that TNF-α suppresses bone formation in models of fracture repair (15,16) while conditional inactivation of NF-κB in osteoblasts promotes bone formation and ameliorates ovariectomy-induced bone loss (17). TNF-α, through NF-κB activation impedes the transforming growth factor-β (TGF-β)-induced commitment of early mesenchymal stem cells along the osteoblast pathway as well as suppressing

BMP-induced bone formation and osteoblast differentiation, by intersecting with and antagonizing the activation of Smad-signal transduction (2-7,14,18). Smad activity in the osteoblast is in turn regulated by Smurf1-induced proteasomal degradation (19) as well as by upregulation of the Smad activation antagonist Smad7 (20).

p-Hydroxycinnamic acid (HCA) is a carotene that is derived from cinnamic acid found in plants and fruits. HCA has been found to stimulate bone formation in rat femoral tissues in organ culture *ex vivo* (21) and to inhibit the differentiation of primary bone marrow cells into osteoclasts *in vitro* (22) suggesting dual anabolic and anti-catabolic activities. Furthermore, HCA has been used to effectively ameliorate ovariectomy-induced bone loss in rats (23) as well as bone loss associated with streptozotocin-induced diabetes in rats (24).

In this study, we examined the mechanism pertaining to the bone anabolic and anticatabolic activity of HCA on bone cells. Our data reveal that like other agents endowed with dual anabolic and anticatabolic activities, HCA functions as a natural NF-κB antagonist, suppressing RANKL-induced NF-κB signaling in osteoclast precursors and relieving the inhibitory actions of TNF-α on the pro-anabolic Smad pathway.

Materials and methods

Materials. α-minimal essential medium (α-MEM) and antibiotics (penicillin and streptomycin) were purchased from Invitrogen Corp. (Carlsbad, CA). Fetal bovine serum (FBS) was from Hyclone. RANKL, TGF-β, TNF-α and BMP-2 were from R&D Systems (Minneapolis, MN). HCA, mouse anti-poly-histidine antibody and all other reagents were purchased from the Sigma Chemical Corporation (St. Louis, MO) unless otherwise specified.

Cell culture. The preosteoblastic cell line MC3T3-E1, clone 14 (MC3T3) and the monocytic cell line RAW264.7 were purchased from the American Type Culture Collection (Manassas, VA) and cultured as described previously (7,14).

Osteoblast differentiation assays and Alizarin Red-S staining. MC3T3 cells or primary bone marrow stromal cells were plated and cultured for 72 h in α-MEM (1.0 ml/well) containing 10% FBS in 12-well dishes at a density of $1.0x10^5$ cells/well. The medium was aspirated and changed to mineralization medium (α-MEM supplemented with 10% FBS, L-ascorbic acid (100 μg/ml) and 4 mM β-glycerophosphate) as previously described (14,25). Vehicle or HCA was added at a dose of 0.01 to 10 μM and cells were replenished with fresh medium every 3 days. At 17-18 days cells were rinsed with PBS and calcium deposition was visualized by fixing the cells in 75% ethanol for 30 min at 4˚C followed by staining with Alizarin Red-S (40 mM, pH 6.2) for 30 min at room temperature. Excess stain was removed by copious washing with distilled water. Plates were imaged using a flatbed scanner (Epson Perfection 1660 Photo), and quantitated using ImageJ (26). To quantify mineralization Alizarin Red-S was eluted from cultures by incubation in 10% cetylpyridinium chloride solution. After complete elution, absorbance was read at 570 nm on a microtiter plate reader.

Osteoclastogenesis assays and TRAP staining. RAW264.7 cells were cultured in 96-well plates in α-MEM supplemented with 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin at a density of $1x10^4$ cells/well. Cells were cultured for 6 days with RANKL (30 ng/ml) pre-incubated for 10 min with crosslinking anti-polyhistidine antibody (2.5 μg/ml) to induce osteoclast formation. HCA was added in the range of 0.1-100 μM. After 6 days of culture, the cells were fixed and stained for tartrate resistant acid phosphatase (TRAP) activity using a leukocyte acid phosphatase kit (Sigma). $TRAP^+$ cells with three or more nuclei were defined as osteoclasts and were quantitated under light microscopy and 5 wells/group averaged.

NF-κB and Smad reporter constructs and luciferase assays. The NF-κB responsive reporter pNF-κB-Luc (BD Biosciences) and the Smad responsive reporter pGL3-Smad were used as previously described (14). The Smad reporter is responsive to both TGF-β- and to BMP-induced Smad species. Briefly, reporter plasmids were transfected into MC3T3 or RAW264.7 cells ($1x10^5$ cells/well) using Lipofectamine 2000 reagent (Invitrogen) in α-MEM without FBS and antibiotics. Five hours later the medium was changed to α-MEM containing 10% FBS plus antibiotics and the cells were treated with TNF-α (MC3T3) or RANKL (RAW264.7) to stimulate NF-κB activity, or with TGF-β (1 ng/ml) or BMP-2 (0.5 μg/ml) to stimulate Smad activity and treated with or without TNF-α. Parallel groups received vehicle or HCA in the dose range of 0.1-100 μM. Cells were extracted with passive lysis buffer (Promega, Madison, WI) 24 h later, and luciferase activity was measured using the Luciferase Assay System of Promega, on a microplate luminometer (Turner Designs, Sunnyvale, CA).

Statistical analysis. Statistical significance was determined using the GraphPad InStat version 3 for Windows XP (GraphPad Software, Inc., La Jolla, CA). Multiple comparisons were performed by one-way analysis of variance (ANOVA) with the Tukey-Kramer multiple comparisons post-test. $P<0.05$ was considered statistically significant.

Results

HCA augments the differentiation of MC3T3 preosteoblasts into mineralizing osteoblasts. HCA has been demonstrated to promote differentiation of primary osteoblasts *in vitro*. To validate the MC3T3 preosteoblastic cell line model for mechanistic investigations of HCA action we treated MC3T3 cells with HCA (0.01-10 μM) in mineralizing medium (MM) for 21 days. Cultures were stained with Alizarin Red-S to visualize calcium deposition (Fig. 1A). Alizarin Red-S was subsequently eluted from the wells and quantified by spectrophotometry (Fig. 1B). HCA dose-dependently increased the mineralization of MC3T3 cells ratifying their use for further detailed mechanistic studies.

HCA alleviates the inhibitory effect of TNF-α on MC3T3 mineralization. Because TNF-α-induced NF-κB signaling potently inhibits osteoblastic differentiation *in vivo* and *in vitro* (13,14) we investigated whether HCA could relieve the inhibitory effect of TNF-α on MC3T3 mineralization. HCA dose dependently reversed the inhibitory effect of TNF-α

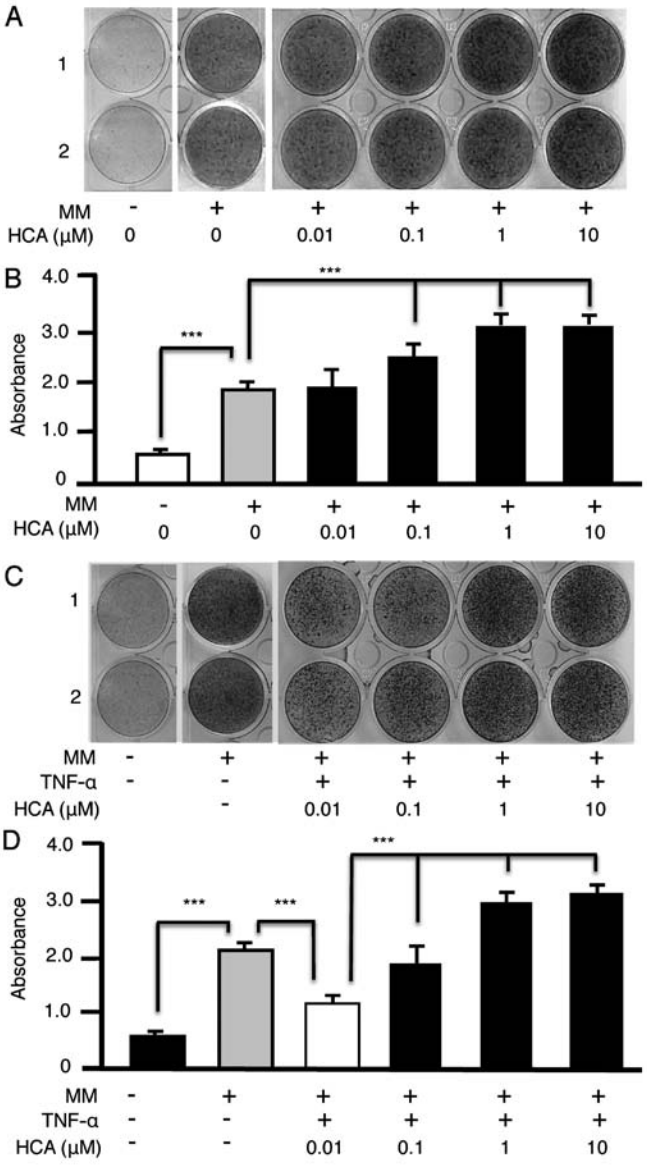

Figure 1. Effect of *p*-hydroxycinnamic acid (HCA) on mineralization in MC3T3 osteoblastic cells. MC3T3 preosteoblastic cells were differentiated in mineralizing medium (MM) containing ascorbic acid (100 ng/ml) and 4 mM β-glycerophosphate in the presence of either vehicle or HCA (0.01-10 μM) for 21 days. (A) Mineralization was visualized by Alizarin Red-S staining and (B) quantified by spectrophotometry (570 nm) following solubilization and elution with 10% cetylpyridinium chloride. (C) MC3T3 preosteoblastic cells were differentiated in mineralizing medium (MM) in the presence of TNF-α (5 ng/ml) and vehicle or HCA (0.01-10 μM) for 21 days. Mineralization was visualized by Alizarin Red-S staining and (D) quantified by spectrophotometry (570 nm) following solubilization and elution with 10% cetylpyridinium chloride. Data are expressed as the mean ± SD of 2 replicate samples from 2 independent experiments. ***P<0.001 vs. the MM-treated control group or the TNF-α negative control group (grey bar) or TNF-α positive control group (white bar).

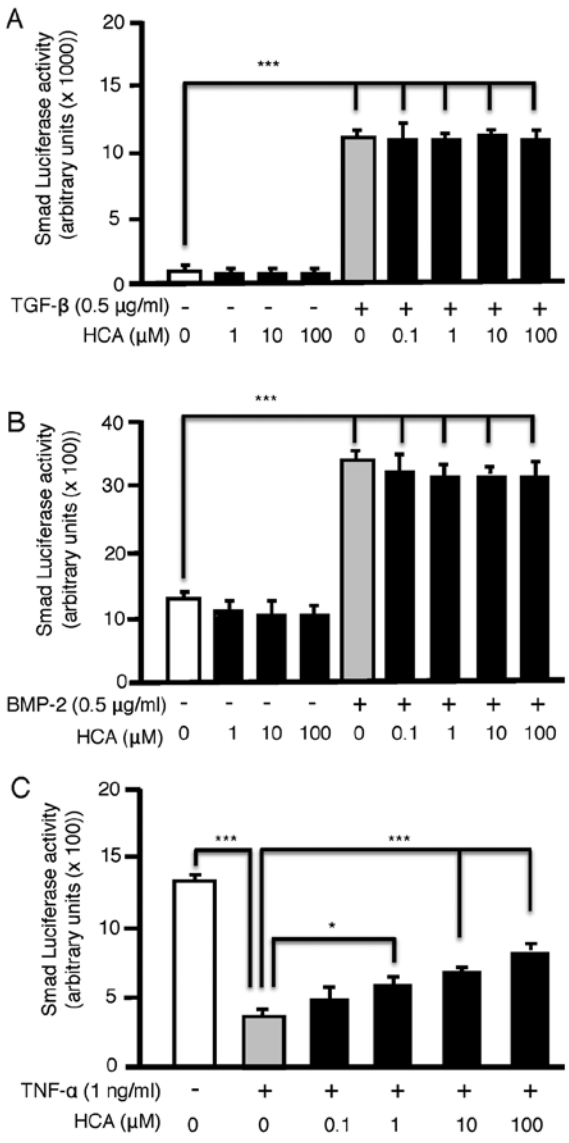

Figure 2. Effect of *p*-hydroxycinnamic acid (HCA) on Smad activation in MC3T3 cells. MC3T3 cells were transfected with Smad luciferase reporter and treated with either vehicle or HCA (0.1-100 μM) in the presence of (A) TGF-β, (B) BMP-2 or (C) without cytokine stimulation, but in the presence or absence of TNF-α. Luciferase activity was quantified 24 h later. Data expressed as mean ± SD of 5 replicate samples per data set, and are representative of 2 independent experiments. *P<0.05, ***P<0.001 vs. the unstimulated control (white bar) or the cytokine-treated group (grey bar).

visualized by Alizarin Red-S staining (Fig. 1C) and quantified by spectrophotometry (Fig. 1D).

HCA has no direct effect on basal or cytokine-induced Smad activity but antagonizes the inhibitory effect of TNF-α on Smad activity. We next investigated whether HCA regulates the bone anabolic Smad signaling pathway in preosteoblasts, either directly by indirectly. To achieve this we transfected MC3T3 cells with a Smad responsive luciferase reporter. The Smad agonists TGF-β (Fig. 2A) and BMP-2 (Fig. 2B) upregulated Smad reporter activity, however HCA had no direct additive effects on baseline or cytokine-induced Smad induction. However, when Smad activation was potently repressed by addition of TNF-α, HCA significantly and dose-dependently (0.1-100 μM) reversed the inhibitory action of TNF-α (Fig. 2C). These data suggest an indirect stimulatory effect of HCA by relieving TNF-α-induced inhibitory effects on Smad signal transduction.

HCA suppresses TNF-α-induced NF-κB activation in MC3T3 cells. Because NF-κB-signal transduction is potently inhibitory to anabolic signals such as Smad (2-7,14,18) and TNF-α

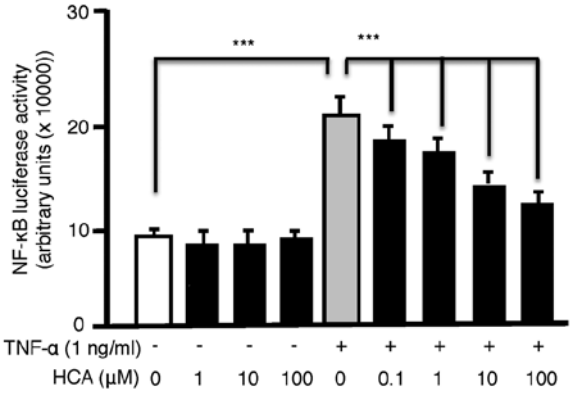

Figure 3. Effect of *p*-hydroxycinnamic acid (HCA) on TNF-α-stimulated NF-κB luciferase activity in MC3T3 cells. MC3T3 cells were transfected with a NF-κB luciferase reported construct and treated with a dose range (0.1-100 μM) of HCA in the presence or absence of TNF-α (1 ng/ml) and luciferase activity was quantified 24 h later. Data are expressed as the mean ± SD of 5 replicate samples per data set, and are representative of 2 independent experiments. ***P<0.001 vs. the unstimulated control (white bar) or the TNF-α treated group (grey bar).

is a potent NF-κB activator we further investigated whether HCA suppresses TNF-α-induced NF-κB activation in MC3T3 pre-osteoblasts. MC3T3 cells were transiently transfected with an NF-κB luciferase reporter and basal or cytokine (TNF-α) stimulated NF-κB activity quantified in the presence or absence of HCA. While HCA failed to modulate basal NF-κB activity it dose-dependently (0.1-100 μM) downregulated TNF-α-induced NF-κB reporter activity (Fig. 3).

These data suggest that HCA promotes osteoblast differentiation *in vivo* by alleviating the inhibitory action of endogenous NF-κB-inducing antagonists such as TNF-α on pro-osteoblastic pathways, such as Smad.

HCA dose-dependently inhibits osteoclast formation in RAW264.7 cells by antagonizing NF-κB activation. Although HCA is known to promote osteoblastic bone formation, its action on osteoclastic bone resorption is reported to be repressive. To further examine the mechanism of HCA action on osteoclasts we induced the RAW264.7 osteoclast precursor cell line to differentiate into osteoclasts by exposure to the key osteoclastogenic cytokine RANKL (30 ng/ml) for 7 days. RANKL elicited significant osteoclast formation, which was significantly and dose-dependently (0.1-100 μM) inhibited by addition of HCA (Fig. 4A). HCA had no observable toxic effect on RAW264.7 cell proliferation in the absence of RANKL, as precursors continued to divide over 7 days filling the wells with TRAP-negative mononucleated cells. These data suggest a specific inhibitory effect of HCA on osteoclast differentiation by RANKL.

As a primary signal mediated via RANKL and required for osteoclast differentiation is NF-κB, a pathway established in the studies above to be antagonized by HCA in osteoblasts, we further examined the effect of HCA on NF-κB activation by RANKL in RAW264.7 cells. HCA had no effect on basal NF-κB activation but dose-dependently (0.1-100 μM) inhibited RANKL-induced NF-κB activation (Fig. 4B).

Taken together our data suggest that HCA promotes osteoblast differentiation and/or mineralizing activity and

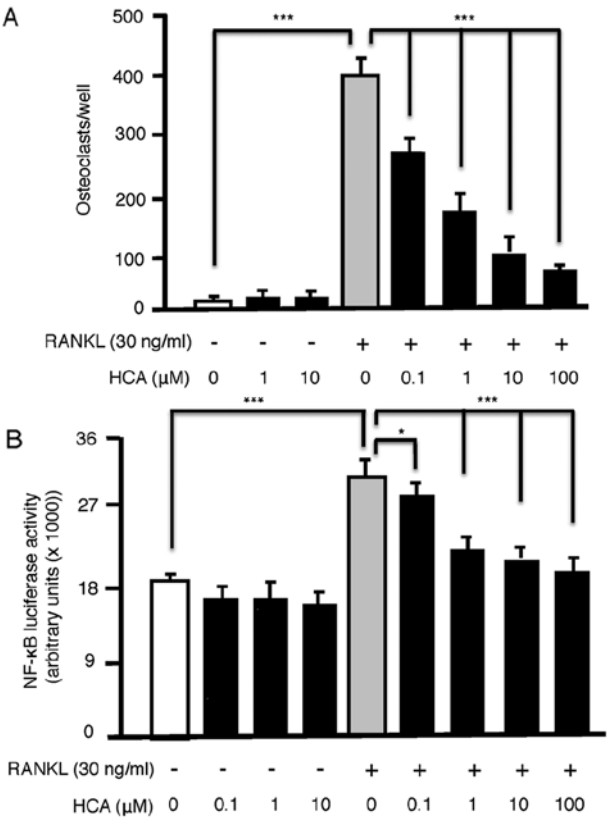

Figure 4. Effect of *p*-hydroxycinnamic acid (HCA) on RANKL-stimulated osteoclastogenesis and NF-κB activation in RAW267.4 osteoclast precursor cells. (A) RAW264.7 cells were induced to differentiation into osteoclasts in the presence of RANKL (30 ng/ml) in the presence or absence of HCA (1-100 μM). Cultures were TRAP stained after 6 days and TRAP$^+$ cells with three or more nuclei counted as osteoclasts. Data are expressed as mean ± SD of 5 replicate samples per data set, and are representative of 2 independent experiments. *P<0.05, ***P<0.001 vs. untreated control (white bar) or vs. RANKL stimulated group (grey bar).

suppresses osteoclast differentiation. These events are likely mediated though suppression of NF-κB activation.

Discussion

How HCA induces bone-anabolic activity and inhibits osteoclastic bone loss is unclear. Our data suggest that HCA functions as a NF-κB antagonist, suppressing RANKL-induced NF-κB signaling in osteoclast precursors while relieving the inhibitory actions of TNF-α on BMP- and TGF-β-induced Smad activation, signals that mediate anabolic effects in osteoblasts.

Although TNF-α was added exogenously in our *in vitro* culture systems as a proof of concept, we have previously demonstrated that in mice *in vivo*, the concentrations of basal TNF-α are of a sufficient magnitude to suppress basal bone formation. These concentrations however, did not achieve a level capable of augmenting RANKL-induced osteoclastic bone resorption (14). By contrast, under inflammatory conditions characteristic of rheumatoid arthritis and estrogen deficiency, TNF-α achieves concentrations that are able to stimulate RANKL-induced osteoclast formation as well as suppress osteoblastic bone formation. In fact, ovariectomy-induced bone loss is a consequence of an imbalance between osteoclastic bone resorption,

which is significantly elevated and osteoblastic bone formation, that although also elevated does not reach a magnitude capable of compensating for the high rates of bone resorption (27). TNF-α may play a central role in both processes. On the osteoclastic side, TNF-α is reported to amplify the activity of RANKL in osteoclast precursors (28,29) by synergizing at the level of signal transduction (30). TNF-α further activates the resorptive activity of osteoclasts independently and synergistically with RANKL (31). At the other end of the spectrum the compensatory rise in bone formation necessary to maintain homeostasis between bone formation and resorption is impeded by inhibitory cytokines, including TNF-α. Our data suggest that HCA may thus prevent the ovariectomy-induced bone loss, as previously reported (23), by not only reducing TNF-α- and RANKL-induced osteoclastic bone resorption, but also by relieving the suppressive effect of TNF-α on bone formation.

In conclusion, our study supports an important protective action of HCA on the skeleton by promoting bone formation under basal conditions and by both suppressing bone resorption and promoting bone formation in inflammatory contexts.

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