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# Curcumin Protects Against Ovariectomy-Induced Bone Loss and Decreases Osteoclastogenesis

W.K. Kim,[1] K. Ke,[1] O.J. Sul,[1] H.J. Kim,[1] S.H. Kim,[1] M.H. Lee,[1] H.J. Kim,[2] S.Y. Kim,[2] H.T. Chung,[1] and H.S. Choi[1*]

[1] *Department of Biological Sciences (BK21 Program), University of Ulsan, Ulsan 680-749, Korea*

[2] *Skeletal Diseases Genome Research Center, School of Medicine, Kyungpook National University, Daegu 700-412, Korea*

## ABSTRACT

Curcumin has anti-oxidative activity. In view of the increasing evidence for a biochemical link between increased oxidative stress and reduced bone density we hypothesized that curcumin might increase bone density by elevating antioxidant activity in some target cell type. We measured bone density by Micro-CT, enzyme expression levels by quantitative PCR or enzyme activity, and osteoclast (OC) formation by tartrate-resistant acid phosphatase staining. The bone mineral density of the femurs of curcumin-administered mice was significantly higher than that of vehicle-treated mice after ovariectomy (OVX) and this was accompanied by reduced amounts of serum collagen-type I fragments, which are markers of bone resorption. Curcumin suppressed OC formation by increasing receptor activator of nuclear factor-κB ligand (RANKL)-induced glutathione peroxidase-1, and reversed the stimulatory effect of homocysteine, a known $H_2O_2$ generator, on OC formation by restoring Gpx activity. Curcumin generated an aberrant RANKL signal characterized by reduced expression of nuclear factor of activated T cells 2 (NFAT2) and attenuated activation of mitogen-activated protein kinases (ERK, JNK, and p38). Curcumin thus inhibited OVX-induced bone loss, at least in part by reducing osteoclastogenesis as a result of increased antioxidant activity and impaired RANKL signaling. These findings suggest that bone loss associated with estrogen deficiency could be attenuated by curcumin administration. J. Cell. Biochem. 112: 3159–3166, 2011. 

**KEY WORDS:** OVARIECTOMY; CURCUMIN; BONE LOSS; OSTEOCLASTOGENESIS; GLUTATHIONE PEROXIDASE-1; RANKL

Curcumin (diferuloylmethane), a major component of turmeric, is a yellow pigment from *Curcuma longa*. It is used as a spice, as well as in some medical preparations. It is employed as an antioxidant against chronic metabolic disease, since free radical-mediated peroxidation of membrane lipids and oxidative damage to DNA and proteins are associated with a variety of chronic pathological complications [Aggarwal and Sung, 2008].

Recent studies have suggested that postmenopausal bone loss may be caused by reactive oxygen species (ROS), which generate a more oxidized bone microenvironment [Maggio et al., 2003]. The fact that isoflavone protects against bone loss and is an antioxidant agent supports this association [Viereck et al., 2002]. It is also supported by in vivo experiments in rats that demonstrate that ovariectomy (OVX) induces oxidative stress and attenuates expression of antioxidants [Muthusami et al., 2005]. In addition, elevated levels of the antioxidant glutathione prevent bone loss during estrogen deficiency, whereas depletion of antioxidants enhances bone loss in mice [Lean et al., 2003].

OCs are highly specialized cells that dissolve bone mineral as well as degrading organic bone matrix. Since OCs play a critical role in bone resorption, and increased OC activity is seen in many osteopenic conditions such as postmenpausal osteoporosis [Rodan, 1991], inhibition of OC differentiation/function has become a major strategy for treating bone loss. OCs originate from hematopoietic stem cells of the macrophage/monocyte lineage, and their differentiation and activity are dependent on macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL) [Suda et al., 1999]. The critical role of

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*Correspondence to: Prof. H.S. Choi, PhD, Department of Biological Sciences, University of Ulsan, Ulsan 680-749, Korea. E-mail: hschoi@mail.ulsan.ac.kr

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RANKL in the development of OC has been demonstrated by the finding that RANKL-deficient mice are severely osteopetrotic due to a defect in OC differentiation [Kong et al., 1999].

The principle objectives of the present study were to investigate a possible protective effect of curcumin on OVX-induced bone loss and to analyze its action on OCs.

## MATERIALS AND METHODS

### ANIMALS AND STUDY DESIGN

Six-week-old C57BL/6J mice purchased from the Jackson Laboratory, and cared for in the University of Ulsan, Immunomodulation Research Center (IRC), were subjected to OVX (n = 14) or sham operation (n = 11). Curcumin was prepared every day as a solution of 5 mg in 13.2 ml phosphate-based saline. Curcumin dose was calculated on the basis of safe levels in animal studies [Goel et al., 2008]. After surgery, vehicle (n = 13) or curcumin (9.5 μg/g of body weight/day) (n = 12) was given intragastrically (0.5 ml/each) through an esophageal cannula, to assure delivery of the correct dose, daily for 8 weeks, and bone density was analyzed. All mice were housed in the specific pathogen-free animal facility of the IRC and were handled in accordance with the guidelines of the Institutional Animal Care and Use Committee of the IRC. The standards were approved by that Committee (2008-028). Micro-CT (μCT) scanning was performed with a GE eXplore Locus SP system (Locus SP; GE Healthcare Company) [Sheng et al., 2009]. For three-dimensional histomorphometry and visualization of long bone structure, femurs were scanned with a high-resolution μCT imaging system set to an effective detector pixel size of 0.008-mm. The software for 3D microstructure analysis was provided by eXplore MicroView version 2.2. Bone resorption marker was measured according to the manufacturer's directions: serum collagen-type I fragments (CTX-1) by RatLaps EIA (Immunodiagnostic Systems, Inc.).

### OSTEOCLASTOGENESIS

Bone marrow cells were isolated from 4- to 5-week-old C57BL/6 mice. Femora and tibiae were aseptically removed and dissected free of adherent soft tissue. The bone ends were cut, and the marrow cavity was flushed out with α-MEM (Life Technologies) from one end of the bone, using a sterile 21-gauge needle. The resulting bone marrow suspension was washed twice, and grown on plates with M-CSF (20 ng/ml) (R&D Systems, Inc.) for 16 h. Non-adherent cells were then removed and culture was continued for two more days, at which time large populations of adherent monocyte/macrophage-like cells had formed on the bottom of the culture dishes. The small numbers of non-adherent cells were removed by washing the dishes with PBS, and the adherent bone marrow-derived macrophages (BMM) were harvested and seeded in 48-well plates. Additional medium containing M-CSF and RANKL (40 ng/ml) (R&D Systems, Inc.) was added, and the medium was replaced on day 3. After incubation for the indicated times, the cells were fixed in 10% formalin for 10 min, and stained for tartrate-resistant acid phosphatase (TRAP) (Sigma Chemical Co.) as described [Shin et al., 2006]. Numbers of TRAP-positive multinucleated cells (MNC; three or more nuclei) were scored.

### MEASUREMENT OF GLUTATHIONE PEROXIDASE (GPX) ACTIVITY

BMM ($2 \times 10^6$ cells) were stimulated with RANKL and M-CSF under the indicated conditions. The Gpx activity of cell extracts was determined with a Gpx cellular activity kit (Sigma Chemical Co.).

### QUANTITATIVE PCR

Total RNA was reverse-transcribed with oligo-dT and Superscript I (Invitrogen). Quantitative PCR (qPCR) was carried out using SYBR Green 1 Taq polymerase (Qiagen) and appropriate primers on a DNA Engine Opticon Continuous Fluorescence Detection System (MJ Research, Inc.). The specificity of each primer pair was confirmed by melting curve analysis and agarose-gel electrophoresis. Relative copy numbers compared to GAPDH were calculated using the expression $2^{-\Delta\Delta C_t}$. The primer sequences used were as follows: 5′-gggactacaccgagatgaacga-3′ and 5′-accattcacttcgcacttctca-3′ (Gpx-1); 5′-agcctttcctactaccattccc-3′ and 5′-tggcactagagacggacaga-3′ (c-Fos); 5′-aataacatgcgagccatcatc-3′ and 5′-tcaccctggtgttcttcctc-3′ (nuclear factor of activated T cells 2, NFAT2); 5′-acccagaagactgtggatgg-3′ and 5′-cacattgggggtaggaacac-3′ (GAPDH).

### IMMUNOSTAINING OF NFAT2

BMM were stimulated on culture plates with M-CSF and RANKL for 0 and 72 h to generate OCs. The cells were fixed in 10% neutral buffered formalin and permeabilized with 0.1% Triton X-100. Immunofluorescence staining was performed using mouse anti-NFAT2 Ab (Santa Cruz Biotechnology) followed by FITC-conjugated anti-mouse IgG (eBioscience), and was determined using a fluorescence microscope (Carl Zeiss). Nuclei were stained with Hoechest (Sigma Chemical Co).

### WESTERN BLOTTING

Western blots were probed with Abs against the phosphorylated and total forms of ERK, JNK, and p38 (Cell Signaling), and then incubated with the appropriate peroxidase-conjugated secondary Ab (Santa Cruz Biotechnology).

### STATISTICAL ANALYSIS

Values are expressed as means ± SEM. Student's *t*-test was used to evaluate differences between samples of interest and the corresponding controls. Differences between groups were assessed by two-way ANOVA, followed by Bonferroni posttests. A *P*-value of less than 0.05 was considered statistically significant.

## RESULTS

### CURCUMIN REDUCES OVX-INDUCED BONE LOSS

To examine the effect of curcumin on OVX-induced bone loss, we used μCT to analyze femurs from OVX mice treated with curcumin or vehicle, and compared the effects with those of sham surgery. Bone mass was evaluated at the level of secondary spongia of distal femurs. μCT comparison of femurs revealed that administration of curcumin partially inhibited bone loss induced by OVX, but had no effect in the case of sham surgery (Fig. 1A). Curcumin-treatment of OVX mice led to an increase of trabecular bone mineral density (BMD) compared with control OVX mice (Fig. 1B; $P < 0.05$). A

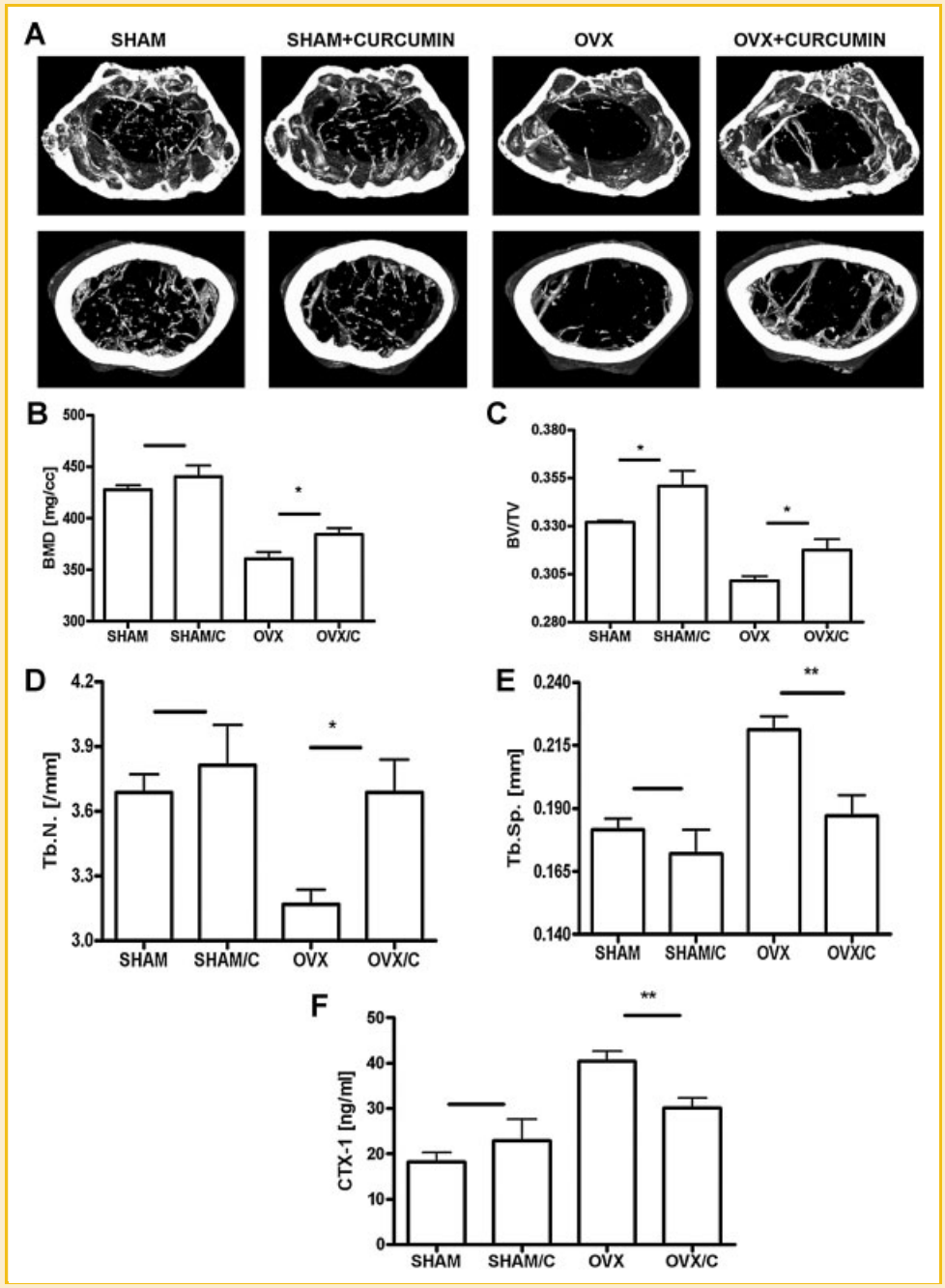


Fig. 1. Curcumin increases bone density in OVX mice. A–D: Bone density of the femurs of vehicle- (OVX, n = 7; sham, n = 6) and curcumin-administered (OVX, n = 7; sham, n = 5) mice 8 weeks after OVX and sham surgery. A: μCT images in upper and lower panels correspond to regions 0.7 and 2.3 mm, respectively, from the growth plates of distal femurs. B–E: Trabecular BMD, trabecular bone volume (BV/TV), Tb.N. (expressed/mm); mean number of trabeculae, and Tb.Sp. (expressed in mm); the distances between two trabeculae of distal femora were measured by μCT as described in Materials and methods. F: OC activity was determined by serum CTX-1. *, $P < 0.05$; **, $P < 0.01$ compared with the vehicle-treated group. Data are expressed as means ± SEM. Differences between groups were analyzed by two-way ANOVA, followed by Bonferroni posttests to compare the effect of curcumin (BMD and serum CTX-1; $P < 0.001$, effect of surgery. $P < 0.05$, effect of treatment. BV/TV and Tb.Sp.; $P < 0.001$, effect of surgery. $P < 0.01$, effect of treatment: Tb.N.; $P < 0.05$, effect of surgery. $P < 0.05$, effect of treatment).

similar pattern was found for trabecular bone volume (BV/TV) and trabecular number (Tb.N.) (Fig. 1C,D; $P < 0.05$). A decrease in trabecular separation (Tb.Sp.) was also observed (Fig. 1E; $P < 0.01$). However, curcumin had no significant effect on sham-operated mice except on BV/TV. Consistent with this, curcumin-treated OVX mice had reduced levels of serum collagen-type I fragments (CTX-1), which are markers of bone resorption (Fig. 1F; $P < 0.01$). OVX also increased body weight compared to sham surgery ($P < 0.001$), and curcumin treatment reduced this elevation of body weight in OVX mice ($P < 0.001$) (Table I), showing that the elevation of bone mass by curcumin was not due to any increase of body mass.

TABLE I. Body Weight Increase in Sham-Operated and OVX Mice With and Without Curcumin Treatment for 8 weeks [Unit: g]

| | Sham | OVX |
|---|---|---|
| Vehicle | 6.85 ± 0.24 | 12.22 ± 0.39 |
| Curcumin | 7.32 ± 0.40 | 7.79 ± 0.39 |

## CURCUMIN INHIBITS OSTEOCLASTOGENESIS BY ENHANCING GPX-1

To elucidate the mode of action of curcumin on bone metabolism, we assessed its effect on BMM, which are OC precursors. Curcumin inhibited OC formation from BMM in a dose-dependent manner (Fig. 2A,B), while total cell numbers were not significantly affected (Fig. 2C), suggesting that the inhibitory effect was not due to cytotoxicity. In addition, the inhibitory effect of curcumin increased with time (Fig. 2D), suggesting that RANKL sensitized the cells to curcumin.

To identify a possible inhibitory mediator, we tested whether curcumin increased the activity of glutathione peroxidase (Gpx), since Gpx-1 is the predominant antioxidant enzyme expressed by OCs upon stimulation with RANKL [Lean et al., 2005]. To determine if curcumin enhances mRNA levels of Gpx-1, we performed qPCR on total cellular mRNA. As shown in Figure 3A, Gpx-1 mRNA reached a maximum level after 24 h incubation with RANKL, and then decreased. However, addition of curcumin increased the expression of Gpx-1 by 29% and 345% after 24 and 48 h, respectively (Fig. 3A), suggesting that it sustains the level of expression of Gpx-1 induced by RANKL. Curcumin also increased Gpx activity by 31% and 49% after 0–48 and 49–96 h treatment, respectively (Fig. 3B). Next, we investigated whether curcumin restored Gpx levels under conditions in which Gpx activity was reduced. Homocysteine, a known generator of $H_2O_2$, directly activates OC formation [Koh et al., 2006] and reduces Gpx-1 activity in endothelial cells [Upchurch et al., 1997]. Homocysteine reduced RANKL-induced Gpx activity, and the addition of curcumin increased the residual activity by 48% ($P < 0.05$) (Fig. 3C). Curcumin also reduced the stimulatory effect of homocysteine on OC formation by 26% (Fig. 3D). Taken together, the data in Figure 3C,D indicate that the enhancement of Gpx-1 by curcumin is closely related to the reduction in OC formation.

## THE REDUCED OSTEOCLASTOGENESIS RESULTS FROM DEFECTIVE RANKL SIGNALING IN THE PRESENCE OF CURCUMIN

c-Fos and NFAT2 play critical and specific roles in OC differentiation induced by RANKL [Takayanagi et al., 2002; Teitelbaum, 2004]. We examined whether curcumin affected the activation of these RANKL-induced signaling pathways. Exposure of BMM to M-CSF

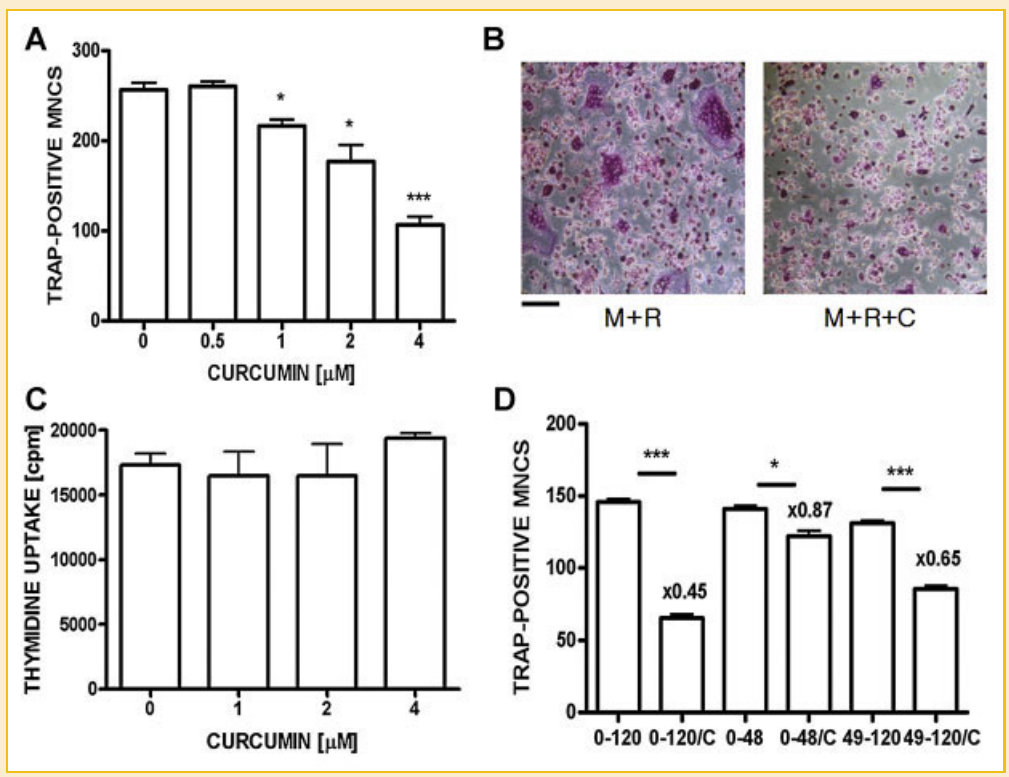


Fig. 2. Curcumin inhibits OC formation. A: BMM were incubated with curcumin (0, 0.5, 1, 2, 4 μM) for 3 days in the presence of M-CSF and RANKL. *, $P < 0.05$; ***, $P < 0.001$ compared with vehicle-treated cells. B: Representative photos of TRAP-positive MNC after treatment with 0 and 4 μM curcumin. Scale bar, 100 μm. C: For the final 18 h of 3 days culture BMM received 1 μCi/well [$^3$H]thymidine (NEN, Boston, MA). Cellular DNA was harvested and counted by liquid scintillation spectroscopy. No significant difference was found between curcumin-treated and non-treated BMM. D: BMM were incubated with curcumin (C; 0, 4 μM) in the presence of M-CSF and RANKL for 0–120, 0–48, and 49–120 h. The culture medium was replaced as indicated after 48 h. *, $P < 0.05$; ***, $P < 0.001$ compared with vehicle-treated cell, n = 3. Numbers above the histograms in (D) are ratios of the number of MNC formed in the presence of curcumin to the number formed in its absence. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

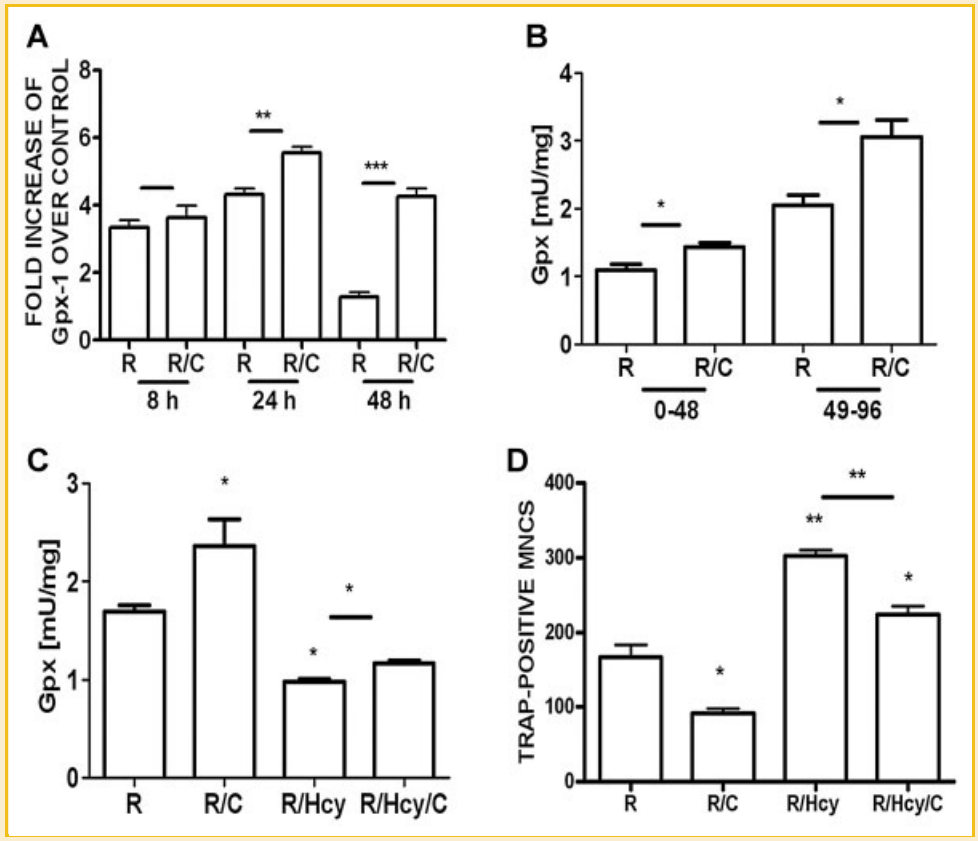


Fig. 3. Curcumin stimulates Gpx-1 induction by RANKL, and opposes the reduction of Gpx by homocysteine. A,B: BMM were stimulated with RANKL and M-CSF in the presence or absence of curcumin (4 μM). At the indicated times, cells were harvested for measurement of Gpx-1 mRNA by qPCR and Gpx activity. Expression before RANKL treatment was set at 1. C,D: BMM were stimulated with RANKL and M-CSF in the presence or absence of curcumin (4 μM) and/or homocysteine (100 μM) for 48 h to measure Gpx activity (C), and for 72 h to measure TRAP-positive MNCs (D). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with vehicle-treated cell. R: RANKL-treated, R/C: RANKL and curcumin-treated, R/Hcy: RANKL and homocysteine-treated, R/Hcy/C: RANKL, homocysteine, and curcumin-treated BMM.

and RANKL increased the expression of c-Fos and NFAT2, and this effect was greatly reduced by curcumin (Fig. 4A). We also examined the expression and cellular localization of NFAT2 after RANKL stimulation, using immunofluorescence staining with an anti-NFAT2 Ab (Fig. 4B). In the absence of RANKL, almost no NFAT2 staining was detectible (left lane) while, in response to RANKL, nuclear localization of NFAT2 was apparent at 72 h (middle lane). By contrast, treatment with curcumin resulted in fewer NFAT2-stained cells (right lane). Since RANKL also activates three mitogen-activated protein kinases (MAPK), p38, ERK, and JNK, by signaling through RANK in OC [Lee and Kim, 2003], we examined whether curcumin affected the activation of these MAPKs. RANKL induced activation of p38, ERK, and JNK after 10 min stimulation, and curcumin strongly inhibited this induction (Fig. 4C). Taken together, our data indicate that curcumin causes defective RANKL signaling by decreased expression of NFAT2 and impaired activation of MAPK.

## DISCUSSION

We showed above that exposure to curcumin reduced OVX-induced bone loss in vivo. Curcumin opposed the substantial reduction in femoral bone density and the elevation of CTX-1 observed after OVX. We have not established whether it affects bone formation after OVX, since OVX causes a marked stimulation of bone resorption as well as a compensatory augmentation of bone formation, although the latter is not enough to compensate for the elevated bone resorption [Pacific, 2007]. OVX increased body weight dramatically [Kim et al., 2010], and curcumin protected against this effect, so eliminating the possibility that the elevated bone mass is due to increased body mass. The mechanism by which curcumin opposes the increase in body weight following OVX is now under investigation.

Bone is a dynamic tissue, involving a balance between formation of new bone by osteoblasts and resorption of old bone by OC [Goltzman, 2002]. Postmenopausal osteoporosis may be an example of imbalance, with bone resorption predominating over bone formation [Rodan, 1991]. Since OCs are primarily responsible for bone resorption, it seems that the increase in bone density brought about by curcumin after OVX is due to inhibition of osteoclastogenesis. This inhibition has been ascribed to several different mechanisms. Curcumin is reported to inhibit OC formation from OC precursors by blocking NF-κB activation [ harti et al., 2004], to stimulate apoptosis of mature OC [Ozaki et al., 2000], and to inhibit osteoclastogenesis by decreasing

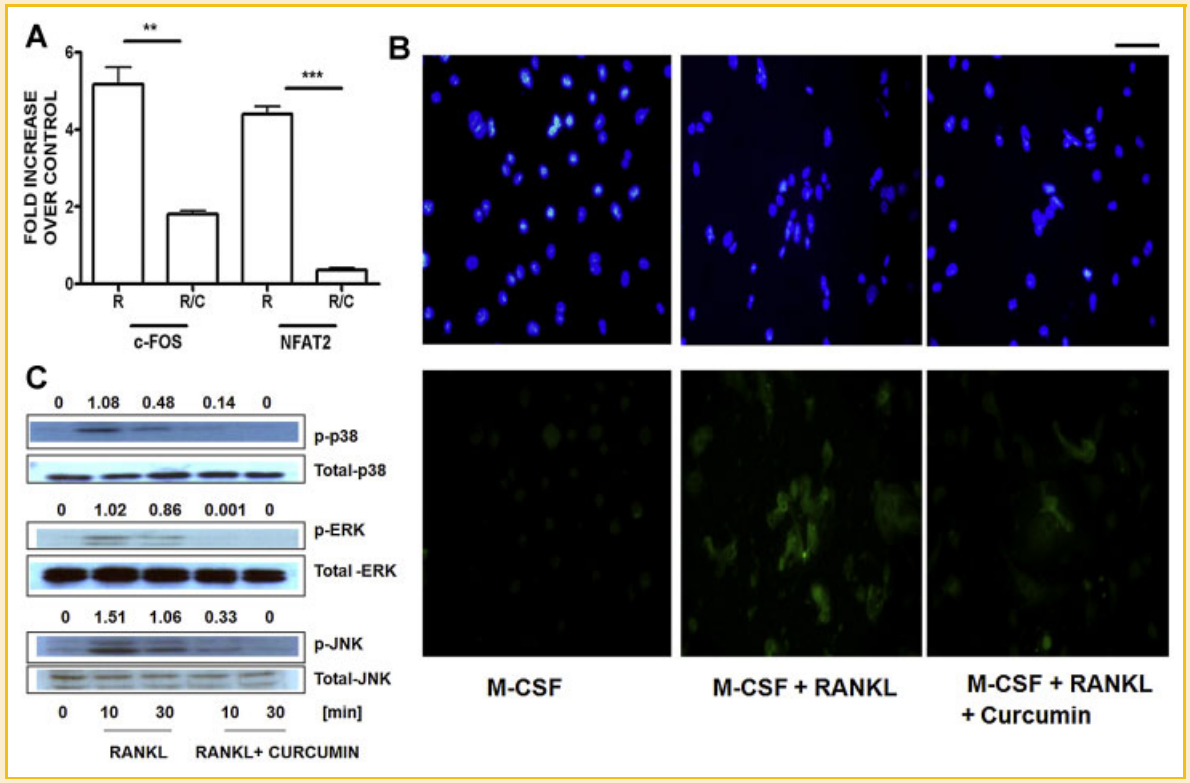


Fig. 4. Curcumin impairs RANKL signaling by reduced expression of NFAT2 and attenuated activation of MAPK. A: BMM were incubated with M-CSF and RANKL in the presence and absence of curcumin (4 μM) for 48 h. Total RNA was extracted and subjected to qPCR for c-Fos and NFAT2. **, $P < 0.01$; ***, $P < 0.001$ compared with vehicle-treated cells. B: BMM were incubated for 72 h in the presence of M-CSF along with vehicle (left panel), RANKL (middle panel), and RANKL plus curcumin (right panel). To evaluate multinucleated cells with the morphologic features of OCs, the cells were stained with anti-NFAT2 Ab and Hoechst. Nuclear localization of NFAT2 was observed at 72 h (middle panel). OC precursors showed little staining of NFAT2 (left panel) and curcumin reduced the number of multinucleated cells stained with NFAT2 (right panel). Scale bar, 200 μm. C: Cytokine- and serum-starved BMM were exposed to RANKL (60 ng/ml). Phosphorylated and total forms of p38, ERK, and JNK were detected by Western blots. Numbers above the box in (C) are ratios of the intensity of the phosphorylated form to that of total enzyme in each case.

RANKL production by bone marrow stromal cells [Oh et al., 2008].

RANKL stimulates BMM to generate ROS, which act as signals for osteoclastogenesis [Lee et al., 2005]. It is likely that curcumin modulates ROS levels by increasing the content of antioxidant enzymes in OC. Indeed we observed that curcumin elevated the activity and the expression of the antioxidant Gpx-1 upon RANKL stimulation, thus augmenting its inhibitory effect on osteoclastogenesis. Gpx-1 is a highly expressed antioxidant enzyme responsible for the intracellular degradation of ROS during the formation of OC, and its transcript levels exceed significantly those of any other antioxidant [Lean et al., 2005]. The induction of Gpx-1 by RANKL could be an antioxidant defense mechanism induced in response to ROS. Over-expression of Gpx-1 in macrophages, the precursors of OC, results in inhibition of OC formation [Lean et al., 2005], suggesting that this could be the mechanism by which curcumin suppresses OC differentiation. Gpx-1 plays a central role in protecting cells from ROS [Galasso et al., 2006]. We also demonstrated that curcumin inhibited the enhanced osteoclastogenesis caused by homocysteine by modulating Gpx levels. Homocysteine is a key intermediate in methionine metabolism, and an increase in its level is associated with osteoporosis [Herrmann et al., 2005], due to enhanced bone resorption resulting from the production of intracellular ROS [Koh et al., 2006]. Hyperhomocysteinemia, which is often associated with aging, also results in bone loss [McLean et al., 2004]. Intracellular Gpx activity and its transcripts are reduced in endothelial cells by treatment with homocysteine [Upchurch et al., 1997]. We showed that exogenously added homocyteine opposed the increase of Gpx activity in OC upon RANKL stimulation. However, co-treatment with curcumin blocked the decrease of Gpx activity caused by homocysteine, confirming that curcumin elevates Gpx.

Our data also showed that the inhibitory effect of curcumin on osteoclastogenesis resulted from aberrant RANKL signaling due to reduced activation of MAPK and decreased expression of NFAT2. The reduced expression of NFAT2 by curcumin has been shown to occur at both the transcriptional and translational levels, but it is not clear whether curcumin inhibits the translocation of NFAT2. An effect of curcumin on NFAT2 activation has not been reported, but blockade of NFAT activation by antioxidants has been demonstrated in several other cell types. *N*-Acetyl-L-cysteine (a general antioxidant) and catalase (a specific $H_2O_2$ inhibitor) prevent the vanadium-induced activation of NFAT mediated by $H_2O_2$ in fibroblasts [Huang et al., 2001], and adenovirally over-expressed Gpx-1 and manganese superoxide dismutase inhibit doxorubicin-induced nuclear NFAT translocation in rat cardiac cells [Kalivendi et al., 2005].

Decreased activation of MAPK by curcumin has been also demonstrated in other situations. For example it protects cardiac cells from ischemia-reperfusion-induced cell death by attenuating JNK activation [Fiorillo et al., 2008], and suppresses adipocyte differentiation by inhibiting MAPK [Ahn et al., 2010].

OVX stimulates accumulation of ROS [Lean et al., 2003], lowers Gpx content in OC [Lean et al., 2005], and results in bone loss, although it is not clear just how oxidative stress causes bone loss. Curcumin has been shown to be a more potent antioxidant than vitamin E [Aggarwal and Sung, 2008] and this could be explained by the finding that curcumin has the ability to scavenge enolic forms of free radicals [Shen and Ji, 2007]. Alternatively, curcumin could induce antioxidant enzymes. It has not been clearly established whether curcumin affects bone metabolism in vivo, although it inhibits osteoclastogenesis in vitro [Ozaki et al., 2000; Bharti et al., 2004; Oh et al., 2008]. Recently several groups have reported a possible bone-sparing effect of curcumin [French et al., 2008; Hie et al., 2009]. Urinary deoxypyrimidinoline, a marker for bone loss, was significantly reduced by orally administered curcumin in streptozotocin-induced diabetic rats [Hie et al., 2009], and bone density tended to increase in response to curcumin in a rat-OVX model, although the effect did not reach statistical significance [French et al., 2008]. The current study extends these findings [French et al., 2008; Hie et al., 2009] by offering a mechanism to help explain the reduced bone loss associated with curcumin administration. We used a mouse-OVX model which displays marked loss of the trabecular bone [Kim et al., 2010], and our data clearly showed that curcumin improves trabecular femur BMD and other bone parameters (BV/TV, Tb.N., and Tb.Sp.) which have been associated with bone loss after OVX. In addition, curcumin showed a (non-significant) tendency to increase BMD and Tb.N. and to decrease Tb.Sp. in sham-operated mice, suggesting that it also affects physiological bone remodeling. Our demonstration of an inhibitory effect of curcumin on osteoclastogenesis due to up-regulation of an antioxidant enzyme and defective RANKL signaling suggests a therapeutic rationale for the use of curcumin to reduce bone loss due to estrogen deficiency.

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