Vitamin K2 stimulates osteoblastogenesis and suppresses osteoclastogenesis by suppressing NF-κB activation

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Abstract. Several bone protective factors are reported to exhibit stimulatory activities on bone formation coupled with inhibitory effects on bone resorption; one such factor is vitamin K2. Vitamin K species [K1 (phylloquinone) and K2 (menaquinone)] have long been associated with bone protective activities and are receiving intense interest as nutritional supplements for the prevention or amelioration of bone disease in humans. However, the mechanisms of vitamin K action on the skeleton are poorly defined. Activation of the nuclear factor κB (NF-κB) signal transduction pathway is essential for osteoclast formation and resorption. By contrast, NF-κB signaling potently antagonizes osteoblast differentiation and function, prompting us to speculate that NF-κB antagonists may represent a novel class of dual anti-catabolic and pro-anabolic agents. We now show that vitamin K2 action on osteoblast and osteoclast formation and activity is accomplished by down-regulating basal and cytokine-induced NF-κB activation, by increasing IκB mRNA, in a γ-carboxylation-independent manner. Furthermore, vitamin K2 prevented repression by tumor necrosis factor α (TNFα) of SMAD signaling induced by either transforming growth factor ß (TGFß) or bone morphogenetic protein-2 (BMP-2). Vitamin K2 further antagonized receptor activator of NF-κB (RANK) ligand (RANKL)-induced NF-κB activation in osteoclast precursors. Our data provide a novel mechanism to explain the dual pro-anabolic and anti-catabolic activities of vitamin K2, and may further support the concept that pharmacological modulation of NF-κB signal transduction may constitute an effective mechanism for ameliorating pathological bone loss and for promoting bone health.

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Introduction

Numerous studies have demonstrated that nuclear factor κB (NF-κB) signal transduction is a critical pathway for osteoclast development, function and survival (1-5), a concept strongly supported by the development of osteopetrosis in p50 and p52 NF-κB double knockout mice, a consequence of defective osteoclast differentiation (6). Additional support for a role of NF-κB in osteoclast formation and activity comes from studies showing that the NF-κB subunit RelB plays a key role in osteoclast differentiation (4), while the p65 NF-κB subunit prevents JNK-mediated apoptosis during receptor activator of NF-κB ligand (RANKL)-induced commitment to the osteoclast phenotype (3). Furthermore, pharmacological agents known to block NF-κB signaling appear to inhibit osteoclast activation (2). Consistent with these data we recently reported that suppression of NF-κB signal transduction using a pharmacological inhibitor blunted osteoclastic bone resorption and ameliorated bone loss induced by ovariectomy in mice (7). Other studies have demonstrated the utility of pharmacological NF-κB suppression to inhibit osteoclast formation and activity associated with multiple myeloma *in vitro* (8) and in an animal model of rheumatoid arthritis *in vivo* (9).

In contrast to the stimulatory effects of NF-κB signaling in osteoclast formation, the p65 NF-κB subunit is reported to inhibit vitamin D-stimulated osteocalcin transcription in osteoblastic cells (10). The potent NF-κB-inducer tumor necrosis factor α (TNFα) is well established to suppress osteoblast formation though multiple poorly understood actions (11,12) and pharmacological suppression of TNFα has been reported to reverse age-related defects in bone formation in a mouse fracture-healing model (13).

Bone morphogenetic proteins (BMPs) stimulate bone formation as they are potent inducers of osteoblast differentiation and angiogenesis (14), while transforming growth factor ß (TGFß) is thought to play important roles in early osteoblast lineage commitment (15), and in the coupling of osteoclasts and osteoblasts as TGFß released during bone resorption induces migration of bone marrow

stromal cells to bone resorptive sites, through a SMAD-dependent signaling pathway (16). We have previously demonstrated that one mechanism by which TNFα suppresses osteoblast differentiation is through NF-κB-mediated antagonism of TGFß- and BMP-2-induced SMAD signaling in differentiating osteoblasts (17). These data are consistent with the demonstration that NF-κB signaling up-regulates SMAD7, an inhibitor of SMAD activation in osteoblastic cells (18) and that TNFα promotes systemic bone loss by promoting proteasomal degradation of bone morphogenetic signaling proteins through up-regulation of SMAD ubiquitination regulatory factor 1 (Smurf1) (19). Recently, a direct inhibitory action of NF-κB on bone formation has been ratified *in vivo* where time- and stage-specific inhibition of the inhibitor of κB kinase (IKK) in differentiated osteoblasts increased trabecular bone mass and bone mineral density (BMD) and ameliorated ovariectomy-induced bone loss by promoting a compensatory increase in bone formation (20).

We recently suggested that pharmacological suppression of NF-κB activation may represent a novel mechanism to enhance bone mass and/or ameliorate pathological bone loss by simultaneously stimulating bone formation and suppressing bone resorption (17). In fact, several known bone protective factors are reported to exhibit such dual pro-anabolic and anti-catabolic effects. The estrogen, 17ß-estradiol is one such agent, and we recently demonstrated that one mechanism by which 17ß-estradiol, but not the closely related phytoestrogen, genistein, promotes bone anabolism is by antagonizing osteoblastic NF-κB activation (21).

Another factor possessing both anti-osteoclastogenic and pro-osteoblastic attributes is vitamin K (22,23). There are two types of natural vitamin K: vitamin K1 (phylloquinone) and vitamin K2 (menaquinone). While vitamin K1 is a single compound widely distributed in green leafy vegetables and vegetable oils, vitamin K2 is found at low doses in animal products, and in high abundance in legumes and fermented soybean products. Vitamin K2 represents a series of isoprenoid unsaturated side-chains of various lengths, with isoprene units varying from 1-14 and is approved in Japan for the treatment of osteoporosis (24,25). The commonly used nutritional supplement menaquinone-4 (MK-4) contains four isoprene units while menaquinone-7 (MK-7) contains 7 (24).

Vitamin K species (both K1 and K2) have long been associated with bone protective activities and are now receiving intense interest as supplements for the prevention or amelioration of bone disease (26). However, the molecular mechanisms of vitamin K2 action on bone cells are poorly defined. Vitamin K is known to support γ-carboxylation of specific glutamic acid residues conferring calcium binding properties to vitamin K-dependent proteins (26). Several protein targets of vitamin K are osteoblastic proteins and components of the bone matrix including osteocalcin, matrix Gla protein, protein S, and Gas 6 (27,28). Under-carboxylated osteocalcin has been reported to correlate with BMD and hip fracture risk in elderly women (29,30), while vitamin K treatment effectively reduces under-carboxylated osteocalcin (31). The association between vitamin K levels and the osteocalcin carboxylation state has led to the suggestion that the primary mechanism underlying the protective influence of vitamin K on bone may involve carboxylation of osteocalcin (26). However, recent clinical data contrast with this notion and suggest that vitamin K treatment at doses capable of correcting under-carboxylated osteocalcin in healthy post-menopausal women do not correlate with altered bone turnover, density, or geometry (31). Consequently, a direct cause-effect relationship between osteocalcin γ-carboxylation and effects of vitamin K on bone mass remains unclear. Furthermore, the mechanism for the anti-osteoclastogenic properties of vitamin K are also poorly defined, although it has been suggested that the geranylgeranyl-like side chain on vitamin K2 species may induce apoptosis of osteoclasts *in vitro* (32) or may act by down-regulating protein kinase C (22).

In this study we demonstrate that the capacity of vitamin K2 to stimulate osteoblast differentiation and mineralization, and suppress osteoclast formation and resorption, may be explained by vitamin K2's tendency to suppress basal and cytokine induced-NF-κB activation, though stimulation of IκBα mRNA production in a γ-carboxylation-independent manner. Our data provide a unified mechanism, centered on NF-κB antagonism, to explain both the anti-resorptive and anabolic actions of vitamin K2 species.

Materials and methods

Materials. α-minimal essential medium (α-MEM) and antibiotics (penicillin and streptomycin) were purchased from Invitrogen Corporation (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Hyclone. MK-7 was from J-Oil Mills (Tokyo, Japan). RANKL, M-CSF, TGFß, TNFα, and BMP-2 were from R&D Systems (Minneapolis, MN). Antibodies for Western blot analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). MK-4, vitamin K1, the mouse anti-poly-histidine antibody, warfarin and all other reagents were purchased from Sigma Chemical Corp., (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 previously described (17,33).

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 ß-glycerophosphate] as previously described (17,34). MK-7 or vehicle were added at a dose of 1 or 10 μM and cells 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 Image J (35).

Osteoclastogenesis assays and tartrate resistant acid phosphatase (TRAP) staining. RAW264.7 cells or primary

mouse monocytes isolated from spleen by adherence to plastic were cultured in 96-well plates in α-MEM supplemented with 10% FBS and 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 cross-linking anti-poly-histidine antibody (2.5 μg/ml) to induce osteoclast formation. In addition, primary monocytes also received M-CSF (25 ng/ml). The dose range of MK-7 added was 0.01-10 μM. After 6 days of culture, the cells were fixed and stained for TRAP activity using a leukocyte acid phosphatase kit (Sigma). $TRAP^+$ cells with three or more nuclei were defined as osteoclasts and were counted under a microscope and 5 wells/group were averaged. Representative wells were photographed under bright field microscopy using a Nikon Eclipse TE2000-S inverted microscope. Images were captured using a digital camera (QImaging Corp., Burnaby, BC, Canada).

Osteoclast activity assays. Resorption was assessed on dentine discs according to the manufacturer's directions (Immunodiagnostic Systems Inc., Scottsdale, AZ). Briefly, mature osteoclasts from primary mouse monocytes were cultured with RANKL and M-CSF as described above, on dentine discs. Osteoclast formation was monitored by light microscopy. MK-7 (10 μM) or vehicle were added after the development of mature cells (~1-2 weeks). Extensive resorption occurs during the third and fourth weeks of culture. Cells were dissociated with 6% bleach for 15 min, followed by 3 washes in water. Resorption pits were visualized by staining with 1% toluidine blue in 0.5% sodium tetraborate solution and digitally photographed under bright field microscopy at x100 magnification.

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 by us (17). The SMAD reporter is responsive to both TGFß- and to BMP-induced SMADs. Briefly, reporter plasmids or empty vector control (pGL3-Basic) were transfected into MC3T3 or RAW264.7 cells ($1x10^5$ cells/well) using the Lipofectamine 2000 reagent (Invitrogen) in α-MEM without FBS or antibiotics. Five hours later the medium was changed to α-MEM containing 10% FBS plus antibiotics and cells were treated with TNFα (MC3T3) or RANKL (RAW264.7) to stimulate NF-κB activity, or with TGFß (1 ng/ml) or BMP-2 (0.5 μg/ml) to stimulate SMAD activity and treated with or without TNFα. Some cultures received warfarin (20 μM) in order to suppress γ-carboxylation activity as previously reported (36). Parallel groups received vehicle or MK-7 (0.01-10 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, USA). Equal transfection efficiency was validated for all plasmids using the *Renilla* luciferase reporter plasmid pRL-SV40 (Promega).

Western blot analysis. Cells were plated in 35 mm wells at a density of 10^6 cells/well in 2 ml of medium (α-MEM + 10% FBS and antibiotics) and cultured for 24 h prior to addition of MK-7 (10 μM) for an additional 24 h, followed by addition of TNFα (10 ng/ml) for 180 min. Cultures were subsequently lysed for preparation of cytosolic and nuclear extracts as previously described (37). Samples 30 μg protein/lane were separated by SDS-PAGE and transferred to nylon membranes for Western blotting using antibodies against p65, IκBα and phospho-specific IκBα (P-IκBα). Loading controls consisted of ß-actin for cytosolic proteins, or proliferating cell nuclear antigen (PCNA) for nuclear proteins. A minimum of 3 blots from independent experiments were scanned on an Epson Perfection 1660 Photo scanner and band intensity was measured using Image J (35). Data from independent experiments were normalized to the respective control before averaging.

Real-time RT-PCR for runt-related transcription factor 2 (Runx2), Osterix, and IκBα mRNA quantitation. Real-time RT-PCR for Runx2 and Osterix was performed on an ABI Prism 7000 instrument (Applied Biosystems, Foster City, CA). IκBα was quantitated using a commercial Applied Biosystems TaqMan Gene Expression Assay probe and primer set (Mm00477798_m1). Changes in relative gene expression following MK-7 stimulation were calculated using the $2^{-\Delta\Delta CT}$ method and normalized to 18S rRNA.

Statistical analysis. Statistical significance was determined using 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 Tukey-Kramer multiple comparisons post test for parametric data, or Kruskal-Wallis post test for non-parametric data, as indicated. $P<0.05$ was considered statistically significant.

Results

MK-7 augmented the differentiation of MC3T3 and primary bone marrow stromal cells into mineralizing osteoblasts and induced expression of Runx2 and Osterix. MK-7 has been reported to promote expression of alkaline phosphatase and Runx2 in MC3T3 preosteoblastic cells (38) and to stimulate mineral accretion in rat bone tissues *in vitro* (39). However, the molecular mechanisms by which MK-7 acts on osteoblasts remain unclear. To validate the MC3T3 model for investigation of MK-7 action we treated MC3T3 cells with MK-7 (1 or 10 μM) in mineralizing medium for 17 days and stained for calcium deposition with Alizarin Red-S. The data show (Fig. 1A and B) that MK-7 augmented the differentiation of MC3T3 cells into mineralizing osteoblasts. Using real-time RT-PCR we further demonstrated that MK-7 potently induced expression of Runx2 (Fig. 1C) and Osterix (Fig. 1D) in MC3T3 cells within 6 h of stimulation before declining, although expression of both genes remained significantly elevated at 24 h.

We further confirmed an identical pro-osteoblastogenic effect of MK-7 on primary mouse bone marrow stromal cell differentiation (Fig. 1E and F), confirming the MC3T3 cell line response as representative of primary cells and ratifying its use for further detailed mechanistic studies.

TNFα is an inhibitor of osteoblastic differentiation *in vivo* and *in vitro* (12,17). Interestingly, we found that MK-7 was

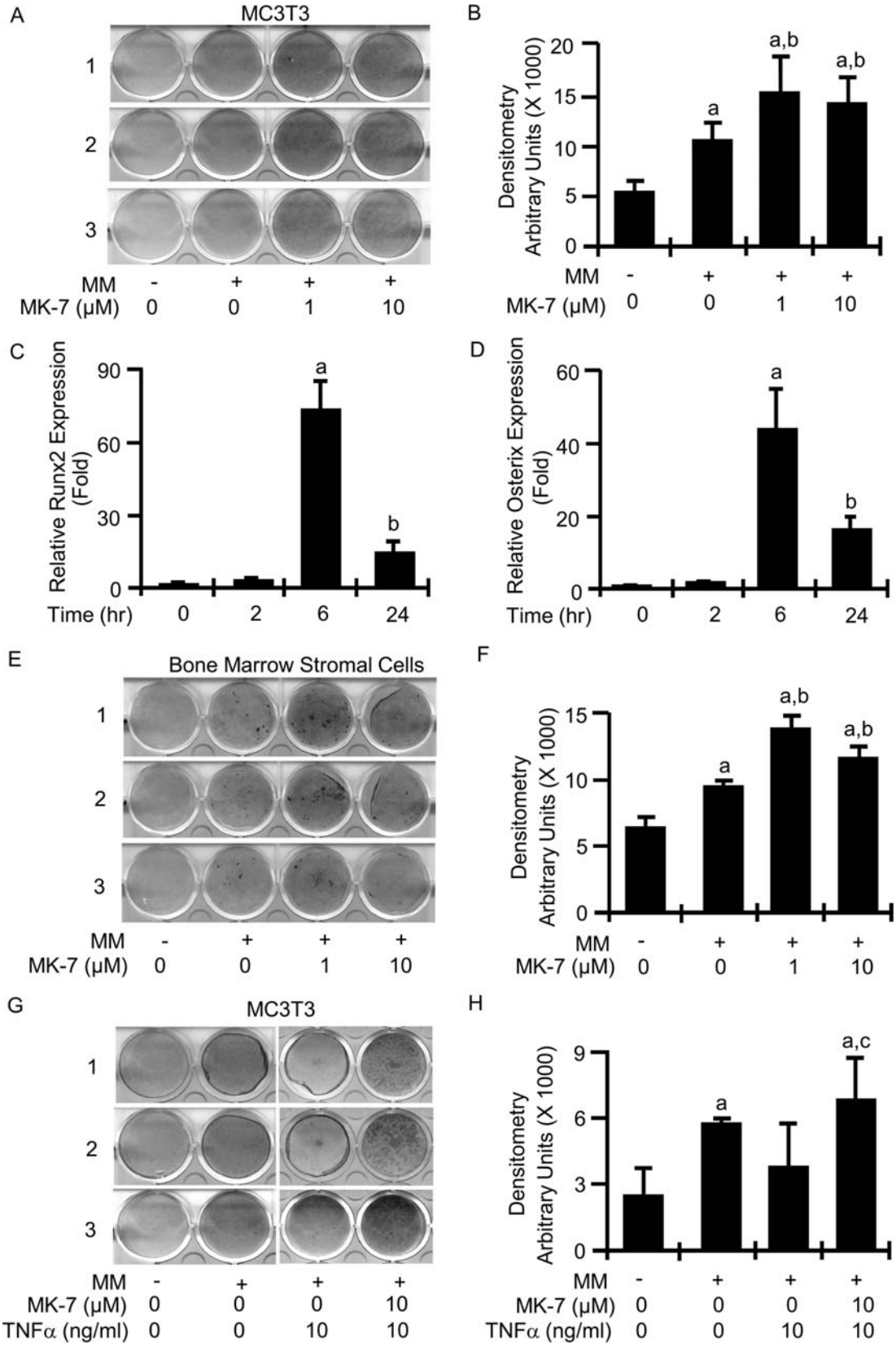

Figure 1. Vitamin K2 promotes osteoblast differentiation and mineralization *in vitro*. (A) MC3T3 cells were cultured in the presence (+) or absence (-) of mineralizing medium (MM) with MK-7 (0, 1 or 10 μM) and cultures were stained with Alizarin Red-S at 18 days for visualization of calcium deposition. (B) Mineralization quantitated by densitometry. Real-time RT-PCR was performed for (C) Runx2 and (D) Osterix expression in MC3T3 cells at 2, 6 and 24 h following addition of MK-7 (10 μM). (E) Primary bone marrow stromal cells were cultured in the presence (+) or absence (-) of MM with MK-7 (0, 1 or 10 μM) and cultures were stained with Alizarin Red-S at 18 days for visualization of calcium deposition. (F) Mineralization quantitated by densitometry. (G) MC3T3 cells were cultured in the presence (+) or absence (-) of MM with (+) or without (-) TNFα (10 ng/ml), and with MK-7 (10 μM) or vehicle and were stained with Alizarin Red-S at 17-18 days. (H) Mineralization quantitated by densitometry. In panel G, each pair of wells were derived from non-contiguous regions on the same plate for each independent experiment (1, 2 and 3) and separated by a white line to indicate this fact. All densitometry represents the mean ± SD of at least 3 independent cultures. For Panels B, F and H; [a]P<0.05 vs. MM (-), [b]P<0.05 vs. MM (+) MK-7 (0 μM), [c]P<0.05 vs. MK-7 (0 μM) TNFα (10 μM). For panels C and D; [a]P<0.01 vs. 0 time, [b]P<0.05 vs. 0 time. One-way ANOVA with Tukey-Kramer post test.

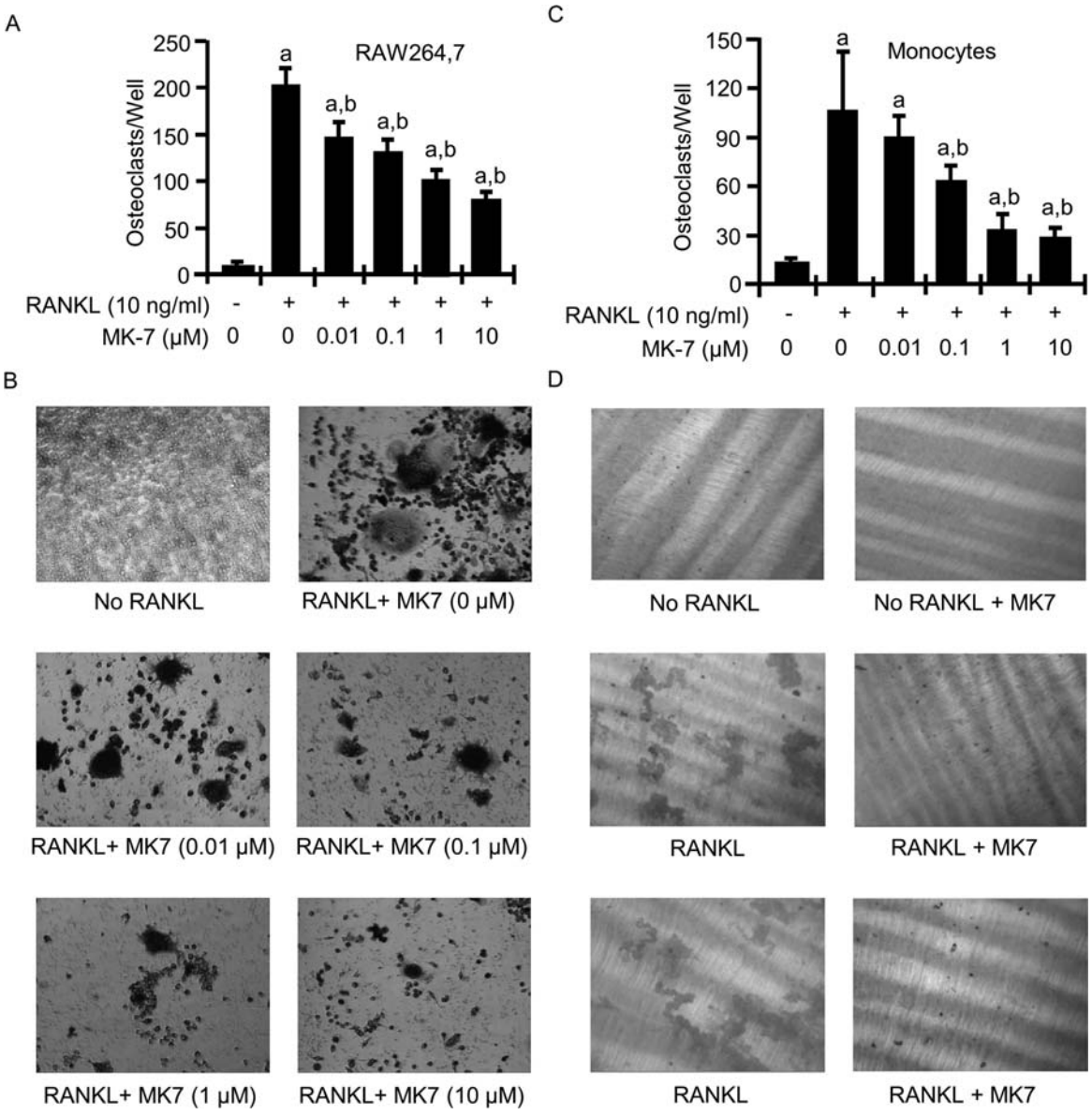

Figure 2. Vitamin K2 suppresses osteoclast differentiation and resorption *in vitro*. (A) RAW264.7 cells or (B) purified primary mouse splenic monocytes were treated with RANKL (10 ng/ml) in the presence or absence of MK-7 (0, 0.01, 0.1, 1 or 10 μM) for 7 days and stained with TRAP. TRAP$^+$ multinucleated cells (3 or more nuclei) were counted and averaged for 5 independent wells for each data point. Data are representative of 3 independent experiments. (B) A representative field for each condition presented in panel A was photographed under bright field microscopy at x200 magnification. (C) Osteoclasts were generated from primary mouse monocytes with RANKL and M-CSF and quantitated as in (A). [a]P<0.01 relative to unstimulated (no RANKL or MK-7); [b]P<0.01 relative to RANKL treated only (no MK-7); by one-way ANOVA with Tukey-Kramer post test. (D) Osteoclasts were generated from primary mouse monocytes with RANKL and M-CSF on dentine discs followed by addition of MK-7 (10 μM) or vehicle. Resorption pits were visualized by staining with toluidine blue and digitally photographed under bright field microscopy at x100 magnification. Pits from 2-4 representative disks are shown for RANKL and RANKL + MK-7.

capable of ameliorating the suppressive action of TNFα on MC3T3 mineralization (Fig. 1G and H). As we have previously reported that basal TNFα concentrations are of a sufficient magnitude to effectively suppress basal bone formation in mice (17), the data suggest that MK-7 may increase basal bone formation *in vivo* by antagonizing TNFα signaling.

MK-7 dose dependently inhibits osteoclast formation by RAW264.7 cells and primary mouse monocytes and suppresses resorptive activity. In contrast to its stimulatory activity on osteoblasts, MK-7 is reported to inhibit osteoclastic bone resorption *in vitro* (22). To establish a model for mechanistic investigations of MK-7 action on *in vitro* osteoclastogenesis, we treated RAW264.7 osteoclast precursors with RANKL for 7 days and quantitated the formation of osteoclasts [TRAP$^+$ multinucleated cells (3 or more nuclei)], in the presence of MK-7 (0, 0.01, 0.1, 1 or 10 μM). MK-7 dose-dependently inhibited osteoclast formation from RAW264.7 cells (Fig. 2A). Photomicrographs of representative fields from the TRAP-stained cultures are shown in Fig. 2B. Identical results were observed when primary mouse monocytes were differentiated into osteoclasts by stimulation with M-CSF and RANKL (Fig. 2C). Finally, we examined the effect of MK-7 on osteoclast resorptive activity *in vitro*, by culturing mature

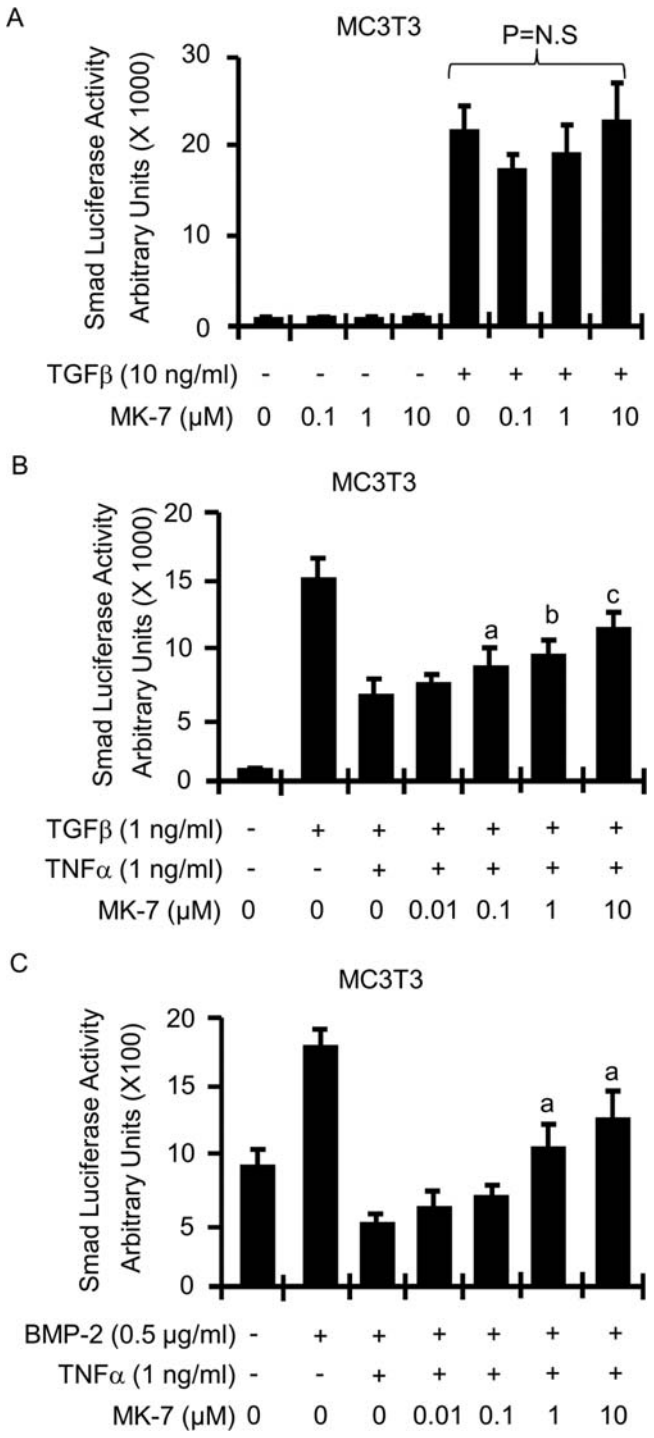

Figure 3. Effect of Vitamin K2 on TGFß and BMP-2-induced SMAD activation in osteoblast precursors in the presence or absence of TNFα. MC3T3 cells were transfected with pGL3-SMAD, a luciferase SMAD activity reporter. (A) The effect of MK-7 (0, 0.1, 1 or 10 μM) on basal or TGFß (10 ng/ml)-induced SMAD activity was quantitated 24 h later. P, not significant (NS) vs. TGFß only (no MK-7) for all data points located within the brackets. (B) SMAD activity was induced with TGFß (10 ng/ml) and cultures were treated with TNFα (10 ng/ml), a SMAD activation inhibitor, in the presence or absence of MK-7 (0, 0.01, 0.1, 1 or 10 μM). SMAD-induced luciferase activity was quantitated 24 h later. [a]P<0.05, [b]P<0.01, [c]P<0.001 relative to TGFß + TNFα (no MK-7). (C) SMAD activity was induced with BMP-2 (0.5 μg/ml) and cultures treated with TNFα (10 ng/ml) in the presence or absence of MK-7 (0, 0.01, 0.1, 1 or 10 μM). SMAD-induced luciferase activity was quantitated 24 h later. [a]P<0.001 relative to BMP-2 + TNFα (no MK-7). For all panels the data represent mean ± SD of five transfections/data point and are representative of 3 or more independent experiments. All statistics in this figure were performed using one-way ANOVA with Kruskal-Wallis post test.

osteoclasts from purified mouse monocytes on dentin discs and then treating with MK-7 (10 μM). MK-7 almost completely abrogated the formation of resorption lacunae (Fig. 2D).

MK-7 antagonizes the inhibitory effect of TNFα on TGFβ- and BMP-2-induced SMAD activation. TGFß is an important early commitment factor for mesenchymal stem cell (MSC) differentiation along an osteoblastic trajectory (15) and for inducing migration of MSCs to bone resorptive sites (16). By contrast, BMPs are potent inducers of osteoblast differentiation (40). The SMAD signaling pathway is a major mechanism by which both TGFß and BMPs mediate their effects on osteoblasts. To investigate if MK-7 regulates SMAD signaling in preosteoblasts we transfected MC3T3 cells with a SMAD responsive luciferase reporter. Our data show that although MK-7 has no additive or synergistic effects on TGFß-induced SMAD activation (Fig. 3A), it strongly antagonized the inhibitory effect of TNFα on TGFß-induced (Fig. 3B) and BMP-2-induced (Fig. 3C) SMAD activation.

MK-7 suppresses basal and cytokine-induced NF-κB activity in MC3T3 and RAW264.7 cells. We (17) and others (18) have reported that TNFα-induced NF-κB signaling antagonizes SMAD activation. Consequently, we next investigated whether MK-7 antagonizes the inhibitory action of TNFα on SMAD activation, and/or inhibits RANKL-induced NF-κB activation in osteoclast precursors. MC3T3 and RAW264.7 cells were transiently transfected with an NF-κB luciferase reporter and basal or cytokine (TNFα or RANKL) stimulated NF-κB activity was quantitated in the presence or absence of MK-7. Our data reveal that MK-7 significantly and dose-dependently (0.01-10 μM) suppressed NF-κB activity induced by TNFα in MC3T3 cells (Fig. 4A) or by RANKL in RAW264.7 cells (Fig. 4B). In addition, MK-7 weakly, but significantly, suppressed basal NF-κB activity in MC3T3 (Fig. 4C) and RAW264.7 cells (Fig. 4D).

MK-4 inhibits NF-κB, but with lower affinity than MK-7, while vitamin K1 displays no detectable anti-NF-κB activity. As the MK-4 derivative of vitamin K2, is widely available as a nutritional supplement and has been the subject of several clinical studies we examined whether MK-4 mediated a similar inhibitory activity on NF-κB activation as MK-7. MK-4 suppressed NF-κB activation by TNFα in MC3T3 cells, but in contrast to MK-7 this effect was only significant at a dose of 10 μM, 10-fold higher than that of MK-7 (Fig. 4E). Finally, as both vitamin K1 and K2 are reputed to have protective effects on bone turnover and are often considered interchangeable, we examined the effect of vitamin K1 on basal and TNFα-induced NF-κB activity in MC3T3 osteoblast precursors. In contrast to vitamin K2 species (MK-7 and MK-4), vitamin K1 displayed no tendency to suppress NF-κB activation at comparable doses (Fig. 4F), suggesting a significantly reduced potency relative to K2 species, or a different mechanism of action on bone cells.

MK-7 suppresses NF-κB activation by a γ-carboxylation-independent mode of action. It is widely accepted that the major action of vitamin K species is to γ-carboxylate

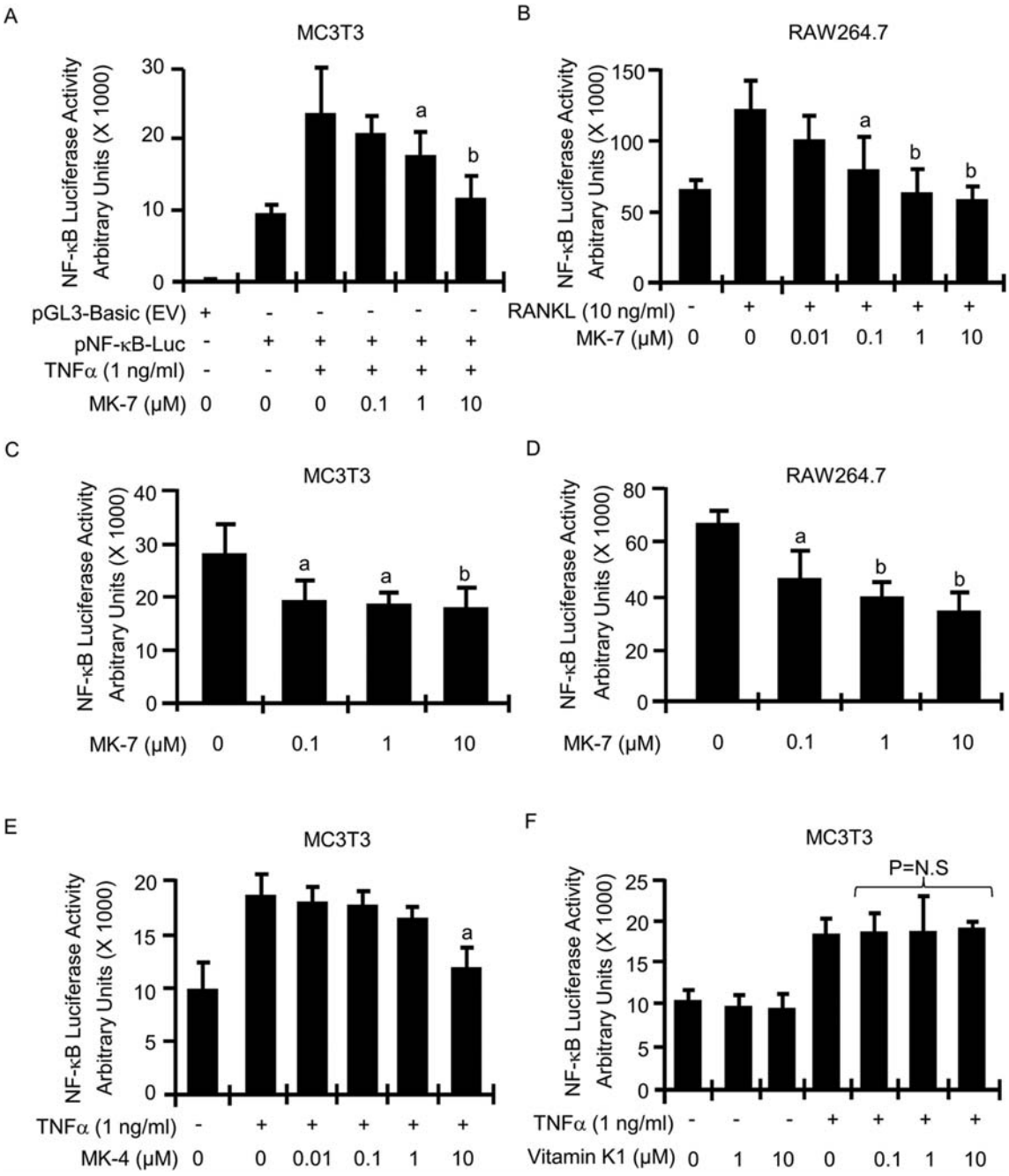

Figure 4. Vitamin K2 suppresses NF-κB activation in osteoclast and osteoblast precursors. (A) MC3T3 cells were transfected with pNF-κB-Luc, an NF-κB activity reporter plasmid or empty vector (EV) and cells cultured in the presence or absence of TNFα (1 ng/ml) and MK-7 (0, 0.1, 1 or 10 μM). (B) RAW264.7 cells were transfected with pNF-κB-Luc and cultured in the presence or absence of RANKL (10 ng/ml) and MK-7 (0, 0.01, 0.1, 1 or 10 μM). (C) MC3T3 and (D) RAW264.7 cells were transfected with pNF-κB-Luc, and the effect of MK-7 (0, 0.1, 1 or 10 μM) on baseline NF-κB transcription was quantitated. (E) MC3T3 cells were transfected with pNF-κB-Luc, and cultured in the presence or absence of TNFα (1 ng/ml) and the vitamin K1 analog MK-4 (0, 0.01, 0.1, 1 or 10 μM) or (F) vitamin K1 (0, 0.1, 1 or 10 μM). Data represent the average ± SD of 5 replicate samples. Panels A, B and E are representative of 3 independent experiments and panels C, D and F of 2 independent experiments. Panels A, B, and E ^{a}P<0.01 or ^{b}P<0.001 relative to TNFα only or RANKL only (no MK-7 or MK-4); panels C and D ^{a}P< 0.01, ^{b}P<0.001 relative to MK-7 (0 μM). Panel F, P=not significant (NS) relative to TNFα only, for all data sets within brackets. All statistics in this figure were performed using one-way ANOVA with the Tukey-Kramer post test.

glutamate rich proteins, and that its action on bone cells likewise involves this activity. In order to determine the role of γ-carboxylation on the capacity of MK-7 to down-regulate NF-κB activity, we performed NF-κB reporter assays in osteoclast and osteoblast precursor cell lines, treated with RANKL or TNFα respectively, in the presence and absence of the potent γ-carboxylation inhibitor warfarin. MK-7 significantly inhibited NF-κB at both low (Fig. 5A) and high (Fig. 5B) doses of RANKL in RAW264.7 cells, and low (Fig. 5C) and high (Fig. 5D) doses of TNFα in MC3T3 cells. Addition of warfarin had no effect on the capacity of MK-7 to suppress NF-κB activation in either preosteoclasts or preosteoblasts, suggesting a γ-carboxylation-independent mode of action.

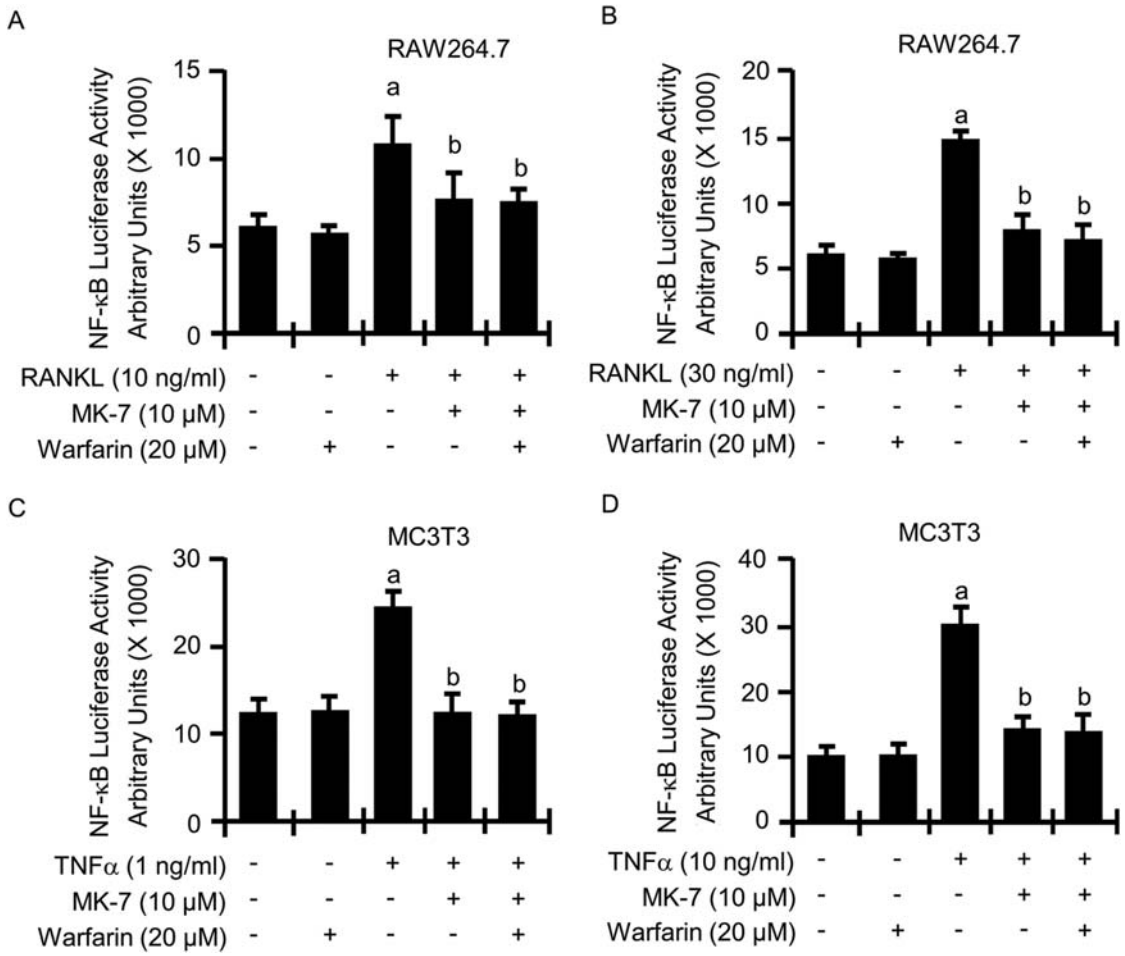

Figure 5. Effect of the γ-carboxylation inhibitor, warfarin on vitamin K2 suppression of TNFα and RANKL-induced NF-κB activation. NF-κB activity was induced in RAW264.7 cells, by stimulation with RANKL at (A) low (10 ng/ml) or (B) high dose (30 ng/ml) in the presence or absence of MK-7 (10 μM), with or without warfarin. NF-κB activity was induced in MC3T3 cells, by stimulation with TNFα at a (C) low (1 ng/ml) or (D) high dose (10 ng/ml) in the presence or absence of MK-7 (10 μM), with or without warfarin. Data represent the average ± SD of 5 replicate samples and are representative of 3 independent experiments. [a]P<0.001 vs. control untreated group, [b]P<0.01 vs. RANKL only or TNFα only groups. One-way ANOVA with the Tukey-Kramer post test.

MK-7 antagonizes TNFα- and RANKL-induced p65 NF-κB subunit translocation from the cytosol to nucleus. Although several NF-κB subunits are recognized as important regulators of osteoclast and osteoblast differentiation and function, the p65 NF-κB subunit is considered as a major species in both cell types (3,12). We consequently performed Western blot analysis for p65 on nuclear and cytosolic extracts from MC3T3 osteoblast precursors, in the presence or absence of TNFα and/or MK-7; and in RAW264.7 osteoclast precursors in the presence or absence of RANKL and/or MK-7. As expected TNFα-induced a translocation of p65 from the cytosol to the nucleus in MC3T3 cells (Fig. 6A) while MK-7 markedly reduced the TNFα-induced p65 translocation. Similarly, RANKL induced the translocation of p65 from the cytosol to the nucleus in RAW264.7 cells, an action antagonized by MK-7 (Fig. 6B).

MK-7 increases IκBα concentration in both osteoblast and osteoclast precursors, but fails to impact the phosphorylation state of IκB. The principal mechanism by which p65 activation is regulated is by its association with IκB. Phosphorylation, followed by ubiquitination and proteosomal degradation of IκB leads to release and nuclear translocation of the active NF-κB species. We consequently performed Western blot analysis for total and P-IκBα (Fig. 7C and D) on cytosolic extracts from MC3T3 osteoblast precursors, in the presence or absence of TNFα and/or MK-7; and in RAW264.7 osteoclast precursors in the presence or absence of RANKL and/or MK-7. While MK-7 led to a significant increase in IκBα concentrations in both osteoblast (Fig. 7A) and osteoclast precursors (Fig. 7B), it did not significantly impact the IκB phosphorylation state (Fig. 7C and D), suggesting a different mechanism of action.

MK-7 significantly enhances IκBα mRNA concentrations. To determine whether MK-7 directly enhances IκBα mRNA levels, we performed real-time RT-PCR for IκBα on MK-7-stimulated MC3T3 and RAW264.7 cells. MK-7 significantly enhanced IκBα mRNA concentrations in MC3T3 cells (Fig. 7E) and in RAW264.7 cells (Fig. 7F).

Taken together our data suggest that MK-7 promotes osteoblast differentiation and activity, and suppresses osteoclast differentiation and activity, by down-regulating NF-κB activation by a mechanism that may involve enhanced

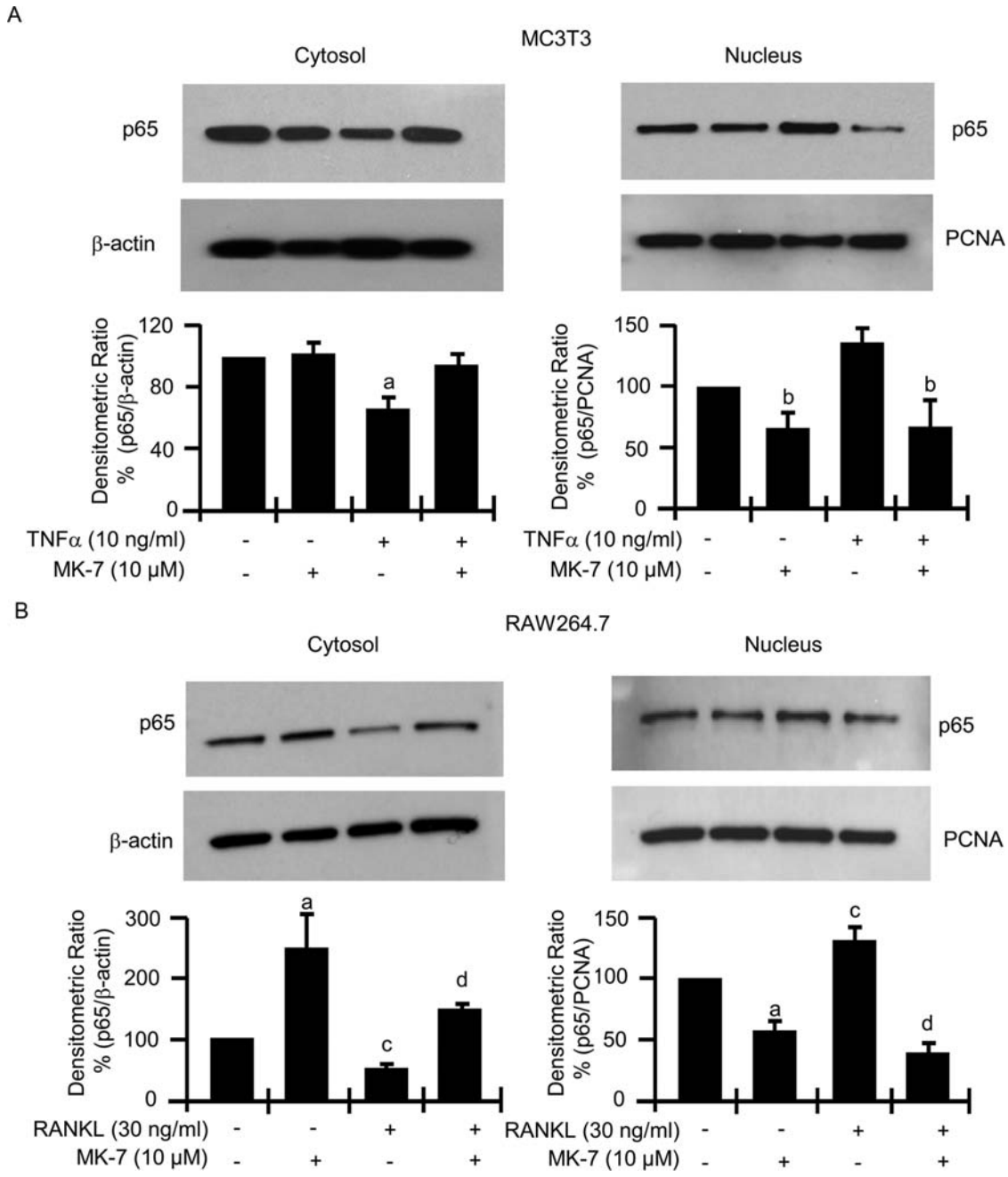

Figure 6. Vitamin K2 suppresses cytosol to nuclear translocation of the NF-κB subunit p65 in osteoblast and osteoclast precursors. (A) MC3T3 or (B) RAW264.7 cells, were treated with MK-7 (10 μM) for 24 h followed by addition of TNFα (10 ng/ml) to MC3T3 cells or RANKL (30 ng/ml) to RAW264.7 cells, for 180 min. Cells were lysed, cytosolic and nuclear proteins were extracted and Western blot analysis was performed for the NF-κB subunit, p65. ß-actin was used as a cytosolic loading control and PCNA as nuclear loading control. Band intensity was quantitated by densitometry and the ratio of p65 to ß-actin, or p65 to PCNA from at least 3 independent experiments is presented as a percentage of control (mean ± SEM) on the bar graph below each Western blot. [a]P<0.05 vs. untreated control, [b]P<0.05 vs. TNFα only. [c]P<0.05 vs. MK-7 only. [d]P<0.05 vs. RANKL only. One-way ANOVA with the Tukey-Kramer post test.

expression of IκBα mRNA leading to increased IκBα protein concentrations.

Discussion

Vitamin K supplements are now widely available in health food stores and are being used indiscriminately at an alarming rate by the public as anti-osteoporotic agents, with little hard scientific data to counsel this use. Indeed, vitamin K2 has long been considered a bone protective factor and an anti-osteoporotic agent in postmenopausal women (25) and has been shown to prevent bone loss in ovariectomized rats (41). Vitamin K2 possesses a rare capacity to stimulate bone formation while simultaneously suppressing bone resorption (22,23,39,42). How this is achieved is not well understood. Our studies presented herein suggest that vitamin K2 promotes bone formation and suppresses bone resorption by antagonizing basal and cytokine-induced activation of NF-κB. We recently reported that endogenous basal levels of TNFα are of a sufficient magnitude to significantly lower maximal

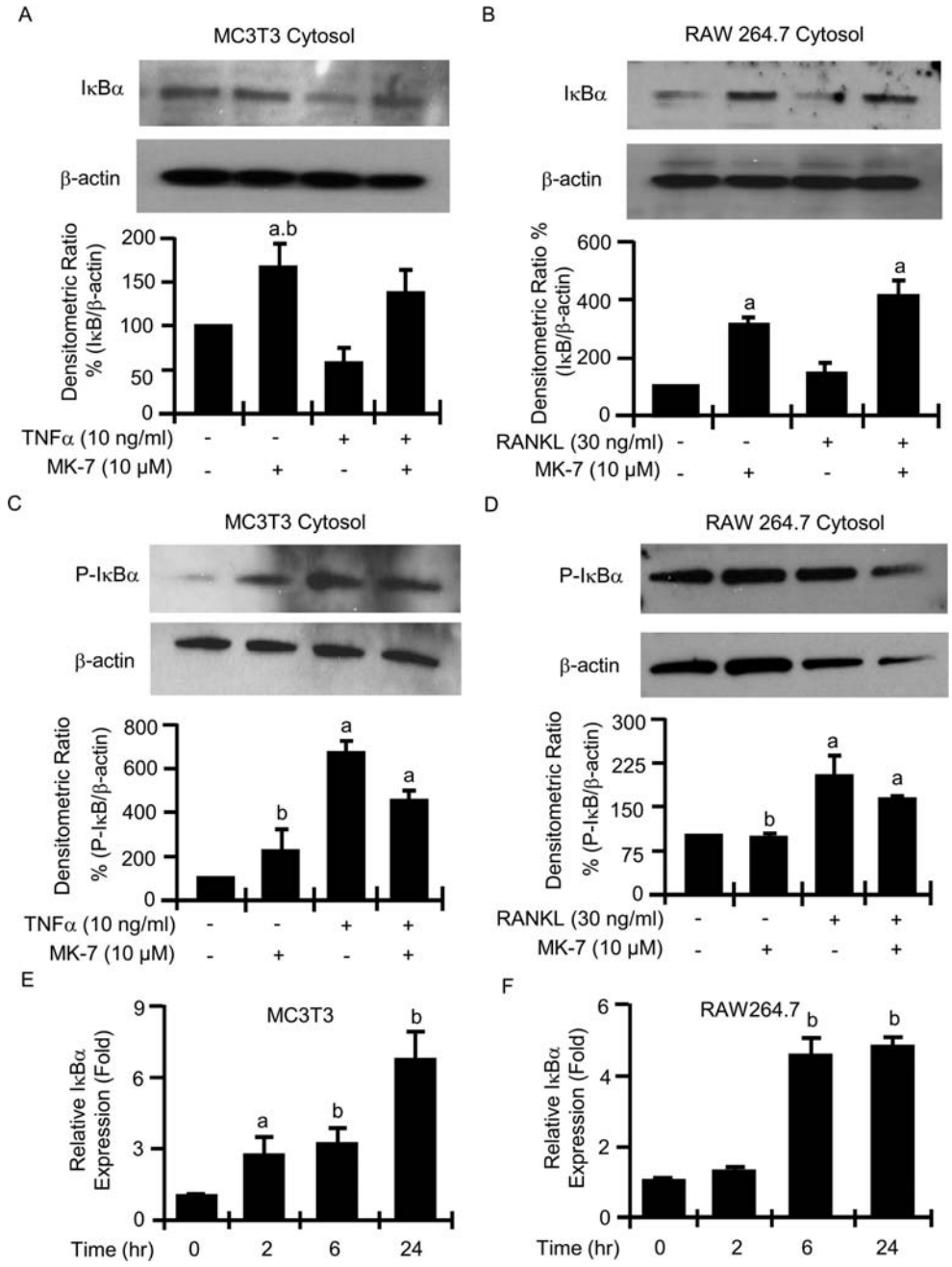

Figure 7. Vitamin K2 promotes the accumulation of IκBα protein and mRNA in osteoblast and osteoclast precursors. MC3T3 cells and RAW264.7 cells were treated with MK-7 (10 μM) for 24 h followed by addition of TNFα (10 ng/ml) to MC3T3 cells or RANKL (30 ng/ml) to RAW264.7 cells, for 180 min. Cells were lysed and cytosolic proteins were extracted and Western blot analysis was performed for the NF-κB translocation inhibitor, IκBα in: (A) MC3T3 cells and (B) RAW264.7 cells; or for phospho-specific IκBα (P-IκBα) in: (C) MC3T3 cells or (D) RAW 264.7 cells. ß-actin was used as a cytosolic loading control and band intensity was quantitated by densitometry. The ratio of IκBα to ß-actin from 3 independent experiments is presented as a percentage of control (mean ± SEM) on bar graph below each blot. [a]P<0.05 vs. untreated control, [b]P<0.05 vs. TNFα or RANKL only. One-way ANOVA with Tukey-Kramer post test. Real-time RT-PCR was performed to quantitate IκBα mRNA from (E) MC3T3 cells and (F) RAW 264.7 cells treated with MK-7 (10 μM), at the indicated time-points. Data points represent mean ± SD of 3 independent experiments with samples run in quadruplicate. [a]P<0.05 vs. 0 h control, [b]P<0.001 vs. 0 h control. One way ANOVA with the Kruskal-Wallis post test.

achievable BMD in mice (17). We now show that MK-7 is capable of ameliorating the suppressive action of TNFα on mineralization, and antagonized both basal and TNFα-induced NF-κB activation in osteoblast precursors. These data are consistent with our previous report, showing that suppression of both basal and cytokine-induced NF-κB activity in osteoblasts, using a specific NF-κB antagonist (TAT-NBD), stimulates osteoblast differentiation *in vitro* (17).

We have demonstrated that MK-7 relieved the suppressive effect of TNFα on TGFß- and BMP-2-induced SMAD activation; however, MK-7 did not appear to have a direct effect on this pathway. Rather, MK-7 appears to antagonize

NF-κB signaling, thus indirectly impacting SMAD activation. We have previously demonstrated that NF-κB intersects with the SMAD signal transduction pathway as NF-κB antagonists block the capacity of TNFα to stimulate SMAD activity (7). Other studies have shown that the capacity of NF-κB to inhibit SMAD signaling is related to the up-regulation of SMAD7, an inhibitory SMAD (18) and the E3-ligases, Smurf1 and Smurf2 (19) which promote proteasomal degradation of SMAD signaling proteins.

These data support the reputed actions of vitamin K2, and specifically of MK-7, in the maintenance of basal bone mass and suggest a mechanism involving TNFα-induced NF-κB in osteoblasts and their precursors. Our data are also consistent with a recent report showing that a vitamin K2 analogue inhibits the proliferation of hepatocellular carcinoma by suppressing cyclin D1 expression through a mechanism involving inhibition of NF-κB activation (43). Taken together this suggests that NF-κB antagonism may be a central feature of vitamin K2 action in multiple systems.

Although MK-4 was also observed to suppress TNFα-induced NF-κB activation in MC3T3 cells, in comparison to MK-7 this effect was much weaker and only achieved statistical significance at a dose 10-fold higher than that required by MK-7. The relative potency of vitamin K2 species may be related to the number of isoprene units; although MK-7 can itself be degraded *in vivo* to MK-4 (41). Given the fact that different vitamin K2 species are often utilized interchangeably in clinical studies, it is possible that some of the conflicting conclusions as to the utility of vitamin K2 in human bone health and amelioration of osteoporosis may stem from different functional potencies of these agents. Since the carboxylation status of vitamin K-dependent proteins is often utilized as a gauge of vitamin K sufficiency (26) this index may not necessarily correlate with optimal NF-κB suppressive activity in bone cells *in vivo*, leading to suboptimal or muted bone sparing effects. Indeed, our data suggest that the γ-carboxylation activity of MK-7 is not required for its anti-NF-κB activities. This is consistent with another study demonstrating that osteoblast-induced mineralization by vitamin K1 was γ-carboxylation-dependent, while vitamins K2 and K3 were less sensitive suggesting γ-carboxylation-dependent and independent pathways (36). γ-carboxylation-independent actions have also been reported for the suppressive effects of vitamin K2 on osteoclastic resorption (44).

Interestingly, in contrast to what was observed for the vitamin K2 species, we failed to identify any anti-NF-κB activity associated with vitamin K1 in our study. While it is generally considered that both vitamins K1 and K2 promote bone health, our data suggest that vitamin K1 may require a much higher dosage or may act via different mechanisms, and should not be considered equivalent or interchangeable with vitamin K2. Many of the contradictory results of recent clinical studies may also reflect, in part, the choice of the vitamin K species utilized.

Our studies show that MK-7 potently suppresses osteoclastogenesis by purified monocytes and clonal RAW264.7 cells in the presence of RANKL, as well as inhibits resorptive activity of purified osteoclasts. These data suggest a direct suppressive effect of vitamin K2 on osteoclast differentiation and activity, as would be expected from an NF-κB antagonist, and are consistent with the known actions of NF-κB in osteoclastogenesis and resorption (1-4). However, it has also been reported that vitamin K2 ameliorates bone destruction in rheumatoid arthritis by down-regulating RANKL production (45). NF-κB signaling is a critical pathway for the activation and function of T cells and B cells (46), both significant sources of RANKL, and RANKL-inducing cytokines such as TNFα and IL-1 under inflammatory conditions (47,48). Our data are consistent with these observations and suggest that vitamin K2-mediated down-regulation of inflammation may also translate into reduced RANKL production by immune and other cells, thus amplifying the direct suppressive effects of this agent on osteoclastogenesis and bone resorption through inhibitory effects on NF-κB activation. In fact vitamin K2 is reported to mediate potent anti-inflammatory effects on arthritis *in vivo* and to suppress the development of arthritis in the collagen-induced arthritis (CIA) animal model (49). The importance of the NF-κB pathway in the development of rheumatoid arthritis is well established and the pharmacological suppression of NF-κB activation itself is reported to ameliorate bone erosions in a rheumatoid arthritis animal model *in vivo* (9). Our data suggest that the anti-NF-κB activities of vitamin K may contribute to these reputed anti-inflammatory properties.

Vitamin K2 has been reported to directly stimulate RANKL expression by MC3T3 osteoblastic cells (50). This may be a consequence of the capacity of vitamin K2 to promote osteoblast differentiation as observed in our study. Despite this action, based on a substantial body of animal and human studies suggesting that vitamin K enhances bone mass, the net effect of vitamin K2 *in vivo* would appear to be in favor of reduced osteoclastogenesis and resorption, possibly because any increased production of RANKL may be counteracted by suppression of NF-κB activation downstream of RANK in osteoclasts and their precursors.

In conclusion, our data provide a novel mechanism to explain the dual pro-anabolic and anti-catabolic activities of vitamin K2, and further support the concept that the pharmacological modulation of the NF-κB signal transduction may constitute an effective mechanism for ameliorating pathological bone loss and for promoting bone health. Our data show that certain natural nutritional factors long-held to promote bone health may already exploit this pathway to modulate bone turnover and mass.

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