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The intact strontium ranelate complex stimulates osteoblastogenesis and suppresses osteoclastogenesis by antagonizing NF-κB activation

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Abstract Strontium ranelate, a pharmaceutical agent shown in clinical trials to be effective in managing osteoporosis and reducing fracture risk in postmenopausal women, is relatively unique in its ability to both blunt bone resorption and stimulate bone formation. However, its mechanisms of action are largely unknown. As the nuclear factor-kappa B (NF-κB) activation antagonists both stimulate osteoblastic bone formation and repress osteoclastic bone resorption, we hypothesized that strontium ranelate may achieve its anabolic and anti-catabolic activities by modulating NF-κB activation in bone cells. In this study, osteoclast and osteoblast precursors were treated with intact strontium ranelate or its individual components sodium ranelate and/or strontium chloride, and its effect on in vitro osteoclastogenesis and osteoblastogenesis and on NF-κB activation quantified. Although the activity of strontium ranelate has been attributed to the release of strontium ions, low dose intact strontium ranelate complex, but not sodium ranelate and/or strontium chloride, potently antagonized NF-κB activation in osteoclasts and osteoblasts in vitro, and promoted osteoblast differentiation while suppressing osteoclast formation. Taken together, our data suggest a novel centralized mechanism by which strontium ranelate promotes osteoblast activity and suppresses osteoclastogenesis, based on suppression of NF-κB signal transduction. We further demonstrate that the biological actions of strontium ranelate may be related to low dose of the intact molecule rather than dissociation and release of strontium ions, as previously thought. These data may facilitate the development of additional novel pharmacological agents for the amelioration of osteoporosis, based on NF-κB blockade.

Keywords Strontium ranelate · Nuclear factor kappa B · Osteoclast · Osteoblast · Bone resorption · Bone formation

Introduction

Strontium ranelate, a divalent strontium salt of ranelic acid, possesses an intriguing capacity to both stimulate bone formation [1] and repress bone resorption [2]. Strontium ranelate is now being utilized extensively for the amelioration of postmenopausal osteoporosis and to increase bone mineral density and reduce bone fracture risk, based on the success of a series of recent clinical trials demonstrating good tolerability and efficacy [3, 4].

Despite the prolific use of strontium ranelate, it's mechanisms of action are poorly defined. Strontium ranelate has been demonstrated to promote differentiation and mineralization of bone building osteoblasts in vitro and to enhance expression of the critical osteoblast transcription factor Runx2 and the specific osteoblast gene product osteocalcin. This induction of an osteoblast differentiation gene program has been suggested as a likely basis underlying the therapeutic bone anabolic actions of strontium ranelate in vivo [5]. Other studies have suggested

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involvement of the calcium-sensing receptor in osteoblast differentiation and mineralization in response to strontium ranelate [6], as well as in its anti-osteoclastogenic properties, by downregulating receptor activator of NF-κB (RANK) ligand (RANKL)-induced osteoclast differentiation and promoting apoptosis of mature osteoclasts [7, 8]. Recently, it was reported that strontium ranelate may act on osteoblasts by upregulating NFAT nuclear translocation and promoting expression of Wnt3a and Wnt5a as well as upregulating β-catenin transcription [9]. Another recent study suggests that strontium ranelate acts on osteoclasts by antagonizing RANKL-induced nuclear translocation of NF-κB and AP-1 transcription factors, [7]. Indeed, NF-κB is well established to be critical for osteoclast development, function and survival [10–16], and we recently reported that pharmacological suppression of NF-κB protects mice from osteoclastic bone loss associated with estrogen deficiency following ovariectomy, a model of postmenopausal osteoporosis [17]. Pharmacological NF-κB suppression has further been demonstrated to inhibit osteoclast formation and activity associated with multiple myeloma in vitro [18] and in an animal model of rheumatoid arthritis in vivo [19].

In contrast to the stimulatory action of NF-κB signal transduction in osteoclast development and activity, we and others have shown that NF-κB activation is potently inhibitory to osteoblast commitment, differentiation and mineralization in vivo and in vitro [20–23]. In fact, tumor necrosis factor alpha (TNFα), a potent NF-κB inducer, has long been recognized as an inhibitor of osteoblast differentiation [24] and activation of the p65 NF-κB subunit by TNFα has been shown to suppress transcription of osteocalcin in osteoblastic cells stimulated with vitamin D [25]. Furthermore, pharmacological suppression of TNFα is reported to reverse age-related defects in bone formation in a mouse fracture healing model [26]. We recently demonstrated that basal endogenous concentrations of TNFα potently diminish maximum achievable peak bone mineral density in mice in vivo and that TNFα-induced NF-κB activation suppresses osteoblast differentiation in vitro and antagonizes transforming growth factor beta (TGFβ)- and bone morphogenetic protein-2 (BMP-2)-induced Smad signaling in differentiating osteoblasts [20]. Furthermore, NF-κB signaling in osteoblasts has also been shown to upregulate Smad7, an inhibitor of Smad activation [21], while TNFα upregulates Smurf1, an E3 ligase that promotes ubiquitination and proteasomal degradation of bone morphogenetic signaling proteins [22]. Recently, a direct inhibitory action of NF-κB on bone formation has been demonstrated 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 and ameliorated ovariectomy-induced bone loss by promoting a compensatory increase in bone formation [23].

As strontium ranelate is a compound that promotes bone formation but suppresses bone resorption we speculated that suppression of NF-κB may provide a unified mechanism to explain these disparate effects. Our data support this notion and demonstrate that strontium ranelate is a potent antagonist of NF-κB activation in both osteoblasts and osteoclasts. Intriguingly, our data further suggest that the anti-NF-κB activities and stimulatory effects on mineralization and suppressive effects on osteoclastogenesis of low dose strontium ranelate are associated with the intact molecule, rather than dissociated strontium ions that have been attributed to its other reported biochemical activities.

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, TGFβ, and TNFα were from R&D Systems (Minneapolis, MN). Strontium ranelate was from Toronto Research Chemicals Inc., (North York, ON, Canada), and sodium ranelate was from Shanghai UCHEM Co., Ltd; Hong Kong, China. Mouse anti-poly-histidine antibody, leukocyte acid phosphatase kits for tartrate resistant acid phosphatase (TRAP) staining, strontium chloride and all other reagents were purchased from the Sigma Chemical Corporation, (St. Louis, MO) unless otherwise specified.

Cell culture

The mouse preosteoblastic cell line MC3T3-E1, clone 14 (MC3T3) and the mouse monocytic cell line RAW264.7 were purchased from the American Type Culture Collection (Manassas, VA) and cultured as previously described [20, 27].

Osteoblast differentiation assays and Alizarin red-S staining

For mineralization assays, MC3T3 cells were plated in 12-well dishes at a density of 1.0×10^5 cells per well in mineralization medium (α-MEM supplemented with 10% FBS, L-ascorbic acid (100 µg/ml) and 4 mM β-glycerophosphate) as previously described [20, 28]. Strontium ranelate, sodium ranelate or strontium chloride were added at the indicated doses and cells replenished with fresh medium every 3 days. At 17–18 days of culture, 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).

Osteoclastogenesis assays and TRAP staining

RAW264.7 cells 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 1×10^4 cells/well. Cells were cultured for 6 days with RANKL (30 ng/ml) preincubated for 10 min with cross-linking anti-poly-histidine antibody (2.5 μg/ml), to induce osteoclast formation. Strontium ranelate, sodium ranelate or strontium chloride were added at the indicated doses. After 6 days of culture, the cells were fixed and stained for TRAP, a specific marker of the osteoclast phenotype, using a leukocyte acid phosphatase kit. $TRAP^+$ cells with three or more nuclei were defined as osteoclasts and were quantitated under light microscopy and normalized for cell size based on number of nuclei. Five wells per group were averaged. Representative wells were photographed under bright field microscopy using a Nikon Eclipse TE2000-S inverted microscope equipped with a digital camera (QImaging Corp., Burnaby, BC, Canada).

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 [20]. Briefly, reporter plasmids were transfected into MC3T3 or RAW264.7 cells (1×10^5 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 MC3T3 cells treated with TNFα (1 or 10 ng/ml as indicated) or RAW264.7 cells treated with RANKL (30 ng/ml) to stimulate NF-κB activity. In some experiments, Smad activity was induced in MC3T3 cells using TGFβ (1 ng/ml). Strontium ranelate, sodium ranelate or strontium chloride were added at the indicated doses. Cells were extracted with passive lysis buffer (Promega Corporation, Madison WI) 24 h later, and luciferase activity measured using the Luciferase Assay System of Promega, on a microplate luminometer (Turner Designs, Sunnyvale, CA, USA).

Data analysis and statistical procedures

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 posttest for parametric data.

Results

Low dose strontium ranelate promotes osteoblast mineralization in vitro, but strontium chloride, sodium ranelate and a combination of strontium chloride and sodium ranelate are inactive

To investigate the mechanism by which strontium ranelate promotes osteoblast differentiation, we established an in vitro model utilizing the MC3T3 mouse preosteoblast cell line that differentiates spontaneously into active osteoblasts within 21 days of culture in medium containing ascorbic acid and β-glycerophosphate (mineralizing medium). In the presence of low dose strontium ranelate (200 μM), we observed a robust enhancement in mineralization at just 17 days of culture, following staining of cultures for calcium deposition using Alizarin red-S (Fig. 1A).

As the strontium component of the strontium ranelate complex has historically been associated with its biological activity in bone, we further compared the relative activity of strontium chloride (200 μM), and using sodium ranelate (200 μM) as a negative control, and a combination of both strontium chloride and sodium ranelate (200 μM each), in parallel cultures. Surprisingly, addition of an equivalent low dose of strontium chloride, sodium ranelate or a combination of strontium chloride and sodium ranelate had no stimulatory effects on osteoblast mineralization (Fig. 1A). Culture with strontium ranelate did not have an effect on the proliferation of osteoblastic cells.

Strontium ranelate antagonizes the suppressive activity of TNFα on osteoblast differentiation in vitro

TNFα is a potent inhibitor of bone formation under basal and pathological conditions in vivo and of osteoblast differentiation and mineralization in vitro. As strontium ranelate promotes bone formation in postmenopausal osteoporosis a condition characterized and driven in part by high levels of TNFα, we examined the capacity of strontium ranelate to mitigate the suppressive action of TNFα on osteoblast differentiation in vitro.

While addition of TNFα (5 ng/ml) suppressed mineralization of MC3T3 cells to unstimulated levels, strontium ranelate at a dose as low as 10 μM completely reversed the

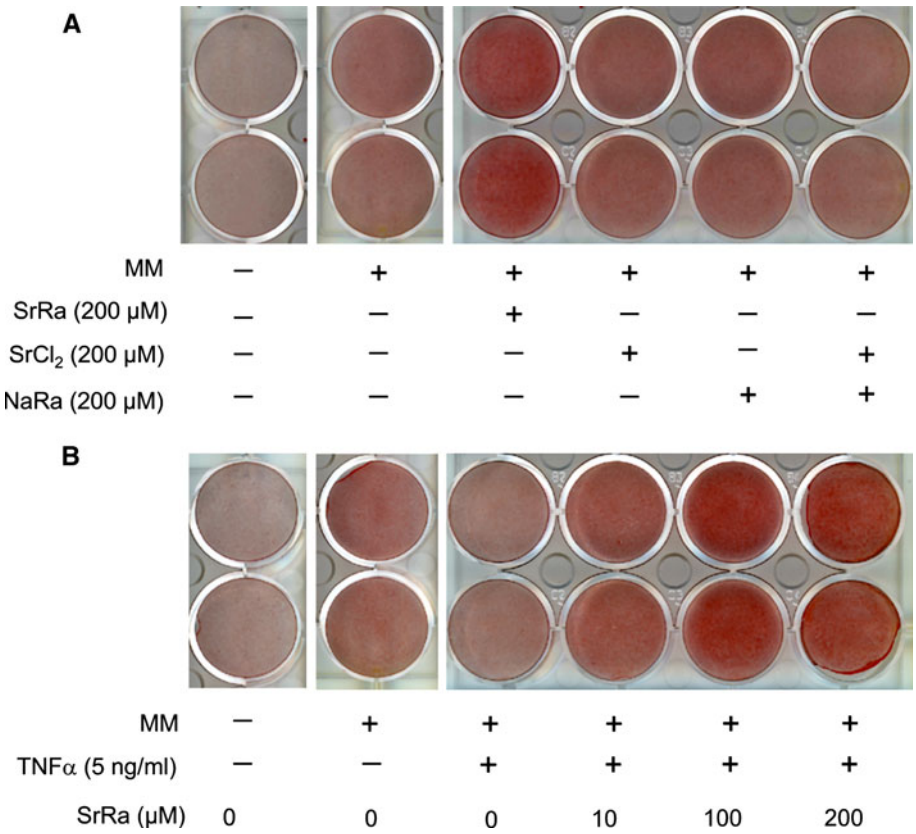

Fig. 1 Effect of strontium ranelate, strontium chloride and sodium ranelate on mineralization of MC3T3 osteoblast precursors and TNFα-induced suppression of mineralization in vitro. **A** MC3T3 cells were cultured in control or mineralizing medium in the presence of absence of 200 μM of strontium ranelate (SrRa), strontium chloride (SrCl) or sodium ranelate (NaRa) for 17 days and mineral deposition visualized by Alizarin red-S staining. **B** MC3T3 cells were cultured in control or mineralizing medium in the presence or absence of the osteoblast differentiation inhibitor TNFα (5 ng/ml). Parallel TNFα-treated wells received a dose range of strontium ranelate (SrRa) from 10 to 200 μM. All wells in each experiment are from the same plate but were digitally separated and reorganized for clarity. Noncontiguous wells are separated by a white space to indicate this fact. Data representative of two independent experiments

suppressive effect of TNFα on basal mineralization and potently augmented mineralization over basal levels at 100 and 200 μM (Fig. 1B).

Strontium ranelate dose dependently suppresses TNFα-induced activation of NF-κB in osteoblast precursors in vitro

A major mechanism by which TNFα mediates its suppressive action on osteoblasts is through NF-κB activation [20, 24]. We consequently hypothesized that strontium ranelate may promote osteoblast mineralization by antagonizing basal and TNFα-induced NF-κB activity. To test this, MC3T3 cells were transiently transfected with an NF-κB luciferase reported plasmid and NF-κB activity upregulated with TNFα (1 ng/ml), a low dose representing basal TNFα levels in vivo or at a higher dose (10 ng/ml) more representative of inflammatory conditions. Addition of strontium ranelate dose dependently suppressed TNFα-induced NF-κB activity in both high (Fig. 2A) and low (Fig. 2B) TNFα-treated cultures.

Strontium ranelate suppresses TNFα-induced NF-κB activation, but strontium chloride, sodium ranelate or a combination of strontium chloride and sodium ranelate are inactive

Our data previously showed that only intact strontium ranelate and not its individual components were capable of inducing osteoblast mineralization. We, consequently, investigated the activity of strontium ranelate relative to strontium chloride, sodium ranelate or a combination of strontium chloride and sodium ranelate on TNFα-induced NF-κB activity. While 200 μM strontium ranelate significantly suppressed TNFα-induced NF-κB activity, strontium chloride and sodium ranelate at equivalent dosage were completely ineffective (Fig. 3). We further added a dose range of strontium chloride

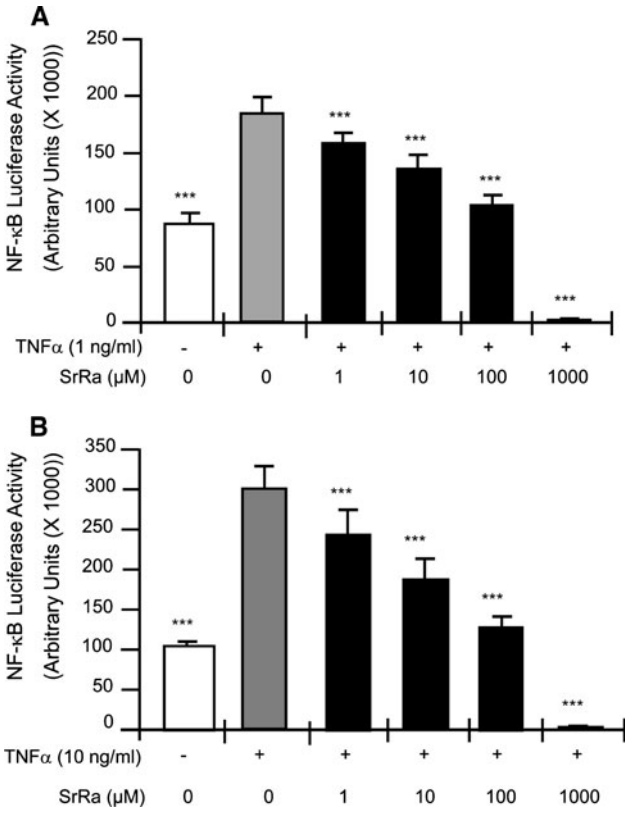

Fig. 2 Effect of strontium ranelate on TNFα-induced NF-κB activation in MC3T3 osteoblast precursors. MC3T3 cells were transfected with NF-κB reporter vector and treated with or without strontium ranelate (SrRa) in a dose range of 1 to 1000 μM, in the absence or presence of: **A** low dose TNFα (1 ng/ml) or **B** 10 ng/ml TNFα. Luciferase activity was quantitated by luminometer 24 h later. Data expressed as mean ± SD of five replicate samples per data set and representative of three independent experiments. ***$P < 0.001$ versus TNFα stimulated only (*gray bar*); one-way ANOVA, Tukey–Kramer posttest)

and sodium ranelate together from 100 μM up to 1000 μM of each. Combined strontium chloride and sodium ranelate produced no suppressive effects on TNFα-induced NF-κB activity at any dose tested.

Strontium ranelate antagonizes the inhibitory effect of TNFα on TGFβ-induced Smad activation

TGFβ is a potent early osteoblast precursor commitment and recruitment factor [29, 30]. We have reported that TNFα antagonizes TGFβ-induced Smad signaling in osteoblast precursors [20] and consequently investigated whether strontium ranelate may alleviate TNFα-induced suppression of TGFβ-induced Smad signaling in preosteoblasts. MC3T3 cells were transiently transfected with a Smad-responsive luciferase reporter and Smad-induced transcription quantitated following stimulation by TGFβ. TGFβ potently stimulated Smad activation and TNFα (1 ng/ml) significantly suppressed basal and TGFβ-induced Smad activation.

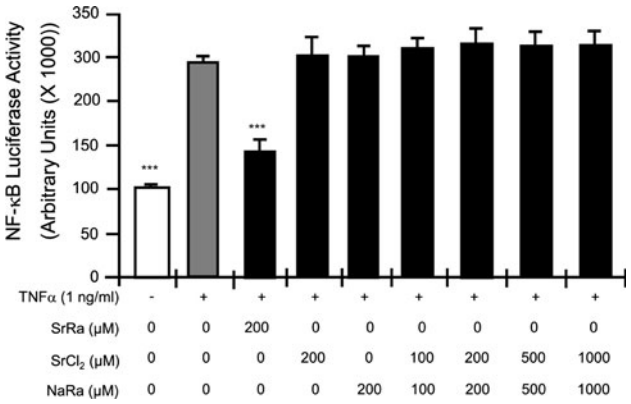

Fig. 3 Effect of strontium ranelate, strontium chloride and sodium ranelate on TNFα-induced NF-κB activation in MC3T3 osteoblast precursors. MC3T3 cells were transfected with NF-κB reporter vector and NF-κB activity stimulated with TNFα (1 ng/ml). Parallel cultures were treated with or without 200 μM strontium ranelate (SrRa) or a combination of strontium chloride ($SrCl_2$) and sodium ranelate (NaRa) from 100 to 1000 μM. Luciferase activity was quantitated by luminometer 24 h later. Data expressed as mean ± SD of five replicate samples per data set and representative of two independent experiments. ***$P < 0.001$ versus TNFα stimulated only (*gray bar*); one-way ANOVA, Tukey–Kramer posttest

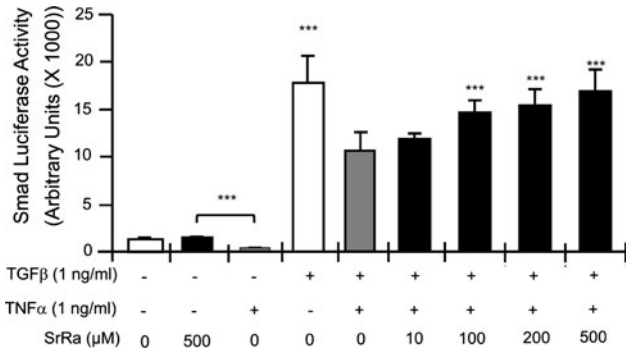

Fig. 4 Effect of strontium ranelate on TNFα-mediated suppression of TGFβ-induced Smad activation. MC3T3 cells were transfected with Smad reporter vector and Smad activity stimulated with TGFβ (1 ng/ml) in the presence or absence of TNFα (1 ng/ml). Parallel cultures were treated with or without strontium ranelate (SrRa) in the range 10–500 μM. Luciferase activity was quantitated by luminometer 24 h later. Data expressed as mean ± SD of five replicate samples per data set and representative of two independent experiments. ***$P < 0.001$ versus TNFα only or TGFβ and TNFα stimulated only (*gray bars*); one-way ANOVA, Tukey–Kramer posttest

Strontium ranelate at 500 μM had no direct effect on basal Smad activity but significantly relieved the suppressive effect of TNFα on TGFβ-induced Smad activity at concentrations of 100 μM and above (Fig. 4).

Strontium ranelate dose dependently suppresses osteoclast differentiation in vitro

In contrast to its stimulatory activity on osteoblast differentiation and activity, strontium ranelate is known to

suppress osteoclast differentiation. To establish an in vitro osteoclastogenesis model suitable for investigation of strontium ranelate activity, we employed the monocytic cell line RAW264.7 and induced it's differentiate into osteoclasts by addition of the key osteoclastogenic cytokine RANKL. The effect of strontium ranelate on osteoclast differentiation was tested over a dose range from 1 to 200 μM, and cultures were stained with TRAP, 7 days later and osteoclast formation quantitated. RANKL induced robust osteoclast formation, which was significantly suppressed by addition of just 1 μM strontium ranelate with a maximal effect observed between 10 and 100 μM (Fig. 5A). Photographs of representative cultures are presented in Fig. 5B.

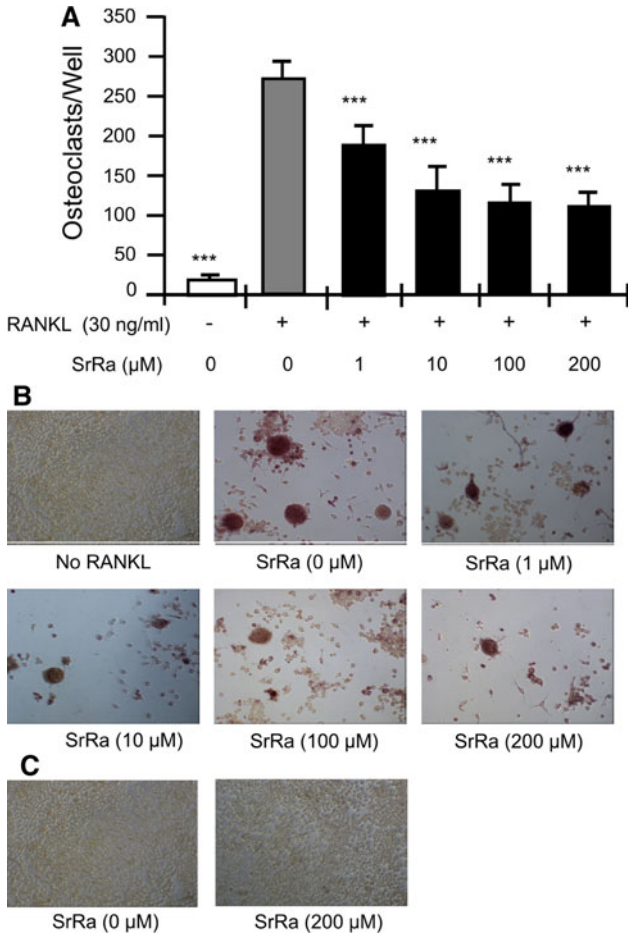

Fig. 5 Effect of strontium ranelate on osteoclast differentiation in vitro. **A** RAW264.7 osteoclast precursors were induced to differentiate into osteoclasts with RANKL (30 ng/ml) in the presence or absence of strontium ranelate (SrRa) in the range 1–200 μM. Cultures were stained with TRAP 7 days later and osteoclasts [TRAP + multinucleated (≥3 nuclei)] cells quantitated. **B** Photographs of representative cultures under bright field (×100 magnification). **C** RAW264.7 osteoclast precursors were culture for 7 days in the absence of RANKL with or without 200 μM strontium ranelate (SrRa). Data expressed as mean ± SD of six replicate wells per data set and representative of two independent experiments. ***$P < 0.001$ versus RANKL stimulated only (*gray bar*), one-way ANOVA, Tukey–Kramer posttest

To assess potential direct toxic effect of strontium ranelate on osteoclast precursors, we treated RAW264.7 cell cultures with vehicle or 200 μM strontium ranelate, the highest dose tested in the osteoclastogenesis experiment (Fig. 5A, B), for 7 days in the absence of RANKL. By 7 days, the low starting density RAW264.7 cells in culture had dramatically proliferated to completely fill the wells in both vehicle and strontium ranelate-treated conditions (Fig. 5C), demonstrating that strontium ranelate does not suppress osteoclastogenesis though direct toxicity or by inhibiting the proliferation of osteoclast precursors.

Strontium ranelate suppresses RANKL-induced osteoclastogenesis, but strontium chloride and sodium ranelate are inactive

It is generally accepted that the mode of action of strontium ranelate is though the generation of strontium ions which then suppress the osteoclasts. We further investigated this mode of action by directly comparing strontium ranelate to strontium chloride, and to sodium ranelate used as a negative control. RAW264.7 cells were differentiated into osteoclasts with RANKL in the presence or absence of 10 or 100 μM strontium ranelate, strontium chloride or sodium ranelate. While strontium ranelate potently suppresses osteoclast formation at both doses, neither strontium chloride nor sodium ranelate had any effect on osteoclast formation (Fig. 6A). Representative TRAP stained cultures are shown in Fig. 6B.

Strontium ranelate dose dependently suppresses activation of NF-κB in osteoclast precursors, but strontium chloride is inactive

The NF-κB signal transduction pathway is essential for the generation of osteoclasts, and strontium ranelate has been reported to antagonize RANKL-induced NF-κB nuclear translocation [7]. As strontium ranelate but not strontium chloride suppressed NF-κB activation in osteoblasts, we further examined the effect of strontium ranelate and strontium chloride on NF-κB activation by RANKL in osteoclast precursors. RAW264.7 cells were transfected with an NF-κB reporter and stimulated with RANKL to induce NF-κB activity, in the presence or absence of a dose range of strontium ranelate (1–1000 μM). Strontium ranelate dose dependently and significantly blunted RANKL-induced NF-κB activity (Fig. 7). As previously observed in osteoblasts, strontium chloride failed to suppress NF-κB activity between 1 and 1000 μM.

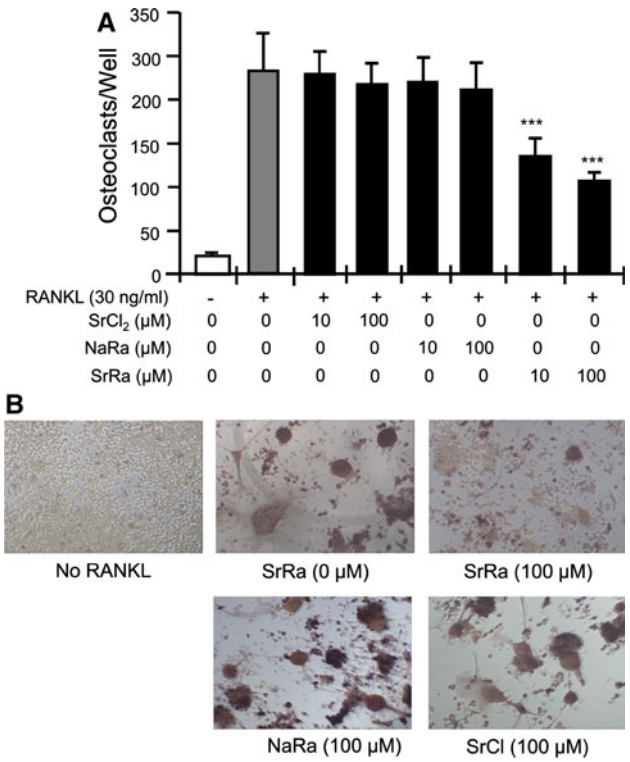

Fig. 6 Effect of strontium ranelate, strontium chloride and sodium ranelate on osteoclast differentiation in vitro. **A** RAW264.7 osteoclast precursors were induced to differentiate into osteoclasts with RANKL (30 ng/ml) in the presence or absence of 10 or 100 μM strontium ranelate (SrRa), strontium chloride ($SrCl_2$) or sodium ranelate (NaRa). Cultures were stained with TRAP 7 days later and osteoclasts (TRAP + multinucleated (≥3 nuclei)) cells quantitated. Data expressed as mean ± SD of six replicate wells per data set and representative of two independent experiments. $***P < 0.001$ versus RANKL stimulated only (*gray bar*), one-way ANOVA, Tukey–Kramer posttest. **B** Photographs of representative cultures under bright field (×100 magnification)

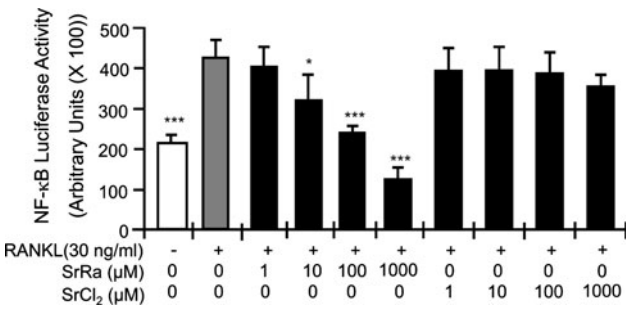

Fig. 7 Effect of strontium ranelate and strontium chloride on RANKL-induced NF-κB activation in osteoclast precursors. RAW264.7 osteoclast precursors were transfected with NF-κB reporter vector and NF-κB activity stimulated with RANKL (30 ng/ml). Parallel cultures were treated with or without a dose range of strontium ranelate (SrRa) or strontium chloride ($SrCl_2$) from 1 to 1000 μM and luciferase activity quantitated by luminometer 24 h later. Data expressed as mean ± SD of five replicate samples per data set and representative of two independent experiments. $*P < 0.05$; $***P < 0.001$ versus RANKL stimulated only (*gray bar*); one-way ANOVA, Tukey–Kramer posttest

Discussion

Strontium ranelate has been shown in clinical trials to protect against bone loss and to reduce fracture risk in postmenopausal women [3, 4] by both stimulating bone formation and simultaneously suppressing bone resorption. However, the molecular processes by which this is accomplished are poorly characterized. Our studies presented herein implicate the suppression of NF-κB activation as a centralized mechanism to achieve these actions by regulating both osteoclasts and osteoblasts.

We demonstrate that strontium ranelate was capable of reversing the suppressive action of TNFα on mineralization in vitro. This was consistent with data demonstrating that strontium ranelate blunted NF-κB activation induced by TNFα in osteoblast precursors. TNFα is a pro-inflammatory cytokine that plays a central role in the pathology of multiple osteoporotic conditions, including postmenopausal osteoporosis and rheumatoid arthritis. Given the capacity of strontium ranelate to antagonize TNFα-induced NF-κB activity in osteoblasts, we speculate that the anabolic effects of this compound in vivo may be achieved, at least in part, by suppressing NF-κB activation induced by TNFα and other inflammatory cytokines in postmenopausal women. Given its action on NF-κB signal transduction, our data further suggest that strontium ranelate may be an effective anti-osteoporotic agent in other inflammatory conditions including rheumatoid arthritis. In fact, pharmacological suppression of NF-κB activation is reported to ameliorate bone erosions in an in vivo animal model of rheumatoid arthritis [19]. In addition, as the NF-κB pathway plays important roles in driving inflammation, strontium ranelate may have additional indirect benefits in reliving inflammation and inflammation-associated sequelae such as skeletal deterioration as a consequence of potential anti-inflammatory properties. Strontium ranelate may further have applications in fracture repair as a recent study reports that pharmacological suppression of TNFα reverses age-related defects in bone formation in a mouse fracture healing model [26].

We have reported that in vivo, basal TNFα levels reach a magnitude capable of suppressing basal bone formation and lowering peak bone mineral density in mice and that suppression of basal NF-κB activity in osteoblasts, using a specific inhibitor, upregulates osteoblast differentiation in vitro even in the absence of any exogenous NF-κB agonists [20]. Consistently, strontium ranelate was observed to promote a robust osteoblast mineralization even in the context of our unstimulated in vitro cultures. In fact, our NF-κB reporter assays do provide evidence of a high rate

of basal NF-κB activity in osteoblast precursors which might explain the capacity of strontium ranelate to augment MC3T3 mineralization under unstimulated conditions. One explanation for this high basal activity is that the FBS necessary to culture the cells may contain NF-κB activators. Alternatively, it is possible that osteoblasts maintain an intrinsically high basal NF-κB state that may act as a natural barrier to spontaneous osteoblast precursor differentiation, thus maintaining the pluripotency of the lineage.

TGFβ is an important early commitment factor for osteoblast differentiation and for targeting and recruitment of osteoblast precursors to sites of bone resorption [29, 30]. We have reported that one mechanism by which TNFα blunts bone formation is by suppressing Smad signal transduction downstream of the TGFβ receptors. Indeed, our data reveal that strontium ranelate significantly alleviates the suppressive effect of TNFα on TGFβ-induced Smad activation in osteoblast precursors. Strontium ranelate showed no direct effects on Smad activity, and its action is thus likely mediated solely through antagonism of TNFα-driven NF-κB activation. We previously showed that NF-κB antagonists block the capacity of TNFα to suppress Smad activity [17] suggesting that NF-κB intersects with the Smad signal transduction pathway. Other studies have further defined these sites of intersection and reveal that NF-κB induces Smad7, an inhibitor of Smad signaling [21] and that the ubiquitin ligase Smurf1 mediates TNFα-induced systemic bone loss by promoting proteasomal degradation of bone morphogenetic signaling proteins [22].

In contrast to osteoblasts, strontium ranelate was found to potently antagonize RANKL-induced NF-κB activation in osteoclast precursors without evidence of anti-proliferative or direct toxic effects. NF-κB activation is critical to osteoclast differentiation and activity, and blunting NF-κB activation could account, in part, for the anti-osteoclastogenic activity of this compound in vivo. Our data are consistent with and support a recent study reporting that the effects of strontium ranelate on osteoclasts may involve, inhibition of RANKL-induced nuclear translocation of NF-κB and AP-1, in the early stages of osteoclastic differentiation [7].

We have further examined the role of intact strontium ranelate relative to that the strontium component of the complex. It has long been believed that the biological activity of strontium ranelate is solely a consequence of the dissociation of the complex in vivo releasing strontium ions that are preferentially targeted to bone and the bone microenvironment. In fact, based on this assumption, multiple varieties of strontium-containing molecules are now being widely touted as effective anti-osteoporotic supplements. To our surprise, our data suggest that the anti-NF-κB activity and anti-osteoclastogenic effects and pro-osteoblastic effects of low dose strontium ranelate may be a direct consequence of the intact complex, rather than the strontium moiety. This data appear to contrast with other studies such as that of Fromigue et al. who reported that addition of a mixture of strontium chloride and sodium ranelate to culture medium at a molar ratio of 100:1 was biologically active in MC3T3 cells and stimulated nuclear factor of activated T cells (NFAT) translocation and Wnt transcription, leading to induction of an osteoblastic gene program [9]. In this study, however, strontium ion concentrations were titrated to 1 or 3 mM. In our studies, strontium ranelate achieved significant biological activities at doses as low as 1 μM and was saturating at 100 μM in the in vitro osteoblast differentiation and mineralization assays. By contrast, we observed no significant effect of strontium chloride on NF-κB activity at doses up to 1 mM. Our studies suggest that in contrast to the potential actions of high dose strontium on osteoblast differentiation, low doses of intact strontium ranelate may further mediate potent effects on osteoblast and osteoclast differentiation, by antagonizing NF-κB activation.

It is not surprising that we observe such effects in vitro as strontium ranelate is an extremely stable complex in neutral buffered solutions typical of tissue culture systems. More perplexing is, how this may translate into an in vivo effect based on current precepts. Although a proof of concept remains to be demonstrated in vivo, we believe that the concept of intact strontium ranelate-mediating skeletal actions all or in part is in fact plausible. Although, when administered orally, stomach acid is believed to dissociate the complex into its component ions, given the relatively high chronic dose of strontium ranelate used clinically (2 g daily), it is highly likely that at least some intact complex is delivered to the intestine undegraded, and given its small size, these molecules are likely to be readily absorbed into the circulation. Because strontium ranelate complex is stable at physiological pH and is not biologically metabolizable in vivo, we speculate that over time a pooling of intact strontium ranelate may accumulate at skeletal sites. Given the capacity of strontium ranelate to mediate anti-NF-κB effects at concentrations that are orders of magnitude lower than those claimed for strontium ion action, it is indeed possible that intact strontium ranelate may mediate anti-NF-κB activity leading to a weak suppression of osteoclastogenesis and a weak stimulation of osteoblastogenesis, as is reported for this agent.

In conclusion, our data provide a novel mechanism to explain the dual pro-anabolic and anti-catabolic activities of strontium ranelate and further support the concept that pharmacological modulation of NF-κB signal transduction may constitute an effective mechanism for ameliorating pathological bone loss.

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