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Strontium ranelate decreases RANKL-induced osteoclastic differentiation in vitro: involvement of the calcium sensing receptor.

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Non standard abbreviations: strontium (Sr^{2+}); extracellular concentration of Sr^{2+} (Sr^{2+}_o); calcium (Ca^{2+}); extracellular concentration of Ca^{2+} (Ca^{2+}_o); alpha modified Minimum Essential Medium ($\alpha-$MEM); Dulbecco's Minimum Essential Medium (DMEM), peripheral blood mononuclear cells (PBMC); tartrate-resistant acid phosphatase (TRAP); calcium-sensing receptor (CaR); receptor activator of NFkB ligand (RANKL); receptor activator of NFkB (RANK); paranitrophenylphosphate (pNPP); paranitrophenol (pNP); high performance liquid chromatography (HPLC), hydroxylysylpyridinoline (HP); 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT); recombinant adeno-associated virus (rAAV); β-galactosidase cDNA (βGal).

Abstract

Strontium ranelate exerts both an anti-catabolic and an anabolic effect on bone cells. To further investigate the mechanism by which strontium ranelate inhibits bone resorption, the effects of varying concentrations of Sr^{2+}_o on osteoclastic differentiation were studied using RAW 264.7 cells and PBMC. We report that increasing concentrations of Sr^{2+}_o down-regulate osteoclastic differentiation and tartrate-resistant acid phosphatase (TRAP) activity, leading to inhibition of bone resorption (- 48% when PBMC were cultured for fourteen days in the presence of 2 mM Sr^{2+}_o). Using a dominant negative form of the CaR and a small interfering RNA approach, we provide evidences that the inhibition of osteoclast differentiation by Sr^{2+}_o is mediated by stimulation of the calcium-sensing receptor (CaR). Moreover, our results suggest that the effects of Sr^{2+}_o on osteoclasts are, at least in part, mediated by inhibition of the RANKL-induced nuclear translocation of NFkB and AP-1, in the early stages of osteoclastic differentiation. In conclusion, our data indicate that Sr^{2+} 1) directly inhibits the formation of mature osteoclasts through down-regulation of RANKL-induced osteoclast differentiation and 2) decreases osteoclast differentiation through the activation of the CaR.

Introduction

Strontium ranelate is a therapeutic agent used in the treatment of post menopausal osteoporosis. It significantly reduces the risk of vertebral and hip fractures in postmenopausal women (Meunier et al., 2004; Reginster et al., 2005; Roux et al., 2006). Strontium ranelate is composed of an organic moiety and two atoms of stable Sr^{2+}. It exerts its effects on bone cells through a novel mechanism of action, which is thought to result directly from the bone-seeking properties of Sr^{2+} (Farlay et al., 2005). Sr^{2+}_o may increase in the bone micro-environment during the process of bone resorption, as shown for Ca^{2+}_o (Silver et al., 1988), and modulate the activity of osteoblasts and osteoclasts within the vicinity (Marie, 2005; Marie, 2006). Thus, strontium ranelate increases osteoblastic replication and synthesis of the collagenous matrix (Barbara et al., 2004; Bonnelye et al., 2008; Canalis et al., 1996) and reduces osteoclastic bone resorption (Barbara et al., 2004; Baron and Tsouderos, 2002; Bonnelye et al., 2008; Takahashi et al., 2003). The process of bone resorption is a complex, multi-step process: 1) osteoclast precursors leave their haematopoietic niches and osteoclast differentiation occurs; 2) mature bone-resorbing osteoclasts adhere tightly to the bone surface, forming a sub-osteoclastic compartment into which both protons and proteolytic enzymes are secreted in order to resorb the osseous mineralized matrix; and 3) the osteoclasts die by apoptosis, ending the process of bone resorption (Horowitz et al., 2001; Manolagas and Jilka, 1995). We and others have demonstrated that strontium ranelate acts on all these steps, by decreasing osteoclast formation and maturation (Bonnelye et al., 2008), disrupting the osteoclast actin-containing sealing zone (Bonnelye et al., 2008) and increasing osteoclast apoptosis (Hurtel-Lemaire et al., 2009). In the present study, we aimed to characterize in greater detail the cellular mechanism(s) by which strontium ranelate inhibits osteoclastic differentiation and consequently bone resorption, using two cellular models, which are widely utilized to study osteoclastic differentiation, *i.e.* human peripheral blood monocytic cells (PBMC) and RAW 264.7 cells. We showed that increasing concentrations of Sr^{2+}_o decrease osteoclastic differentiation in a dose dependent manner, resulting in a reduction of *in vitro* bone resorption. Moreover, our results suggest that the effects of Sr^{2+}_o on osteoclasts are, at least in part, mediated by stimulation of the CaR, which

may, in turn, inhibit the RANKL-induced nuclear translocation of NFkB and AP-1 in the early stages of osteoclastic differentiation.

Materials and Methods

Reagents - Due to the limited solubility of strontium ranelate in the culture medium employed here, strontium chloride (MO, USA) mixed with sodium ranelate (Technologie Servier, Orléans, France) was used to test levels of Sr^{2+}_o up to 24 mM. A 1/100 ratio between ranelic acid and Sr^{2+} was used to reproduce the therapeutic circulating ratio observed in patients treated with strontium ranelate. As no appreciable differences were observed when strontium chloride was mixed with sodium ranelate (as a source of ranelic acid) or when strontium chloride was used alone, results are simply expressed in terms of the divalent strontium ion. R&D systems (MN, USA) provided both murine and human recombinant RANKL and human recombinant macrophage colony-stimulating factor (M-CSF). Culture plates were purchased from Corning (NY, USA). All other reagents were purchased from Sigma-Aldrich (MO, USA). It is of note that α-MEM and DMEM contain 1.8 mM of Ca^{2+}.

RAW 264.7 cell culture– The mouse monocyte cell line RAW 264.7 (TIB-71) was obtained from the American Type Culture Collection (ATCC) (Molsheim, France), and was routinely cultured in DMEM, supplemented with 10% heat-inactivated foetal calf serum (FCS), 2 mM L-glutamine and 100 U/mL penicillin/streptomycin (PS). For osteoclast differentiation, RAW 264.7 cells were seeded in 96-well plates (10^3 cells/well) and were cultured for 5 days in α-MEM supplemented with 10% FCS, L-glutamine, PS and murine recombinant RANKL (30 ng/mL). The culture media were replaced with fresh media on day three. RAW 264.7 cells were treated with Sr^{2+} during the full culture period (5 days), from day 0 to day 3, or from day 3 to day 5, depending of the experiment.

PBMC isolation and culture- Blood samples from healthy male donors were provided by a blood transfusion center (Etablissement Francais du Sang, Lille, France). To isolate PBMC, blood was diluted 1:1 (v/v) in RPMI culture medium, layered 2:1 (v/v) over Histopaque® 1077 (Sigma-Aldrich,

MO, USA) and centrifuged (400g for 30 min). The PBMC layer was collected and washed in RPMI, isolated again by centrifugation (250g) and resuspended in RPMI containing 10% FCS, glutamine and PS. PBMC were then seeded directly on bovine bone slices (6 mm diameter) in 48-well plates at a density of 500,000 cells per well. After 2 hours, bone slices were vigorously rinsed in order to remove non-adherent cells. The adherent cells were then cultured for 14 days in α-MEM containing human recombinant M-CSF (30 ng/mL) and human recombinant RANKL (25 ng/mL). Culture media were replaced twice a week.

Osteoclastogenesis assays - Osteoclast differentiation was assessed by both cytochemical staining and measurement of TRAP activity. Both RAW 264.7 and PBMC-differentiated osteoclasts were fixed for 10 min with 3.7% paraformaldehyde, washed with PBS at room temperature, and then stained for Tartrate Resistant Acid Phosphatase (TRAP) using the Leucocyte Acid Phosphatase kit (Sigma-Aldrich), following the manufacturer's instructions. Only TRAP-positive multinucleated (>3 nuclei) cells were considered to be osteoclasts. For the measurement of TRAP activity, cells were lysed and incubated for 1 hour with a reaction buffer containing paranitrophenylphosphate (pNPP). The reaction was stopped with NaOH (0.3 N), and optical densities were read using a microplate spectrophotometer (405 nm). Optical densities were compared to a standard curve calibrated with paranitrophenol (pNP). Protein content was quantified using the Biorad Protein Assay with serum albumin as a standard. Results were obtained as micromoles pNP/mg protein, and then converted to percentage of control. TRAP activity was measured in lysates obtained from both cellular models after five days (RAW 264.7 cells) or fourteen days (PBMC) of culture.

Bone resorption assay – 1- Pit area measurement: When all adherent cells were stripped from the bone slices, the osseous surface was stained with toluidine blue, allowing visualization of resorption lacunae. The percentage of the bone surface that was resorbed per slice was quantified using a computerized method (Software Biocom CounScan®). 2- Biochemical measurement: bone resorption was also assessed by evaluating collagen degradation products in culture supernatants. Culture

supernatants were harvested from wells when media were changed and pooled prior to assessment by HPLC for their HP content, following a previously described protocol (Lorget et al., 2000).

Cell viability - Cell viability was measured by the MTT assay. Briefly, RAW 264.7 cells were cultured with different concentrations of Sr^{2+}_o for 5 days in the absence of RANKL. Then, MTT was added to the culture and the plates were incubated at 37°C for 1 hour to allow the transformation of MTT into water-insoluble formazan crystals. Formazan was dissolved in isopropanol-0.1N HCl. Absorbance at both 570 nm and 660 nm (background) were then assessed using a microplate reader, in order to assess cellular viability. No significant differences were observed between the cells cultured in the presence or absence of Sr^{2+}_o, at concentrations ranging from 0.1 mM to 6 mM, and a culture duration as long as 120 h (data not shown).

Gene Delivery by Recombinant Adeno-Associated Virus - High-efficiency gene transfer into RAW 264.7 cells was accomplished using a rAAV-based method. A bovine CaR sequence with a naturally occurring dominant-negative mutation, R186Q (DN-CaR), or the same vector encoding βGal (as a control for non-specific effects of viral infection) were placed under the control of a cytomegalovirus immediate-early promoter element and packaged in the same vector as previously described (Tfelt-Hansen et al., 2003). Prior to being exposed to the virus, RAW 264.7 cells were cultured overnight in α-MEM supplemented with 10% FCS. Cells were then washed once with serum-free α-MEM, and about 1,000 viral particles/cell were used to infect each well (as optimized by pilot studies). Cells were incubated for 90 min in serum-free medium at 37°C in a cell culture incubator. Then, equal volumes of α-MEM containing 20% FCS were added to the cells to achieve a final serum concentration of 10%. The cells were then differentiated into osteoclasts following the procedure previously described.

Small interfering RNA – CaR SiRNA was designed and synthesized in collaboration with Eurogenetec (Liege, Belgium) based on the human reference sequence NM_000388. CaR SiRNA were designed in regions of the cDNA that are homologous with the murine sequence, and sequences will be provided

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by R.M. upon request. SiRNA transfections were carried out in triplicate on RAW 264.7 cells. Briefly, 0.2 µl of SiRNA (100 µM) was diluted into 50 µl α-MEM and added to each well. Next, 2.5 µl NeoFx (Ambion, Inc.) was diluted into 50 µl α-MEM for each sample, incubated for 10 min at room temperature, and 50 µl of diluted transfection mixture was added. Finally the mixture (100 µl) was added to wells that contained the RAW 264.7 cells, which were incubated for an additional 90 min at 37°C with 5% CO_2. Then a volume of α-MEM containing 20% serum that was equal to that in the wells was added to the cells to achieve a final serum concentration of 10%. The cells were finally cultured for 36 h prior to being used for the experiments described in subsequent sections. As negative controls, parallel experiments were carried out using scrambled SiRNA, which did not match the sequences of any mammalian mRNAs (Ambion, INC.). Depletion of endogenous mRNAs encoding CaR by SiRNA was confirmed by real time PCR and transcripts were knocked down by 80% in RAW 264.7 cells.

Flow cytometry analysis - 5×10^5 cells were incubated with CaR monoclonal antibodies or with isotype control antibodies for 30 min on ice in the dark. After washes in PBS-3% FBS and 0.1% sodium azide, cells were incubated with PE-conjugated IgG mouse antibodies for 30 min. After several washes, expression of cell surface CaR was analyzed by FACS (FACS Aria cytometer). Cytometry analysis has demonstrated that cell surface CaR is expressed by 96% of the RAW 264.7 cells (Supp Fig. 1).

Annexin V assay for determination of apoptosis/necrosis ratio was performed as follows: RAW 264.7 cells were washed twice with cold PBS; resuspended in 10 mM HEPES (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl2; and incubated for 15 min at room temperature with Annexin V-PE and 7-AAD (PE Annexin V Apoptosis Detection Kit I – from BD Pharmingen). Cells were analyzed within 1 h, by flow cytometry, using a FACS Aria cytometer.

Analysis of specific binding of AP-1 and NFκB to DNA by ELISA - Preparation of nuclear extracts of RAW 264.7 cells was performed using a nuclear extract kit (Active Motif, Carlsbad, CA) according to the instructions given by the manufacturer. Binding of c-Jun or p65 to their consensus oligonucleotides was determined using the ELISA-based Trans-AM AP-1 or NFκB kit (Active Motif), respectively. Briefly, 20 µg of nuclear proteins was incubated in a 96-well plate, which was pre-coated with oligonucleotide containing the AP-1 or NFκB consensus binding site. The 96-well plate was then treated with a primary antibody specific for the activated form of c-Jun or p65. Subsequent incubation with a secondary antibody (anti-IgG HRP-conjugated antibody) and a developing solution provided a colorimetric reaction, which was quantified at 450 nm with a reference wavelength at 655 nm. The specificity of the observed c-Jun and p65 binding was confirmed by incubation of nuclear extracts with the immobilized AP-1 or NFκB consensus binding probes in the presence of excess wild-type or mutated oligonucleotide (data not shown).

Statistical analysis - The results are expressed as mean ± SEM. The statistical differences among groups were evaluated using the Kruskal-Wallis test. The Mann-Whitney U-test was then used to identify differences between the groups when the Kruskal-Wallis test indicated a significant difference ($p < 0.01$).

Results

Effects of Sr^{2+}_{o} on osteoclastic differentiation

In both the RAW 264.7 cells and PBMC, increasing concentrations of Sr^{2+}_{o} were shown to decrease osteoclast differentiation in a dose-dependent manner (Fig. 1A and 1D). This effect was statistically different from the control cultures at concentrations of Sr^{2+}_{o} as low as 2 and 6 mM, in experiments completed with PBMC and RAW 264.7 cells, respectively. Sr^{2+}_{o}-induced effects were maximal when

the cells were cultured in the presence of 24 mM Sr^{2+}_o. At this concentration, Sr^{2+}_o was shown to reduce the differentiation of RAW 264.7 cells by 80% ($p < 0.001$) and PBMC differentiation by 100% ($p < 0.001$). As observed on photomicrographs, when RAW 264.7 cells were cultured in the presence of 6 mM Sr^{2+}_o (Fig. 1C), osteoclasts appeared smaller in size and a smaller number of nuclei was observed compared to control osteoclasts (Fig. 1B). In the PBMC model no morphological differences were observed at 6 mM Sr^{2+} (Fig. 1F) compared to control cultures (Fig. 1E). These effects on osteoclastic differentiation were confirmed by measurement of TRAP activity, another marker used to track the differentiation of mature osteoclasts (Fig. 2). Thus, when cells were differentiated in the presence of 24 mM Sr^{2+}_o, TRAP activity was significantly decreased by 85% (RAW 264.7 cells) and 97% (PBMC) compared to controls. Significant differences were observed at concentrations of Sr^{2+}_o as low as 6 mM ($p < 0.05$) and 1 mM ($p < 0.05$) in the murine and human models, respectively.

Effects of Sr^{2+}_o on bone resorption

As shown in the photomicrographs presented in figure 3, Sr^{2+}_o (6 mM) (Fig. 2B) reduced PBMC-differentiated osteoclastic bone resorption compared to that observed in control cultures (treated with RANKL and M-CSF alone) (Fig. 3A). Herein, bone resorption was assessed by measurement of both pit area and HP release. Sr^{2+}_o decreased osteoclastic bone resorption in a dose-dependent manner. Bone resorption was reduced by 48% when PBMC were cultured for fourteen days in the presence of 2 mM Sr^{2+}_o ($p < 0.01$, Fig. 3C). As observed when assessing the osteoclast differentiation, the effects of Sr^{2+}_o were maximal when the cells were cultured in the presence of 24 mM Sr^{2+}_o. At this concentration, osteoclastic bone resorption was completely blocked ($p < 0.001$, compared to control). These results were confirmed by HP measurement. However, the reduction of bone resorption was quantitatively smaller by HP measurement than by the measurement of pit area. Significant inhibition of HP release into the culture supernatant was observed at concentrations of Sr^{2+}_o as low as 6 mM (-54%, $p < 0.01$), reaching -70% when cells were cultured in the presence of 24 mM Sr^{2+}_o ($p < 0.01$, Fig. 3D).

Sequential effects of Sr^{2+}_{o} on osteoclastic differentiation

Due to the results obtained when we assessed the effects of Sr^{2+}_{o} on both osteoclast differentiation and bone resorption, we decided to pursue our investigation by adding Sr^{2+}_{o} (6 mM) at various times during the differentiation of osteoclast precursors to osteoclasts. When Sr^{2+}_{o} (6 mM) was added to late-stage (days 3-5) or early-stage (days 0-3) cultures of RAW 264.7 cells, its inhibitory effect was observed in the early-stage cultures rather than in the late-stage ones (Fig 4). Thus, introduction of Sr^{2+}_{o} during the early stages of the differentiation of RAW 264.7 cells (first 3 days) led to an inhibition of osteoclastic differentiation (-33%) similar to that observed when Sr^{2+}_{o} was added for the full 5 days of culture (-39%). Only a weak (-9%) but significant difference ($p < 0.05$) in osteoclast-like cell numbers was observed when Sr^{2+}_{o} was added for only the last two days of culture compared to control (RAW 264.7 cells treated with RANKL alone), suggesting that Sr^{2+}_{o} exerts its inhibitory effect on osteoclastogenesis during the first days of differentiation.

Intracellular events that mediate the effects of Sr^{2+}_{o} on osteoclastic differentiation

Osteoclast precursors sense RANKL through RANK, which then triggers an intracellular signaling mechanism responsible for the differentiation of osteoclast precursors through the interactions of TNF related activated factors (TRAFs) and rapid activation of transcription factors such as NFkB and AP-1. To test the role of NFkB and AP-1 in Sr^{2+}_{o}-induced effects, RAW 264.7 cells were treated for 45 min with RANKL (30 ng/ml), and then NFkB and AP-1 translocation into the nuclei was assessed using an ELISA-based technique (Trans-AM from Active motif). In either case, RANKL was shown to promote the translocation of these transcription factors compared to cells not treated with RANKL (Fig 5). Pre-incubation of RAW 264.7 cells for 4 h with Sr^{2+}_{o} (6 mM) prevented the RANKL-induced nuclear translocation of NFkB and AP-1 (Fig 5A and 5B, $p < 0.01$). Sr^{2+}_{o} alone did not modify the nuclear translocation of NFkB and AP-1, suggesting that Sr^{2+}_{o}-induced effects on these transcription factors depend on stimulation of the cells by RANKL.

Role played by the calcium-sensing receptor in Sr^{2+}_{o}-induced effects on osteoclast-like cells

Because Sr^{2+} is an agonist of the CaR (Chattopadhyay et al., 2007; Coulombe et al., 2004) and because we recently showed that Sr^{2+}_{o} induces osteoclast apoptosis through activation of the CaR (Hurtel-Lemaire et al., 2009), we hypothesized that Sr^{2+}_{o} might inhibit osteoclast differentiation directly via stimulation of the CaR. βGal- and DN-CaR-transfected RAW 264.7 cells were cultured for five days in the presence of RANKL (30 ng/ml). TRAP-positive multinucleated cells were then counted in wells in which the cells had been cultured in the presence or absence of Sr^{2+}_{o} (6 mM). As shown in figure 6A, when βGal-transfected RAW 264.7 cells were cultured for five days in the presence of Sr^{2+}_{o} (6 mM), osteoclast differentiation was reduced by 50% ($p < 0.001$) compared to cells cultured in the absence of Sr^{2+}_{o}. As previously observed (Mentaverri et al., 2006), transfection of the cells with DN-CaR led to a significant reduction in the number of TRAP-positive RAW cell-derived osteoclasts. A decrease of more than 50% compared to βGal-transfected cells treated with RANKL (30 ng/ml) alone was observed ($p < 0.001$), confirming the role played by the CaR, and calcium sensing mechanism, in the control of osteoclast differentiation process. The number of osteoclasts observed with DN-CaR-transfected RAW cells cultured for five days, in the presence of 6 mM Sr^{2+}_{o} was not reduced further and was not significantly different from that observed when DN-CaR cells were cultured in the absence of Sr^{2+}_{o} ($p = 0.15$). Thus, compared to the effects exert by Sr^{2+}_{o} on βGal-transfected cells, DN-CaR-transfection appears to protect RAW 264.7 cells from Sr^{2+}_{o}. Similar results were obtained when RAW 264.7 cells were transfected with CaR SiRNA, confirming the role played by the CaR in Sr^{2+}-induced effects (Figure 6B). The functionality of the SiRNA sequences utilized in this study was assessed by flow cytometry analysis that has demonstrated that while 74.6% of scramble SiRNA-transfected cells expressed cell surface CaR, only 23.1% of CaR-SiRNA-transfected cells expressed the receptor (Supp Fig. 1). Moreover, the ratio of receptor expression between CaR-SiRNA vs scramble SiRNA-transfected RAW 264.7 cells was even greater when total fluorescence intensity was calculated, with receptor expression of around 1:4.

Discussion

Bone volume and micro-architecture are under the control of both osteoblasts and osteoclasts, which, under normal conditions, are engaged in a continuous process of bone remodeling. It is accepted that this mechanism is controlled by both systemic and local factors, which tightly regulate the cellular differentiation, activity and apoptosis of both osteoblasts and osteoclasts (Manolagas and Jilka, 1995). In osteoporosis, this equilibrium is disrupted in favor of increased activity of osteoclastic cells, without the compensatory synthesis of new bone by osteoblasts (Hofbauer and Schoppet, 2004). Over the past decade, important insights have been made in the bone field, giving a new awareness of the regulation of bone remodeling. In this respect, growing evidence has confirmed the major role played by the triad RANK-RANKL-OPG (Katagiri and Takahashi, 2002; Martin, 2004) in the regulation of osteoclastic activity and in the communication between osteoclasts and osteoblasts. Numerous compounds formerly or currently used in the treatment of osteoporosis target at least one of the three cytokines involved in this triad, through mechanisms that for some of these compounds are not completely understood.

$Sr^{2+}{}_o$ previously deposited in bone may possibly be released from the bone matrix and, therefore, be present in the bone micro-environment during the process of bone resorption, as previously observed for Ca^{2+} (Silver et al., 1988). Thus, Sr^{2+} modulates directly the activity of osteoblasts and osteoclasts, *in vitro* (Marie, 2005; Marie, 2006) and probably *in vivo*. In 2002, Baron et al. (Baron and Tsouderos, 2002) first showed that strontium ranelate reduces the differentiation of osteoclasts in a dose dependent manner, using chicken bone marrow cells as a model. As recently demonstrated by Bonnelye using osteoclast precursors isolated from the spleens of mice (Bonnelye et al., 2008), we found that Sr^{2+}, at concentrations as low as 2 mM, significantly reduces osteoclastic differentiation in both RAW 264.7 and PBMC models. Altogether these data indicate that Sr^{2+} inhibits the osteoclastic differentiation process through a direct effect on osteoclast precursor cells. It is of note, however, that significant inhibitory effects were obtained at concentrations of about 1-2 mM in the human (PBMC) and the chicken bone marrow models, while higher concentration of $Sr^{2+}{}_o$ (6 mM) was necessary to reach the same effects in the murine model (RAW 264.7 cells). Because of the complexity of the

intracellular signaling and the intrinsic differences that exist between each cell type, it is conceivable that Sr^{2+}_o might be more effective at reducing osteoclast differentiation of some precursors than others. Thus, the relative effectiveness may depend on factors in the transduction pathways, available for activation in each cell type, which requires further investigation.

Having demonstrated that Sr^{2+} significantly inhibits osteoclast formation, we showed that Sr^{2+} also interferes with their function. These results corroborate those obtained by Baron et al., (Baron and Tsouderos, 2002) and by Bonnelye et al., (Bonnelye et al., 2008), and confirm that Sr^{2+}-induced effects on osteoclastogenesis may be directly associated with a significant and dose dependent reduction in bone resorption. Alternatively, Sr^{2+} may induce its effects directly on both osteoclast precursors and mature resorbing cells. Hence, these effects could also be related to a decreased of mature osteoclast number, to disruption of the osteoclast actin-containing sealing zone (Bonnelye et al., 2008), as well as to other mechanisms that directly affect bone resorption and remain to be identified. We have also established that Z-VAD-fmk, a caspase cascade inhibitor peptide had no effect on Sr^{2+}_o-induced inhibition of osteoclastogenesis when introduced in culture at concentrations ranging from 10 to 50 µM, and that cell viability was not modified in the presence of Sr^{2+}_o, at concentrations up to 6 mM or at cell culture incubation times as long as 120 h (data not shown). This finding was further confirmed by the annexin V / 7-AAD method (Supp Fig. 2) and lead us to conclude that apoptosis of osteoclast precursors, or any toxic effects of Sr^{2+}_o, is not brought into play in the effects induced by strontium concerning their differentiation into mature osteoclasts, at least when cells were exposed to concentrations of Sr^{2+}_o up to 6 mM.

Clearly, osteoclasts, as calcium-mobilizing cells, are under the control of Ca^{2+}_o, which have been shown to modulate osteoclastogenesis, osteoclast bone resorption and osteoclast apoptosis. As observed for Ca^{2+} (Takahashi et al., 2002), in the murine model, treatment with 6 mM Sr^{2+}_o at an early stage of culture (the initial 72 hours) reduced the number of TRAP-positive, multinucleated cells. This is in contrast to the data obtained when Sr^{2+} or Ca^{2+} were introduced for the last two days of culture. In these culture conditions Ca^{2+} was shown to stimulate the osteoclastic differentiation process (Takahashi et al., 2002), while, in our study, Sr^{2+} did not. Taken together, these

data suggest that Sr^{2+} and Ca^{2+} may inhibit the osteoclast differentiation process through, at least to some degree, different mechanisms of action.

The interaction between the RANKL and its receptor is a mandatory signal that activates, among other factors, NFkB and the AP-1. Increasing doses of Sr^{2+}_o were shown to down-regulate RANKL-induced nuclear translocation of both NFkB and AP-1, probably inhibiting the RANKL-induced osteoclast differentiation *in vitro*. From these results, one can hypothesize that an adaptative mechanism may take place in osteoclast precursors when they are stimulated by Sr^{2+}. Nonetheless, as observed for Ca^{2+} (Takami et al., 2000), Sr^{2+}_o is also likely to act on other cells present in the microenvironment of osteoclasts, such as osteoblasts or stromal cells. Therefore Sr^{2+}_o may affect osteoclast differentiation *in vivo* by modulating the production of osteoclastogenic cytokines, such as RANKL, OPG and/or M-CSF in the bone microenvironment, as was recently suggested (Atkins et al., 2008, Brennan, 2009).

Activation of the CaR is known to contribute to Ca^{2+}- or Sr^{2+}_o-induced effects on bone cells (Brown, 2003; Mentaverri et al., 2006). Since the presence of the CaR has been demonstrated on osteoclast precursors (House et al., 1997; Kanatani et al., 1999; Yamaguchi et al., 1998), we hypothesized that the CaR was involved in the Sr^{2+}_o-induced inhibition of osteoclast differentiation. Our studies, in which we utilized the dominant negative form of the CaR (R186Q) and CaR SiRNA, confirm this hypothesis and provide new evidence for the involvement of the CaR 1) in osteoclast differentiation and 2) in Sr^{2+}_o-induced effects on osteoclast differentiation. Since we have shown that different signaling pathways downstream of the CaR exist for Sr^{2+} and Ca^{2+} and that both ions modulate RANKL-induced NFkB nuclear translocation in mature rabbit osteoclasts (Hurtel-Lemaire et al., 2009), it would be very interesting to assess whether a similar mechanism takes place in osteoclast precursors. In a more integrative manner, these results are in line with those recently published by Chang et al (Chang et al., 2008), who utilized conditional knockout of the CaR in osteoblasts to make an important step forward in understanding the role played by the CaR in osteoblasts under non pathological conditions. Taken together, these recent finding confirm the CaR as one of the key actors that control the activities of bone cells. These data also suggest that more studies need to be carried out in order to assess the exact roles played by the CaR expressed on bone cells in both normal and pathological processes that take place or involve bone tissue.

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In conclusion, in the present study we confirmed that Sr^{2+}_o is a potent inhibitor of osteoclastic differentiation *in vitro*. Thus osteoclast precursors can sense Sr^{2+}_o, which is now known to regulate both osteoclast differentiation and bone resorption. As indicated in a schematic illustrating the data we published so far, through stimulation of the CaR, Sr^{2+}_o modulates different stages of the osteoclast lifespan (Fig. 7). It is conceivable that Sr^{2+}, acting through the CaR, may significantly modulate bone cell activity, leading to a reduction in the risk of fracture in osteoporotic subjects.

Acknowledgements

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Footnotes

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Legends for figures

Figure 1. *Sr^{2+}_o inhibits RANKL-induced osteoclastic differentiation of both RAW 264.7 cells and PBMC*. A) RAW 264.7 cells were cultured for 5 days in the presence of RANKL (30 ng/ml) with or without increasing levels of Sr^{2+}_o (from 0 to 24 mM). Cells were then fixed with paraformaldehyde and stained for TRAP activity. Photomicrographs are representative of the cellular population obtained when RAW 264.7 cells were cultured for five days either B) in the absence or C) in the presence of Sr^{2+}_o (6 mM). Data are expressed as the percentage of TRAP-positive cells derived from cultures treated with RANKL alone, in each experiment. Data are representative of three independent experiments (4 wells per condition were counted for each experiment). ** $p < 0.01$ and *** $p < 0.001$ compared to the number of TRAP-positive cells derived from cultures treated with RANKL alone. D) PBMC were cultured for 14 days in the presence of 30 ng/ml M-CSF, 25 ng/ml RANKL, with or without various concentrations of Sr^{2+}_o, ranging from 0 to 24 mM. The photomicrographs are representative of the cellular population obtained when PBMC were cultured for fourteen days, either E) in the absence or F) in the presence of Sr^{2+}_o (6 mM). Data are expressed as the percentage of TRAP-positive multinucleated cells counted in wells in which PBMC were cultured in the presence of RANKL and M-CSF alone (Ct). Data are representative of three independent experiments (4 wells per condition were counted for each experiment). * $p < 0.05$ and *** $p < 0.001$ compared to control.

Figure 2. *Sr^{2+}_o inhibits TRAP activity in both RAW 264.7 cells and PBMC*. RAW 264.7 cells and PBMC were cultured for 5 days in the presence of RANKL (30 ng/ml) and for 14 days in the presence of RANKL and M-CSF, respectively. Simultaneously, cells were exposed to increasing levels of Sr^{2+}_o, from 0 to 24 mM. Cells were then lysed, and TRAP activity was measured as described in the Materials and Methods. Data are expressed as the percentage of TRAP activity measured in cells derived from each model in the absence of Sr^{2+}_o (Controls). Data are representative of three independent experiments (4 wells per condition were counted for each experiment). * $p < 0.05$ and *** $p < 0.001$ compared to controls (Ct).

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Figure 3. *Increasing Sr^{2+}_o inhibits the osteoclastic bone resorption.* Formation of resorption pits on bone slices by PBMC-derived OCLs cultured for 14 days in the presence of M-CSF (30 ng/mL) and RANKL (25 ng/mL). PBMC were cultured for 14 days in the presence of increasing concentrations of Sr^{2+}_o, from 0 to 24 mM. Pictures are representative of the bone resorbing activity exerted by the PBMC-differentiated osteoclasts on the osseous surface, when cultured either A) in the absence or B) in the presence of Sr^{2+}_o (6 mM). The bone resorbing activity was assessed C) by pit area measurement as well as D) by determination of hydroxylysylpyridinoline (HP) levels in the culture supernatant. Results are expressed as percent of control and represent the mean ± SEM of three independent experiments (4 wells per condition were counted for each experiment). ** $p < 0.01$ and *** $p < 0.001$ compared to controls (Ct).

Figure 4. *Sr^{2+}_o affects osteoclastogenesis at an early stage of differentiation.* The effects of Sr^{2+}_o on osteoclast differentiation were assessed in RAW 264.7 cells treated for five days with RANKL (30 ng/ml). Sr^{2+}_o (6 mM) was introduced into the culture for the first three days, for the last two days, or for the full length of the differentiation (5 days). Data are expressed as the percentage of TRAP-positive cells derived from RAW 264.7 cells treated with RANKL alone (Ct). Data are representative of four independent experiments (5 wells per condition were counted for each experiment). * $p < 0.05$ and *** $p < 0.001$ compared to cultures treated with RANKL alone. ns = non significant.

Figure 5: *Effect of Sr^{2+}_o on the RANKL-induced promoter binding of the transcription factors AP1 and NFkB.* Prior to stimulation, the RAW 264.7 cells were pre-incubated for 4h in α–MEM and in the absence of FCS. The cells were then incubated with or without 6 mM Sr^{2+}_o for 4 hours in the absence of FCS. Finally, RANKL (30 ng/ml) was added for 30 min before the preparation of nuclear extracts. The promoter binding activity was determined with ELISA kits provided by Active Motif®. Results are expressed as the percentage of control and are representative of five independent experiments. ns = non significant.

Figure 6. *Role played by the CaR in Sr^{2+}_o-induced effects on OC differentiation.* A) βGal- and DN-CaR-transfected RAW 264.7 cells were cultured for 5 days with 30 ng/ml of RANKL. Cells were cultured in the presence or absence (Ct) of Sr^{2+}_o(6 mM). Cells were then fixed with 3.7% paraformaldehyde and stained for TRAP activity. Data represent the mean ± SEM of the number of TRAP-positive osteoclast-like cells (OCLs) counted in each well and are representative of three independent experiments (4 wells per condition were counted for each experiment). *** $p < 0.001$ compared to the number of cells derived from βGal-transfected RAW 264.7 cells treated with RANKL alone. B) Similar experiments where conducted using CaR or scramble SiRNA-transfected RAW 264.7 cells. Data represent the mean ± SEM of the number of TRAP-positive osteoclast-like cells (OCLs) counted in each well and are representative of three independent experiments (4 wells per condition were counted for each experiment). * $p < 0.05$, ** $p < 0.01$ compared to the number of cells derived from Scramble SiRNA-transfected RAW 264.7 cells treated with RANKL alone. NS = non significant.

Figure 7. *Schematic representation of the role played by strontium on osteoclasts through the stimulation of the CaR.* Among potential actions, Sr^{2+}_o exerts its effects mainly through activation of the CaR for reduced the osteoclastic differentiation and bone resorption at the same time as stimulating apoptosis of mature osteoclasts. All essential steps of the osteoclast lifespan appear to be affected by the compound. Thus, under the effect of Sr^{2+}_o, a decrease in osteoclast differentiation is observed. This decrease was the direct consequence of reduced osteoclastic bone resorption by decreasing the number of mature active osteoclasts. Simultaneously, an increase of osteoclast apoptosis is observed. This cell death contributes to reduce bone resorption, at least *in vitro*.

RAW 264.7 cells differentiation

A

B

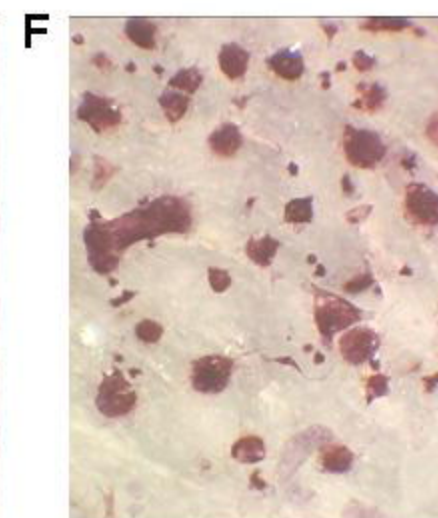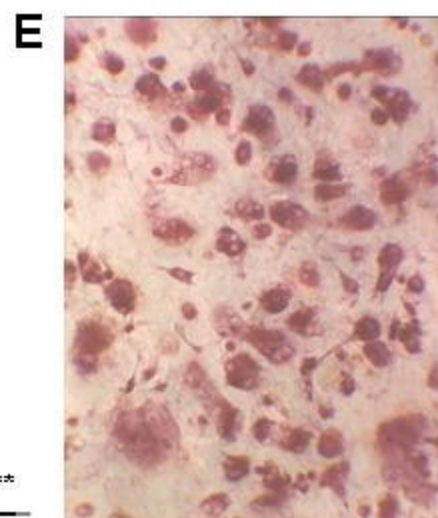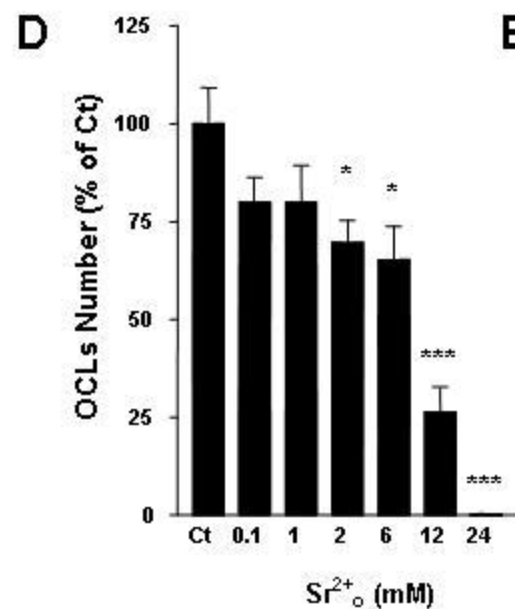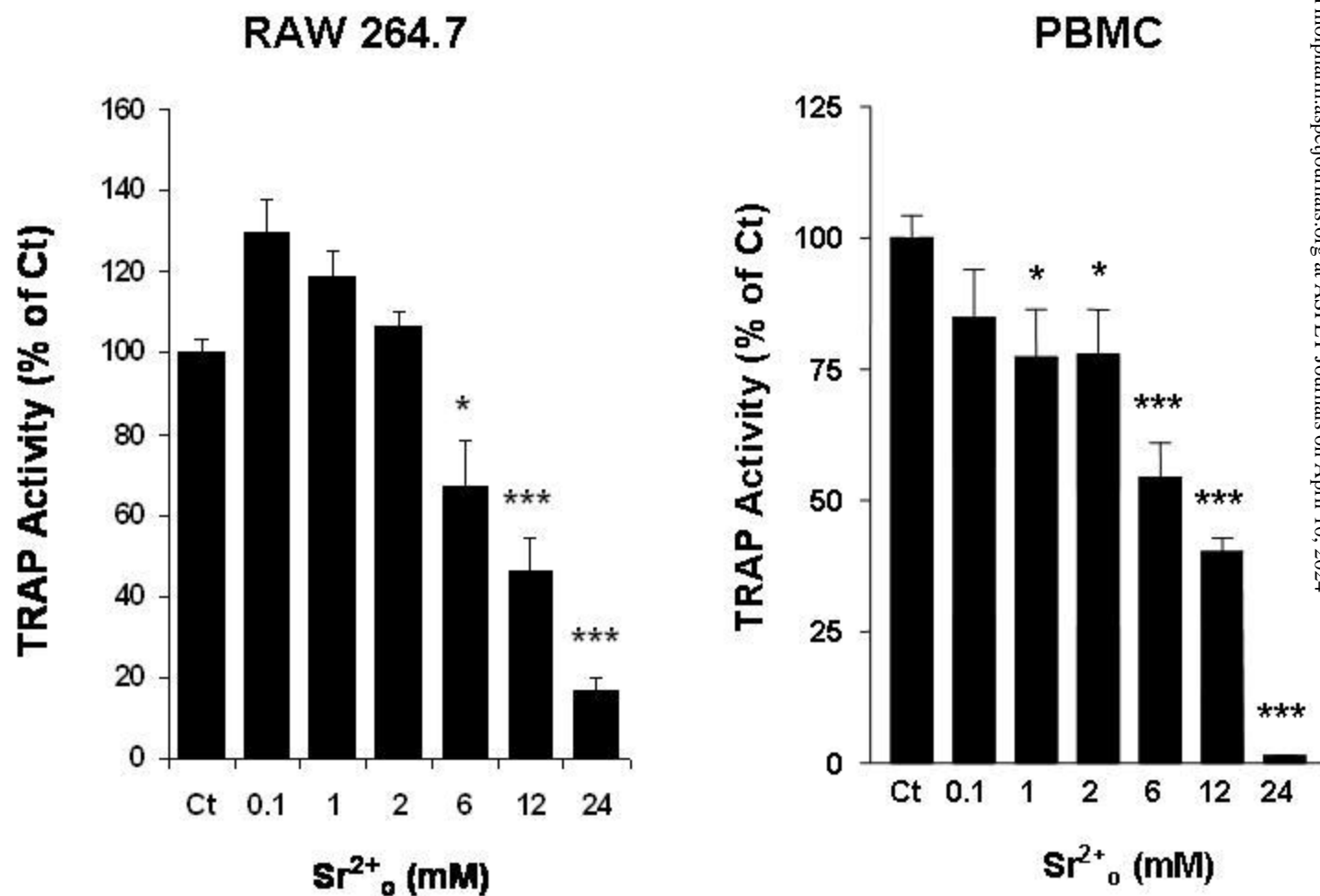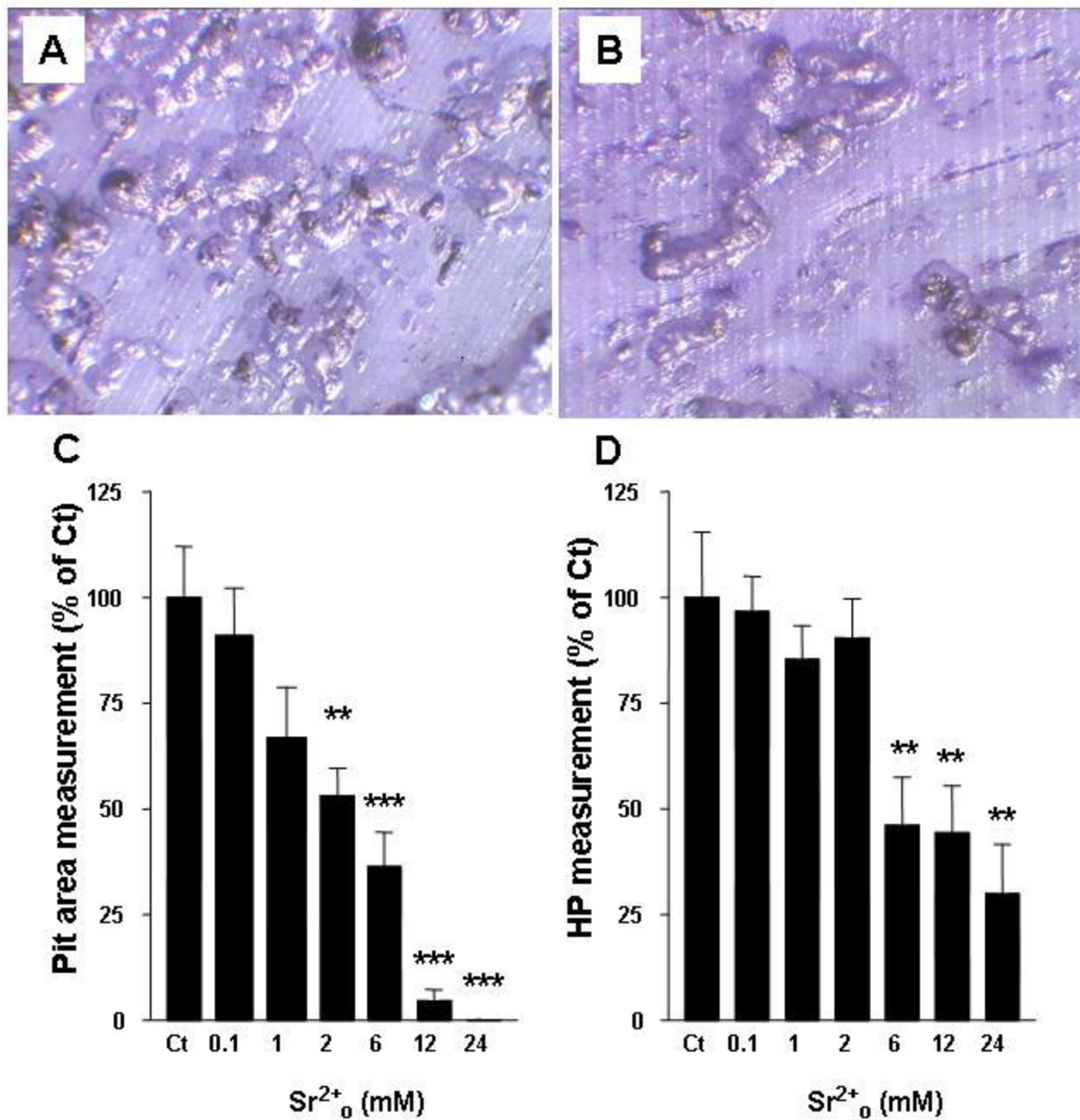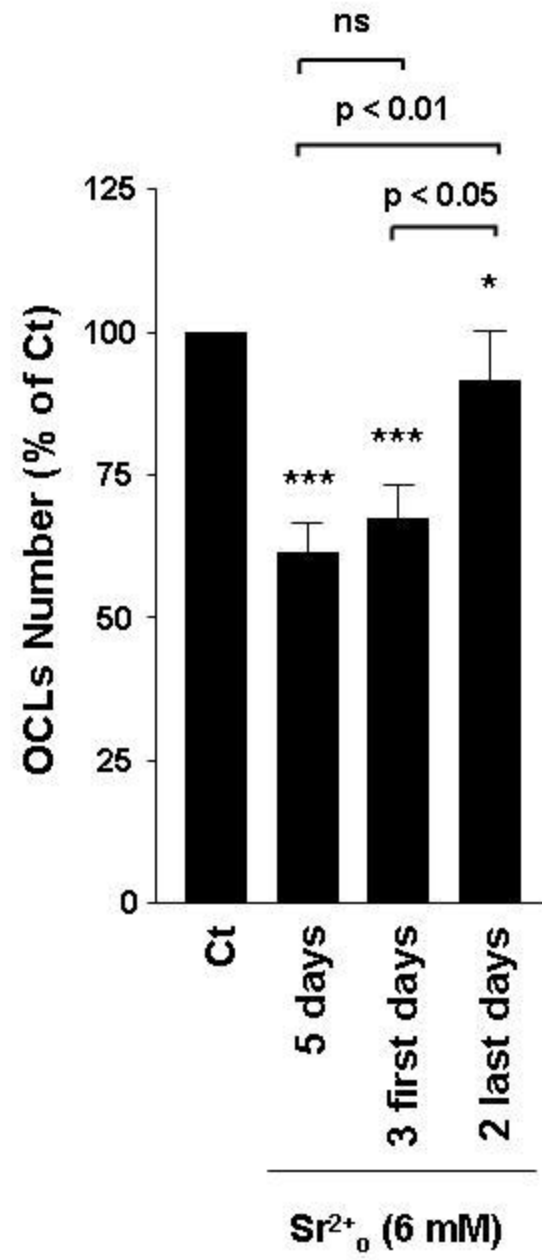**C**

PBMC differentiation
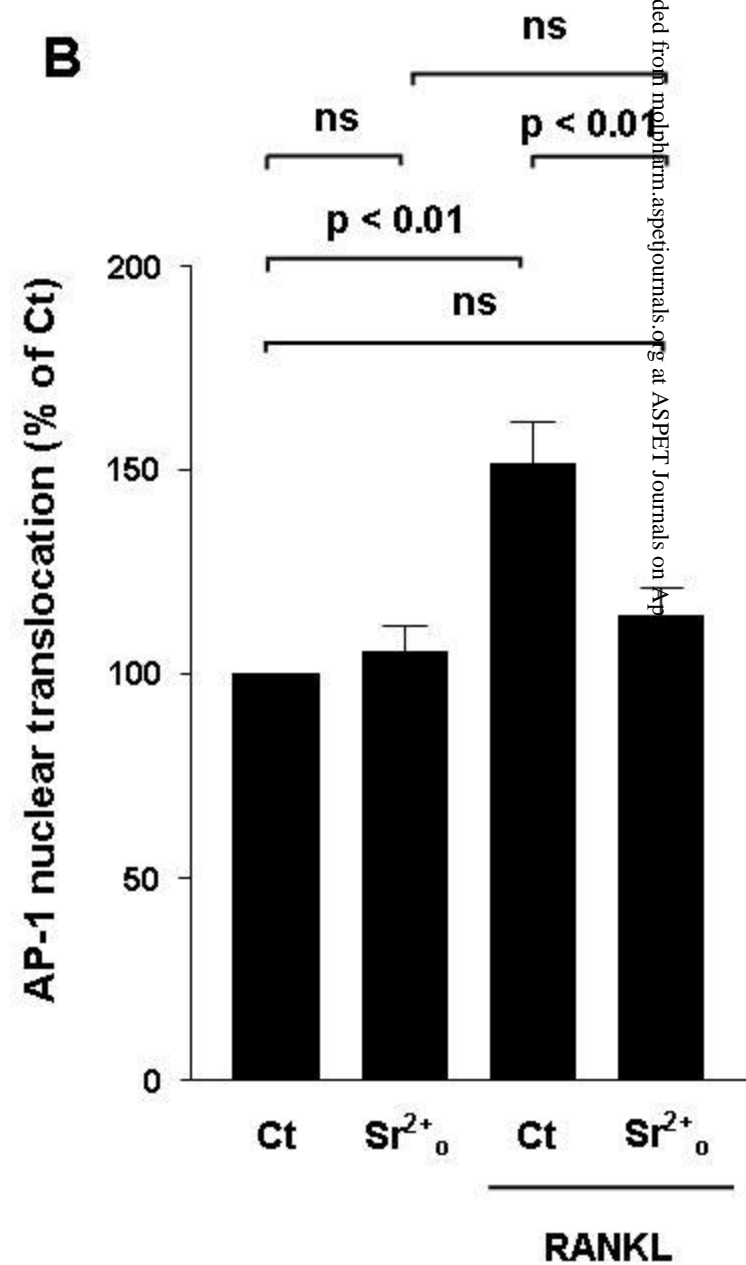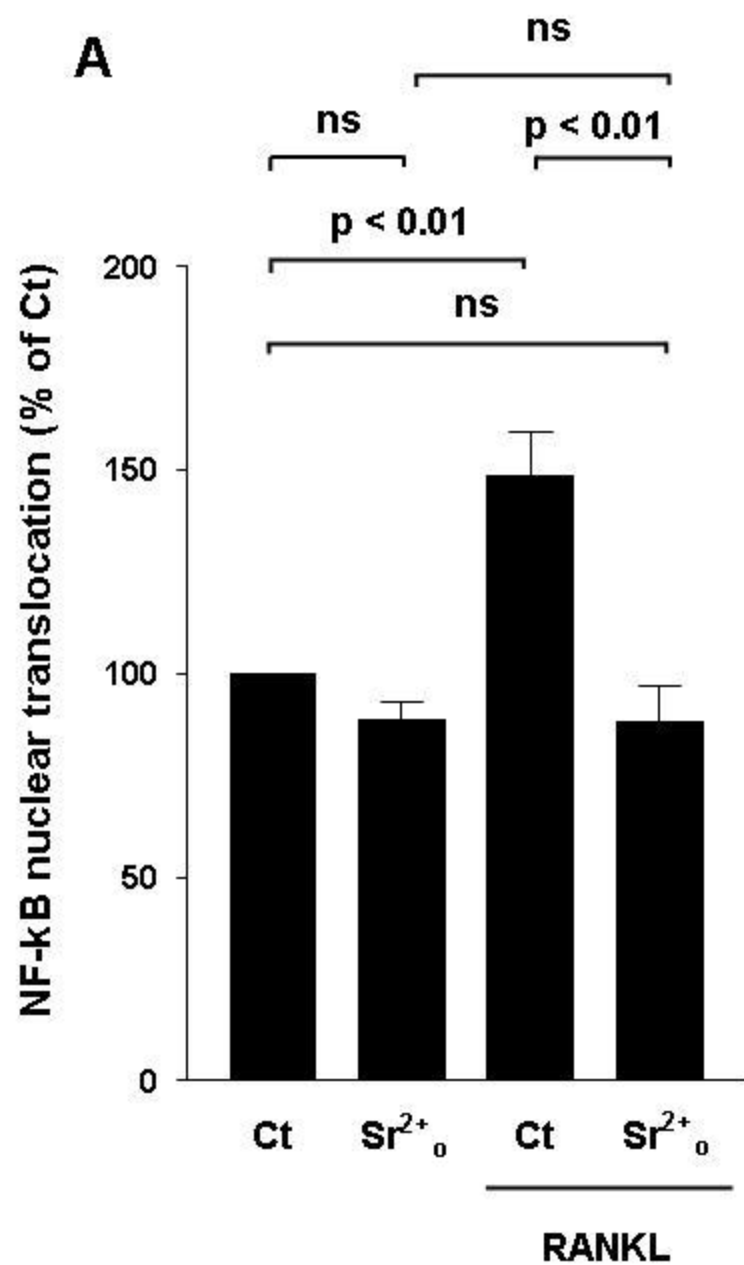

D

E

F

Figure 1

RAW 264.7
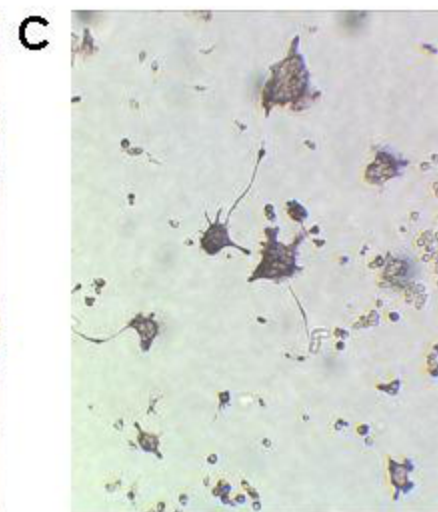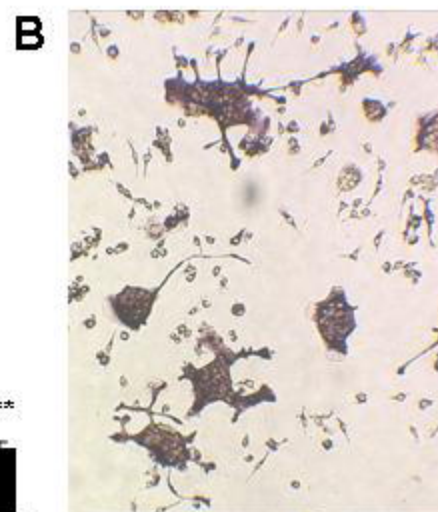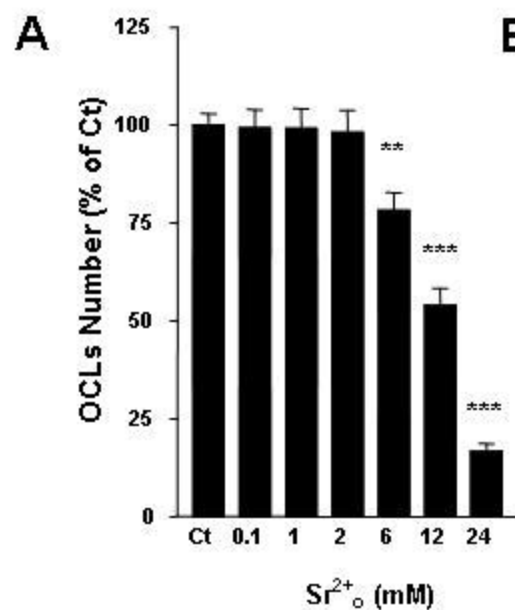
PBMC

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7