Inhibitory effects of eugenol on RANKL-induced osteoclast formation via attenuation of NF-κB and MAPK pathways

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Abstract

Bone loss diseases are often associated with increased receptor activator of NF-κB ligand (RANKL)-induced osteoclast formation. Compounds that can attenuate RANKL-mediated osteoclast formation are of great biomedical interest. Eugenol, a phenolic constituent of clove oil possesses medicinal properties; however, its anti-osteoclastogenic potential is unexplored hitherto. Here, we found that eugenol dose-dependently inhibited the RANKL-induced multi-nucleated osteoclast formation and TRAP activity in RAW264.7 macrophages. The underlying molecular mechanisms included the attenuation of RANKL-mediated degradation of IκBα and subsequent activation of NF-κB pathway. Furthermore, increase in phosphorylation and activation of RANKL-induced mitogen-activated protein kinase pathways (MAPK) was perturbed by eugenol. RANKL-induced expression of osteoclast specific marker genes such as TRAP, cathepsin K (CtsK), and matrix metalloproteinase-9 (MMP-9) was remarkably downregulated by eugenol. These findings provide the first line of evidence that eugenol mediated attenuation of RANKL-induced NF-κB and MAPK pathways could synergistically contribute to the inhibition of osteoclast formation. Eugenol could be developed as therapeutic agent against diseases with excessive osteoclast activity.

Keywords: Eugenol; RAW264.7 macrophages; Osteoclast; RANKL; NFκB pathway; MAPK pathway

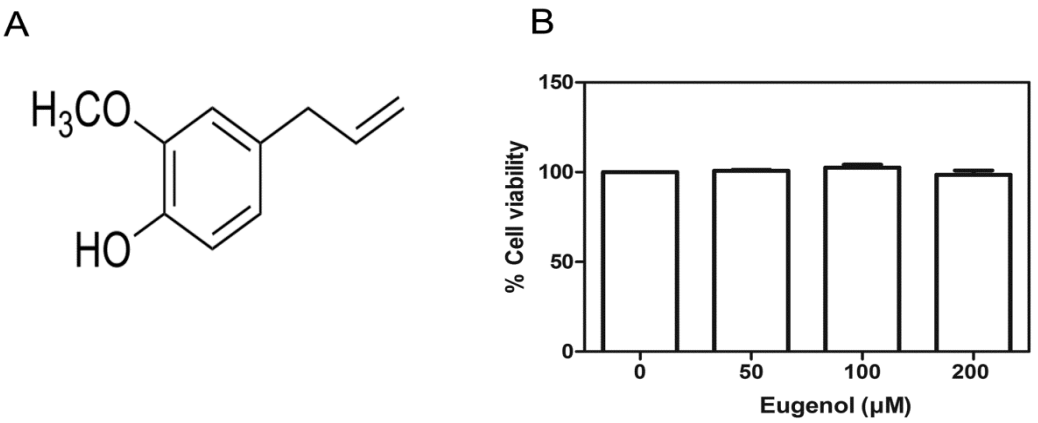
Introduction

Osteoclasts are large multinucleated cells that originate from mononuclear precursor cells of the monocyte-macrophage lineage. These are the only cells known to be capable of resorbing bone (1). Osteoblasts arise from mesenchymal stem cells and are responsible for bone formation and mineral deposition (2). The activity of osteoclasts is counterbalanced by osteoblasts and is often perturbed during diseases such as osteoporosis, rheumatoid arthritis, osteomyelitis, periodontitis, cancer-related hypercalcemia and bone metastases (3).

RANKL (receptor activator of NF-κB ligand) is a type II homotrimeric transmembrane protein expressed by osteoblasts as a membrane-bound and as a secreted protein along with a soluble decoy receptor of RANK, osteoprotegerin (4). RANKL binds to its receptor on osteoclast progenitor cells and initiates downstream signalling cascades indispensable for osteoclastogenesis. TNF Receptor-Associated Factor (TRAF) proteins transmit the RANK signals to downstream targets and thereby activate the NF-κB pathway and the three mitogen-activated protein kinase pathways (MAPK): JNK, ERK and p38 (5, 6). Activation of these pathways leads to osteoclast differentiation and activation. Hence, targeting the RANKL-pathway may be an effective approach for developing therapy against diseases characterised by excessive osteoclast activity.

Plant-derived natural compounds have been reported to be a promising source for discovery of new drugs targeting RANKL-induced osteoclast formation (7-10). Eugenol (4-allyl-2-methoxyphenol) is a biologically active phenolic constituent of *Syzigium aromaticum/ Eugenia aromaticum* (cloves; Fig. 1A) (11). Cinnamon, nutmeg and bay leaves also contain eugenol. Eugenol is used as a flavouring agent in the cosmetic and food industry as well as a cavity filling cement in dentistry (11). Several lines of evidence suggest that eugenol possesses potent anti-cancer, anti-inflammatory and anti-oxidant activities (11, 12). In this study the effects of eugenol on RANKL-induced osteoclast formation in RAW264.7 macrophages and underlying molecular mechanisms were investigated.

Figure 1. (A) Molecular structure of eugenol. **(B)** Cell viability of eugenol-treated RAW264.7 macrophages. Cells were treated with indicated concentrations of eugenol for 48 h and cell viability was measured using alamar blue assay. Data are expressed as mean ±SEM percent of control and are representative of three independent experiments.



Materials and methods

Reagents and materials

Dulbecco's Modified Eagle Medium (DMEM) and heat inactivated fetal bovine serum (FBS) were obtained from GIBCO (Grand Island, NY, USA). Penicillin/Streptomycin/Fungizone-Mix (#226) was supplied by Highveld Biological (Sandringham, South Africa). RANKL (#462-TEC) was purchased from Research and Diagnostic Systems (Minneapolis, MN, USA). Eugenol (#E51791), protease cocktail inhibitors (#P2714), TRI® reagent (#T9424) and Leukocyte Acid Phosphatase assay (TRAP) kit (#387A-KT) were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). Alamar blue reagent (#DAL1100) and cell extraction buffer (#FNN0011) were acquired from Life Technologies (Carlsbad, CA, USA). The bicinchoninic acid (BCA) protein assay kit (#23227) was supplied by Thermo Scientific (Rockford, IL, USA). M-MuLV reverse transcriptase (#M0253S) was purchased from New England Biolabs (Hitchin, UK). KAPA SYBR FAST qPCR Kit Master Mix (2X) (#KK4600) was bought from Kapa Biosystems (Cape Town, South Africa). Rabbit polyclonal antibodies against JNK (#22928), (p)-JNK-Thr^{183}/Tyr^{185} (#11504), ERK (#29162), (p)-ERK-Thr^{202}/Tyr^{204} (#12082), p38 (#21683), (p)-p38-Thr^{180}/Tyr^{182} (#11581), p65 (#21014), (p)-p65-Ser^{536} (#11014), IκBα (#21122), (p)-IκBα-Ser$^{32/36}$ (#11152) were procured from Signalway Antibody LLC. (College Park, MD, USA) and to that of GAPDH (#37168) were supplied by Abcam (Cambridge, MA, USA). Goat-anti-rabbit alkaline-phosphatase-conjugated secondary antibodies and NBT-BCIP chromogenic substrate were purchased from Life Technologies (Carlsbad, CA, USA).

Cell culture and maintenance

RAW264.7 murine macrophages (#TIB-71) were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in DMEM with 10% FBS. All media were supplemented with penicillin (100 U ml^{-1}), streptomycin (100 µg ml^{-1}) and fungizone (0.25 µg ml^{-1}) and were regarded as complete culture medium. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. Eugenol was prepared in DMSO at a stock concentration of 1M and was stored in the dark at -80 ^{o}C until further use. Stock solutions were freshly diluted to working

concentrations in complete culture medium before experiments. The final DMSO concentration in the culture medium did not exceed 0.02% (v/v). All of the cell culture experiments, including eugenol as well as controls were vehicle (DMSO) treated. Cells were pre-exposed to various concentrations of eugenol or vehicle for 4h followed by RANKL alone or RANKL in combination with eugenol throughout the experiments.

Alamar Blue assay

RAW264.7 macrophages were plated in 96-well plates in DMEM containing 10% FBS at a density of 5 x 10^3 cells/well. After 12h of attachment, cells were exposed to increasing concentrations of eugenol (50 µM, 100 µM and 200 µM) and further incubated for 2 days. Alamar blue assay was conducted as per manufacturer's instructions (Life Technologies). Fluorescence was measured at 590 nm on a microplate reader (Biotek, VT, USA). Results were evaluated by plotting the fluorescent signal versus compound concentration and expressed as percentage of control.

Osteoclast differentiation and TRAP activity assay

RAW264.7 macrophages were differentiated into osteoclasts as described earlier (13). Briefly, cells were suspended in DMEM containing 10% FBS and seeded into sterile 24-well culture plates at a density of 1.5 x 10^4 cells per well. Cells were pre-treated with increasing concentrations of eugenol (50 µM, 100 µM and 200 µM) or vehicle for 4 h, followed by stimulation with RANKL (15 ng ml^{-1}) alone or in combination with eugenol for 5 days. Cell culture media and factors were replenished every third day. After 5-days of differentiation, cells were simultaneously assessed for TRAP activity and TRAP-staining for osteoclast formation. Assessment of TRAP activity was performed as described earlier (14). The absorbance was measured at 405 nm on a microplate reader and expressed as percentage of control (Epoch, Biotek, VT, USA). TRAP-staining was conducted using a Leukocyte Acid Phosphatase assay kit according to the manufacturer's instructions (Sigma-Aldrich). TRAP-positive multinucleated cells containing more than 3 nuclei were counted as osteoclasts. Photographs were taken with a Discovery V20 Stereo-microscope equipped with an axiocam MRc5 camera (Zeiss,

Oberkochen, Germany). Total number of TRAP-positive cells per well of a 24-well plate (at least three wells per group) were counted and quantified.

Osteoclast specific gene expression analysis

RAW264.7 macrophages (4 x 10^4 cells per well) were seeded in 24-well plates and pre-exposed to 200 µM of eugenol or vehicle for 4h followed by treatment with RANKL alone or in combination with eugenol for 72h. Total cellular RNA was extracted from the cells using TRI® reagent (Sigma-Aldrich). One microgram of the extracted RNA was reverse transcribed into cDNA using M-MuLV reverse transcriptase (New England Biolabs). The quantitative real-time PCR (qPCR) assay was performed using KAPA SYBR FAST qPCR Kit Master Mix (Kapa Biosytems). The relative gene expression levels were calculated and analyzed by $2^{-\Delta\Delta CT}$ method (15). Results were normalized to the housekeeping gene GAPDH. Primers for osteoclast specific genes used in this study are provided in Table 1.

Western blot analysis

RAW264.7 macrophages (2 x 10^5 cells per well) were seeded in 6-well plates and pre-exposed to 200 µM eugenol or vehicle for 4h followed by exposure to RANKL alone or in combination with eugenol for 30 min. Cells were lysed in ice-cold cell extraction buffer supplemented with protease cocktail inhibitors for 30 min on ice, with vortexing at 10 minute intervals. Resultant cell lysates were centrifuged at 15,000 g for 30 minutes at 4°C and the supernatant was stored in aliquots at -80°C until further use. Purified proteins were quantified using a BCA protein assay kit as per manufacturer's directions (Sigma-Aldrich). Equal amount of proteins were loaded onto each lane and resolved on a 12% polyacrylamide gel and electrotransferred to nitrocellulose membranes with Tris-glycine transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol v/v). Membranes were blocked with 3% BSA in PBS containing 0.05% Tween-20 for 1h and were probed with indicated antibodies at 4°C overnight followed by incubation with goat-anti rabbit-alkaline-phosphatase-conjugated secondary antibody for 1h. Blots were developed using NBT/BCIP substrate and digital images of the blots were acquired using a flatbed scanner (Ricoh Aficio, Johannesburg, South Africa). The densitometric data for band

intensities in different sets of experiments were generated by analysing the digital images on ImageJ software (16).

Statistical analysis

Data are representative of three independent experiments unless otherwise stated and are represented as mean ±SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc multiple comparison test using Graph Pad Prism Software (Graph Pad software Inc, CA, USA). *P*-value <0.05 was regarded as statistically significant.

Results

Eugenol does not inhibit cell viability of RAW264.7 macrophages

RAW264.7 macrophages were treated with 50 to 200 µM of eugenol for 48h and cell viability was assessed by alamar blue assay. Eugenol at tested concentrations had no cytotoxic effects on RAW264.7 macrophages (Fig. 1B). Therefore, concentrations within this range were chosen to investigate the effects of eugenol on osteoclast formation and TRAP activity.

Eugenol inhibits RANKL-induced osteoclast formation and TRAP activity in RAW264.7 macrophages

RAW264.7 macrophages were pre-treated with indicated concentrations of eugenol or vehicle for 4h followed by exposure to RANKL alone or in combination with eugenol and allowed to differentiate into osteoclasts for 5 days. As shown in Fig. 2A, RANKL-induced the formation of osteoclasts in vehicle control cells. In contrast, osteoclast differentiation was markedly reduced in eugenol treated cells (Fig. 2 B, C, D). Furthermore, eugenol significantly inhibited the RANKL-induced osteoclast differentiation in RAW264.7 macrophages in a dose-dependent manner as evidenced by fewer osteoclasts formed (Fig. 2E). Additionally, TRAP activity (an osteoclast marker enzyme) significantly declined in the presence of eugenol (Fig. 2 F). Eugenol at a concentration of 200 µM showed the maximum inhibitory effects on osteoclast formation and TRAP activity, hence this concentration was chosen for downstream experiments.

Figure 2. Effects of eugenol on RANKL-induced osteoclast formation. **(A-D)** RAW264.7 macrophages were pre-treated with indicated concentrations of eugenol or vehicle for 4 h followed by stimulation with RANKL (15 ng ml-1) or RANKL plus eugenol for 5 days. TRAP-positive cells containing more than 3 nuclei were counted as osteoclasts. Osteoclasts stain purple/red in the presence of TRAP (Scale bars: 20 µm). **(E)** The numbers of TRAP positive multi-nucleated osteoclasts werecounted in each well. **(F)** TRAP activity was quantitatively measured and is represented as percent of control. The results are mean ±SEM and are representative of three independent experiments. (*P<0.05, **P <0.01, ***P <0.001 vs control).

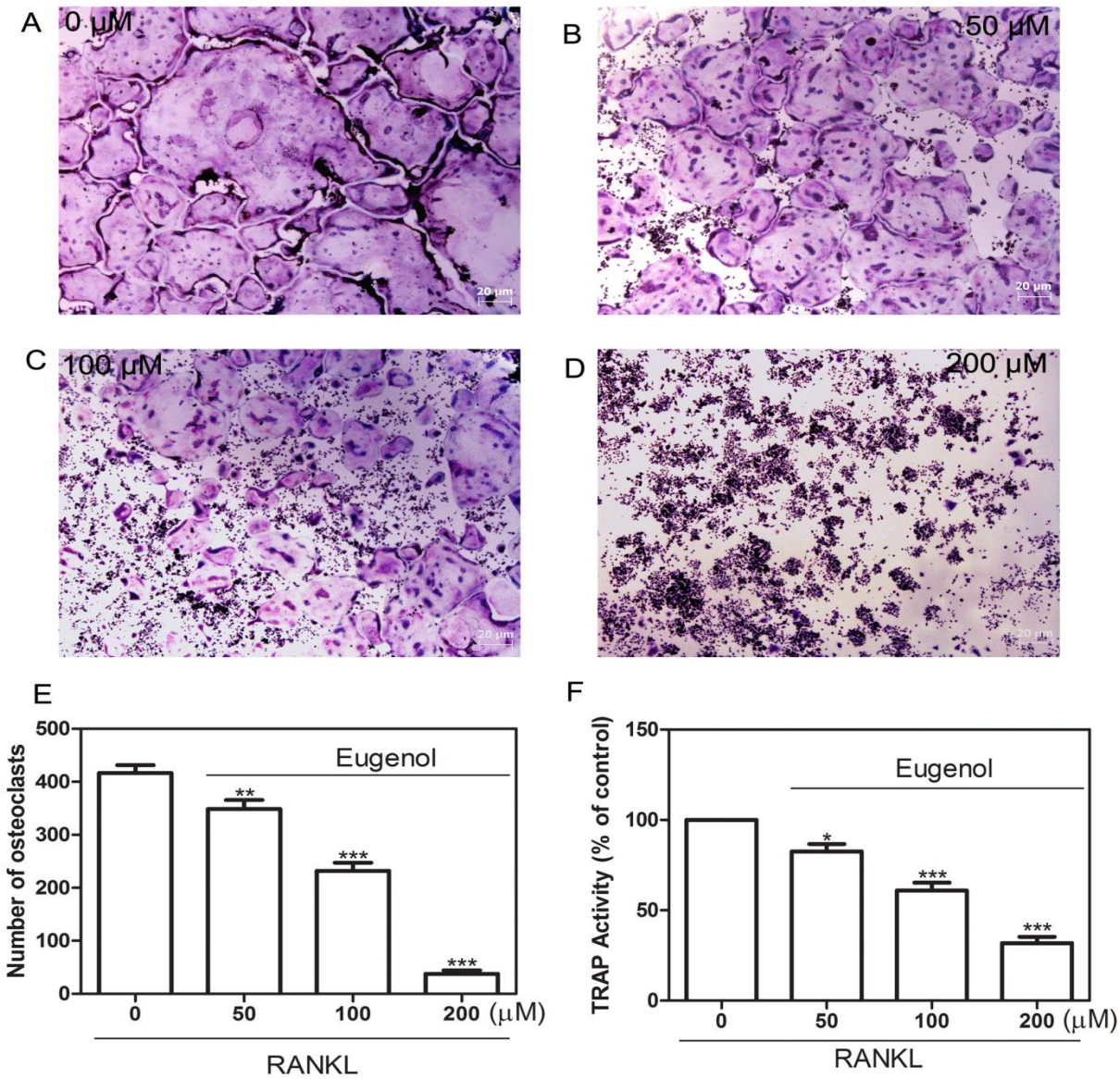
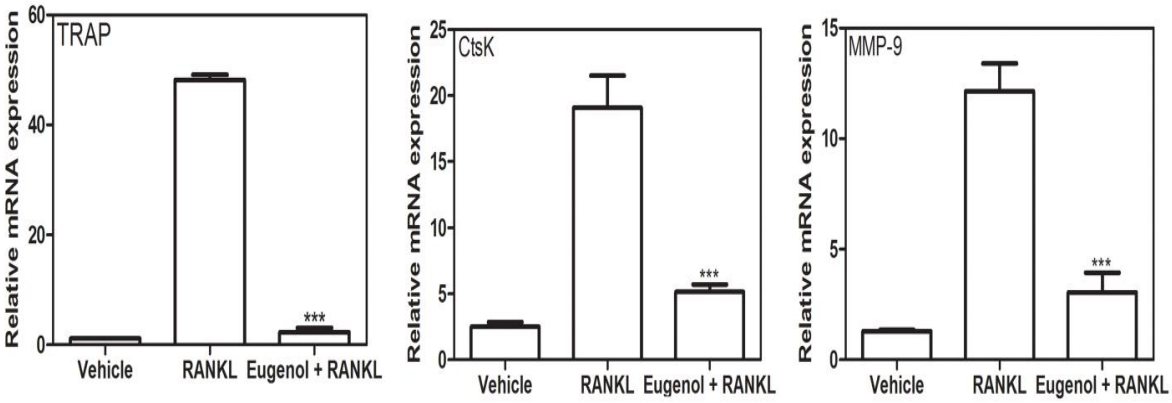


Figure 3. Effects of eugenol on RANKL-induced osteoclast specific gene expression. RAW264.7 macrophages were pre-treated with 200 µM eugenol or vehicle for 4 h followed by stimulation with RANKL (15 ng ml-1) or RANKL plus eugenol for 72 h. mRNA expression levels of TRAP, CtsK and MMP-9 were analysed by qPCR and were normalized against GAPDH levels. The results are mean ±SEM and are representative of three independent experiments. (***P <0.001 vs RANKL)



Eugenol inhibits RANKL-induced osteoclast-specific gene expression in RAW264.7 macrophages

The effects of eugenol on RANKL-induced osteoclast-specific gene expression were examined by qPCR. The expression of osteoclast-specific genes TRAP, MMP-9 and CtsK were gradually induced by RANKL in RAW264.7 macrophages at 72h (Fig. 3). However, pre-treatment with eugenol before RANKL-exposure dramatically suppressed the RANKL-induced osteoclastogenic gene expression in RAW264.7 macrophages (Fig. 3). Collectively, these data further supported the potential inhibitory role of eugenol on RANKL-mediated osteoclast formation.

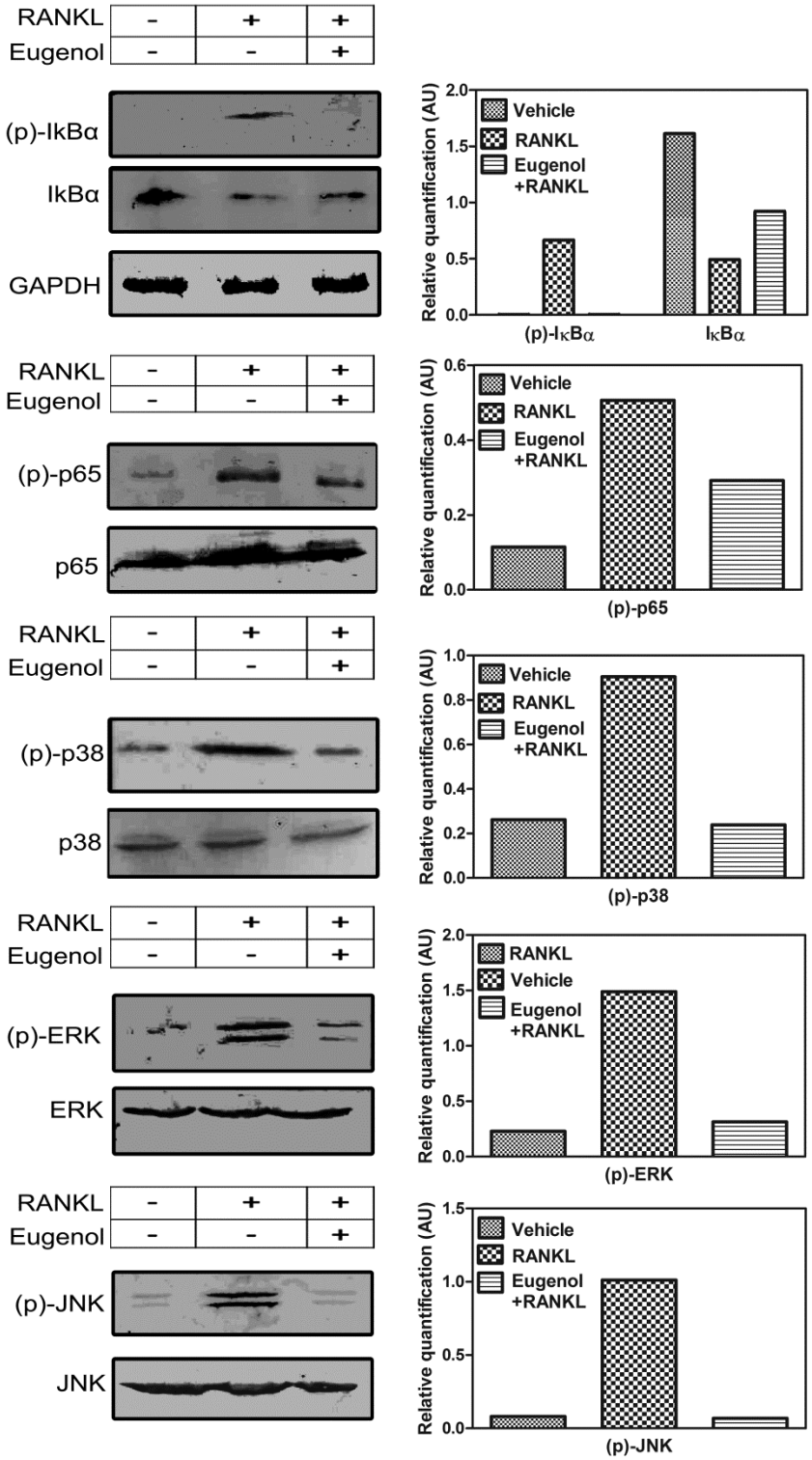
Eugenol inhibits RANKL-induced NF-κB activation in RAW264.7 macrophages

RANKL-mediated activation of NF-κB is indispensable for osteoclast formation. In an inactive state IκBα remains bound with NF-κB, and only become active after IκBα is phosphorylated and subsequently degraded. Thus, we investigated whether eugenol inhibits RANKL-induced phosphorylation and degradation of IκBα. RAW264.7 macrophages were pre-treated with eugenol for 4h and IκBα-protein levels were analysed after 30 min of RANKL-exposure. Eugenol markedly suppressed the RANKL-induced phosphorylation and degradation of IκBα (Fig. 4A). Moreover, RANKL-mediated phosphorylation of p65-subunit of NF-κB was drastically reduced by eugenol in RAW264.7 macrophages (Fig. 4B).

Eugenol inhibits the activation of RANKL-induced MAPK pathways in RAW264.7 macrophages

RANKL-mediated activation of MAPK pathways plays an important role in osteoclast formation. To further elucidate the mechanism of action underlying the inhibitory effects of eugenol on RANKL-induced osteoclastogenesis; phosphorylation and activation of MAPK pathways- p38, JNK and ERK were examined. Treatment of RAW264.7 macrophages for 30 min with RANKL remarkably induced the phosphorylation of these pathways. Intriguingly, pre-treatment with eugenol for 4h before RANKL stimulation markedly inhibited the RANKL-mediated phosphorylation of ERK, JNK and

Figure 4. Effects of eugenol on RANKL-induced activation of NF-κB and MAPK pathways. RAW264.7 macrophages were pre-treated with indicated 200 μM eugenol or vehicle for 4 h followed by stimulation with RANKL (15 ng ml-1) or RANKL plus eugenol for 30 min. Cell lysates were subjected to western blot analysis. Expression levels of (A) (p)-IκBα, IκBα and GAPDH (B) (p)-NF-κB-p65 subunit and NF-κB-p65-subunit (C) (p)-p38 and p38 (D) (p)-ERK and ERK (E) (p)-JNK and JNK are shown. The AU values were obtained by quantification through ImageJ software. AU values of (p)-IκBα and IκBα were normalised to GAPDH and to that of (p)-NF-κB-p65, (p)-p38, (p)-ERK, (p)-JNK were normalised to total p65, p38, ERK and JNK respectively. The results are representative of two independent experiments. (p) denotes phosphorylated form.



p38 (Fig. 4 C, D, E). Therefore, attenuation of NFκB and MAPK pathways by eugenol synergistically contributes to its inhibitory effects on RANKL-induced osteoclastogenesis.

Discussion

Osteoclasts are responsible for bone resorption (1). During certain diseases such as arthritis, osteoporosis, cancer and other chronic inflammatory disorders the fragile balance between bone formation (mediated by osteoblasts) and bone resorption (mediated by osteoclasts) is perturbed (3). It has been reported that osteoclasts attain a significant increase in their activity and function during these illnesses indicating the importance of osteoclasts in bone loss (17). Systemic hormones and cytokines regulate the formation and activation of osteoclasts (18). Presence of RANKL is vital for osteoclast differentiation as RANKL-knockout mice not only developed a severe form of osteopetrosis; the rodents were completely devoid of osteoclasts (19). In certain pathological conditions RANKL is often overexpressed and drives excessive osteoclast activity contributing to a vicious cycle of bone loss (17). Hence, effective and affordable compounds that can attenuate RANKL-induced osteoclast formation are much needed. Eugenol is a polyphenol shown to possess potent anti-inflammatory activity, anti-oxidative activity and anti-cancer activity (20-23). Here, we investigated effects of this bioactive compound on RANKL-induced osteoclastogenesis. We found that eugenol dose-dependently inhibited TRAP-activity and osteoclast formation induced by RANKL, mediated in part by abolishing the activation of NF-κB and MAPK pathways.

In this study, we used RAW264.7 murine macrophages to investigate the direct effects of eugenol on RANKL-induced osteoclast formation. The RAW264.7 model provides an edge over other osteoclast differentiation models as these cells express c-fms receptor for macrophage-colony stimulating factor (M-CSF) as well as M-CSF of their own and does not contain osteoclast/bone marrow stromal cells. RAW264.7 macrophages respond by differentiating to osteoclasts in the presence of RANKL (24, and references therein). Hence, this model provides an excellent experimental setup to focus specifically on RANKL-signalling in osteoclast progenitors. In this study, eugenol dose-dependently inhibited the RANKL-induced formation of osteoclasts and TRAP activity in RAW264.7 macrophages without

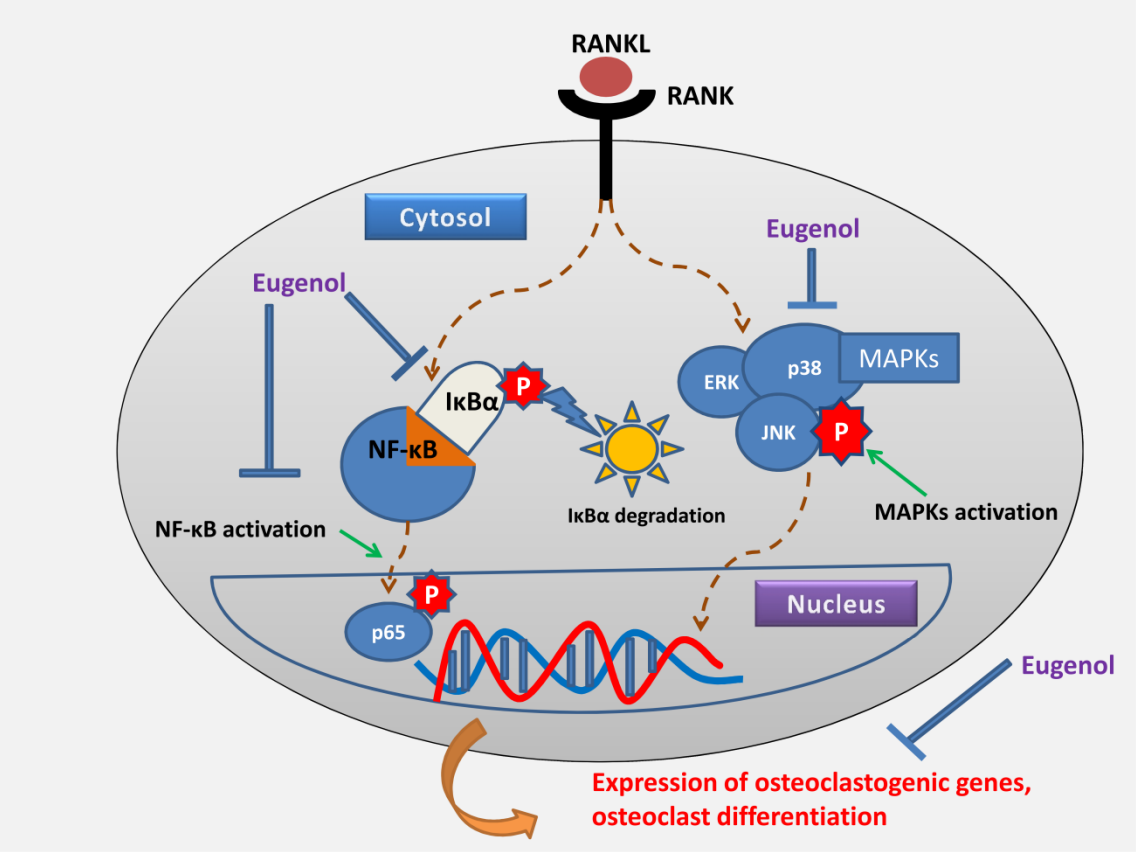
cytotoxicity. Eugenol at a concentration of 200 μM was most effective in abrogating osteoclast formation. This phenolic compound has been reported to inhibit iNOS, COX-2 and expression of proinflammatory cytokines namely IL-6, TNF-α and PGE_2 (20, 25). Eugenol has also been reported to have potent anti-oxidant activity (26). Oxidative damage of lipid, protein and DNA is known to cause chronic inflammation (22). Eugenol is effective in scavenging reactive oxygen species (ROS), inhibits both iron and Fenton reagent mediated lipid peroxidation (11, 22, 26-28). ROS are important for osteoclast formation and function and regulate RANKL-induced osteoclast differentiation (29). Since, these factors play a crucial role in elevated osteolysis along with RANKL this may explain in part the inhibitory effects of eugenol on RANKL-induced osteoclast formation.

TRAP, CtsK and MMP-9 are osteoclast specific markers and are expressed at abundant levels by differentiating and mature osteoclasts (8). Proteolytic enzymes such as TRAP, CtsK and MMP-9 play an important role in osteoclast-mediated bone resorption and are considered as markers of osteoclast formation (30). Targeted disruption of TRAP and CtsK in mice resulted in remarkable loss in osteoclast activity causing osteopetrosis (31, 32). Recently, Lotinun et al, by generating osteoclast- and osteoblast-targeted CtsK knockout mice have demonstrated that bone formation rate is increased by elevated expression of sphingosine-1-phosphate in osteoclasts (33). In humans, pycnodysostosis (a disorder in which bone becomes severely dense) accompanied by critical loss in osteoclast activity occurs due to mutation in CtsK gene (34). Eugenol is used as a component in endodontic sealers. Rodriguez et al, in their studies demonstrated the inhibitory effects of Tubliseal, a zinc-oxide-eugenol based sealer on *in vitro* human osteoclastogenesis and in co-culture of osteoblastic and osteoclast precursor cells. They reported anti-osteoclastogenic effects of this product that correlated with reduced osteoclast-specific marker gene expression (35, 36). In the present study we found that eugenol significantly downregulated the RANKL-induced expression of key osteoclastogenic genes TRAP, CtsK and MMP-9.

RANKL/RANK signalling plays a pivotal role in osteoclast activity and osteoclast formation (18). RANKL binds to its receptor RANK, a member of tumor necrosis factor receptor (TNFR) superfamily and initiates a signalling cascade by recruiting TRAF-proteins that subsequently transmit the signals

downstream and activate NF-κB and MAPK pathways (37). Certain derivatives of eugenol have been reported to inhibit LPS-induced activation of NF-κB by blocking the phosphorylation and degradation of IκBα and phosphorylation of ERK, JNK and p38-MAPK pathways in RAW264.7 macrophages (25, 38). In another study it was shown that eugenol not only supressed the activation of NF-κB but also attenuated TNF-α release in alveolar macrophages from BALB/c mice and bronchoalveolar lavage fluid respectively (39). Kaur et al. reported the inhibitory effects of eugenol during chemical carcinogenesis on proinflammatory cytokine production and activation of NF-κB in a mouse skin cancer study (40). In a rat model of gastric carcinogenesis induced by N-Methlyl-N′-nitro-N-nitrosoguanidine, eugenol suppressed NF-κB activation, IκBα phosphorylation and degradation thereby modulating the expression of target genes involved in cancer progression (21). It is well established that RANKL signalling can activate NF-κB as well as p38, JNK and ERK-MAPK pathways, critical for osteoclast differentiation (41). Inhibition of NF-κB activation by reduced phosphorylation of IκBα and p65subunit of NF-κB and subsequent degradation of IκBα is lethal for osteoclast formation (41). In this study we found that, eugenol inhibited the RANKL-induced phosphorylation and degradation of IκBα. p65 (RelA) is one of the members of the NF-κB family that regulates the survival of osteoclasts. p65 knock-out mice show decreased osteoclast number and severely blunted osteoclastogenic response to RANKL. In this study, RANKL-mediated phosphorylation of p65-subunit was found to be impaired by eugenol indicating that downstream activation of NF-κB members was exacerbated leading to abrogation of NF-κB signalling. Dominant-negative form of JNK and p38 has been reported to prevent RANKL-induced osteoclastogenesis of RAW264.7 macrophages (42, 43). Genetic disruption of Erk1 results in reduced osteoclast progenitor cell numbers and decreased osteoclast formation and RANKL has been shown to be the major activator of JNK (44-46). Multiple intracellular signalling pathways contribute to the complex process of osteoclast formation and the sum of the individual pathways ultimately lead to the osteoclastogenesis in a given experimental condition (1, 36). In the present study, eugenol inhibited the RANKL-mediated activation and phosphorylation of ERK, JNK and p38 MAPK pathways. Thus, eugenol modulates RANKL-mediated activation of early signalling pathways such as MAPKs and NF-kB which correlates with inhibition of RANKL-induced osteoclastogenesis.

Figure 5. A schematic representation of the inhibitory effects of eugenol on RANKL-induced osteoclast formation and underlying molecular mechanisms.



Conclusions

In conclusion, the results of the present study demonstrate for the first time that eugenol could inhibit RANKL-induced osteoclast formation by impairing the signalling pathways downstream of RANKL including NF-κB and MAPKs (Fig. 5). Eugenol has the potential to suppress osteoclast differentiation and may be developed as a therapeutic agent to overcome RANKL-mediated osteoclast activation in diseases such as osteoporosis and rheumatoid arthritis.

Declaration of interest

The authors declare that they have no competing interests.

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