Aldehydic components of Cinnamon bark extract suppresses RANKL-induced osteoclastogenesis through NFATc1 downregulation

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A R T I C L E   I N F O

Article history:
Received 24 July 2008
Revised 5 September 2008
Accepted 6 September 2008
Available online 14 September 2008

Keywords:
Cinnamomum zeylanicum
Osteoclastogenesis
NFATc1
RANKL

A B S T R A C T

Several major bone diseases are directly attributable to bone loss, including osteoporosis, bone metastasis, and rheumatoid arthritis. The nuclear factor of activated T cell 1 (NFATc1), a transcription factor, has recently been shown to play an essential role in osteoclastogenesis. In this study, we found that of several herbs, Cinnamomum zeylanicum (C. zeylanicum) exhibited the strong inhibitory effects on osteoclastogenesis and that its mechanism of action involves the suppression of NFATc1-mediated signal transduction. C. zeylanicum dose-dependently inhibited osteoclast-like cell formation at concentrations of 12.5-50 μg/ml without affecting cell viability. Resorption pit assays have shown that C. zeylanicum also inhibits the bone-resorbing activity of mature osteoclasts. Treatment with C. zeylanicum inhibited the receptor activator of nuclear factor-κB ligand (RANKL)-induced NFATc1 and c-fos expression. Additionally, C. zeylanicum moderately inhibited phosphorylation of IκB-α, suggesting that the c-fos/NFATc1 pathway, rather than the nuclear factor-κB (NF-κB) pathway, is the primary target of C. zeylanicum during RANKL-induced osteoclastogenesis. Using an HPLC-DAD system, we identified three major peaks for four characteristic components in the C. zeylanicum extract and identified an unknown peak as 2-methoxycinnamaldehyde via HPLC and a 2D-COSY 1H NMR study. We identified cinnamaldehyde and 2-methoxycinnamaldehyde as active components reducing osteoclast-like cell formation and inhibiting NFATc1 expression. Notably, in a resorption pit assay, 2-methoxycinnamaldehyde exhibited remarkable inhibition rates of 95% at 2 μM on bone resorption. In summary, this study points to the conclusion that C. zeylanicum inhibits RANKL-induced osteoclastogenesis. This finding raises prospects for the development of a novel approach in the treatment of osteopenic disease.

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1. Introduction

The balance between bone resorption (by osteoclasts) and bone formation (by osteoblasts) maintains bone homeostasis in a process called bone remodeling. Incremental changes in the rate of bone resorption can lead to bone disruption and cause major bone diseases, including osteoporosis, bone metastasis, and rheumatoid arthritis. Understanding the mechanism that regulates osteoclastogenesis would pose significant clinical implications and raise prospects for finding ways to control this mechanism.

Large tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs) that are hematopoietic in origin, osteoclasts are capable of resorbing bone. Osteoclastogenesis progresses through multiple stages, including differentiation, fusion, and activation (maturation) regulated by various factors, including cytokines, hormones, and other cells in the bone microenvironment. A key factor is RANKL, a member of the tumor necrosis factor family. RANKL mediates osteoclastogenesis by binding to its receptor RANK on osteoclast precursor cells. Analyses of RANK-deficient mice show that an intrinsic defect in the osteoclasts causes serious osteopetrosis, implicating RANKL as an essential factor in modulating osteoclast differentiation and activation.

The association of RANKL with RANK recruits tumor necrosis factor receptor-associated factors such as NF-κB-inducing kinase, activating NF-κB signaling pathways. Other essential regulatory factors include c-fos; c-fos-deficient mice also exhibit deficient osteoclast formation and have an osteopetrosic phenotype. Recent reports indicate that ectopic expression of NFATc1 causes precursors to undergo efficient differentiation in the absence of RANKL and that NFATc1-deficient embryonic stem cells fail to differentiate into osteoclasts in response to RANKL. The induction of NFATc1 is regarded as a hallmark event in the cell fate determination of osteoclasts. In a genome-wide search for the RANKL-inducible genes specifically required for terminal differentiation of osteoclasts, NFATc1 was shown to be strongly induced by RANKL. NFATc1 expression depends on the NF-κB and c-fos pathways activated by RANKL, suggesting NFATc1’s integral role in RANKL signaling.

Several recent reports whose goal is to identify patterns for preventing osteoporosis through daily diet examined the effects of food components and their bioactive components on bone metabolism. We also focused on identifying inhibitors of osteo-

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clastogenesis among medicinal food stuffs, screening an extract library prepared from 33 plants for effective dual inhibitors of both TRAP⁺ MNC formation and NFATc1 expression in RANKL-induced osteoclastogenesis systems. In preliminary experiments, we performed a screening test using a TRAP assay with a finding that seven herbal extracts (Chaenomeles sinensis, C. zeylanicum, Artemisia princeps, Citrus sinensis var. daidai, Crocus sativus, Citrus limon, and Mallotus japonicus) strongly inhibit TRAP⁺ MNC formation. We subsequently screened these herbal extracts for strong inhibitors of NFATc1 expression. Of these extracts, the molecular target-based screening tests identified C. zeylanicum extract as the most potent inhibitor.

Cinnamon bark or cortex is a popular natural spice and herb commonly used in traditional Chinese medicine to treat gastritis, blood circulation disturbances, and inflammatory disease.¹⁹–²⁰ In recent years, their pharmacological effects in the treatment of type 2 diabetes have attracted wide attention.²¹ Choi et al. has also reported that Cinnamomum cassia (C. cassia) stimulates bone formation by osteoblasts in vitro,²² but little work has been done to evaluate the effects on osteoclast differentiation and bone resorption. In our study, we demonstrate for the first time that cinnamon bark strongly inhibits osteoclastic activity and that its mechanism of action is attributable to the suppression of NFATc1-mediated signal transduction. We also identified cinnamaldehyde (CA) and 2-methoxycinnamaldehyde (2-MCA) as its active components. Our results raise the possibility that C. zeylanicum may be used to facilitate bone modulation in osteopenic disease.

2. Results

2.1. Cinnamon bark extract suppresses RANKL-induced osteoclastogenesis by restriction of NFATc1 expression in RAW264.7 cells

NFATc1, an essential transcriptional factor for osteoclast differentiation, is upregulated after RANKL stimulation.¹² In preliminary experiments, to explore effective inhibitors of RANKL-induced osteoclastogenesis, we investigated the effects of seven herbal extracts on both TRAP⁺ MNC formation and NFATc1 expression. This molecular target-based screening identified C. zeylanicum extract as the most potent inhibitor among these extracts (data not shown). To confirm the effects of C. zeylanicum extract on osteoclast differentiation, we evaluated the number of TRAP⁺ MNCs formed after RANKL stimulation in the presence or absence of the extract. As shown in Figure 1A and B, C. zeylanicum extract inhibited the formation of TRAP⁺ MNCs in a dose-dependent manner at 12.5–50 µg/ml but had no effects on the growth or survival of RAWs (Fig. 1C). In addition, the morphology of C. zeylanicum extract-treated RAW cells was nearly identical to that of the control cells.

Next, to determine the effects of C. zeylanicum extract on bone resorption by mature osteoclasts, RAWs were cultured on calcium phosphate apatite-coated plates with either vehicle or with C. zeylanicum extract for 8 days, and the level of resorption activity was monitored using the BD BioCoat™ Osteologic™ Bone Cell Culture System. Treatment resulted in a dose-dependent depression of resorption activity. Complete inhibition was observed at concentrations of 50 µg/ml of C. zeylanicum extract (Figs. 1D and E). These data indicate that C. zeylanicum extract has the ability to reduce osteoclastic bone resorption associated with osteoclast differentiation.

As shown in Figure 2A, RANKL increased NFATc1 expression, with maximum induction at 48 h, indicating that C. zeylanicum extract at concentrations of 50 µg/ml completely blocks NFATc1 expression at all time points. We also observed that adding C. zeylanicum extract reduced NFATc1 expression in a dose-dependent manner and found that C. zeylanicum extract exerts potent inhibitory effects on both TRAP activation and NFATc1 induction, key events in osteoclast differentiation. These results suggest that the inhibition of RANKL-induced NFATc1 induction is the critical target of C. zeylanicum extract.

2.2. Effects of cinnamon bark extract on expression of c-fos, and on NF-κB signaling pathway in RANKL-stimulated RAW264.7 cells

To investigate the molecular mechanisms of C. zeylanicum extract in osteoclast precursors, we determined the level of expression of c-fos, essential factors in NFATc1 gene expression. In particular, as is well-known, osteoclastogenesis requires c-fos, a transcription factor for AP-1.¹³ As shown in Figure 3A, C. zeylanicum extract at 24 h after RANKL stimulation completely restrained expression of both c-fos and NFATc1.

We further examined the effects of C. zeylanicum extract on the NF-κB signaling pathway. NF-κB activation is known to progress through the activation of IkB kinase and the subsequent phosphorylation of IkB for ubiquitin-dependent degradation. The results in Figure 3B show that untreated RAW264.7 cells significantly increased phosphorylation of IkB-a and degradation of IkB-α via stimulation by RANKL. C. zeylanicum extract treatment barely suppressed phosphorylated IkB-α via stimulation by RANKL. Pretreatment of RAW264.7 cells with C. zeylanicum extract for 1 hour tended to reduce IkB-α phosphorylation, IkB-α degradation at higher concentrations compared to un-pretreated RANKL-stimulated controls (Fig. 3C), although phosphorylated IkB-α at 50 µg/ml of C. zeylanicum extract was still observed. These results suggest that the c-fos/NFATc1 pathway, not the NF-κB pathway, is the primary target of C. zeylanicum extract during RANKL-induced osteoclastogenesis.

2.3. Chemical profiling of C. zeylanicum extract and specification of its active components as effective inhibitors of RANKL-induced osteoclastogenesis

In several previous studies, the chemical components of Cinnamomum species have been clearly defined by HPLC analysis.²³,²⁴ Figure 4A shows an HPLC chromatogram for C. zeylanicum extract. Using an HPLC-DAD system, we identified three characteristic peaks as cinnamyl alcohol (peak 1), cinnamic acid (peak 2), and CA (peak 3) by comparing corresponding retention times and UV spectra for the standards and by consulting the literature.²³,²⁴ One undetermined peak (peak 4) was also detected in C. cassia (data not shown) and was previously treated as an unknown component.²⁴ This compound was not inconsistent with the peak of eugenol. Therefore, the fraction of peak 4 was collected and processed without further purification by the RP-HPLC isolation method (Fig. 4B). Structural identification of this compound isolated from C. zeylanicum extract was characterized as 2-MCA by ¹H NMR and 2D-COSY (Fig. 4C) and confirmed by comparison against the ¹H NMR spectra of the standard. 2-MCA has already been isolated and purified from cinnamon bark extract with preparative thin-layer chromatography.²⁵–²⁷ However, this data may prove useful in a systematic study involving authentication and assessment of the related Cinnamomum species, since we determined the HPLC profile for 2-MCA, in addition to well-defined compounds.

To determine the active component(s) of C. zeylanicum extract, we investigated the effects of four components on both NFATc1 expression and TRAP activity. As shown in Figure 5A, CA and 2-MCA strongly inhibited NFATc1 expression in 48 h cultures of RAW cells, implying that these compounds are the active components in C. zeylanicum extract and that the inhibitory effects of 2-
MCA exceed those of CA. The other components exhibited no suppressive effects at high concentrations.

We also demonstrated that 2-MCA completely inhibits both TRAP+ MNCs formation and bone resorption in RANKL-stimulated cells. These results indicate that CA and 2-MCA are the active components of *C. zeylanicum* extract.

### 3. Discussion and conclusions

Several recent reports indicate the potential role of certain herbs and their components in providing protection against osteoporosis, suggesting that consumption of herbs may contribute to bone remodeling.13–17 Cinnamon bark or cortex is a popular natu-
Cinnamon bark is a spice and herb commonly used in traditional Chinese medicine, with numerous putative health benefits, but little is known about the effects of cinnamon bark on bone metabolism. Our study is the first to indicate that cinnamon bark extract, as C. zeylanicum extract, inhibits the RANKL-induced NFATc1 pathway in mouse RAWs and prevents osteoclastogenesis.

The RANKL-induced NFATc1 pathway plays an integral role in osteoclastogenesis and can be regarded as a promising target for therapeutic intervention in bone diseases. Urushibara et al. reported that inhibiting this pathway blocked osteoclast differentiation and bone destruction. In our study, we initially used a screening approach to identify the effects of several herbal extracts on NFATc1-mediated signal transduction in a RAW264.7 murine cell line. We then discovered that C. zeylanicum extract suppressed to a remarkable degree the expression of NFATc1 in osteoclastogenesis and that the inhibitory effects of C. zeylanicum extract correlate with its modulation in TRAP+ MNC formation, known to be an osteoclast-specific marker protein expressed in functionally mature osteoclasts. Thus, C. zeylanicum extract is likely to inhibit RANKL-induced osteoclast formation by selectively inhibiting NFATc1 expression.

Several plant extracts and compounds have the capacity to prevent bone loss at menopause by inducing osteoclast apoptosis. This suggests that modulating osteoclast lifespan may be a key step in regulating bone resorption. However,
the effects of C. zeylanicum extract may involve another mechanism, since C. zeylanicum extract-treatment cells were not observed to influence cell viability in either the absence or presence of RANKL-stimuli (data not shown).

We found that C. zeylanicum extract strongly suppressed expressed c-fos, which plays an exclusive role among the AP-1 proteins. Its signal pathway is known to be centrally involved in RANKL-induced NFATc1 expression. Putative AP-1 binding sites have been found in the promoter region of NFATc1, and osteoclast precursors lacking c-fos are free of NFATc1 expression. Our results raise the possibility that C. zeylanicum extract inhibits RANKL-induced osteoclast formation at the early stages of osteoclastogenesis in RAWs, inhibiting NFATc1 expression.

Suppressing NF-κB also affects NFATc1 expression. Takatsuha et al. have shown that NF-κB inhibitor (−)-dehydroxymethyllepoxyquinomic regulates NFATc1 expression and inhibits bone resorption.32 NF-κB activation depends on two pathways:4 The classic NF-κB pathway involves phosphorylations and ubiquitination-dependent degradation of IkBs; the alternative pathway is responsible for activating p52:RelB dimers. In a genomic study, p50/p52-deficient mice, not p50- or p52-deficient mice, exhibited bone abnormalities due to defects in osteoclast differentiation.33,34 suggesting the function of cross-talk between the classic and alternative pathways. To explore the possibility that C. zeylanicum extract may inhibit NF-κB activation, we investigated the effects of C. zeylanicum extract on phosphorylation of IkB. We found that C. zeylanicum extract barely inhibits NF-κB activation of classic pathways at a dose of 12.5–50 μg/ml, which is insufficient for both NFATc1 expression and osteoclast formation. Thus, the inhibitory effects of C. zeylanicum extract on NFATc1 expression may be attributable primarily to the inhibition of c-fos expression, not NF-κB activation, since the inhibition of the classical pathway is an essential NF-κB inhibitor function. In this study, we demonstrated that C. zeylanicum extract also suppresses bone resorption, suggesting that C. zeylanicum extract may modulate the bone remodeling process by controlling bone resorption through inhibition of NFATc1 expression.

We found first that CA and 2-MCA may behave as inhibitors in the RANKL-induced NFATc1 pathway and of osteoclastogenesis. In particular, 2-MCA appears to strongly inhibit at low concentrations (1–2 μM), although the content of CA is higher than that of 2-MCA in C. zeylanicum extract (Fig. 4A). Kim et al. report that CA and 2-MCA have been identified as NF-κB inhibitors from C. cassia using LPS-stimulated RAW 264.7 cells and that these compounds show IC50 values of 43 and 31 μM, respectively.29 However, the only NF-κB inhibitor function of C. zeylanicum extract has difficulty in accounting for the inhibitory effects of C. zeylanicum extract on osteoclastogenesis. This is because C. zeylanicum extract inhibits NF-κB activation weakly at doses of 12.5–50 μg/ml, despite a marked osteoclastic inhibition at the same concentrations. In addition, we observed virtually no inhibitory effects of 2-MCA on NF-κB activation at concentrations as low as 1–2 μM in the RANKL-stimulated RAWs model (Supplementary data). Thus, to understand the inhibitory effects of these compounds on osteoclastogenesis, we have also been compelled to focus on other essential factors, including c-fos. In contrast, we found that cinnamic acid and cinnamal alcohol fail to inhibit TRAP+ MNCs formation in RANKL-stimulated RAWs (data not shown), implying that the aldehye functional groups of CA and 2-MCA may be essential for the inhibition of osteoclastogenesis through the suppression of NFATc1 expression. In addition, our findings raise the possibility that the derivatives of benzene ring in CA, including 2-MCA, might enhance the potent inhibitory effects on osteoclastogenesis. We have demonstrated that the inhibitory action of 2-MCA would be more potent than that of CA, a conclusion in agreement with several reports that have shown varying inhibitory effects between CA and the derivatives of its benzene ring with respect to antimicrobial activity and effectiveness as NF-κB inhibitors.35,36

Takayanagi et al. have shown that administering FK506, a calcineurin inhibitor, induces bone mass reductions despite a blockade of osteoclast differentiation.37 The modulation of NFAT proteins likely affects the normal homeostasis of the skeletal system, since NFAT cooperates with Osterix, an essential osteogenic factor, and accelerates osteoblast differentiation and bone formation. However, recent reports indicate that extract of the bark of C. cassia blume, a species related to C. zeylanicum, has estrogenic effects and may accelerate bone formation, indicating that the complex mechanisms involved in the regulation of NFATc1 expression may evoke homeostasis of the skeletal system.32 Additional study is needed to understand the molecular events involved, including evaluations of potential active components having estrogenic effects.

In summary, our study clearly demonstrates that cinnamon bark strongly inhibits osteoclastic activity. The mechanism of action involves the suppression of NFATc1-mediated signal transduction. We also identified CA and 2-MCA as its active components. The results of this study suggest that C. zeylanicum extract may be highly effective in the treatment of pathological bone disruption-related diseases, including osteoporosis, bone metastasis, and rheumatoid arthritis.

4. Materials and methods

4.1. Materials

All media components were purchased from Invitrogen (Carlsbad, CA). Antibodies against NFATc1 and nuclear inhibitory κB-α...
A polyclonal antibody to c-fos was obtained from Abcam, Ltd. (Cambridgeshire, UK). Antibodies against GAPDH were purchased from Ambion, Inc. (Austin, TX). Antibodies against phospho-IkBα were purchased from Cell Signaling Technology (Beverly, MA). Goat anti-rabbit horseradish peroxidase conjugate was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA); goat anti-mouse horseradish peroxidase and BioCoat™ Osteologic™ Bone Cell Culture System from BD Biosciences (San Jose, CA); and CA, 2-MCA, cinnamyl alcohol, cinnamic acid, and MTT from Sigma–Aldrich (St. Louis, MO). RANKL was purchased from Pepro Tech EC, Ltd. (London, UK). The sample of C. zeylanicum was prepared from the bark of C. zeylanicum Blume purchased commercially (Mimuro, Inc., Shizuoka, Japan) and pulverized. C. zeylanicum (100 mg) extract was obtained by extraction with 1 ml of 99.5% ethanol at room temperature for 3 days. The resultant solution was centrifuged and the supernatant removed, after which the solution was evaporated to dryness under vacuum conditions. The residue was dissolved in dimethylsulfoxide.

4.2. Cell cultures

The RAW264.7 mouse macrophage cell line was obtained from the American Type Culture Collection (Manassas, VA). RAW264.7 cells were maintained in DMEM medium supplemented with 10% FBS and 2 mM glutamine, and antibiotics. Incubation was performed at 37 °C in 5% CO2 in humidified air. For osteoclast generation and all other experiments, α-MEM medium was used.

4.3. TRAP staining

After 3 days of culturing, RAW 264.7 cells treated with RANKL were fixed with a solution containing acetone and citrate for 30 s and washed twice with double-distilled water. TRAP staining was performed using a kit purchased from Sigma according to the manufacturer’s instructions. Graphical displays of cell morphology (B top) and relative number of TRAP+ MNCs (C). (B bottom and D) Effects of 2-MCA on bone resorption by mature osteoclasts; RAW 264.7 cells on calcium phosphate apatite-coated plate (BioCoat™ Osteologic™ Bone Cell Culture System) were incubated with RANKL in the presence or absence of C. zeylanicum extract (25–50 μg/ml) for 8 days. Resorption pits (magnification ×100) (B bottom) and, total resorbed area (square millimeters) (D). The values represent means ± SD of triplicate determinations; * p < 0.01 compared to control group.

4.4. Resorption pit assay

This assay was performed according to the manufacturer’s instructions. Briefly, to determine the effects of cinnamon bark extract (or its active components) on pit formation, RAW cells were pretreated with cinnamon bark extract (or active components) for an hour, before adding RANKL (50 ng/ml) to calcium phosphate apatite-coated plates (BioCoat™ Osteologic™ Bone Cell Culture System).


