Cetylpyridinium Chloride Inhibits Receptor Activator of Nuclear Factor-κB Ligand-Induced Osteoclast Formation

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Osteoclasts are responsible for bone erosion in diseases as diverse as osteoporosis, periodontitis, and rheumatoid arthritis. Antiseptic products have received recent attention as potential therapeutic and preventive drugs in human disease. The purpose of this study was to investigate the effect of the antiseptic cetylpyridinium chloride (CPC) on osteoclast formation using mouse bone marrow-derived macrophages (BMMs). CPC inhibited receptor activator of nuclear factor (NF)-κB ligand (RANKL)-induced osteoclast formation in a dose-dependent manner without causing cytotoxicity. The mRNA expression of cathepsin K, calcitonin receptor (CTR), and Prdm1 in osteoclasts was reduced by CPC. In experiments to elucidate its mechanism of action, CPC was found to suppress RANKL-induced expression of c-Fos and nuclear factor of activated T cells (NFATc1), transcription factors that are essential for osteoclast differentiation. CPC also inhibited RANKL-induced activation of extracellular signal-regulated kinase (ERK) and NF-κB and expression of cyclooxygenase (COX)-2. These results collectively suggest that CPC inhibits osteoclast differentiation by suppressing the activation of ERK and NF-κB and reducing the expression of COX-2, c-Fos, and NFATc1. CPC may therefore be a useful drug in the prevention of bone loss.

Key words osteoporosis; osteoclast; cetylpyridinium chloride

Bone remodeling depends on continual resorption of bone by osteoclasts and its replacement by osteoblasts. Imbalances in this process disrupt the maintenance of bone homeostasis, which in turn leads to pathogenic conditions such as osteoporosis, rheumatoid arthritis, lytic bone metastases, and Paget’s bone disease. Osteoclasts are bone-resorbing, multinucleated cells that are derived from the monocyte-macrophage lineage. Excessive osteoclastic bone resorption plays a critical role in bone destruction in pathological bone diseases such as osteoporosis, rheumatoid arthritis, periodontal disease, and some metastatic cancers. Thus, the development of drugs that can regulate osteoclast differentiation is clinically important.

The interaction between receptor activator of nuclear factor (NF)-κB (RANK) and RANK ligand (RANKL) is essential for osteoclast differentiation and activation. The binding of RANKL to RANK on osteoclast precursor cells triggers the activation of tumor necrosis factor receptor-associated factor 6 (TRAF6) and the subsequent activation of NF-κB and mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase 1/2 (ERK1/2), p38, and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK). NF-κB-induced cyclooxygenase (COX)-2 expression plays a key role in the RANKL-induced osteoclast differentiation.

Nuclear factor of activated T cells (NFATc1) is a downstream transcription factor in the RANKL/RANK signaling pathway and plays a crucial role in osteoclastogenesis. As a key molecule of osteoclastogenesis, it induces the expression of a series of osteoclast-specific genes, including those encoding cathepsin K, tartrate-resistant acid phosphatase (TRAP), calcitonin receptor, and osteoclast-associated receptor. NFATc1 has been suggested to be the downstream target of c-Fos during osteoclastogenesis. A member of the AP-1 transcription factor family, c-Fos induces NFATc1 expression by directly binding to the NFATc1 promoter region and positively regulates osteoclastogenesis.

Cetylpyridinium chloride (CPC), a cationic quaternary ammonium compound, is used in some types of mouthwashes, toothpastes, lozenges, throat sprays, breath sprays, and nasal sprays (Fig. 1A). It is an antiseptic agent with a broad antimicrobial spectrum and has rapid bactericidal effects on Gram-positive pathogens and fungicidal effects on yeasts. It is assumed that it kills bacteria by disturbing membrane function, thereby causing leakage of cytoplasmic material and ultimately the collapse of the intracellular equilibrium. Research has demonstrated that CPC is effective in preventing gingival inflammation, thereby helping to prevent periodontitis. Although osteoclasts have been shown to resorb alveolar bone in periodontitis, little is known about the direct effects of CPC on osteoclasts. Thus, in the present study, we investigated the effects of CPC on osteoclast formation.

MATERIALS AND METHODS

Reagents CPC was purchased from Sigma-Aldrich Corporation (St. Louis, MO, U.S.A.). Antibodies against ERK, phospho-ERK, inhibitor of kappa B (IκB) and c-Fos were purchased from Cell Signaling Technology (Danvers, MA, U.S.A.). Antibody against COX-2 was purchased from CAYMAN chemical company (MI, U.S.A.). Antibody against β-actin was purchased from Abcam (Cambridge, MA, U.S.A.). Antibody against NFATc1 was purchased from Santa Cruz (Santa Cruz, CA, U.S.A.). All other reagents were from Sigma-Aldrich Corporation.

Cells and Culture System Mouse bone marrow cells were isolated from the ICR mice (Samtako, Inc., Korea) as described before. Bone marrow cells were cultured in alpha-minimal essential medium (MEM) containing 10% fetal bovine serum (FBS) with 10ng/mL macrophage colony stimulating factor (M-CSF) overnight. Non-adherent cells were harvested and cultured with 30ng/mL M-CSF for 3 d. Floating cells were removed and adherent cells (bone marrow-derived macrophages (BMMs)) were used as osteoclast precursors. All

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cells were cultured in alpha-MEM with 10% FBS at 37°C in 5% CO₂ incubator.

**Osteoclast Differentiation** BMMs suspended in alpha-MEM containing 10% FBS were seeded at 1×10⁵/well in 96-well plate and differentiated into osteoclasts by further treatment with indicated concentrations of CPC in the presence of M-CSF (30ng/mL) and RANKL (100ng/mL) for 4d. Then cells were washed with phosphate-buffered saline (PBS), fixed with 3.7% formalin for 15 min, fixed again with ethanol–acetone (50:50, v/v) for 1 min, and the fixed cells were stained for tartrate-resistant acid phosphatase (TRAP) as described previously. The number of TRAP-positive cells with more than 3 nuclei was counted under a light microscope.

**RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)** Total RNA was extracted from BMMs by Easy-Blue (iNtRON Biotechnology, Inc. Korea). cDNA was synthesized from total RNA by using Revert Aid™ first strand cDNA synthesis Kit (Fermentas, EU) and

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**Fig. 1. Effect of CPC on RANKL-Induced Osteoclast Formation of BMMs**

(A) The structure of CPC. (B) BMMs were cultured with various concentration of CPC in the presence of M-CSF (30ng/mL) and RANKL (100ng/mL) for 4d and stained for TRAP activity. TRAP-positive (+) multinucleated cells containing more than 3 nuclei were counted as osteoclasts in each well. (C) TRAP staining of BMMs in 96-well plates is also shown. (D) CPC (30nm) was added during the indicated culture days in the presence of RANKL and M-CSF. The TRAP-positive (+) multinucleated cells of each well was counted. (E) BMMs were cultured with or without CPC (30nm) in the presence of RANKL and M-CSF for 4d. Total RNA was isolated from cells, and RT-PCR analysis was performed to determine the expressions of osteoclast-associated gene. Data are expressed as mean±S.D. of three independent experiments. Veh, vehicle; R, RANKL. *p<0.05 vs. Veh; Scale bar=200μm.
amplified using PCR. Primers for osteoclastogenic genes used in this study are as follows: Prdm1, 5′-tgcttatcccagcacc-3′ (forward), 5′-cttcaggttggagagctgacc-3′ (reverse); calcitonin receptor (CTR), 5′-tttcaagaaccttagctgccagag-3′ (forward), 5′-caaggcacggacaatgttgagaag-3′ (reverse); cathepsin K, 5′-cttcgctatacgtgcagcaga-3′ (forward), 5′-acgcaccaatatcttgacc-3′ (reverse); β-actin, 5′-tttgatgtcacgcacgatttcc-3′ (forward), 5′-tgtgatggtgggaatgggtcag-3′ (reverse); The PCR program was as follows: Prdm1, 35 cycles, after an initial denaturation step at 94°C for 5 min, then denaturation at 94°C for 30 s, annealing at 52°C for 45 s, and extension at 72°C for 40 s, with a final extension at 72°C for 10 min; CTR 28 cycles, cathepsin K and β-actin 22 cycles, after an initial denaturation step at 94°C for 3 min, then denaturation at 94°C for 30 s, annealing at 58°C for 45 s, and extension at 72°C for 60 s, with a final extension at 72°C for 10 min. Products were separated by 1% agarose gel electrophoresis, stained with ethidium bromide. Results were normalized to β-actin for each sample.

**Immunoblot Analysis** Total cell lysates were isolated, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto immobilon-P membranes (Millipore, Bedford, MA, U.S.A.). The membranes were blocked with 5% nonfat-milk in PBS-T (PBS, 0.1% Tween 20), and then immunostained with specific antibodies and immuno-reactivity was detected with ECL solution (Amersham Biosciences, Piscataway, NJ, U.S.A.) using an LAS3000 luminescent image analyzer (FUJIFILM Co., Tokyo, Japan).

**Bone Resorption Assay** BMMs were differentiated on dentin slices with M-CSF (30 ng/mL) and RANKL (100 ng/mL) in the presence or absence of CPC (30 nM) for 10 d. Cells were removed from the dentin slice by wiping the surface of it, then slices were stained with toluidine blue (J.T. Baker, U.K., 1 µg/mL). And the numbers of pit on the dentin slices were counted.

**Statistical Analysis** Data represent the means and the ±S.D. from at least three experiments. Statistical analysis was performed by one-way analysis of variance followed by the Student’s t-test with p-value < 0.05 considered significant.

**RESULTS**

**CPC Inhibits RANKL-Induced Osteoclast Formation and Expression of Osteoclastogenic Genes** RANKL induces osteoclast formation in mouse bone marrow macrophage cultures. To investigate whether CPC regulates osteoclast formation, we used a mouse bone marrow-derived macrophage (BMM) culture system. BMMs were efficiently induced to differentiate into TRAP-positive multinucleated cells in the presence of M-CSF and RANKL. CPC reduced the formation of TRAP-positive multinucleated cells in a concentration-dependent manner, causing complete inhibition at a concentration of 30 nM (Figs. 1B,C). In a time course study, CPC inhibited RANKL-induced osteoclast formation at both early
stages (days 0–2) and late stages (days 2–4) (Fig. 1D). These results suggest that CPC inhibits both the differentiation of precursor cells into mononuclear osteoclasts and the fusion of mononuclear osteoclasts to form multinucleated osteoclasts. At the working concentration, CPC did not affect cell viability or morphology, as observed under a light microscope (data not shown), suggesting that CPC inhibits the differentiation of osteoclasts without causing cytotoxicity.

We next analyzed the effects of CPC on the expression profiles of various genes that are putatively involved in RANKL-mediated osteoclastogenesis (Fig. 1E). When BMMs were cultured with M-CSF and RANKL for 4 days, the expression of various genes related to osteoclast function, including those encoding CTR and cathepsin K, was stimulated. This stimulation was dramatically suppressed by CPC. Furthermore, CPC also reduced RANKL-induced expression of Prdm1, which encodes Blimp-1. Blimp-1 acts as a global repressor of multiple anti-osteoclastogenic genes, including the genes encoding interferon regulatory factor-8 (IRF-8) and v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (MafB), and thereby induces the transformation of monocyte/macrophage precursor cells into terminally differentiated osteoclasts. These results suggest that CPC regulates the expression of osteoclast differentiation- and function-related genes during RANKL-mediated osteoclastogenesis.

CPC Suppresses Pit Formation on Bone Slices Functional osteoclasts have the ability to generate resorption pits on bone. To resorb the bone, mature multinucleated osteoclasts must maintain a proper actin cytoskeletal organization, which facilitates resorptive activity.11 We examined whether the effects of CPC on osteoclastogenesis affect bone-resorption activity by performing an in vitro pit formation assay using a dentin slice. Many resorption pits were generated in wells containing RANKL-treated cells. In contrast, CPC treatment significantly inhibited pit formation (Fig. 2). Taken together, these results suggest that CPC inhibits osteoclast differentiation, which in turn reduces bone-resorption activity.

CPC Suppresses Pit Formation on Bone Slices

CPC Inhibits RANKL-Induced Expression of NFATc1 and c-Fos by Modulating ERK and NF-κB Signaling To verify the molecular mechanism by which CPC inhibits osteoclast formation, we investigated the effect of CPC on RANKL-induced NFATc1 and c-Fos expression in BMMs (Fig. 3). CPC almost completely blocked the induction of NFATc1 and c-Fos by RANKL. These data suggest that CPC inhibits RANKL-induced osteoclast differentiation by suppressing the induction of NFATc1 and c-Fos.

We then examined the effects of CPC on signaling pathways activated by RANKL to define the molecular mechanism of CPC action. We first evaluated the effect of CPC on ERK activation following stimulation of BMMs with RANKL. CPC reduced the phosphorylation of ERK by RANKL (Fig. 4A), suggesting that ERK pathways are mediators of the anti-osteoclastogenic effect of CPC.

Next, we determined whether CPC affects the activation of NF-κB by RANKL. Under normal conditions, NF-κB dimers form an inactive complex with inhibitor κB (IκB) in the cytoplasm. In RANKL-stimulated cells, IκB kinase (IKK) phosphorylates IκB, thereby targeting IκB for ubiquitin-dependent proteasome degradation and releasing the active NF-κB dimer.18 When we stimulated BMMs with RANKL, rapid degradation of IκB was observed on Western blots; this response was significantly inhibited by CPC (Fig. 4B). These results indicate that CPC inhibits RANKL-induced NF-κB activity by reducing the degradation of IκB.

CPC Reduces RANKL-Induced COX-2 Expression We finally examined the effect of CPC on COX-2 expression in BMMs following RANKL stimulation. We found that RANKL increases COX-2 expression in BMMs, and CPC significantly inhibits this response (Fig. 4C). These findings suggest that the suppression of osteoclastogenesis by CPC is partly mediated by the attenuation of COX-2 expression.

**Fig. 3. Effect of CPC on RANKL-Induced NFATc1 and c-Fos Expression**

(A–B) BMMs were pre-incubated with CPC (30 nm) for 30 min, and then treated with or without 300 ng/mL of RANKL for 24 h in the presence of M-CSF (30 ng/mL). Cell lysates were then subjected to Western blotting analysis with the indicated antibodies. The intensities of protein bands were analyzed and normalized. Data are expressed as mean ± S.D. of three independent experiments. Veh, vehicle; R, RANKL.*p < 0.05 vs. Veh.
CPC is a cationic member of the quaternary ammonium family and is easily adsorbed onto oral surfaces. As a surface-active agent, it has a broad antimicrobial spectrum and is particularly effective at killing Gram-positive pathogens and yeasts. \(^{12}\) CPC has been shown to significantly improve plaque-associated gingivitis, which can precede periodontitis. \(^{19}\) Alveolar bone resorption as a result of enhanced osteoclast activity is commonly observed in periodontitis. Accumulating evidence demonstrates that periodontitis involves bacterially derived factors that stimulate the differentiation, maturation, and survival of osteoclasts, leading to bone loss. \(^{20}\)

In the present study, we showed that CPC inhibits RANKL-induced osteoclast formation \textit{in vitro} without causing cytotoxicity (Figs. 1B, C). In agreement with this, RT-PCR analysis revealed that CPC decreased the mRNA expression of the osteoclastogenic marker genes encoding CTR and cathepsin K (Fig. 1E) as well as Prdm1, which encodes Blimp-1, a transcriptional repressor of anti-osteoclastogenic genes. Furthermore, CPC suppressed osteoclastic pit formation on dentin slices (Fig. 2). These results indicate that CPC inhibits RANKL-induced osteoclast formation and prevents bone resorption \textit{in vitro}.

The binding of RANKL to RANK leads to the rapid activation of many signaling molecules, including MAPKs (ERK, JNK, and p38 MAPK) and NF-\(\kappa\)B, and subsequently induces the expression of the transcription factors c-Fos and NFATc1, which play key roles in osteoclastogenesis. \(^{21}\) Our mechanistic experiments revealed that CPC inhibits the RANKL-induced
expression of the master transcription factors c-Fos and NFATc1, possibly through suppression of early signaling events such as the phosphorylation of ERK and IκB (Figs. 3, 4). This observation is consistent with the finding that CPC affected early mononuclear osteoclast formation (Fig. 1D). CPC also suppressed late multinuclear osteoclast formation, which occurs by cell fusion. Although NFATc1 signaling has been reported to be involved in late-stage osteoclastogenesis, further studies on the effects of CPC may reveal details of its mechanism of action in osteoclast cell fusion.

COX-2 is an essential mediator of RANKL-induced osteoclastogenesis, and its level transiently increases in BMMs. Inhibition of COX-2 expression suppresses the differentiation of BMMs into osteoclasts. It was previously shown that the ability of RANKL to regulate COX-2 expression depends on NF-κB, a key regulator of COX-2 transcription. CPC has been reported to reduce COX-2 mRNA expression in IL-1β- and TNF-α-stimulated cells. Moreover, it has been suggested that NF-κB signaling may be impaired in the presence of CPC. In agreement with these earlier findings, COX-2 protein levels were reduced by CPC (Fig. 4C). These results suggest that CPC may inhibit osteoclast formation via MAPK, NF-κB, and COX-2 pathways.

M-CSF also has been found to be a key regulator of osteoclast formation. Several studies have shown that M-CSF is necessary for the proliferation, differentiation, and survival of osteoclast precursors. Thus, we could not exclude the possibility that CPC acts on M-CSF signaling in BMMs. Further studies are needed to elucidate this point.

In conclusion, we showed that CPC inhibits osteoclastogenesis by suppressing the RANKL-induced expression of c-Fos and NFATc1 via ERK and NF-κB pathways. Several lines of evidence indicate that modulation of osteoclastogenesis through RANKL/RANK signaling can be an effective therapeutic strategy for bone disorders. Thus, CPC may be useful for the treatment of bone diseases characterized by excessive bone resorption, including periodontitis.

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REFERENCES