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Inhibition of osteoclast differentiation and bone resorption by a novel lysophosphatidylcholine derivative, SCOH

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Abstract

Osteoclasts are multinucleated cells formed by multiple steps of cell differentiation from progenitor cells of hematopoietic origin. Intervention in osteoclast differentiation is considered as an effective therapeutic approach to the treatment for bone diseases involving osteoclasts. In this study, we found that the organic compound (*S*)-1-lyso-2-stearoylamino-2-deoxy-*sn*-glycero-3-phosphatidylcholine (SCOH) inhibited osteoclast differentiation. The inhibitory effect of SCOH was observed in mouse bone marrow cell cultures supported either by coculturing with osteoblasts or by adding macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL). M-CSF and RANKL activate the ERK, Akt, and NF-κB signal transduction pathways, and SCOH suppressed this activation. SCOH also inhibited the bone resorptive activity of differentiated osteoclasts. It attenuated bone resorption, actin ring formation, and survival of mature osteoclasts. Reduced activation of Akt and NF-κB and decreased induction of XIAP were observed in mature osteoclasts treated with SCOH. Thus, this novel phosphatidylcholine derivative may be useful for treating bone-resorption diseases.

Keywords: Osteoclast; SCOH; Bone resorption; ERK; Akt; NF-κB

1. Introduction

Bone remodeling is a process that continues throughout life and involves the resorption of mineralized matrix followed by new bone formation. A good balance between resorption and new bone formation maintains overall bone mass, whereas loss of this balance results in bone metabolic diseases, such as osteoporosis. Osteoporosis, which enhances fracture risk on trauma, can be provoked by estrogen deficiency, hyperparathyroidism, hyperthyroidism, and corticosteroid hormone; these factors are associated with an increase in the number and activity of the bone-resorbing cells, osteoclasts [1].

Osteoclasts originate from hematopoietic precursor cells of the phagocyte lineage and differentiate into multinucleated cells by the fusion of mononuclear progenitors [2]. Osteoclasts are the only cell type capable of resorbing mineralized bone, and they act under the control of numerous cytokines, hormones, and growth factors produced by supporting cells such as osteoblasts and stromal cells [3]. Among cytokines, RANKL was found to be a key osteoclastogenic molecule that directly binds to its cognate receptor, RANK, on osteoclast precursor cells [4,5]. Upon RANKL binding, RANK recruits tumor necrosis factor receptor (TNFR)-associated factors (TRAFs), especially TRAF6, which are membrane-proximal adaptor molecules [6–9]. TRAFs induce a strong activation of NF-κB.

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Abbreviations: SCOH, (*S*)-1-lyso-2-stearoylamino-2-deoxy-*sn*-glycero-3-phosphatidylcholine; TRAP, tartrate-resistant acid phosphatase; RANKL, receptor activator of nuclear factor kappa B ligand; ERK, extracellular signal-regulated kinase; M-CSF, macrophage colony stimulating factor.

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The essential roles of RANKL, RANK, TRAF6, and NF-κB were clearly demonstrated in gene knockout mice that displayed osteopetrotic bone architecture resulting from the lack of functional osteoclasts [10–13]. Also, PI 3-kinase/Akt, p38, and ERK signaling pathways are involved in osteoclast differentiation through participation in RANKL signaling [14,15].

To resorb bone, mature osteoclasts attach to the mineralized bone surface and build a sealing zone forming an actin ring structure. Wortmannin, a specific inhibitor of PI 3-kinase, disrupts the actin ring in osteoclasts and blocks osteoclastic bone resorption [16]. Several other inhibitors that disrupt the actin ring have been developed as candidates for treatment of osteoporosis [17,18]. Once terminally differentiated, osteoclasts die by apoptosis in the absence of supporting cells or osteoclast survival factors [19]. RANKL, M-CSF, TNF-α, and IL-1α promote osteoclast survival [19–22], whereas estrogen and transforming growth factor-β increase osteoclast apoptosis [23,24]. Miyazaki *et al*. reported that ERK activation is indispensable for osteoclast survival and up-regulation of bone-resorption by osteoclasts [25]. Altering osteoclast survival has been considered as a strategy for development of therapeutic reagents for the treatment of osteoclast-mediated bone diseases.

To search for compounds that reduce bone resorption, we performed random screening of chemical libraries. We found a compound that inhibits osteoclast differentiation, SCOH. This compound also inhibited osteoclast survival, formation of actin rings, and osteoclastic bone resorption. SCOH suppressed the activation of signaling molecules associated with osteoclast differentiation and survival, including Akt, ERK, and NF-κB.

2. Materials and methods

2.1. Osteoclast culture

Osteoclasts were generated by coculturing mouse bone marrow cells with supporting osteoblasts. Briefly, primary osteoblasts were obtained by growing calvarial cells from ICR newborn mice for 1 day in α-minimum essential medium (α-MEM; Gibco BRL) containing 10% (v/v) fetal bovine serum (FBS; Gibco BRL), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37° in a humidified chamber with 5% CO_2. Bone marrow cells were obtained by flushing tibiae from 6- to 7-week-old ICR mice and suspended in α-MEM/10% FBS. Then 1×10^7–2×10^7 bone marrow cells and 1×10^6 osteoblasts were seeded on a 90-mm collagen-coated culture dish and incubated for 6–7 days in the presence of 10^{-8} M VtD_3 and 10^{-6} M PGE_2. To obtain mature osteoclasts, the cocultured cells were detached by treating with 0.2% collagenase (Wako) at 37° for 10 min, replated on 60-mm dishes, and culturing for another day. The dishes were then treated with 0.1% collagenase at 37° for 15 min and intensely pipetted to remove osteoblasts. The remaining cells were more than 95% pure osteoclasts, as determined by TRAP cytochemistry.

Osteoclast differentiation was also induced from bone marrow-derived macrophage preparations. Bone marrow cells were cultured in α-MEM/10% FBS for 24 hr. The non-adherent cells were collected and cultured in α-MEM/10% FBS containing 50 ng/mL M-CSF (Peprotech EC) for 3 days. Cells at this stage were considered bone marrow-derived macrophages. For osteoclast generation, cells were further incubated in medium containing 30 ng/mL M-CSF and 50 ng/mL RANKL (Peprotech EC) for 5 days.

2.2. Western blotting

Bone marrow-derived macrophages or osteoclasts purified from the coculture (see Section 2.1) were lysed in a buffer containing 20 mM Tris–HCl, 150 mM NaCl, 1% Triton X-100, and protease and phosphatase inhibitors. The lysates (30–40 μg) of cellular proteins were separated by 10% SDS–PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). After blocking with 5% skim milk, the membrane was probed with anti-phospho Akt, ERK, JNK, and p38 (Cell Signaling Technology). The same membranes were stripped and reprobed with anti-Akt, ERK, JNK, and p38 (Cell Signaling Technology).

2.3. Electrophoretic mobility shift assay (EMSA)

EMSA was performed as previously described [21]. Osteoclasts purified from the coculture were lysed in a hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF) containing 0.6% NP-40 and microfuged at 1700*g* for 15 min. The pellet was lysed in 15 μL of a high salt buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT) for 20 min on ice. After adding 75 μL of storage buffer (20 mM HEPES, pH 7.9, 100 mM NaCl, 20% glycerol, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT), samples were agitated for 10 s by vortexing, and then microfuged at 20,000*g* for 20 min. Nuclear extracts (10 μg) were incubated with approximately 20,000 cpm of ^{32}P-labeled NF-κB binding site oligomer 5′-AGTTGAGGGGACTTTCC-CAGGC-3′ (Santa Cruz Biotechnology) for 30 min at 20°. The DNA-bound NF-κB proteins were subjected to 4–5% polyacrylamide gel electrophoresis followed by autoradiography.

2.4. Resorption pit assay

Cocultured cells were detached as described above from the collagen-coated dishes. Cells were replated on dentine

slices that had been placed in 96-well plates and treated with SCOH for 24 hr. After the incubation period, attached cells were completely removed from dentine slices by abrasion with a cotton tip, and resorption pits were visualized by staining with hematoxylin solution (Sigma). Photographs were taken under a light microscope at 40× magnification, and total areas of resorption pits were analyzed by the Image Pro-Plus program version 4.0 (Media Cybernetics).

2.5. Survival assay

Osteoclasts purified from the coculture were pretreated with SCOH for 30 min, RANKL was added to 100 ng/mL, and incubation was continued for 24 hr. After washing twice with PBS, attached cells were stained for TRAP enzyme with the Leukocyte Acid Phosphatase Assay Kit (Sigma) as per the manufacturer's instructions. TRAP-positive cells with 5 or more nuclei were counted.

2.6. 4-6-Diamidino-2-phenylindole (DAPI) staining

Purified osteoclasts were pretreated with or without SCOH for 30 min and further incubated for 12 hr after the addition of 100 ng/mL RANKL. Cells were washed once in PBS, fixed with 10% formaldehyde for 5 min at room temperature, and then incubated with 1 μg/mL DAPI in PBS for 5 min. Cells showing fragmented chromatin morphology were considered apoptotic.

2.7. Actin ring formation

The cocultured cells were detached from collagen dishes and replated on glass cover slips. Osteoblasts were removed with 0.1% collagenase. Purified osteoclasts were starved with α-MEM containing 0.1% FBS and pretreated with or without 10 μM SCOH for 15 min. 100 ng/mL of RANKL were added and cells were further incubated for 30 min. Cells were fixed and stained with rhodamine-conjugated phalloidin.

2.8. Caspase assay

Purified osteoclasts were pretreated with SCOH or the vehicle for 30 min and further treated with 100 ng/mL RANKL for 8 hr. After rinsing in PBS, cells were lysed in the Cell Lysis Buffer included in the Caspase 3 Colorimetric Assay Kit (R&D Systems). The enzymatic reaction for caspase 3 activity was carried out with the *p*-nitroanilide-conjugated DEVD peptide (DEVD-pNA) substrate as described by the manufacturer.

2.9. Statistical analysis

All quantitative data are presented as mean ± SD. Each experiment was performed 3–5 times, and results from one representative experiment are shown. Statistical differences were analyzed by Student's *t*-test.

3. Results

3.1. Effects of SCOH on osteoclast differentiation

We screened chemical libraries derived from natural products to identify compounds that inhibit osteoclast differentiation in cocultures of mouse bone marrow cells and osteoblasts. We found a compound that effectively reduced the formation of TRAP-positive multinuclear osteoclasts. This compound, (*S*)-1-lyso-2-stearoylamino-2-deoxy-*sn*-glycero-3-phosphatidylcholine, is termed SCOH, based on its chemical structure (Fig. 1). SCOH decreased osteoclast generation by 67.52 ± 5.14% at 5 μM and 95.62 ± 0.46% at 10 μM in a coculture system (Fig. 2A). The inhibitory effect of SCOH on osteoclast generation in the coculture could be either from direct effects on osteoclast progenitors or from indirect effects on osteoblasts that support the differentiation of osteoclast progenitors. To determine whether SCOH has direct effects on osteoclast progenitors, we utilized a culture system that does not require supporting cells. TRAP-positive osteoclasts can be generated when bone marrow macrophages are cultured in the presence of RANKL and M-CSF. Mouse bone marrow-derived macrophages were cultured in the presence of various concentrations of SCOH with 50 ng/mL RANKL and 30 ng/mL M-CSF for 5 days. SCOH inhibited the formation of TRAP-positive multinuclear osteoclasts in a dose dependent manner (Fig. 2B and C). SCOH also suppressed the generation of mononuclear TRAP-positive cells (data not shown). Addition of SCOH during only the early or late period of the culture also had inhibitory effects on osteoclast formation (data not shown). These results suggest that SCOH may affect both the commitment and cell fusion steps of osteoclast differentiation from bone marrow progenitor cells. The inhibitory effect of SCOH on osteoclast differentiation could be due to cytotoxicity. To exclude this possibility, we examined whether SCOH has an adverse effect on cell proliferation. We used the MTT assay to test various cell types including osteoblasts, bone marrow cells, bone marrow-derived macrophages, and 293T cells. SCOH did not show any cytotoxicity (data not shown). In summary, SCOH is an effective inhibitor of osteoclast differentiation in the coculture as well as bone marrow-derived macrophage culture systems.

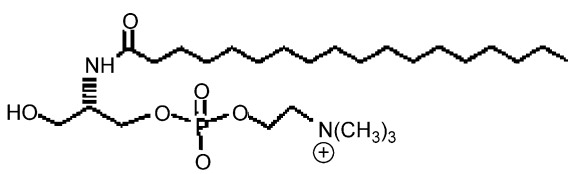

Fig. 1. Chemical structure of SCOH.

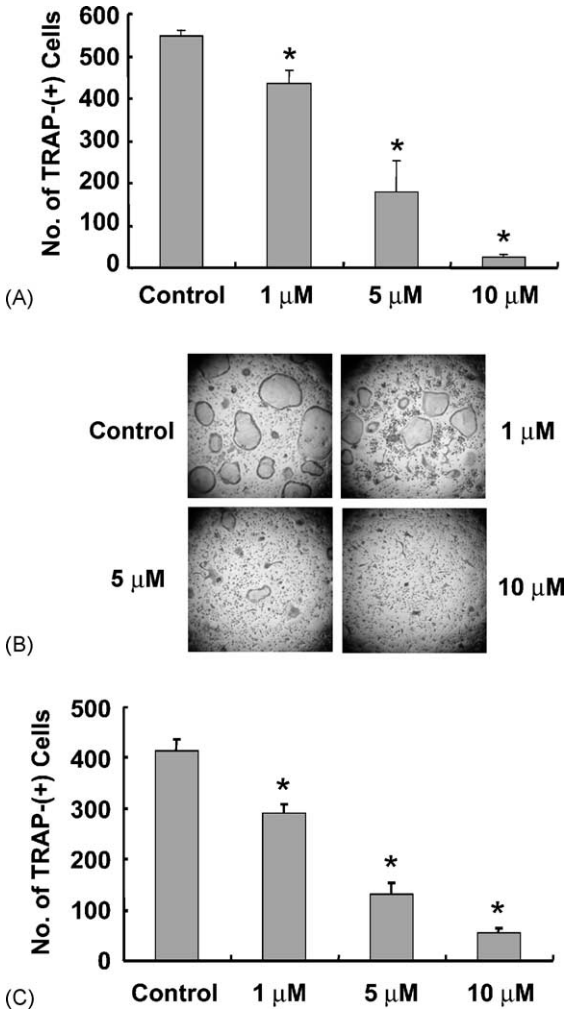

Fig. 2. Effects of SCOH on osteoclast differentiation. Mouse bone marrow cells and calvarial osteoblasts were cultured in 48-well plates in the presence of 10^{-8} M VtD_3 and 10^{-6} M PGE_2 for 5 days. The indicated concentration of SCOH was added in the culture. TRAP-positive multinuclear cells containing 5 or more nuclei were counted as osteoclasts (A). Bone marrow-derived macrophages were cultured in 48-well plates in the presence of 30 ng/mL M-CSF and 50 ng/mL RANKL for 5 days in the presence of indicated concentrations of SCOH. Cells were fixed and stained for TRAP (B and C). TRAP-positive multinuclear cells were counted (C). Data from one experiment are presented as mean ± SD of triplicate samples and similar results were obtained in two other experiments. $^{*}P < 0.01$, significant differences from the control.

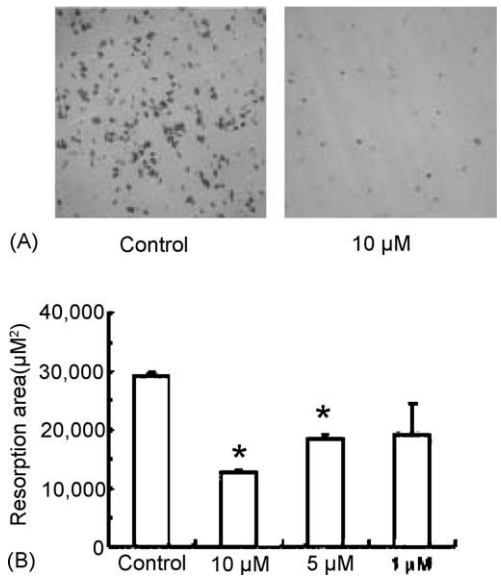

Fig. 3. Inhibition of bone resorption activity of osteoclasts by SCOH. Bone marrow cells were cultured with calvarial osteoblasts as described in Section 2. Cells were detached from the collagen-coated dish and replated on dentine slices. Cells were cultured for 24 hr in the presence of the indicated concentrations of SCOH. Resorption pits on dentine slices were visualized by staining with hematoxylin (A). Resorption pit areas were analyzed by the Image Pro-Plus program version 4.0 (B). The mean ± SD of five dentine slice samples from one experiment is shown. Similar results were observed in two other experiments. $^{*}P < 0.01$, significant differences from the control.

3.2. Inhibition of bone-resorbing activity of osteoclasts by SCOH

Mature differentiated osteoclasts undergo a morphological and functional polarization, and begin to resorb mineralized bone surface. The extent of bone resorption *in vivo* is influenced by various factors that govern osteoclast number and activity. The results of *in vitro* experiments on osteoclast differentiation suggested that SCOH might *in vivo* reduce osteoclast number. We next explored the possibility that SCOH may directly affect the bone resorption activity of differentiated mature osteoclasts. Mature osteoclasts were generated by the coculture, placed on dentine slices, and cultured in the presence of various concentrations of SCOH for 24 hr. In the presence of SCOH, bone resorption was inhibited in a dose-dependent manner (Fig. 3). This result shows that SCOH not only inhibits osteoclast formation, but also decreases their bone resorption activity.

3.3. SCOH blocks actin ring formation by osteoclasts

To resorb bone, osteoclasts must form a sealing zone by generating a ring structure made of F-actin. We investigated whether SCOH affects the formation of the actin ring structure. Staining F-actin with rhodamine-phalloidin showed the actin ring in mature osteoclasts (Fig. 4). In the absence of RANKL, the actin ring was loose and fuzzy (Fig. 4A and B, left), while upon addition of RANKL, the actin ring became more dense and smooth (Fig. 4A and B, middle). SCOH treatment abolished the effect of RANKL on the ring structure (Fig. 4A and B, right). These results indicate that SCOH could affect cytoskeletal organization, which is necessary for bone resorption by osteoclasts.

3.4. Inhibition of osteoclast survival by SCOH

Once terminally differentiated, osteoclasts undergo apoptotic cell death. The 24 hr-survival rate of differentiated multinuclear osteoclasts was below 20% in the absence of any survival factors (data not shown). RANKL has been

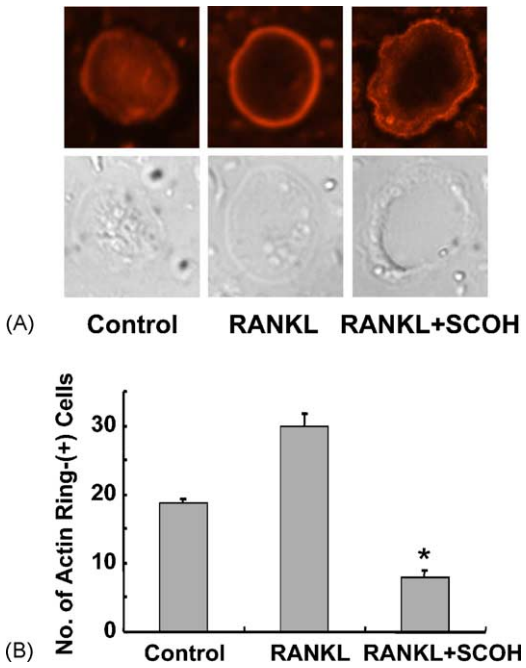

Fig. 4. Inhibition of actin ring formation by SCOH. Cocultured cells were detached from the collagen-coated dish and reseeded on glass cover slips. After removal of osteoblasts, osteoclasts were deprived of serum in medium containing 0.1% FBS for 5 hr. Cells were pretreated with or without 10 μM SCOH for 15 min and incubated with 100 ng/mL RANKL for 30 min. Cells were fixed and stained with rhodamine-phalloidin (A). The number of cells showing clear actin ring structure in triplicate samples are presented as mean ± SD (B). This result was reproduced in three independent experiments. $^{*}P < 0.01$, significant differences from the control.

reported to be one of the major factors that promote osteoclast survival [26]. To examine whether SCOH affects osteoclast survival, we incubated purified mature osteoclasts in the presence or absence of 10 μM SCOH with or without RANKL. Addition of RANKL greatly reduced the death of mature osteoclasts (Fig. 5A). SCOH attenuated the effect of RANKL on osteoclast survival. As SCOH decreased the survival of purified osteoclasts, we next investigated the effect of SCOH on apoptosis of these cells. When nuclear DAPI staining was performed, apoptotic osteoclasts showed condensed chromatin and fragmented nuclei in the absence of the survival factor (Fig. 5B). RANKL greatly reduced the percentage of cells with apoptotic nuclei (Fig. 5B and C), but treatment with SCOH significantly reversed the RANKL-induced inhibition of osteoclast apoptosis (Fig. 5B and C). Caspases play an important role in apoptosis. We examined the effect of SCOH on caspase 3 activity in osteoclasts. Differentiated osteoclasts were incubated with or without SCOH in the presence of 100 ng/mL RANKL for 8 hr. The addition of SCOH significantly increased the caspase 3 activity (Fig. 5D). These results suggest that SCOH may interfere with the function of RANKL in osteoclast survival and thus accelerate osteoclast apoptosis through caspase activation.

3.5. Inhibition of osteoclast differentiation-associated signaling pathways by SCOH in osteoclast precursors

Previously, we provided evidence for the involvement of PI 3-kinase/Akt, ERK, and p38 in osteoclast differentiation [15]. Consequently, the Akt, ERK, and p38 signaling pathways have emerged as therapeutic targets in a variety of bone-resorbing diseases. As SCOH was found to suppress osteoclast differentiation (Fig. 2), we next determined whether SCOH affects signaling pathways involving these kinases. When bone marrow-derived macrophages, osteoclast precursor cells that can differentiate to osteoclasts upon culturing in the presence of M-CSF and RANKL, were stimulated with M-CSF or RANKL, the phosphorylation of Akt and ERK increased (Fig. 6A and B). SCOH treatment blocked the phosphorylation of Akt and ERK induced by either M-CSF or RANKL (Fig. 6A and B). On the other hand, SCOH itself greatly increased the phosphorylation of JNK and p38 (Fig. 6A and B). M-CSF or RANKL did not affect the SCOH-induced phosphorylation of JNK and p38 (Fig. 6A and B). The activation of NF-κB pathway is a prerequisite for osteoclast differentiation [27]. To activate NF-κB, IκB should be degraded through phosphorylation process. SCOH prevented IκB degradation and phosphorylation induced by RANKL (Fig. 6B). Taken together, SCOH disrupted multiple signaling pathways that have been implicated in osteoclast differentiation.

3.6. SCOH blocks Akt, NF-κB, and XIAP activation induced by RANKL in mature osteoclasts

SCOH suppressed the survival and increased the apoptosis of differentiated mature osteoclasts (Fig. 5). Our previous study suggested that the signaling pathway of the serine/threonine kinase Akt plays an important role in the survival of mature osteoclasts [21]. Therefore, we investigated the effect of SCOH on the extent of Akt phosphorylation in purified mature osteoclasts. As previously reported, RANKL induced Akt activation in mature osteoclasts (Fig. 7A, lane 3). SCOH treatment attenuated the RANKL-induced Akt activation (lane 4). NF-κB is one of the most important transcription factors for promoting cell survival by inducing anti-apoptotic genes [28]. When assessed by EMSA, the activation of NF-κB was observed in purified mature osteoclasts stimulated with RANKL for 30 min (Fig. 7B). Treatment of the cells with SCOH inhibited the RANKL-induced NF-κB activation (Fig. 7B). In addition to these observations of Akt and NF-κB involvement in osteoclast survival, it was also important to assess the level of the inhibitor of apoptosis (IAP) family of anti-apoptotic proteins in mature osteoclasts. When mature osteoclasts were treated with RANKL, the expression level of XIAP, the most potent inhibitor of caspases and apoptosis among the IAPs [29], increased in a time-dependent way (Fig. 7C, lanes 1–3). The level of XIAP was lowered by SCOH treatment (Fig. 7C, lanes 4–6). By contrast, the level

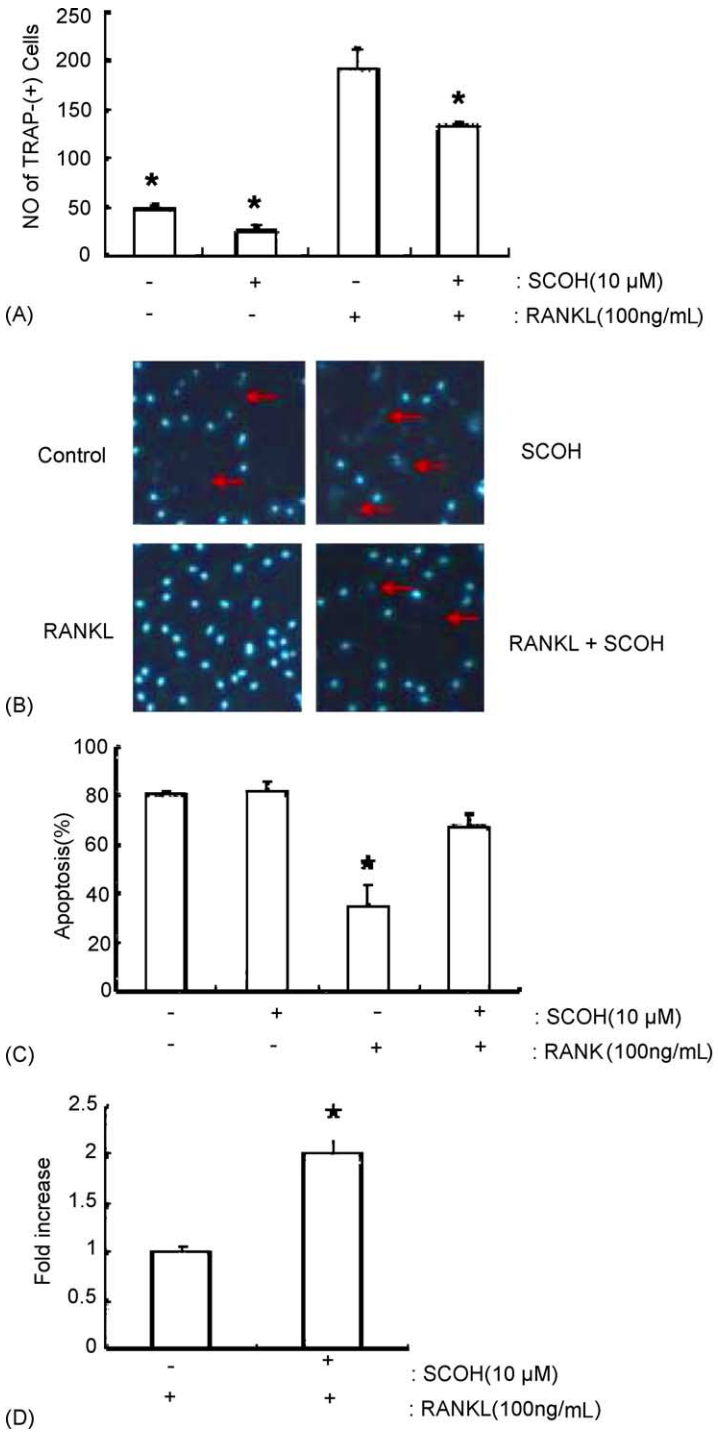

Fig. 5. Effect of SCOH on osteoclast apoptosis. Differentiated mature osteoclasts were purified from the coculture as described in Section 2. Cells were pretreated with 10 μM SCOH or the control vehicle for 30 min and incubated for 24 hr after the addition of RANKL to 100 ng/mL. Survival of osteoclasts was measured as the number of TRAP-positive multinuclear cells (A). Purified osteoclasts were pretreated with 10 μM SCOH for 30 min and incubated in the presence of 100 ng/mL RANKL for 12 hr. The nuclei were stained with DAPI to identify apoptotic cells. Cells with apoptotic nuclear staining are indicated by arrows. (B). Percentage of the apoptotic cells nuclei are shown (C). Cells were pretreated with 10 μM SCOH for 30 min and further treated with 100 ng/mL RANKL for 8 hr. Caspase 3 activity in lysates was measured using the DEVD-pNA substrate (D). Data from one experiment are presented as mean ± SD of triplicate samples and similar results were obtained in two other experiments. $^{*}P < 0.01$, significant differences from the control.

of c-IAP1 and c-IAP2 was not affected by SCOH (data not shown). Overall, our findings suggest that SCOH reduces osteoclast survival by interfering with the activation of Akt and NF-κB and the induction of XIAP activation by RANKL in differentiated osteoclasts.

4. Discussion

Bone mass in adults decreases with age because of the imbalance between the rate of bone formation and that of bone resorption. Osteoclasts are the only known cell type

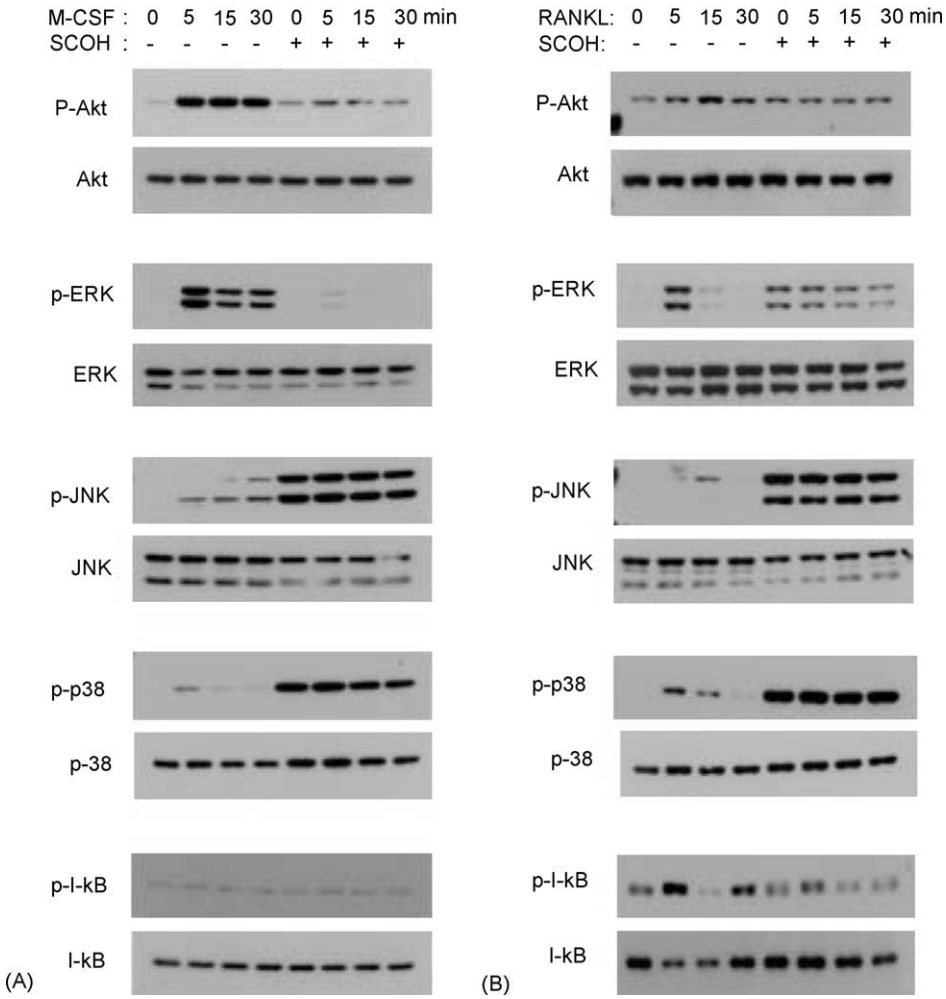

Fig. 6. Effect of SCOH on signal transduction induced by RANKL or M-CSF. Bone marrow-derived macrophages were deprived of serum in medium containing 0.1% FBS for 5 hr, pretreated with 10 μM SCOH for 30 min, and then stimulated by the addition of 50 ng/mL M-CSF (A) or 500 ng/mL RANKL (B) for the indicated times. Cells were lysed and analyzed by western blotting.

capable of resorbing mineralized bone. Bone resorption is influenced by various factors that govern osteoclast number and activity. We have conducted a random screening for compounds that reduce the generation or activation of osteoclasts and thus may have therapeutic applications for bone-resorbing diseases such as osteoporosis, Paget's disease, and periodontal diseases. In this study, we report a novel inhibitor of osteoclast differentiation, activity, and survival. This compound, SCOH, inhibits both the differentiation of precursor cells to TRAP-positive multinucleated osteoclasts and also the bone resorption activity and survival of differentiated osteoclasts (Figs. 2, 3 and 5).

For bone resorption, osteoclasts undergo an active cytoskeletal reorganization to generate an exclusive resorption compartment by forming a circumferential sealing zone [30]. At the sealing zone the actin filament forms an attachment ring structure [31]. Consequently, in osteoclasts actively involved in bone resorption, a compact ring structure is usually observed when F-actin is stained. We found that SCOH significantly reduced the bone resorption activity of differentiated mature osteoclasts (Fig. 3) and blocked the actin ring formation induced by RANKL (Fig. 4). The total amount of bone degradation also reflects the rate at which osteoclasts differentiate and die. Many osteoclastic factors such as RANKL, IL-1, TNF-α, and M-CSF, prolong osteoclast survival and inhibit osteoclast apoptosis, leading to elevated bone resorption [21,22,32]. Bisphosphonate derivatives, which are the strongest inducers of osteoclast apoptosis *in vitro*, are therapeutically effective for in diseases of increased bone turnover, such as Paget's disease and hypercalcemia of malignancy [33]. SCOH also displayed an apoptotic effect on mature osteoclasts, resulting in a decrease in cell survival (Fig. 5). Therefore, SCOH may reduce bone resorption by osteoclasts both by disrupting cytoskeletal architecture necessary for the resorption process *per se* and also by reducing the life span of differentiated osteoclasts.

To elucidate the action mechanism of SCOH on differentiation and resorption, we examined its effects on signaling pathways in osteoclasts at various differentiation stages. In differentiation of bone marrow-derived osteoclast precursors into mature TRAP-positive osteoclasts, PI

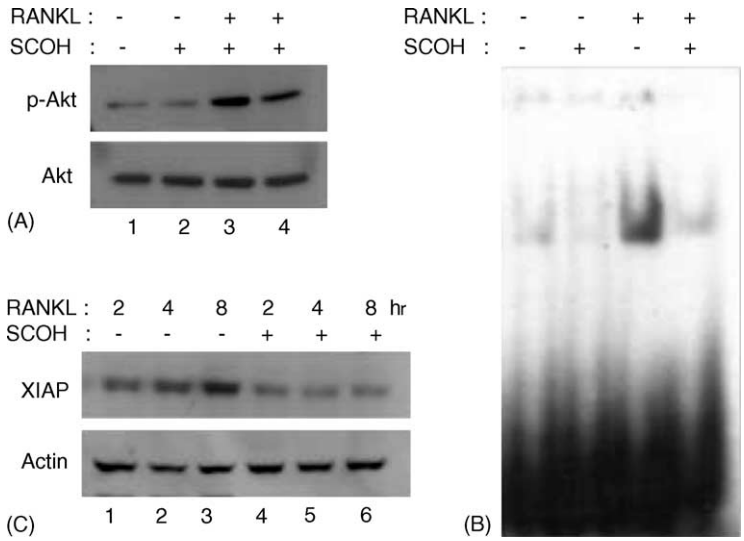

Fig. 7. Effect of SCOH on RANK signaling in mature osteoclasts. Mature osteoclasts purified from the coculture were deprived of serum in medium containing 0.1% FBS for 5 hr, pretreated with 10 μM SCOH for 30 min. RANKL was added to 1 μg/mL and incubation was continued for 15 min. Cells were lysed and analyzed by western blotting (A). Purified osteoclasts were pretreated with 10 μM SCOH for 30 min and further incubated for 30 min after the addition of RANKL to 500 ng/mL. Cells were harvested and nuclear extracts were prepared as described under Section 2. NF-κB activity was measured by EMSA with 7 μg of nuclear extracts (B). Cells were incubated with 100 ng/mL RANKL in the absence or presence of SCOH for the indicated times. Cell lysates were prepared and subjected to western blotting with anti-XIAP (top). The same membrane was stripped and reprobed with anti-actin (bottom) (C).

3-kinase and, subsequently, Akt are activated through Src family kinases [9]. In our previous report, the ERK pathway was also shown to be important in osteoclast differentiation [15]. SCOH suppressed the activation of Akt and ERK in response to M-CSF and RANKL (Fig. 6). In addition, the activation of NF-κB has an essential role in osteoclast differentiation [10,27]. SCOH treatment blocked the degradation of IκB and decreased the phosphorylation of IκB induced by RANKL (Fig. 6B). Taken together, the effects of SCOH on suppression of osteoclast differentiation may result from the blockade of these pathways.

Several signaling molecules have been demonstrated to play roles in counteracting apoptotic processes. Akt exerts anti-apoptotic effects by phosphorylating and inactivating components of apoptotic machinery such as Bad and caspase 9 and transcription factors necessary for the induction of genes required for cell death [34]. NF-κB activation has been extensively characterized and shown to induce anti-apoptotic genes including IAP family members [29]. The mechanisms by which RANKL increases the survival of differentiated mature osteoclasts appear to involve Akt and NF-κB activation and XIAP induction (Fig. 7). SCOH interfered with all these responses (Fig. 7). The direct target of SCOH may be a component involved in early events of RANK signaling, which occur in or proximal to the plasma membrane.

Interestingly, SCOH itself increased the activation of JNK and p38 in osteoclast precursor cells (Fig. 6A and B). JNK and p38 MAPKs are activated by cell injurious stresses, such as heat shock, UV, and osmotic stress. The involvement of JNK and p38 in apoptotic stress responses have been reported [35]. Lysophosphatidylcholine, SCOH-related compound, has been shown to activate JNK in bovine aortic endothelial cells [36] and to induce apoptosis in human endothelial cells through a p38 MAPK-dependent pathway [37]. Given these reports, it is logical to think that SCOH might have stimulated the apoptosis of osteoclast precursor cells and thereby contributed to the suppression of differentiation to osteoclasts. However, we did not observe any cytotoxicity of SCOH with our osteoclast precursor cell preparations. In addition, the activation of JNK and p38 by SCOH was not detected in mature osteoclasts (data not shown), which underwent apoptosis. Therefore, it appears that the activation of JNK and p38 is not associated with apoptosis during osteoclast differentiation.

Whether SCOH needs to enter the cell for its action or it acts on components in the plasma membrane, if it enters the cell, how it enters, and the precise mechanism by which SCOH elicits its responses are questions to be resolved. A recent report demonstrated that exogenously added lysophosphatidylcholine rapidly was inserted into the outer leaflet of the plasma membrane and traversed the lipid bilayer [38]. Lysophosphatidylcholine can then be converted to phosphatidylcholine, which can be further processed for generation of arachidonic acid. Besides JNK and p38 activation mentioned above, lysophosphatidylcholine has been reported to produce reactive oxygen species [39], increase intracellular free calcium concentration [40], and activate adenylate cyclase [41]. The molecular mechanisms for these diverse signaling effects of lysophosphatidylcholine remain elusive. While whether SCOH enters the cell and exerts its effect by a way analogous

to lysophosphatidylcholine is an intriguing question, the relationship between lysophosphatidylcholine or phosphatidylcholine and bone turnover is yet another important issue to be investigated.

In summary, we found that SCOH, a compound identified from chemical libraries, suppressed osteoclast differentiation and bone resorption. Also, it inhibited survival of osteoclasts. Therefore, SCOH might be a candidate for drug development for the treatment of bone-resorbing diseases such as osteoporosis, bone-erosive rheumatoid arthritis, and advanced periodontal disease.

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