EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 43, No. 11, 605-612, November 2011

Simvastatin inhibits osteoclast differentiation by scavenging reactive oxygen species

Ho-Jin Moon[1], Sung Eun Kim[1], Young Pil Yun[1], Yu-Shik Hwang[1], Jae Beum Bang[2], Jae-Hong Park[3,4]* and Il Keun Kwon[1,4]*

[1]Department of Maxillofacial Biomedical Engineering and Institute of Oral Biology
[2]Department of Dental Education
[3]Department of Pediatric Dentistry
School of Dentistry
Kyung Hee University
Seoul 130-701, Korea
[4]Corresponding authors: Tel, 82-2-958-9379;
Fax, 82-2-965-7247; E-mail, pedopjh@khu.ac.kr (J.H.P.)
Tel, 82-2-961-0350; Fax, 82-2-967-5019;
E-mail, kwoni@khu.ac.kr (I.K.K.)
*These authors contributed equally to this work.
http://dx.doi.org/10.3858/emm.2011.43.11.067

Accepted 9 August 2011
Available Online 10 August 2011

Abbreviations: M-CSF, macrophage-colony stimulating factor; RANKL, receptor activator of nuclear factor-κB ligand; ROS, reactive oxygen species; TRAFs, TNF receptor-associated factors; TRAP, tartrate-resistant acid phosphatase

Abstract

Osteoclasts, together with osteoblasts, control the amount of bone tissue and regulate bone remodeling. Osteoclast differentiation is an important factor related to the pathogenesis of bone-loss related diseases. Reactive oxygen species (ROS) acts as a signal mediator in osteoclast differentiation. Simvastatin, which inhibits 3-hydroxy-3-methylglutaryl coenzyme A, is a hypolipidemic drug which is known to affect bone metabolism and suppresses osteoclastogenesis induced by receptor activator of nuclear factor-κB ligand (RANKL). In this study, we analyzed whether simvastatin can inhibit RANKL-induced osteoclastogenesis through suppression of the subsequently formed ROS and investigated whether simvastatin can inhibit H_2O_2-induced signaling pathways in osteoclast differentiation. We found that simvastatin decreased expression of tartrate-resistant acid phosphatase (TRAP), a genetic marker of osteoclast differentiation, and inhibited intracellular ROS generation in RAW 264.7 cell lines. ROS generation activated NF-κB, protein kinases B (AKT), mitogen-activated protein kinases signaling pathways such as c-JUN N-terminal kinases, p38 MAP kinases as well as extracellular signal-regulated kinase. Simvastatin was found to suppress these H_2O_2-induced signaling pathways in osteoclastogenesis. Together, these results indicate that simvastatin acts as an osteoclastogenesis inhibitor through suppression of ROS-mediated signaling pathways. This indicates that simvastatin has potential usefulness for osteoporosis and pathological bone resorption.

Keywords: bone resorption; mitogen-activated protein kinases; osteoclast; RANK ligand; reactive oxygen species; simvastatin

Introduction

Osteoclasts are known to participate in bone remodeling by removing the bone's mineralized matrix resulting in bone resorption. It has been well documented that excessive osteoclast formation is the cause of pathological bone diseases such as rheumatoid arthritis and osteoporosis (Boyle *et al*., 2003). Osteoclast differentiation of precursors can be induced by the presence of both receptor activator of NF-κB ligand (RANKL), a key factor which controls function and survival of mature osteoclasts (Yasuda *et al*., 1998; Suda *et al*., 1999; Teitelbaum and Ross, 2003) and macrophage-colony stimulating factor (M-CSF), which is produced by osteoblasts or T cells (Lacey *et al*., 1998; Fata *et al*., 2000; Teitelbaum, 2000). It is well known that the binding of RANKL to its receptor, receptor activator of nuclear factor κB (RANK), begins the induction of TNF receptor-associated factors (TRAFs) and activates multiple downstream signaling pathways such as NF-κB, protein kinases B (AKT), c-JUN n-terminal kinases (JNK), p38 MAP kinases (p38) and extracellular signal-regulated kinase (ERK) (Lee *et al*., 2001; Lee *et al*., 2003; Kwak *et al*., 2008).

Various growth factors and cytokines such as TNF-α have been shown to bind to receptors and generate reactive oxygen species (ROS) thus increasing intracellular ROS levels in various cells (Thannickal and Fanburg, 2000). At high concentrations, ROS oxidation can result in the inflammatory

response, aging, apoptosis, and cancer. However, low levels of ROS may play a role as a secondary messenger in various signaling pathways (Sundaresan *et al*., 1995; Bae *et al*., 1997; Forman *et al*., 2004). Osteoclast lineages are very susceptible to oxidative stress, as osteoclasts are produced by increased-generation of intracellular ROS (Garrett *et al*., 1990; Steinbeck *et al*., 1994) and osteoclasts are activated by ROS (Garrett *et al*., 1990; Fraser *et al*., 1996). RANKL-induced intracellular ROS production serves to regulate the RANKL signaling pathways required for osteoclast differentiation and act as an upstream component of signaling pathways that mediate osteoclast activation and survival (Ha *et al*., 2004; Lee *et al*., 2005). Such signaling pathways also induce bone resorption by mature osteoclasts (Armstrong *et al*., 2002).

Simvastatin is one of the lipid-lowering drugs used to inhibit 3-hydroxy-3 methylglutaryl coenzyme A (HMG Co-A) reductase (Jamal *et al*., 2004). Recently, it has been reported that simvastatin also has an effect on bone metabolism. Simvastatin has been found to increase bone formation (Mundy *et al*., 1999; Staal *et al*., 2003; Jadhav and Jain, 2006) by promoting osteoblast differentiation and mineralization (Maeda *et al*., 2001). Simvastatin may also inhibit the RANKL-induced NF-κB activation pathway that leads to suppression of osteoclastogenesis (Ahn *et al*., 2008) and inhibits induction of matrix metalloproteinase-9 which is related to bone resorption (Kim *et al*., 2009). However, the exact signal mechanism of simvastatin in osteoclasts which leads to decreased activation has yet to be elucidated. Simvastatin has also been shown to be a potent antioxidant *in vitro* (Giroux *et al*., 1993; Girona *et al*., 1999; Mason, 1999) and *in vivo* (Tomás *et al*., 2000; Davignon *et al*., 2004).

In this study, we investigated the effect of simvastatin on osteoclast differentiation and we found that simvastatin acts as a RANKL-mediated osteoclastogenesis inhibitor. This inhibitory effect appears to be due to suppression of ROS generation and simvastatin also blocked RANKL-induced signaling pathways (MAPKs, AKT and IκBα) which were required for osteoclast differentiation. It was showed that simvastatin inhibits osteoclast differentiation and osteoclast activity by decreasing the generation of intracellular ROS.

Results

Simvastatin inhibited RANKL-induced osteoclast differentiation

As a control, RANKL-induced osteoclast differentiation

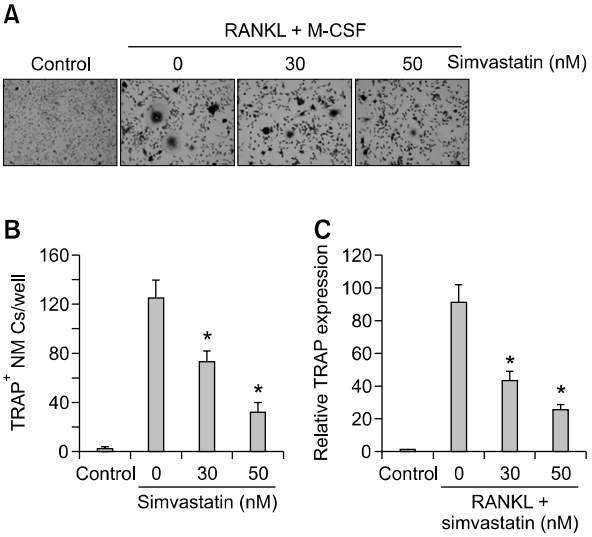

Figure 1. Inhibitory effect of simvastatin on osteoclast differentiation from RAW 264.7 cell-induced by RANKL. RAW 264.7 cells were cultured with the indicated dose of simvastatin osteoclastogenesis for 7 days. Photographs of TRAP-stained osteoclasts generated from RAW 264.7 cells (A). Simvastatin reduced TRAP+ multinuclear osteoclast formation from RAW 264.7 cells (B). TRAP down-regulation of RANKL-induced gene expression with simvastatin in real-time PCR (C). *$P < 0.01$, significantly different from without simvastatin.

was tested in order to evaluate the effect of simvastatin on RANKL-induced osteoclast differentiation. RAW 264.7 cells were allowed to differentiate to osteoclast in the presence of RANKL and M-CSF for 7 days. Upon TRAP staining, the number of TRAP stained cells, indicating degree of osteoclast differentiation, increased in a time-dependent manner for 7 days. This is consistent with results from RT-PCR and real-time PCR analysis which showed a time-dependent increase of TRAP gene expression. In order to ensure that the added simvastatin would not have a cytotoxic effect, an MTT assay was conducted. The MTT assay showed no cytotoxic effect of simvastatin on cell within the used range in this study (< 50 nM, data not shown). After verifying the validity of culture condition for osteoclast differentiation, simvastatin was added to RAW 264.7 cells at varying concentrations during RANKL-mediated osteoclast differentiation. As shown in Figure 1A, simvastatin inhibited RANKL-induced osteoclast differentiation. This is shown by a dose-dependent decrease in the formation of TRAP positive cells and simvastatin inhibited TRAP+ MNC generation by 73.7 ± 8.1 and 32.3 ± 8.0 at 30 and 50 nM concentrations, respectively (Figure 1B). Also, there were by a dose-dependent down-regulation of TRAP gene expression in RT-PCR (data not shown) and

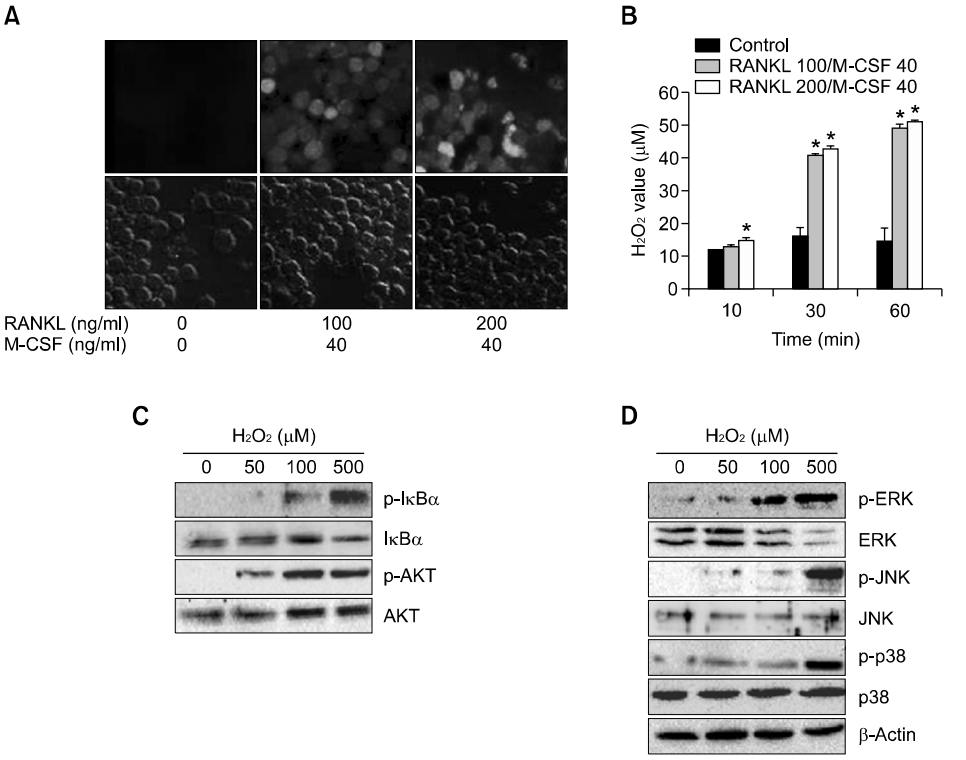

Figure 2. Production of ROS following RANKL stimulation and effects of H_2O_2 on p-IκBα, p-AKT, p-MAPKs signals activation in RAW 264.7 cells. Cells were stimulated with RANKL/M-CSF and Intracellular ROS production was detected by CM-DCF fluorescence (A). The relative level of H_2O_2 to produce this process was quantified the Amplex Red reagent (B). The phosphorylation of IκBα and AKT activation was induced in a dose-dependent manner by exogenous treatment with H_2O_2 (C) and MAPK signals activation also was detected in H_2O_2 treated cells (D). *$P < 0.01$, significantly different from control.

real-time PCR analysis (Figure 1C).

Intracellular ROS generation upon RANKL-stimulated osteoclast differentiation and related signal pathways

To investigate whether ROS production was induced during RANKL-stimulated osteoclast differentiation, intracellular ROS levels were analyzed with a cell-permeable, oxidation-sensitive dye DCFH-DA. ROS oxidation of this dye was detected by using laser-scanning confocal microscopy. RANKL and M-CSF stimulation was showed an increase in the intensity of DCF fluorescence indicating oxidation by H_2O_2 in RAW 264.7 cells (Figure 2A). This dose-dependent ROS production was also indicated by quantification analysis with Amplex Red reagent which reacted with H_2O_2 to produce a red-fluorescent oxidation product (Figure 2B).

After verifying ROS generation within cells during RANKL-stimulated osteoclast differentiation, the relationship between H_2O_2 and various downstream signaling pathways of osteoclast differentiation such as IκBα, AKT, JNK, p38 and ERK were evaluated without RANKL stimulation. In western blot analysis, IκBα and AKT activation was induced in a dose-dependent manner by exogenous treatment with H_2O_2 (Figure 2C). The phosphorylation of IκBα by degradation of IκBα and AKT signal activity were all observed to reach their maximum levels when cells were exposed to a dose of 500 uM H_2O_2. Additionally, MAPK signals activity was detected in H_2O_2 treated cells (Figure 2D), and the phosphorylation of ERK, JNK, and p38 increased significantly with exposure to H_2O_2 in a dose-dependent manner.

Simvastatin's antioxidant activity and ROS inhibition effects in osteoclast precursors

To assess the antioxidant effect of simvastatin, we measured the scavenging activity of simvastatin against exogenous H_2O_2 at different concentrations with Amplex Red reagent. As shown in Figure 3A, content of the exogenous H_2O_2 45 μM concentrations significantly decreased when 50 nM of simvastatin was added. However, simvastatin had little antioxidant effect on H_2O_2 when used at a concentration of 30 μM. This indicates a dose-dependent antioxidant effect of simvastatin against H_2O_2 at concentrations of 45 μM or higher. Therefore, our results confirm that simvastatin acts as an antioxidant above some H_2O_2 concentrations.

After verifying the effect of simvastatin against exogenous H_2O_2, the ability of simvastatin to scavenge intracellular ROS was investigated on cells undergoing RANKL-stimulated osteoclast differentiation. There was a marked decrease in

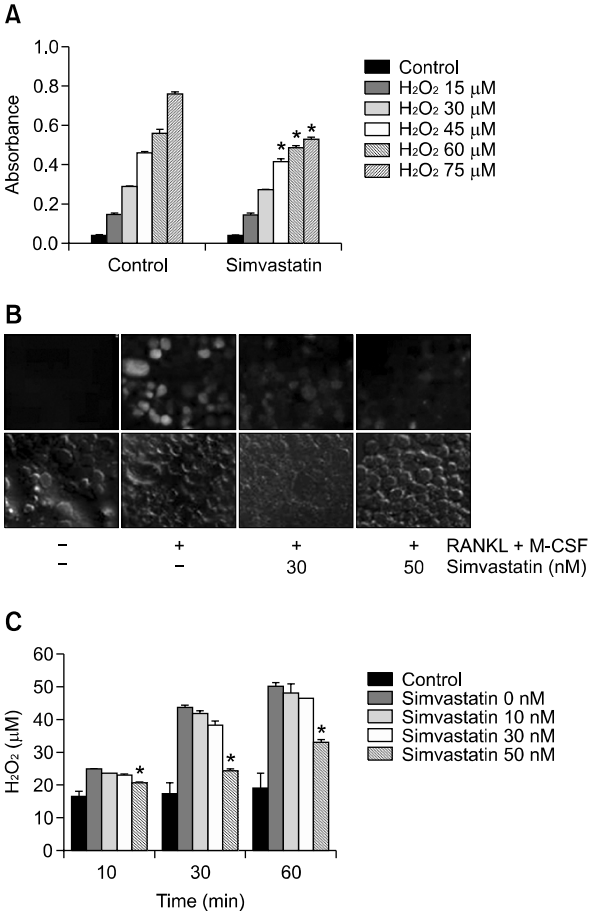

Figure 3. Inhibition of ROS by simvastatin following exogenous H_2O_2 stimulation and RANKL stimulation in osteoclast differentiation. The amount of H_2O_2 was measured by scavenging activity of simvastatin against exogenous H_2O_2 at different concentrations with Amplex Red reagent (A). The cells were pretreated with simvastatin for 30 min, and then stimulated with RANKL/M-CSF for 60 min. Intracellular ROS production was detected by CM-DCF fluorescence (B). The relative level of H_2O_2 to produce was quantified the Amplex Red reagent (C). *$P < 0.01$, significantly different from control.

the intensity of DCF fluorescence, a ROS indicator, within these cells. This was found to occur in a dose-dependent manner in simvastatin treated cell cultures during RANKL-induced osteoclast differentiation (Figure 3B). Additionally, an Amplex Red hydrogen peroxide Assay was performed in order to quantify the inhibitory effect of simvastatin on ROS generation during RANKL-induced osteoclast differentiation (Figure 3C). As opposed to the gradual increase of H_2O_2 amount within RANKL-stimulated cells for 60 min, the amount of H_2O_2 decreased significantly in a dose-dependent manner during RANKL-induced osteoclast differentiation when the cells were treated with simvastatin. The highest inhibitory effect of simvastatin on ROS generation was observed at a dose of 50 nM simvastatin.

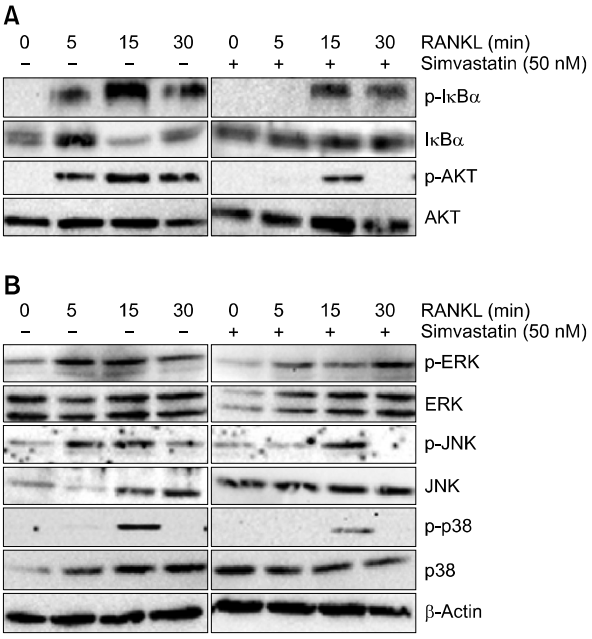

Figure 4. Effects of simvastatin on RANKL-induced p-IκBα, p-AKT, MAPKs activation in osteoclasts. RAW 264.7 cells were pretreated with simvastatin for 30 min, and then stimulated with RANKL for the indicated times. Whole cell lysates were immunoblotted to western blot analysis for phosphorylation of IκBα and AKT signals proteins (A) and MAPKs signals proteins such ERK, JNK and p38 (B) as indicated.

Simvastatin's impact on downstream ROS signaling molecules and phenotypic cellular changes

In order to elucidate the underlying mechanism of simvastatin's inhibitory effect on osteoclast differentiation through ROS degeneration, we investigated the activation/inactivation of multiple downstream signaling molecules such as MAPKs, AKT and IκBα. These molecules are known to participate in osteoclast differentiation pathways during RANKL-stimulated osteoclast differentiation. RAW 264.7 cells were stimulated with RANKL in either the presence (50 nM concentration) or absence of simvastatin. Each signaling activity was detected with specific phospho-antibodies of the respective molecule's active form. As shown in Figure 4A, the highest phosphorylation of IκBα was detected after 15 min of RANKL treatment. However, co-incubation with simvastatin reduced such RANKL-induced phosphorylation of IκBα. In addition, RANKL-induced AKT activation was also blocked by simvastatin. Similarly, RANKL-induced activations of ERK, JNK and p38 were detectable within 15 min, but simvastatin reduced these RANKL-induced MAPKs signal activities (Figure 4B).

Discussion

Bone resorption is a process of bone remodeling which is known to be mediated by osteoclasts, which result in bone loss by eliminating the bone's mineralized matrix. It has been well reported that excessive bone resorption can be related to bone loss diseases such as osteoporosis and rheumatoid arthritis (Gough *et al*., 1994). To date, various drugs have been developed and applied to treat such bone loss related diseases. Simvastatin is known to be one of these drugs used to suppress bone resorption by inhibiting osteoclastogenesis (Yamashita *et al*., 2010). However, the underlying biological mechanism of simvastatin's inhibitory effect on osteoclast differentiation has yet to be elucidated. Therefore, we studied the inhibitory effect of simvastatin on osteoclast differentiation and its underlying mechanism.

In this study, simvastatin-mediated inhibition of RANKL-induced osteoclast differentiation was proven. This was shown by a dose-dependent decrease of the formation of TRAP positive cells, as well as a dose-dependent down-regulation of TRAP gene expression. Simvastatin prevents the production of ROS and this action attenuates the H_2O_2-induced early signaling activities including AKT, JNK, p38, ERK and NF-κB signaling pathways, which subsequently suppresses osteoclast formation.

It is well known that low concentrations of ROS mediated signals are required for the activation of downstream signaling pathways (Rhee, 1999; Dröge, 2002) and the generation of ROS is controlled by the binding of various cytokines to receptors (Bonizzi *et al*., 1999). In the osteoclast differentiation pathway, RANKL binds to the RANK receptor and then stimulates intracellular ROS generation. This ROS is known to stimulate osteoclast differentiation and participate in early signaling events associated with osteoclast activation for bone resorption (Hall *et al*., 1995). Here, we show that ROS was generated within RAW 264.7 cells during RANKL/M-CSF-simulated osteoclast differentiation. This ROS production during RANKL-simulated osteoclast differentiation decreased distinctly when the RAW 264.7 cells were co-treated with simvastatin. Previous studies have reported on the inhibitory function of simvastatin on osteoclast differentiation by blocking the RANKL-induced signaling pathway (Ahn *et al*., 2008; Yamashita *et al*., 2010). It has also been reported that simvastatin has antioxidant activity (Giroux *et al*., 1993; Girona *et al*., 1999; Mason, 1999; Tomás *et al*., 2000; Davignon *et al*., 2004). From these findings, we hypothesized that simvastatin might suppress osteoclast differentiation by scavenging the generated intracellular ROS which acts as a secondary messenger in the RANKL osteoclast differentiation signaling pathway.

Previous studies have shown that simvastatin inhibited RANKL-induced NF-κB pathway activation through the suppression of IκBα phosphorylation, IκBα degradation, and IκBα kinase activity (Ahn *et al*., 2008). This study confirmed these results that simvastatin down-regulated IκBα phosphorylation and IκBα degradation both of which are known to participate in the RANKL-induced osteoclast differentiation signaling pathway. In this study, the main intracellular signal pathways engaged by RANK were also investigated in order to elucidate the underlying mechanisms of simvastatin's inhibitory effects on this pathway. It has been previously found that RANKL-induced osteoclast differentiation is mediated by RANK. RANK mainly activates NF-κB, AKT, JNK, P38 and ERK signaling pathways (Kim *et al*., 2009; Hasegawa *et al*., 2010; Lee *et al*., 2010). Other drugs have been found to suppress these RANKL-induced signaling pathways. Curcumin, a natural polyphenolic derogate extracted from turmeric, has also been found to suppress osteoclast differentiation and osteoclast function. These effects were accompanied by the inhibition of IκBα phosphorylation and NF-κB activation (Von Metzler *et al*., 2009). Luteolin has also been found to inhibit osteoclasts, however, this does not affect RANKL-induced MAPKs expression and activation. Instead, this antioxidant was found to affect the phosphorylation of ATF2, which is downstream of the p38 MAPK signaling pathway (Lee *et al*., 2009). Sauchinone also attenuates RANKL-induced NF-κB, ERK and p38 MAPKs activation in osteoclast differentiation. However, the activation of JNK by RANKL was not affected by sauchinone (Han *et al*., 2007). In our study, we also detected inhibition effects of RANKL-induced signaling pathways by simvastatin. Simvastatin inhibited not only IκBα signal but also the AKT, and MAPKs such as JNK, p38 and ERK signaling pathways which are normally activated through ROS in osteoclast differentiation. Simvastatin strongly attenuates RANKL-induced AKT and JNK signaling.

In conclusion, we analyzed the effect of osteoclast differentiation by simvastatin and identified one mechanism by which simvastatin acts as an inhibitor. These results show that simvastatin suppresses RANKL-induced osteoclastogenesis through inhibition of ROS-induced signaling pathways. This study elucidated the mechanism by which simvastatin acts as an osteoclast inhibitor by blocking ROS and help contribute to further clarification of the mechanisms underlying osteoporosis and other pathological bone-loss diseases. Also, antioxidants may have therapeutic implications for inhibiting

bone resorption.

Methods

Reagents and materials

RANKL and M-CSF were obtained from R&D Systems (Minneapolis, MN). The Leukocyte Acid Phosphatase Assay kit was purchased from Sigma-Aldrich (St. Louis, MO). The 2',7'-dichlorofluorescein diacetate (DCFH-DA) and Amplex® Red Hydrogen Peroxide/Peroxidase Assay kit were obtained from Molecular Probes (Eugene, OR). All antibodies for JNK, phospho JNK, ERK, phospho ERK, p38, phospho p38 (Thr180/Tyr182), AKT, phospho AKT, IκBα and phospho IκBα were purchased from Cell Signaling Technology (Beverly, MA). Simvastatin was obtained from Chong kun Dang pharm (Seoul, Korea).

Cell culture and differentiation into osteoclasts

RAW 264.7 (mouse macrophage) cells were obtained from the Korean Cell Lines Bank (KCLB, Seoul, Korea) and were cultured in Dulbecco's modified Eagles medium (DMEM, GIBCO, NY) supplemented with 10% fetal bovine serum (FBS, GIBCO) and 1% antibiotic-antimycotic in a humidified 5% CO_2 atmosphere. The cells were incubated in the presence of RANKL (200 ng/ml) and M-CSF (40 ng/ml) with or without varying doses of simvastatin for 7 days. The culture medium was replaced every 3 days.

Tartrate-resistant acid phosphatase (TRAP) staining

Cells were fixed by soaking in 3.7% formaldehyde for 20 min and were then placed in 0.1% triton X-100 for 10 min. Following this, the cells were stained using the Leukocyte Acid Phosphatase Assay kit following the manufacturer's instruction. TRAP-positive multinucleated cells containing three or more nuclei were considered to be osteoclasts.

RT-PCR and real-time quantitative PCR

Total RNA was purified using an RNeasy Plus Mini Kit (Qiagen, CA) and cDNA was synthesized from 1 μg of total RNA using *AccuPower*® RT PreMix (Bioneer, Daejeon, Korea). All PCR amplifications were performed using *AccuPower*® PCR PreMix (Bioneer). The primers for TRAP were 5'-ACT TCC CCA GCC CTT ACT ACC G-3' (sense) and 5'-TCA GCA CAT AGC CCA CAC CG-3' (antisense), and the primers for GAPDH were 5'-ACT TTG TCA AGC TCA TTT CC -3' (sense) and 5'-TGC AGC GAA CTT TAT TGA TG -3' (antisense). To confirm RT-PCR results in this study, the same cDNA samples were analyzed using real-time PCR. Primers were selected using the same primers as used in RT-PCR. For relative quantitation, the reactions were tested using DyNAmo™ SYBR® Green qPCR Kit (Finnzymes, Espoo, Finland) and PCR processing was carried out in an iCycler (Bio-Rad, Hercules, CA). The copy number of the target gene was normalized to an endogenous reference, GAPDH. The fold change from normal samples was set at 1-fold and the ratio of the normalized fold change was calculated.

Reactive oxygen species (ROS) assay

Intracellular ROS images were measured using the fluorescence of DCF, which is the oxidized product of 2',7'-dichlorofluorescein diacetate (DCFH) in stained cells. Pre-osteoclasts were stimulated with only RANKL and M-CSF or with these signal molecules combined with simvastatin. Afterwards, the cells were washed in Hank's balanced salt solution (HBSS) and then incubated in the dark for 30 min with 10 μM DCFH-DA. The fluorescence of DCF was detected by confocal microscopy. Also, the amount of H_2O_2 in each lysate was measured by using an Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes). In the presence of peroxidase, the Amplex Red reagent reacted with H_2O_2 to produce a red-fluorescent oxidation product and this process was performed per manufacturer's instruction.

Western blotting

Cells were lysed by the addition of cold RIPA lysis buffer containing 0.5 M Tris-HCl, pH 7.4, 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 mM EDTA along with protease and phosphatase inhibitors. An equal amount of protein (30 μg) was subjected to SDS-PAGE and transferred to a nitrocellulose transfer membrane. After blocking with 5% skim milk, the membrane was probed with anti-phospho JNK, phospho ERK, phospho p38, phospho AKT, phospho IκBα followed by incubation with an appropriate secondary antibody conjugated to horseradish peroxidase. The membranes were then stripped and reprobed with anti-JNK, ERK, p38, AKT, IκBα and β-actin. Signals were detected using a ChemiDoc XRS System (Bio-Rad).

Statistical analysis

Each experiment was performed at least three times. All quantitative data are expressed as mean ± S.D. Statistical differences were analyzed by Student's *t*-test (*$P < 0.01$).

Acknowledgements

This Research was supported by the Program of Kyung Hee University for the Young Researcher of Medical Science in 2007 (KHU-20071500).

References

Ahn KS, Sethi G, Chaturvedi MM, Aggarwal BB. Simvastatin, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, suppresses osteoclastogenesis induced by receptor activator of nuclear factor-κB ligand through modulation of NF-κB pathway. Int J Cancer 2008;123:1733-40

Armstrong AP, Tometsko ME, Glaccum M, Sutherland CL, Cosman D, Dougall WC. A RANK/TRAF6-dependent signal transduction pathway is essential for osteoclast cytoskeletal organization and resorptive function. J Biol Chem 2002;277:

44347-56

Bae YS, Kang SW, Seo MS, Baines IC, Tekle E, Chock PB, *et al*. Epidermal growth factor (EGF)-induced generation of hydrogen peroxide. Role in EGF receptor-mediated tyrosine phosphorylation. J Biol Chem 1997;272:217-21

Bonizzi G, Piette J, Schoonbroodt S, Greimers R, Havard L, Merville MP, *et al*. Reactive oxygen intermediate-dependent NF-κB activation by interleukin-1β requires 5-lipoxygenase or NADPH oxidase activity. Mol Cell Biol 1999;19:1950-60

Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. Nature 2003;423:337-42

Davignon J, Jacob RF, Mason RP. The antioxidant effects of statins. Coron Artery Dis 2004;15:251-8

Dröge W. Free radicals in the physiological control of cell function. Physiol Rev 2002;82:47-95

Fata JE, Kong YY, Li J, Sasaki T, Irie-Sasaki J, Moorehead RA, *et al*. The osteoclast differentiation factor osteoprotegerin-ligand is essential for mammary gland development. Cell 2000;103:41-50

Forman HJ, Fukuto JM, Torres M. Redox signaling: thiol chemistry defines which reactive oxygen and nitrogen species can act as second messengers. Am J Physiol Cell Physiol 2004;287:246-56

Fraser JH, Helfrich MH, Wallace HM, Ralston SH. Hydrogen peroxide, but not superoxide, stimulates bone resorption in mouse calvariae. Bone 1996;19:223-6

Garrett IR, Boyce BF, Oreffo RO, Bonewald L, Poser J, Mundy GR. Oxygen-derived free radicals stimulate osteoclastic bone resorption in rodent bone *in vitro* and *in vivo*. J Clin Invest 1990;85:632-9

Girona J, La Ville AE, Solà R, Plana N, Masana L. Simvastatin decreasesaldehyde production derived from lipoprotein oxidation. Am J Cardiol 1999;83:846-51

Giroux LM, Davignon J, Naruszewicz M. Simvastatin inhibits the oxidation of low-density lipoproteins by activated human monocyte-derived macrophages. Biochim Biophys Acta 1993;1165:335-8

Gough AK, Lilley J, Eyre S, Holder RL, Emery P. Generalised bone loss in patients with early rheumatoid arthritis. Lancet 1994;344:23-7

Ha H, Kwak HB, Lee SW, Jin HM, Kim HM, Kim HH, Lee ZH. Reactive oxygen species mediate RANK signaling in osteoclasts. Exp Cell Res 2004;301:119-27

Hall TJ, Schaeublin M, Jeker H, Fuller K, Chambers TJ. The role of reactive oxygen intermediates in osteoclastic bone resorption. Biochem Biophys Res Commun 1995;207:280-7

Han KY, Yang D, Chang EJ, Lee Y, Huang H, Sung SH, *et al*. Inhibition of osteoclast differentiation and bone resorption by sauchinone. Biochem Pharmacol 2007;74:911-23

Hasegawa S, Yonezawa T, Ahn JY, Cha BY, Teruya T, Takami M, *et al*. Honokiol inhibits osteoclast differentiation and function *in vitro*. Biol Pharm Bull 2010;33:487-92

Jadhav SB, Jain GK. Statins and osteoporosis: new role for old drugs. J Pharm Pharmacol 2006;58:3-18

Jamal SM, Eisenberg MJ, Christopoulos S. Christopoulos, rhabdomyolysis associated with hydroxymethylglutaryl-coenzyme A reductase inhibitors. Am Heart J 2004;147; 956-65

Kim HJ, Chang EJ, Kim HM, Lee SB, Kim HD, Su Kim G, Kim HH. Antioxidant α-lipoic acid inhibits osteoclast differentiation by reducing nuclear factor-κB DNA binding and prevents *in vivo* bone resorption induced by receptor activator of nuclear factor-κB ligand and tumor necrosis factor-α. Free Radic Biol Med 2006;40:1483-93

Kim SE, Thanh Thuy TT, Lee JH, Ro JY, Bae YA, Kong Y, *et al*. Simvastatin inhibits induction of matrix metalloproteinase-9 in rat alveolar macrophages exposed to cigarette smoke extract. Exp Mol Med 2009;41:277-87

Kwak HB, Sun HM, Ha H, Lee JH, Kim HN, Lee ZH. AG490, a Jak2-specific inhibitor, induces osteoclast survival by activating the Akt and ERK signaling pathways. Mol Cells 2008;26:436-42

Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, *et al*. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 1998;93:165-76

Lee JH, Jin H, Shim HE, Kim HN, Ha H, Lee ZH. Epigallocatechin-3-gallate inhibits osteoclastogenesis by down-regulating c-Fos expression and suppressing the nuclear factor-κB signal. Mol Pharmacol 2010;77:17-25

Lee JW, Ahn JY, Hasegawa S, Cha BY, Yonezawa T, Nagai K, *et al*. Inhibitory effect of luteolin on osteoclast differentiation and function. Cytotechnology 2009;61:125-34

Lee NK, Choi YG, Baik JY, Han SY, Jeong DW, Bae YS, Kim N, Lee SY. A crucial role for reactive oxygen species in RANKL-induced osteoclast differentiation. Blood 2005;106: 852-9

Lee SE, Chung WJ, Kwak HB, Chung CH, Kwack KB, Lee ZH, *et al*. Tumor necrosis factor-α supports the survival of osteoclasts through the activation of Akt and ERK. J Biol Chem 2001;276:49343-9

Lee ZH, Kim HH. Signal transduction by receptor activator of nuclear factor κ B in osteoclasts. Biochem Biophys Res Comm 2003;305:211-4

Maeda T, Matsunuma A, Kawane T, Horiuchi N. Simvastatin promotes osteoblast differentiation and mineralization in MC3T3-E1 cells. Biochem Biophys Res Commun 2001;280: 874-7

Mason RP. Direct antioxidant effect of atorvastatin and an active metabolite: a comparative analysis. Circulation 1999;100:I-541

Mundy G, Garrett R, Harris S, Chan J, Chen D, Rossini G, *et al*. Stimulation of bone formation *in vitro* and in rodents by statins. Science 1999;286:1946-9

Rhee SG. Redox signaling: hydrogen peroxide as intracellular messenger. Exp Mol Med 1999;31:53-9

Staal A, Frith JC, French MH, Swartz J, Güngör T, Harrity TW, *et al*. The ability of statins to inhibit bone resorption is directly related to their inhibitory effect on HMG-CoA reductase

activity. J Bone Miner Res 2003;18:88-96

Steinbeck MJ, Appel WH Jr, Verhoeven AJ, Karnovsky MJ. NADPH-oxidase expression and in situ production of superoxide by osteoclasts actively resorbing bone. J Cell Biol 1994;126:765-72

Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ. Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. Endocr Rev 1999;20:345-57

Sundaresan M, Yu ZX, Ferrans VJ, Irani K, Finkel T. Requirement for generation of H2O2 for platelet-derived growth factor signal transduction. Science 1995;270:296-9

Teitelbaum SL, Ross FP. Genetic regulation of osteoclast development and function, Nat Rev Genet 2003;4:638-49

Teitelbaum SL. Bone resorption by osteoclasts. Science 2000;289:1504-8

Thannickal VJ, Fanburg BL. Reactive oxygen species in cell signaling. Am J Physiol Lung Cell Mol Physiol 2000;279: L1005-28

Tomás M, Sentí M, García-Faria F, Vila J, Torrents A, Covas M, Marrugat J. Effect of simvastatin therapy on paraoxonase activity and related lipoproteins in familial hypercholesterolemic patients. Arterioscler Thromb Vasc Biol 2000;20:2113-9

Von Metzler I, Krebbel H, Kuckelkorn U, Heider U, Jakob C, Kaiser M, *et al*. Curcumin diminishes human osteoclastogenesis by inhibition of the signalosome-associated I κB kinase. J Cancer Res Clin Oncol 2009;135:173-9

Yamashita M, Otsuka F, Mukai T, Yamanaka R, Otani H, Matsumoto Y, *et al*. Simvastatin inhibits osteoclast differentiation induced by bone morphogenetic protein-2 and RANKL through regulating MAPK, AKT and Src signaling. Regul Pept 2010;162:99-108

Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, *et al*. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Natl Acad Sci USA 1998;95:3597-602