academicJournals

Vol. 5(7), pp. 348-355, July 2013
DOI: 10.5897/IJMMS2013.0932
ISSN 2006-9723 ©2013 Academic Journals
http://www.academicjournals.org/IJMMS

International Journal of Medicine and Medical Sciences

Full Length Research Paper

Anti-osteoclastogenic activity of butanol fraction of rice bran extract via downregulation of MAP kinase activity and c-Fos/NFATc1 expression

Jungsun Moon[1#], Seong-Hee Moon[2#], Sik-Won Choi[2], Sookyeon Lee[3], Seong Hwan Kim[2*] and Dongsool Yim[1]

[1]College of Pharmacy, Sahmyook University, Seoul 139-742, Korea.
[2]Laboratory of Translational Therapeutics, Pharmacology Research Center, Bio-Organic Science Division, Korea Research Institute of Chemical Technology, Daejeon 305-600, Korea.
[3]Slowfood Research Institute, Slowfood Culture Center, Namyangjoo 472-872, Korea.

Accepted 31 May, 2013

Osteoclasts are responsible for bone metabolic diseases including osteoporosis, rheumatoid arthritis, multiple myeloma and peritonitis. Recently, the anti-osteoporotic activities of natural product extracts have become the subject of research interest. Rice bran (RB) extracts exhibits anti-inflammatory activity and ameliorates anti-oxidative stress. However, the effects of RB extracts on osteoclastogenesis-related diseases such as osteoporosis have not been investigated. Here, we investigated the effects of RB extracts and of its fractions on RANKL-induced osteoclast differentiation. Interestingly, the butanol extracts of RB (RB-BuOH) dose-dependently inhibited osteoclast differentiation by down-regulating the RANKL-induced activations of mitogen-activated protein (MAP) kinases. Moreover, the mRNA expression of osteoclast-mediating molecules such as c-Fos, NFATc1, DC-STAMP and cathepsin K were dose-dependently attenuated by RB-BuOH during osteoclast differentiation. Furthermore, RB-BuOH depressed the protein levels of NFATc1 and its promoter activity. The findings of this study show that RB-BuOH and its components might prevent osteoclast-related bone loss.

Key words: Rice bran, osteoclast differentiation, NFATc1, mitogen-activated protein (MAP) kinase.

INTRODUCTION

A tight balance between osteoclast-mediated bone resorption (or destruction) and osteoblast-mediated bone formation is required to maintain bone homeostasis. However, an imbalance induced by increasing osteoclastic bone resorptive activity or decreasing osteoblastic bone-forming activity can lead to a variety of bone metabolic diseases, such as osteoporosis (Boyle et al., 2003). Therefore, two strategies are adopted to reduce the incidence of osteoporosis; that is, the reduction of bone resorption using anti-resorptive agents, like bisphosphonates, and the induction of bone formation using anabolic agents, like parathyroid hormone (PTH). Apparently, anti-resorptive agents still remain the therapeutic mainstay for osteoporosis, but the most common anti-resorptive agents, bisphosphonates, pose risks of side effects like bisphosphonate-related osteonecrosis of the jaw (Dannemann et al., 2007) and atypical femoral fracture (Meier et al., 2012). Anabolic PTH is currently available for stimulating bone formation, but its use is limited by its costs and concerns regarding its long-term safety. Thus, there is an urgent need for new anti-osteoporotic agents that are both effective and. safe for long-term

*Corresponding author. E-mail: hwan@krict.re.kr. Tel: +82-42-860-7687. Fax: +82-42-861-4246.

#Authors contributed equally to this study.

management.

Rice is the most important food in Asia, including Korea, and generally white rice which has the bran removed from brown rice is usually consumed. Rice bran (RB) has long been considered an agricultural waste, but recently, it has been reported to contain various nutrients including vitamins, minerals and amino acids that could be used to prevent and treat chronic diseases. Therefore, RB extracts have been reported to exhibited anti-inflammatory, anti-oxidative, anti-diabetic, anti-mutagenic and anti-carcinogenic activities (Nam et al., 2005; Roschek et al., 2009; Norazalina et al., 2010; Rao et al., 2010; Jun et al., 2012; Kaup et al., 2012). These studies have attracted considerable research attention regarding the biological activity of RB, and its evident safety assessment has accelerated the commercial use of RB and its ingredients.

The biological activity of RB could result from components such as γ-oryzanols, tocopherols, tocotrienols and phenolic compounds which have been shown to have potential roles in disease prevention and treatment (Henderson et al., 2012; Verschoyle et al., 2007; Flight and Clifton, 2006). Among the bioactive components in RB, several substances such as ferulic acid, hydroxycinnamic acid, tocopherols and tocotrienols have been shown to inhibit osteoclastogenesis or prevent bone loss (Sassa et al., 2003; Lai and Yamaguchi, 2007; Ha et al., 2011). However, the effect of RB extracts on osteoclast differentiation has not been previously studied. Therefore, in this study, we investigated the effects of RB extracts on osteoclast differentiation and sought to determine the molecular mechanism responsible for its action.

MATERIALS AND METHODS

Preparation of rice bran extracts

Rice bran from grain rice (*Orysa sativa* L., Japanica) obtained by milling rice cultivated in Pocheon, Kyunggi Province, Korea, was used in this study. Totally, 8 kg of rice bran was extracted with 90% (v/v) hot ethanol (EtOH) three times for 4 h. The extract was filtered and concentrated in a vacuum to obtain the EtOH extract (yield 1,787 g). The concentrated extract was suspended in water and partitioned sequentially with *n*-hexane (Hx; yield 1,049 g), ethyl acetate (EA; yield 8 g) and *n*-butanol (BuOH; yield 20 g) to afford three fractions and an aqueous residue. These fractions were evaporated to dryness in a rotary vacuum evaporator (Büchi, Swiss). The dried extracts were dissolved in dimethyl sulfoxide (DMSO) to the final concentration of 200 mg/ml. Further dilution was done by phosphate buffered saline (PBS).

Osteoclast differentiation

Bone marrow cells were obtained from 5 to 8-week-old male imprinting control region (ICR) mice by flushing femurs and tibias with α-minimum essential medium (MEM) supplemented with antibiotics (100 units/ml penicillin, 100 µg/ml streptomycin; Hyclone, UT). Cells were cultured for 1 day in 10 cm culture dishes in α-MEM containing 10% fetal bovine serum (FBS; Gibco, Paisley, UK), antibiotics, and macrophage colony stimulating factor (M-CSF; 10 ng/ml; Peprotech, NJ). Non-adherent bone marrow cells were plated on 9 cm petri dishes and cultured for 3 days in the presence of M-CSF (30 ng/ml). After removing non-adherent cells by washing, remaining adherent cells were bone marrow-derived macrophages (BMMs). BMMs were induced to differentiate into osteoclasts by culture (1×10^4 cells/well in a 96-well plate or 3×10^5 cells/well in a 6-well plate) for 4 days in the presence of M-CSF (30 ng/ml) and receptor activator of nuclear factor kappa-B ligand (RANKL; 5 ng/ml; R&D Systems, MN).

Tartrate-resistant acid phosphatase (TRAP) staining

To visualize mature osteoclasts, we stained cells with label tartrate-resistant acid phosphatase (TRAP), a biomarker of osteoclast differentiation. Cells were then fixed with 10% formalin for 10 min and 0.1% Triton X-100 for 10 min, and stained with the leukocyte acid phosphatase kit 387-A (Sigma, MO). Images were captured with a microscope equipped with the DP controller (Olympus Optical, Tokyo).

Cell viability assay

BMMs were suspended in α-MEM containing 10% FBS and plated in a 96-well plate at a density 1×10^4 cells/well. Cells were treated with various concentrations of RB-BuOH in the presence of M-CSF (30 ng/ml) and incubated for 1 or 3 days. Cell viability was then measured with the cell counting Kit-8 (Dojindo Moleculer Technologies, MD) according to the manufacturer's protocol. Measured absorbances were converted to cell numbers using a standard curve.

Western blot analysis

Briefly, cells were homogenized and centrifuged at 10,000 × *g* for 15 min. Supernatants were collected for the isolation of cytoplasmic proteins. Denatured proteins were separated on the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, CA). After incubation with antibody, the membranes were developed using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce), with the LAS-3000 luminescent image analyzer (Fuji Photo Film Co., Ltd., Japan). All antibodies used in this study were purchased from Santa Cruz (CA).

Real-time PCR

Primers were chosen using the on-line primer 3 design program (Rozen and Skaletsky, 2000), and are listed in Table 1. Total RNA was isolated using TRIzol reagent, according to the manufacturer's instructions. First strand cDNA was synthesized using the Omniscript RT kit (Qiagen, CA) and 1 µg of total RNA, 1 µM of oligo-dT_{18} primer, 10 units of RNasin (RNase inhibitor; Promega, WI), according to the manufacturer's instructions. Quantitative polymerase chain reaction (PCR) (QPCR) was performed using the Stratagene Mx3000P Real-Time PCR system and Brilliant SYBR Green Master Mix (Stratagene, CA). Briefly, first-strand cDNA was diluted 1:10, and primers (10 pmol) were added according to the manufacturer's instructions. The thermocycling protocol consisted of three parts. The first part involved incubation at 95°C for 10 min to activate the polymerase; the second involved 40 amplification cycles of 94°C for 40 s (denaturation), 53°C for 40 s (annealing), and 72°C for 1 min (extension); and the third incubation at 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s to generate PCR product temperature-dissociation curves (also called 'melting curves'). All reactions were run in triplicate, and data were analyzed using the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001). Glyceraldehyde-3-

Table 1. Primer sequences used in this study.

Target gene	Forward (5'-3')	Reverse (5'-3')
c-Fos	CCAGTCAAGAGCATCAGCAA	AAGTAGTGCAGCCCGGAGTA
NFATc1	GGGTCAGTGTGACCGAAGAT	GGAAGTCAGAAGTGGGTGGA
Cathepsin K	GGCCAACTCAAGAAGAAAAC	GTGCTTGCTTCCCTTCTGG
DC-STAMP	CCAAGGAGTCGTCCATGATT	GGCTGCTTTGATCGTTTCTC
GAPDH	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA

phosphate dehydrogenase (GAPDH) was used as an internal standard. Statistical significances were determined using the Student's *t*-test using GAPDH-normalized $2^{-\Delta\Delta C}{}_{T}$ values.

Luciferase activity assay

Human embryonic kidney 293T cells were plated in a 24-well plate, and then transfected with different amounts of the following reporter plasmids: (1) the DNA-binding sequence of the nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) fused to the firefly-luciferase sequence and, (2) the sequence of receptor activator of nuclear factor κB (RANK) fused to the renilla-luciferase sequence in pGL4 vector. After 48 h, transfected cells were lysed with lysis buffer (Promega, Madison, WI, USA), and luciferase activity was measured using the dual-luciferase assay system (Promega). Luciferase activity was normalized versus renilla luciferase activity in each sample.

Statistical analysis

All experiments were performed in triplicates and quantitative values are presented as mean ± standard deviation (SD). The significances of differences were determined using the Student's *t*-test. Statistical significance was accepted for p values < 0.05.

RESULTS

RB-BuOH inhibits RANKL-induced osteoclast differentiation

Rice bran ethanol extract (RB-EtOH) was further fractionated to obtain hexane (RB-Hx), ethyl acetate (RB-EA) and butanol (RB-BuOH) fractions. The anti-osteoclastogenic effects of all four extracts were evaluated during the RANKL-induced osteoclast differentiation of primary mouse BMMs. At 50 μg/ml, RB-EtOH and RB-Hx did not exhibit anti-osteoclastogenic activity, but RB-BuOH did inhibit the formation of TRAP-positive multinucleated osteoclasts (Figure 1A). RB-EA was cytotoxic to BMMs. RB-BuOH significantly and dose-dependently inhibited the RANKL-induced formation of TRAP-positive multinucleated osteoclasts (Figure 1B), numbers of multinucleated osteoclasts (Figure 1C) and the mRNA expression of TRAP (Figure 1D). To ascertain that the inhibitory effect of RB-BuOH on RANKL-induced osteoclast differentiation was not due to its cytotoxicity, we evaluated its effect on cell viability. At the concentrations used in this study, RB-BuOH did not have any cytotoxic effect on BMMs (Figure 1E).

RB-BuOH inhibits RANKL-induced MAP kinase activation

To gain insight of the mechanism by which RB-BuOH blocks osteoclast differentiation, we investigated the effect of RB-BuOH on the activities of signaling molecules involved in osteoclast differentiation. First, we considered MAP kinases because they are known to be involved in the progress (Asagiri and Takayanagi, 2007). By evaluating the effect of RB-BuOH on the activations of MAP kinases, we found that RANKL strongly induced the activations of p38, JNK, and ERK, but these activations were strongly inhibited by RB-BuOH at 40 μg/ml (Figure 2).

RB-BuOH inhibits the RANKL-induced expressions of c-Fos and NFATc1 and the expressions of their regulatory genes

The inhibitory effect of RB-BuOH on osteoclast differentiation was further explored by evaluating the expressions of the transcription factors, c-Fos and nuclear factor of activated T cells (NFATc1) which are known to regulate genes required for osteoclast differentiation. In addition, we investigated the NFATc1-regulated mRNA levels of dendrite cell-specific transmembrane protein (DC-STAMP) and cathepsin K. As shown in Figure 3, mRNA expression levels of c-Fos and NFATc1 were strongly induced by RANKL, but these inductions were significantly inhibited by RB-BuOH. The expression levels of DC-STAMP and cathepsin K were also strongly elevated by RANKL, but these inductions were also significantly inhibited by RB-BuOH.

RB-BuOH inhibits RANKL-induced NFATc1 activity

We hypothesized that the anti-osteoclastogenic activity of RB-BuOH might result from its ability to inhibit the expression and activity of NFATc1 via down regulation of MAP kinase activity. To explore this hypothesis, the effect of RB-BuOH on the protein expression and activity of NFATc1 was further evaluated in BMMs. As shown in Figure 4A, western blot analysis revealed that the RANKL-mediated induction of NFATc1 protein was inhibited by RB-BuOH (Figure 4A). Furthermore, the NFATc1-

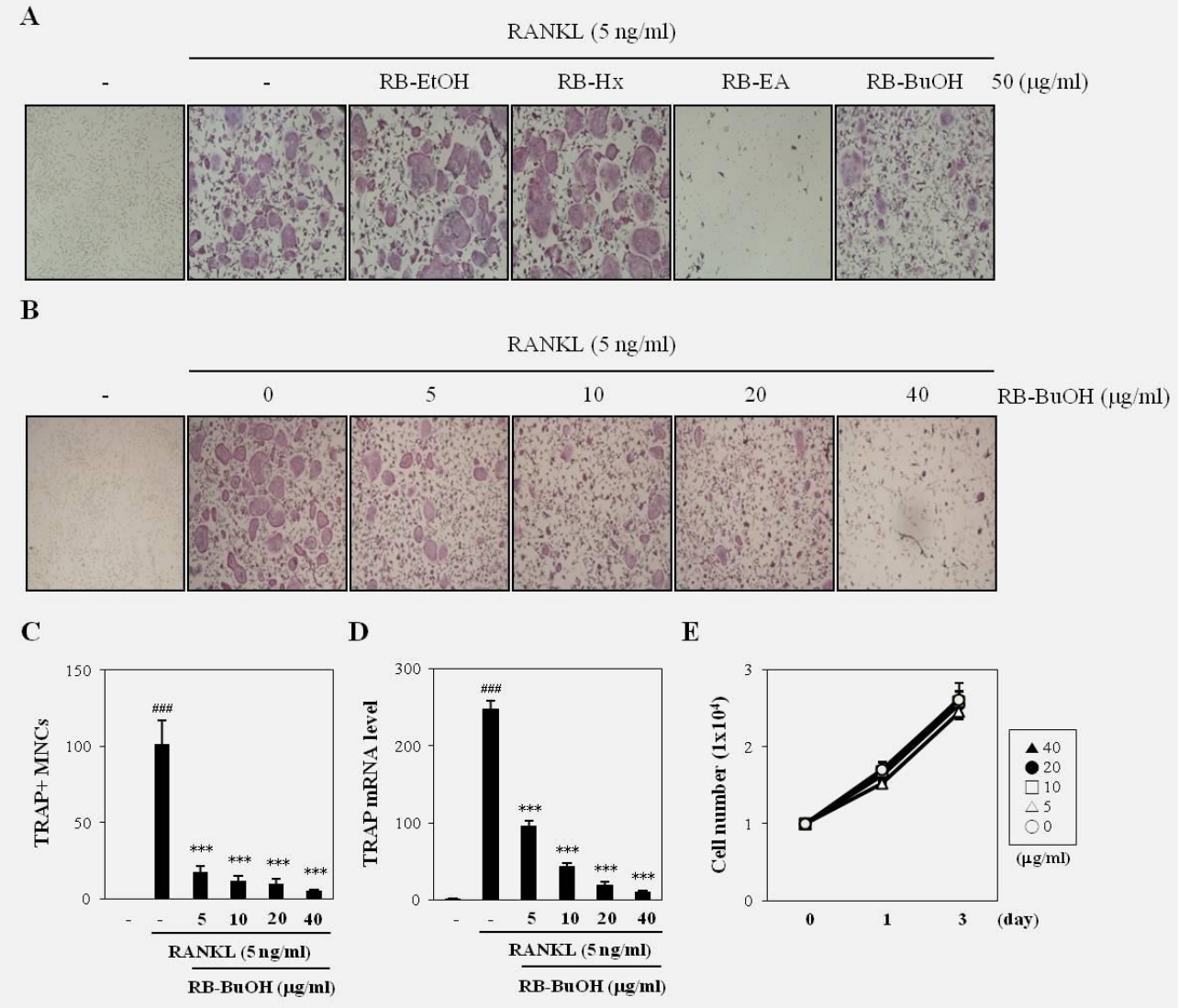

Figure 1. RB extracts inhibit RANKL-induced osteoclast differentiation. (A) BMM cells were cultured for 4 days in the presence of RANKL (5 ng/ml) and M-CSF (30 ng/ml) with RB extracts. Multinucleated osteoclasts were visualized by TRAP staining. (B) RB-BuOH dose-dependently inhibited RANKL-induced osteoclast differentiation. (C) RB-BuOH dose-dependently inhibited RANKL-induced formation of TRAP positive-multinucleated osteoclasts ($TRAP^+$MNCs). $TRAP^+$MNCs were counted. $^{###}p < 0.001$ (versus the negative control); ***$p < 0.001$ (versus the RANKL-treated group). (D) RB-BuOH suppressed RANKL-induced mRNA expression of TRAP. BMMs were treated with RANKL (5 ng/ml) and RB-BuOH for 4 days then mRNA expression level of TRAP was analyzed by the real-time PCR. $^{###}p < 0.001$ (versus the negative control); ***$p < 0.001$ (versus the RANKL-treated group). (E) The effect of RB-BuOH on the viability of BMMs was evaluated by CCK-8 assay.

luciferase reporter activity assay showed that the RANKL-mediated activation of NFATc1 was dose-dependently inhibited by RB-BuOH (Figure 4B). These results suggest that NFATc1 could be a major mediator of the anti-osteoclastogenic activity of RB-BuOH.

DISCUSSION

The incidence of osteoporotic fracture can be reduced by prescribing anti-resorptive agents or anabolic agents, but their uses are limited by their side effects, costs and by concerns regarding long-term safety (Dannemann et al., 2007; Meier et al., 2012). An alternative strategy for reducing those limitations is to find natural compounds with anti-osteoporotic potentials. Natural products have historically yielded a variety of therapeutic agents, and healthy nutrients or foods with medicinal properties are both effective and safe for the long-term management of disorders. In fact, recent studies have aimed to identify natural products or healthy foods that prevent and/or ameliorate osteoporosis with minimal adverse effects (Morabito et al., 2002).

In the present study, we found that RB-BuOH

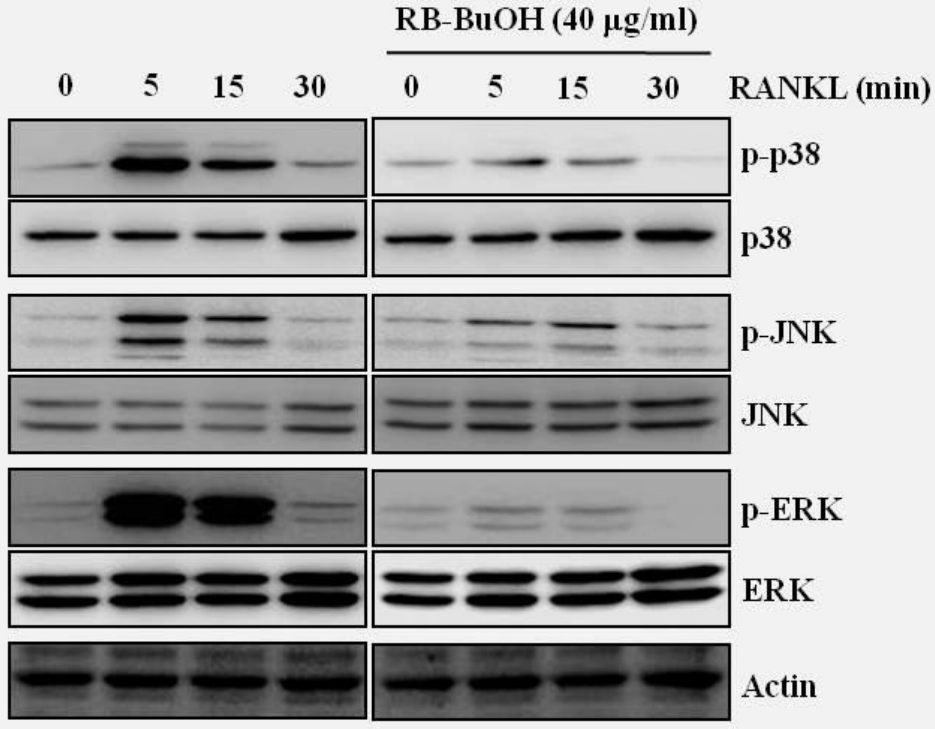

Figure 2. RB-BuOH inhibits RANKL-induced MAP kinase activity. BMMs were pretreated with or without RB-BuOH (40 μg/ml) for 1 h prior to RANKL stimulation (5 ng/ml) at the indicated times. Protein expression levels were evaluated by western blot analysis.

significantly inhibited the RANKL-induced differentiation of BMMs into osteoclasts without any cytotoxicity at the concentration used in this study. Since osteoclasts play an essential role in bone resorption, the modulations of osteoclast differentiation and functional maturation are considered a major strategy for treating bone metabolic diseases such as osteoporosis (Raisz, 2005). Osteoclast differentiation and subsequent maturation are mainly triggered by RANKL signaling, which is considered an important target for preventing pathological bone loss. Docking of RANKL to its receptor, RANK, rapidly activates MAP kinases such as p38, ERK and JNK, and these MAP kinases are essential for the differentiation, survival and activation of osteoclasts (Boyle et al., 2003; Lee and Kim, 2003). Interestingly, RB-BuOH strongly attenuated the RANKL-induced activation of MAP kinases in the present study, and others have reported that RB and its components such as γ-tocotrienol inhibit MAP kinase activations (Bi et al., 2010; Hoshino et al., 2010; Kannappan et al., 2010; Tanaka et al., 2012).

The activations of MAP kinases lead to the stimulation of transcription factors such as activator protein (AP)-1 and NFATc1 (Boyle et al., 2003; Lee and Kim, 2003). c-Fos (an AP-1 family member) is essential for osteoclast differentiation (Wang et al., 1992), and NFATc1 has been shown to rescue osteoclastogenesis in cells that lacked c-Fos (Matsuo et al., 2004). Interestingly, c-Fos is expressed during the early stages of osteoclast differentiation and regulates NFATc1 expression by binding to its promoter region (Matsuo et al., 2004; Asagiri et al., 2005). In the present study, RB-BuOH significantly attenuated the RANKL-induced mRNA expressions of c-Fos and NFATc1. In a previous study, the antioxidant α-tocotrienol in RB was shown to inhibit RANKL-induced osteoclast differentiation by suppressing c-Fos expression, and this anti-osteoclastogenic effect was reversed when c-Fos or an active form of NFATc1 was overexpressed (Xu et al., 2001; Ha et al., 2011).

After NFATc1 is expressed during the middle or late stages of osteoclast differentiation, it subsequently regulates a number of osteoclast-specific genes such as DC-STAMP and cathepsin K (Kim et al., 2008; Balkan et al., 2009). In the present study, RB-BuOH also significantly attenuated the RANKL-induced mRNA expressions of DC-STAMP and cathepsin K, probably due to the inhibition of NFATc1 mRNA expression. Thus, considering DC-STAMP is essential for osteoclast fusion (Kukita et al., 2004; Yagi et al., 2005; Asagiri and Takayanagi, 2007) and cathepsin K is a major player in osteoclastic bone resorption (Gelb et al., 1996; Ishikawa et al., 2001), RB-BuOH might have the potential to inhibit osteoclast fusion and resorptive activity as well as osteoclastogenesis.

Finally, our hypothesis that RB-BuOH inhibits osteoclastogenesis by down regulating MAP kinase-c-Fos-NFATc1 signaling axis was confirmed by our observations

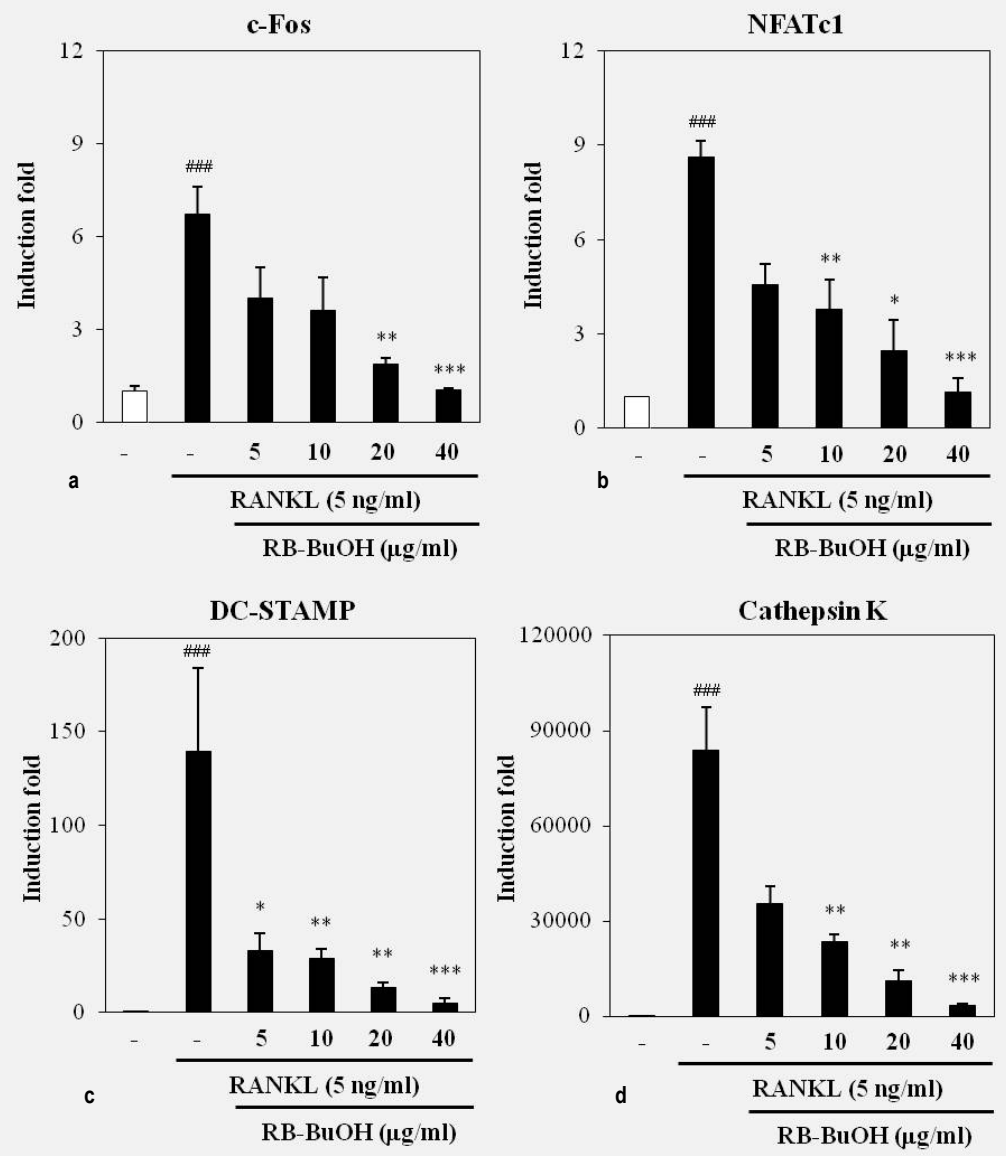

Figure 3. RB-BuOH suppresses RANKL-induced mRNA expression of c-Fos, NFATc1, DC-STAMP and Cathepsin K. BMMs were treated with RANKL (5 ng/ml) and RB-BuOH for 4 days and then mRNA expression levels were analyzed by real-time PCR. $^{###}p < 0.001$ (versus the negative control); $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (versus the RANKL-treated group).

of its effect on the protein expression and activity of NFATc1. Interestingly, RB-BuOH was found to inhibit the protein expression and activity of NFATc1 dose-dependently.

To the best of our knowledge, this is the first study to report that RB-BuOH has the potential to inhibit RANKL-induced osteoclast differentiation. The ability of RB-BuOH to attenuate the RANKL-induced activations of MAP kinases could block the inductions of c-Fos and NFATc1 that down regulate the expressions of NFATc1-controlled osteoclast-specific genes such as DC-STAMP and cathepsin K, which are essential factors for cell fusion and bone resorption. Further study is required to identify the components in RB-BuOH that confer anti-osteoclastogenic activity and to evaluate their anti-resorptive activity in mature osteoclasts. Finally, the anti-osteoclastogenic property of RB-BuOH could provide benefits for bone health, and we believe it should be considered as a potential treatment for osteoclast-related disorders.

ACKNOWLEDGEMENTS

This work was supported by the ‘Food Functionality Evaluation Program’ funded by the Korean Ministry of

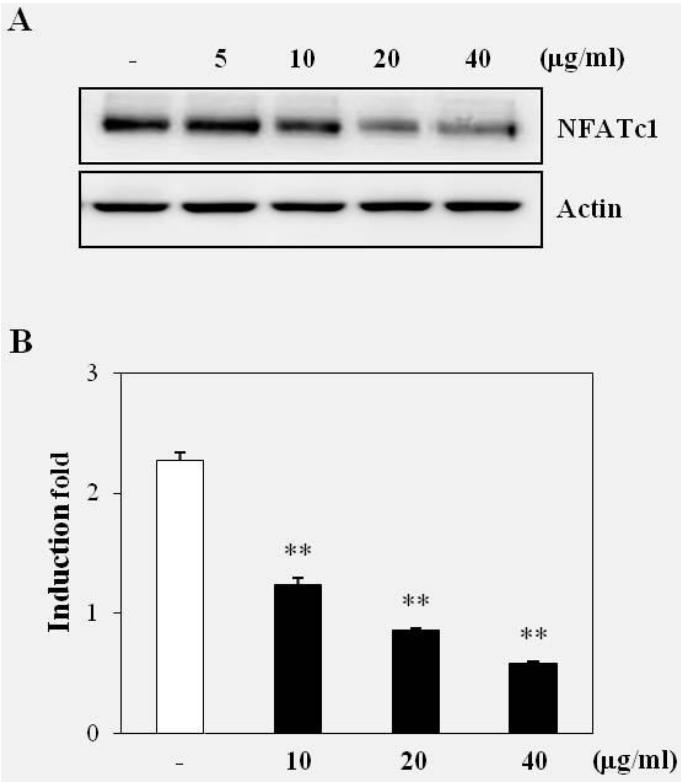

Figure 4. RB-BuOH suppresses NFATc1 expression and its activity. (A) BMMs were treated with RANKL (5 ng/ml) and RB-BuOH for 4 days. The protein expression level of NFATc1 was evaluated by western blot analysis. (B) 293T cells were co-transfected with NFAT firefly-luciferase (100 ng) and RANK (100 ng) together with pGL4 renila-luciferase (20 ng). After 6 h, transfected cells were co-treated with RANKL (50 ng/ml) and RB-BuOH. Treated cells were cultured for 48 h and lysed; luciferase activity is expressed as fold inductions versus the activity of NFAT luciferase only. pGL4 renila-luciferase activity was used to normalize transfection efficiency and activity. **$p < 0.01$ (versus the control).

Food, Agriculture, Forestry, and Fisheries, and the SI-1304 project of KRICT funded by the Korean Ministry of Knowledge Economy.

REFERENCES

Asagiri M, Sato K, Usami T, Ochi S, Nishina H, Yoshida H, Morita I, Wagner EF, Mak TW, Serfling E, Takayanagi H (2005). Autoamplification of NFATc1 expression determines its essential role in bone homeostasis. J. Exp. Med. 202:1261-9.

Asagiri M, Takayanagi H (2007). The molecular understanding of osteoclast differentiation. Bone 40:251–264.

Balkan W, Martinez AF, Fernandez I, Rodriguez MA, Pang M, Troen BR (2009). Identification of NFAT binding sites that mediate stimulation of cathepsin K promoter activity by RANK ligand. Gene 446:90-8.

Bi S, Liu JR, Li Y, Wang Q, Liu HK, Yan YG, Chen BQ, Sun WG (2010). gamma-Tocotrienol modulates the paracrine secretion of VEGF induced by cobalt(II) chloride via ERK signaling pathway in gastric adenocarcinoma SGC-7901 cell line. Toxicology 274:27-33.

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

Dannemann C, Gratz KW, Riener MO, Zwahlen RA (2007). Jaw osteonecrosis related to bisphosphonate therapy: A severe secondary disorder. Bone 40:828–834.

Flight I, Clifton P (2006), Cereal grains and legumes in the prevention of coronary heart disease and stroke: a review of the literature. Euro. J. Clin. Nutr. 60:1145-1159.

Gelb BD, Shi GP, Chapman HA, Desnick RJ (1996). Pycnodysostosis, a lysosomal disease caused by cathepsin K deficiency. Science 273:1236-8.

Ha H, Lee JH, Kim HN, Lee ZH (2011). α-Tocotrienol inhibits osteoclastic bone resorption by suppressing RANKL expression and signaling and bone resorbing activity. Biochem. Biophys. Res. Commun. 406:546-51.

Henderson AJ, Ollila CA, Kumar A, Borresen EC, Raina K, Agarwal R, Ryan EP (2012). Chemopreventive properties of dietary rice bran: current status and future prospects. Adv. Nutr. 3(5):643-53.

Hoshino Y, Hirashima N, Nakanishi M, Furuno T (2010). Inhibition of degranulation and cytokine production in bone marrow-derived mast cells by hydrolyzed rice bran. Inflamm. Res. 59:615-25.

Ishikawa T, Kamiyama M, Tani-Ishii N, Suzuki H, Ichikawa Y, Hamaguchi Y, Momiyama N, Shimada H (2001). Inhibition of osteoclast differentiation and bone resorption by cathepsin K antisense oligonucleotides. Mol. Carcinog. 32:84-91.

Jun HI, Song GS, Yang EI, Youn Y, Kim YS (2012). Antioxidant activities and phenolic compounds of pigmented rice bran extracts. J. Food Sci. 77:C759-64.

Kannappan R, Ravindran J, Prasad S, Sung B, Yadav VR, Reuter S, Chaturvedi MM, Aggarwal BB (2010). Gamma-tocotrienol promotes TRAIL-induced apoptosis through reactive oxygen species/ extracellular signal-regulated kinase/p53-mediated upregulation of death receptors. Mol. Cancer Ther. 9:2196-207.

Kaup RM, Khayyal MT, Verspohl EJ (2012). Antidiabetic Effects of a Standardized Egyptian Rice Bran Extract. Phytother. Res. doi: 10.1002/ptr.4705. [Epub ahead of print]

Kim K, Lee SH, Ha Kim J, Choi Y, Kim N (2008). NFATc1 induces osteoclast fusion via up-regulation of Atp6v0d2 and the dendritic cell-specific transmembrane protein (DC-STAMP). Mol. Endocrinol. 22:176-85.

Kukita T, Wada N, Kukita A, Kakimoto T, Sandra F, Toh K, Nagata K, Iijima T, Horiuchi M, Matsusaki H, Hieshima K, Yoshie O, Nomiyama H (2004). RANKL-induced DC-STAMP is essential for osteoclastogenesis. J. Exp. Med. 200:941-6.

Lai YL, Yamaguchi M (2007). Phytocomponent p-hydroxycinnamic acid inhibits osteoclast-like cell formation in mouse bone marrow cultures. Int. J. Mol. Med. 19:123-8.

Lee ZH, Kim HH (2003). Signal transduction by receptor activator of nuclear factor kappa B in osteoclasts. Biochem. Biophys. Res. Commun. 305:211-4.

Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}_{T}$ Method. Methods 25:402-8.

Matsuo K, Galson DL, Zhao C, Peng L, Laplace C, Wang KZ, Bachler MA, Amano H, Aburatani H, Ishikawa H, Wagner EF (2004). Nuclear factor of activated T-cells (NFAT) rescues osteoclastogenesis in precursors lacking c-Fos. J. Biol. Chem. 279:26475-80.

Meier RPH, Perneger TV, Stern R. Rizzoli R, Peter RE (2012). Increasing occurrence of atypical femoral fractures associated with bisphosphonate use. Arch. Intern. Med. 172:930-6.

Morabito N, Crisafulli A, Vergara C, Gaudio A, Lasco A, Frisina N, D'Anna R, Corrado F, Pizzoleo MA, Cincotta M, Altavilla D, Ientile R, Squadrito F (2002). Effects of genistein and hormone-replacement therapy on bone loss in early postmenopausal women: A randomized double-blind placebo-controlled study. J. Bone Miner. Res. 17:1904–1912.

Nam SH, Choi SP, Kang MY, Kozukue N, Friedman M (2005). Antioxidative, antimutagenic, and anticarcinogenic activities of rice bran extracts in chemical and cell assays. J. Agric. Food Chem. 53:816-22.

Norazalina S, Norhaizan ME, Hairuszah I, Norashareena MS (2010). Anticarcinogenic efficacy of phytic acid extracted from rice bran on azoxymethane-induced colon carcinogenesis in rats. Exp. Toxicol. Pathol. 62:259-68.

Raisz LG (2005). Pathogenesis of osteoporosis: concepts, conflicts, and prospects. J. Clin. Invest. 115:3318-25.

Rao AS, Reddy SG, Babu PP, Reddy AR (2010). The antioxidant and antiproliferative activities of methanolic extracts from Njavara rice bran. BMC Complement Altern. Med. 10:4.
Roschek B Jr, Fink RC, Li D, McMichael M, Tower CM, Smith RD, Alberte RS (2009). Pro-inflammatory enzymes, cyclooxygenase 1, cyclooxygenase 2, and 5-lipooxygenase, inhibited by stabilized rice bran extracts. J. Med. Food 12:615-23.
Rozen S, Skaletsky H (2000). Primer3 on the WWW for general users and for biologist programmers. Methods Mol. Biol. 132:365-86.
Sassa S, Kikuchi T, Shinoda H, Suzuki S, Kudo H, Sakamoto S (2003). Preventive effect of ferulic acid on bone loss in ovariectomized rats. In Vivo 17:277-80.
Tanaka J, Nakamura S, Tsuruma K, Shimazawa M, Shimoda H, Hara H (2012). Purple rice (*Oryza sativa* L.) extract and its constituents inhibit VEGF-induced angiogenesis. Phytother. Res. 26:214-22.
Verschoyle RD, Greaves P, Cai H, Edwards RE, Steward WP, Gescher AJ (2007). Evaluation of the cancer chemopreventive efficacy of rice bran in genetic mouse models of breast, prostate and intestinal carcinogenesis. Br. J. Cancer 96:248-254.
Wang ZQ, Ovitt C, Grigoriadis AE, Möhle-Steinlein U, Rüther U, Wagner EF (1992). Bone and haematopoietic defects in mice lacking c-fos. Nature 360:741-5.
Xu Z, Hua N, Godber JS (2001). Antioxidant activity of tocopherols, tocotrienols, and gamma-oryzanol components from rice bran against cholesterol oxidation accelerated by 2, 2'-azobis(2-methylpropionamidine) dihydrochloride. J. Agric. Food Chem. 49:2077-81.
Yagi M, Miyamoto T, Sawatani Y, Iwamoto K, Hosogane N, Fujita N, Morita K, Ninomiya K, Suzuki T, Miyamoto K, Oike Y, Takeya M, Toyama Y, Suda T (2005). DC-STAMP is essential for cell-cell fusion in osteoclasts and foreign body giant cells. J. Exp. Med. 202:345-51.