Ginsenoside Rh2(S) induces the differentiation and mineralization of osteoblastic MC3T3-E1 cells through activation of PKD and p38 MAPK pathways

Do Yeon Kim1,*, Mi Song Jung1,*, Young Guk Park2, Hai Dan Yuan1, Hai Yan Quan1,3 & Sung Hyun Chung1,3,*

1Department of Pharmacology and Clinical Pharmacy, College of Pharmacy, 2Department of Orthodontics, School of Dentistry, 3Department of Life and Nanopharmaceutical Science, Graduate School of Kyung Hee University, Seoul 130-701, Korea

As part of the search for biologically active anti-osteoporotic agents that enhance differentiation and mineralization of osteoblastic MC3T3-E1 cells, we identified the ginsenoside Rh2(S), which is an active component in ginseng. Rh2(S) stimulates osteoblastic differentiation and mineralization, as manifested by the up-regulation of differentiation markers (alkaline phosphatase and osteogenic genes) and Alizarin Red staining, respectively. Rh2(S) activates p38 mitogen-activated protein kinase (MAPK) in time- and concentration-dependent manners, and Rh2(S)-induced differentiation and mineralization of osteoblastic cells were totally inhibited in the presence of the p38 MAPK inhibitor, SB203580. In addition, pretreatment with Go6976, a protein kinase D (PKD) inhibitor, significantly reversed the Rh2(S)-induced p38 MAPK activation, indicating that PKD might be an upstream kinase for p38 MAPK in MC3T3-E1 cells. Taken together, these results suggest that Rh2(S) induces the differentiation and mineralization of MC3T3-E1 cells through activation of PKD/p38 MAPK signaling pathways, and these findings provide a molecular basis for the osteogenic effect of Rh2(S).

INTRODUCTION

Osteoporosis is a common disorder that is characterized by low bone mineral density and compromised bone strength, and predisposes the patient to increased risk of fracture (1). In normal bone remodeling or bone turnover, osteoblastic bone formation and osteoclastic bone resorption are coupled in a precise and orchestrated manner. The management of osteoporosis is among the greatest challenges faced by modern medicine. Traditional therapeutic agents for osteoporosis have been estrogen, calcitonin, and bisphosphonates, which inhibit bone resorption. These drugs seem to be the most effective method to reduce the rate of postmenopausal bone loss, but may be accompanied by severe side-effects such as breast cancer, venous thromboembolism, rhinitis, or esophageal ulcer (2, 3). Recently, attempts have been made to use a combination of anti-resorptive agents and bone formation-stimulating agents (4). However, the available bone-forming agents have serious adverse effects, may not improve bone quality, or may not reduce the susceptibility to fracture. Therefore, there is an increasing need for safer therapeutic agents with efficacy comparable to commercially available drugs for treating disorders of bone remodeling.

Ginseng, the root of Panax ginseng C.A. Meyer (Araliaceae), has been used as a traditional Chinese medicine for >2,000 years in Asia. In humans and animals, ginseng and its active ingredients show widely beneficial effects including improving immune function, preventing cancer, enhancing sexual function, and inhibiting adipocyte differentiation (5-8). However, few studies have been conducted to examine the anti-osteoporosis effect of ginseng (9, 10). Liu et al. speculated whether ginsenosides affect the differentiation of osteoclasts, and they found that ginsenosides Rh2(R) and Rh2(S) significantly depress osteoblast formation, and Rh2(R) shows a stronger inhibitory effect on osteoclast formation than Rh2(S) (10).

As part of the search for biologically active anti-osteoporotic agents which enhance differentiation and mineralization of osteoblastic MC3T3-E1 cells, we found ginsenoside Rh2(S) to be one of the most active ginsenosides. This effect was mediated by PKD and p38 mitogen-MAPK signaling pathways.

RESULTS

Effects of Rh2(S) on the differentiation and mineralization of MC3T3-E1 cells

To examine the effect of Rh2(S) on the differentiation and mineralization of osteoblasts, MC3T3-E1 osteoblast cells were incubated with Rh2(S) as indicated during osteoblast differen-
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Fig. 1. Effect of Rh2(S) on the differentiation and mineralization in MC3T3-E1 cells. Cells were cultured in osteogenic medium with or without Rh2(S) as indicated for 14 days. Differentiation and mineralization were evaluated by ALP activity and Alizarin Red/von Kossa staining, respectively (A-C). In panel A, vehicle denotes dimethyl sulfoxide in which Rh2(S) was dissolved. Quantification of Alizarin Red staining was performed by extraction with ethylpyridium chloride. The levels of gene expression were analyzed by real-time RT-PCR (D and E). Total RNA was collected on days 3, 7, and 14. Each value represents the mean ± SEM of the fold increase over the control. *P < 0.05, **P < 0.01, and ***P < 0.001 compared to the control group.

The ALP activity, an early-stage osteoblast differentiation marker, reached a maximum level at day 7 and then declined (Fig. 1A), and the activity increased in a concentration-dependent manner (Fig. 1B). Alizarin Red and von Kossa stainings showed that mineralized matrix in MC3T3-E1 cells was formed in a concentration-dependent manner by Rh2(S) on day 14 (Fig. 1C). Rh2(S) (40 μM) markedly increased ALP activity and mineralization by 4.8-fold and 2.5-fold, respectively, when compared to those activities in the control group. To examine the molecular mechanism underlying the promotion of mineralization, gene expression profiles of osteogenic markers, such as ALP, OCN, Osx, and Col-I were investigated after 3, 7 and 14 days of treatment with 40 μM of Rh2(S). As shown in Fig. 1D and 1E, Rh2(S) significantly increased the mRNA expression levels of ALP, OCN, Osx, and Col-I mRNA in time-dependent manners. The highest level of ALP mRNA expression was observed on day 3, and then the expression level abated, whereas OCN, Osx, and Col-I mRNA were gradually up-regulated until day 14. On day 7, Rh2(S) significantly increased the gene expression of OCN, Osx, and Col-I in concentration-dependent manners (Fig. 1E).

Rh2(S) induces differentiation and mineralization of MC3T3-E1 cells via p38 MAPK activation
We investigated whether p38 MAPK is associated with Rh2(S)-induced differentiation and mineralization of MC3T3-E1 cells. As shown in Fig. 2A and 2B, Rh2(S) markedly stimulated the phosphorylation of p38 MAPK in time- and concentration-dependent manners. Next, to confirm that Rh2(S)-induced differentiation and mineralization resulted from activation of p38 MAPK, a pharmacological approach using a p38 MAPK inhibitor (SB203580) was explored. As shown in Fig. 2C, phosphorylation of activating transcription factor 2 (ATF2), a specific target protein for p38 MAPK, was markedly decreased when MC3T3-E1 cells were pretreated with SB203580. In addition, Rh2(S)-induced stimulation of osteogenic gene expression, ALP activity, and mineralization were all attenuated in the presence of SB203580 (Fig. 2D, E and F). These results suggest...
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Fig. 2. Role of p38 MAPK in Rh2(S)-induced osteoblast differentiation. Cells were starved in serum-free medium for 2 h and treated with 40 μM Rh2(S) for up to 60 min or with 10 to 40 μM Rh2(S) for 60 min (A, B). Cells were pretreated with the indicated concentrations of SB203580 for 2 h, exposed to 40 μM Rh2(S) for 1 h, and then the phosphorylated form of ATF2 was determined (C). Cells were incubated in osteogenic medium containing Rh2(S) in the absence or presence of SB203580 for 14 days. ALP activity and gene expression levels were analyzed on day 7, and the mineralized nodules were stained by Alizarin Red/von Kossa on day 14 (D, E, and F). Quantification of Alizarin Red staining was performed by extraction with ethylpyridium chloride. Each value represents the mean ± SEM of the fold increase over the control. *P < 0.05 and **P < 0.01 compared to the Rh2(S) treatment group.

that stress-activated protein kinase p38 is a mediator for Rh2(S)-induced differentiation and mineralization of osteoblastic MC3T3-E1 cells.

Rh2(S) activates p38 MAPK via PKD pathway in MC3T3-E1 cells

Next, we examined whether PKD is associated with Rh2(S)-induced differentiation and mineralization of MC3T3-E1 cells. Fig. 3A and B show that Rh2(S) markedly phosphorylated PKD in time- and concentration-dependent manners, and this phosphorylation was blunted in the presence of Go6976, a PKCμ/PKD inhibitor (Fig. 3C). Pretreatment of cells with Go6976 significantly attenuated the phosphorylation of p38 MAPK, ALP activity, and mineralization (measured by Alizarin Red staining) in a concentration-dependent manner (Fig. 3D), suggesting that Rh2(S) could induce differentiation and mineralization of osteoblastic MC3T3-E1 cells via activating PKD and p38 signaling pathways.

DISCUSSION

Ginsenoside Rh2(S) is a steroidal saponin belonging to the protopanaxadiol type found in Korean red ginseng. Although ginseng generally shows various nutritional effects (11), Rh2(S) mostly exhibits beneficial impacts on cancer prevention (12, 13). Recently, Liu et al. reported that Rh2(R) and Rh2(S) both significantly depressed osteoclast formation and that Rh2(R) showed a stronger inhibitory effect on osteoclast formation than Rh2(S) (10). Recently, we found that Rh2(S) induces the differentiation and mineralization of osteoblastic MC3T3-E1 cells. However, the molecular mechanism of Rh2(S)-induced differentiation and mineralization of osteoblast cells has been unclear. Here, we demonstrate that Rh2(S) induces the differentiation and mineralization of osteoblastic MC3T3-E1 osteoblastic cells via PKD and stress-activated protein kinase p38 MAPK pathways.

Osteogenic differentiation is a complicated process and regulated by a large number of regulators such as ALP, OPN, OCN, Oxs, and Col-I. ALP is the most widely recognized biochemical marker for osteoblastic activity. Although its precise mechanism of action has been poorly understood, this enzyme is believed to play a pivotal role in bone differentiation and mineralization. As shown in Fig. 1, Rh2(S) increases ALP enzyme activity and mRNA gene expression as well as expression of osteogenic genes such as OCN, Oxs, and Col-I. Recently, statins have been shown to induce osteoblast differentiation by stimulating the expression of bone morphogenetic protein-2 (BMP-2) in MC3T3-E1 cells, leading to positive ef-
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Fig. 3. PKD is an upstream kinase for p38 MAPK activated by Rh2(S) in MC3T3-E1 cells. Cells were starved in serum-free medium for 2 h and then stimulated with 40 μM Rh2(S) for up to 60 min or with 10 to 40 μM Rh2(S) for 60 min (A and B). Cells were pretreated with the Go6976 for 2 h as indicated, exposed to 40 μM Rh2(S) for 1 h, and then phosphorylated forms of PKD and p38 were determined (C). Cells were incubated in osteogenic medium containing Rh2(S) in the absence or presence of Go6976 for 14 days. ALP activity was analyzed on day 7, and the mineralized nodules were stained by Alizarin Red on day 14 (D). Quantification of Alizarin Red staining was performed by extraction with ethylpyridium chloride. Each value represents the mean ± SEM of the fold increase over the control. **P < 0.01 compared to the control group.

Effects on bone formation (14). We tested whether Rh2(S) stimulates BMP-2 gene expression in MC3T3-E1 osteoblastic cells. Rh2(S) had no effect on the expression of BMP-2 and this observation was confirmed by using a noggin (extracellular BMP-2 antagonist), suggesting that Rh2(S) induces the differentiation of MC3T3-E1 osteoblastic cells by a mechanism other than the BMP pathway (data not shown).

Erk1/2 (also known as p44/p42 MAPK) and p38 MAPK are members of MAPK and known to be involved in osteoblast differentiation (15, 16). To investigate the involvement of MAPKs in the Rh2(S)-induced osteoblast differentiation, PD98059, a specific inhibitor of upstream kinase that activates Erk1/2 MAPK (17), was employed. However, it did not affect the ALP activity and mineralization (data not shown). Therefore, we tried to determine whether p38 MAPK is associated with Rh2(S)-induced osteoblast differentiation. Western blot analyses revealed that Rh2(S) was able to phosphorylate p38 MAPK in MC3T3-E1 cells in time- and concentration-dependent manners (Fig. 2A and B). In addition, Rh2(S)-induced differentiation and mineralization of MC3T3-E1 cells was reversed in the presence of SB203580, a specific p38 MAPK inhibitor. These results suggest that p38 MAPK is responsible for Rh2(S)-induced osteoblastic differentiation, mineralization, and osteogenic gene expression (Fig. 2D, E and F). Lemoine et al. reported that PKD is involved in BMP-2-induced activation of stress MAPK p38 and osteoblastic cell differentiation (18). We also observed that Rh2(S) stimulates PKD phosphorylation in time- and concentration-dependent manners (Fig. 3A and B). Furthermore, when the cells were treated with Go6976, a PKCμ/PKD inhibitor, the phosphorylated forms of PKD and p38 were decreased in a concentration-dependent manner (Fig. 3C). Rh2(S)-induced ALP activation and mineralization were also suppressed in the presence of Go6976, suggesting that PKD might be an upstream kinase for p38 MAPK.

In summary, we found that ginsenoside Rh2(S) induces the differentiation and mineralization of osteoblastic MC3T3-E1 cells via activation of PKD and p38 MAPK pathways. Ginsenoside Rh2(S) may possess a therapeutic potential for osteoporosis by promoting bone formation.

MATERIALS AND METHODS

Chemicals

The ginsenoside Rh2(S) was obtained from the EMBO Institute (Seoul, Korea), and dissolved in 0.1% DMSO. Antibodies against p38 MAPK, phospho-p38 MAPK, PKCμ/PKD, and phospho-PKCμ/PKD were purchased from Cell signaling Technology (Beverly, MA, USA) and anti-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The p38 MAPK and PKD inhibitors (SB203580, Go6976) were purchased from Calbiochem (San Diego, CA, USA). The Bradford protein assay, RNA and protein extraction, cDNA synthesis and ECL kits were supplied from Intron Biotechnology Inc. (Beverly, MA, USA). The Bradford protein assay, RNA and protein extraction, cDNA synthesis and ECL kits were supplied from Intron Biotechnology Inc. (Beverly, MA, USA). The Bradford protein assay, RNA and protein extraction, cDNA synthesis and ECL kits were supplied from Intron Biotechnology Inc. (Beverly, MA, USA). The Bradford protein assay, RNA and protein extraction, cDNA synthesis and ECL kits were supplied from Intron Biotechnology Inc. (Beverly, MA, USA). The Bradford protein assay, RNA and protein extraction, cDNA synthesis and ECL kits were supplied from Intron Biotechnology Inc. (Beverly, MA, USA).
Cell culture and differentiation
MC3T3-E1 cells, a clonal osteoblastic cell line isolated from the calvariae of a late-stage mouse embryo (19), were obtained from the Riken Cell Bank (Ibaragi, Japan). Cells were cultured in an alpha modification of Eagle Medium (α-MEM without ascorbic acid, Gibco BL, Gland island, NY, USA), with 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin at 37°C. To induce differentiation, cells were seeded onto a 6- or 12-well culture dish and allowed to reach confluency. At confluency (day 0), cells were transferred to α-MEM containing 10% FBS, 1% penicillin-streptomycin, 10 mM β-glycerophosphate, and 100 μg/ml ascorbic acid, and cultured for an additional 7 to 14 days.

Alkaline phosphatase (ALP) enzyme assay
Following treatment, the cells were washed twice with PBS, scraped into 500 μl of 10 mM Tris-HCl (pH 7.6) buffer containing 0.1% Triton-X on ice and sonicated. Protein concentrations were determined using the Bradford protein assay. ALP activity in the cellular fraction was measured by a fluorometric detection kit (Nanjing Jiancheng Biotechnology Co., Ltd., Nanjing, China). A standard curve was created using nitrilotrihydroxamine as a standard, and each value was normalized to the protein concentration. ALP activity of each sample was normalized by protein concentration.

Mineralization analysis
Mineralization of MC3T3-E1 cells was determined in 12-well plates using Alizarin red staining. The cells were fixed with ice-cold 70% ethanol and stained with Alizarin Red to detect calcification. For quantification, cells were destained with ethylenediamine tetraacetate acid (EDTA) and transferred to a 96-well plate, and the cells were destained with 1% acetic acid for 30 min. After washing with distilled water, cells were fixed with 4% formaldehyde in PBS for 30 min. After washing with distilled water, cells were rinsed with PBS and scraped into 500 μl of 10 mM Tris-HCl (pH 7.6) buffer containing 0.1% Triton-X on ice and sonicated. Protein concentrations were determined using the Bradford protein assay. ALP activity in the cellular fraction was measured by a fluorometric detection kit (Nanjing Jiancheng Biotechnology Co., Ltd., Nanjing, China). A standard curve was created using nitrilotrihydroxamine as a standard, and each value was normalized to the protein concentration. ALP activity of each sample was normalized by protein concentration.

Western blot
Cells were rinsed with ice-cold PBS and lysed in Intron 1x cell lysis buffer containing a protease inhibitor mixture and cleared by centrifugation at 8,000 g for 20 min. Cell extracts (40 μg of protein) were loaded onto 8% SDS gels and subjected to SDS-PAGE. Gels were blotted onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) and probed for binding to the anti-phospho-p38 (1 : 1,000), anti-p38 (1 : 1,000), anti-phospho-PKCα/PKD (1 : 1,000), anti-PKCα/PKD (1 : 1,000), anti-ATF2, anti-phospho-ATF2, and anti-actin (1 : 20,000) in 5% non-fat dry milk containing 0.1% Tween 20-Tris-buffered saline. Membranes were washed and incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1 : 2,000) for 2 h. Following washing in 0.1% Tween 20-Tris-buffered saline, the membranes were developed with chemiluminescence detection reagents according to manufacturer’s instructions and exposed to Biomax Kodak film.

Real-time RT-PCR
SYBR green chemistry was used to perform quantitative determinations of the mRNAs for ALP, osteocalcin (OCN), type I collagen (Col-I), BMP-2, and a housekeeping gene (36B4) according to an optimized protocol. Total RNA was taken from cultured MC3T3-E1 cells using EASY BLUE reagent. Total RNA (2 μg) was employed for the synthesis of single-stranded cDNA. The sense and antisense primers used were as follows: mouse ALP sense, 5’-GACTGTACCTCGATACGAGA-3’, and antisense, 5’-CTCATGATGCTCGTGTTGACATGC-3’; mouse OCN sense, 5’-GCAGCTTGTGACACCTGAC-3’, and antisense, 5’-GGAGCTGCTGTGACATCCTAC-3’; mouse Col-I sense, 5’-ACCCTCGAGTGCCTGTTGAC-3’, and antisense, 5’-AGGTCTTCTGAGGCCAGAGGG-3’; mouse 36B4 sense, 5’-AACCCGCTTCCTCGATTGCT-3’, and antisense, 5’-CCGCAGGGGCAAGGATGCT-3’. Real-time PCR was performed using 1 μl of cDNA in a 25 μl reaction volume with LightCycler Real-time PCR System (Roche Applied Science, Indianapolis, IN). The double-stranded DNA-specific dye SYBR Green I was incorporated into the PCR buffer provided in the SYBR Premix Ex TaqTM reagent. The temperature profile of the reaction was 95°C for 15 min, 40 cycles of denaturation at 95°C for 30 s, annealing at 54°C (ALP), or 58°C (OCN, Col-I) for 30 s, and extension at 72°C for 1 min. The 36B4 gene was used to correct for differences in RNA isolation, RNA degradation, and the efficiencies of the reverse transcription.

Statistical analysis
The results are representative of at least 3 independent experiments and are expressed as the mean ± SEM. Comparison of the control and treatment groups was made by ANOVA variance analysis, and the statistical significance was analyzed by Tukey’s test. Differences of P < 0.05 were considered statistically significant.

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REFERENCES
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