Ginsenoside Rh2(S) induces differentiation and mineralization of MC3T3-E1 cells through activation of the PKD/AMPK signaling pathways

DO YEON KIM1*, KI HO PARK2*, MI SONG JUNG1, BO HUANG1,3, HAI-DAN YUAN1, HAI-YAN QUAN1,3 and 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, Kyung Hee University, Seoul 130-701, Republic of Korea

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Abstract. As part of our search for biologically active anti-osteoporotic agents that enhance differentiation and mineralization of osteoblastic MC3T3-E1 cells, we identified the ginsenoside Rh2(S). Mostly known to exhibit beneficial effects in cancer prevention and metabolic diseases, Rh2(S) is one of the most active ginsenosides. Here, we show that Rh2(S) stimulates osteoblastic differentiation and mineralization, manifested by the up-regulation of differentiation markers (alkaline phosphatase and osteogenic genes) and von Kossa/Alizarin Red staining, respectively. Rh2(S) also activated protein kinase D (PKD) and AMP-activated protein kinase (AMPK) in a time- and concentration-dependent manner, and Rh2(S)-induced differentiation and mineralization of osteoblastic cells were significantly abolished in the presence of specific inhibitors; Go6976 for PKD and Ara-A for AMPK. Furthermore, Go6976 suppressed Rh2(S)-mediated activation of AMPK, indicating that PKD may be an upstream signal for AMPK in Rh2(S)-induced differentiation and mineralization of MC3T3-E1 cells. Taken together, these results indicate that Rh2(S) induces the differentiation and mineralization of MC3T3-E1 cells through activation of PKD/AMPK signaling pathways. These findings provide a molecular basis for the osteogenic effect of Rh2(S).

Introduction

Osteoporosis is a systemic skeletal disease characterized by loss of bone mass and micro-architectural deterioration of bone tissue. This disease currently affects more than 200 million people worldwide. Bone formation is a tightly regulated process of lineage-specific differentiation events, and bone homeostasis is maintained by a balance between bone resorption by osteoclasts and bone formation by osteoblasts (1). The management of osteoporosis is among the greatest challenges faced by modern medicine. New targets have been identified via research on bone pathophysiology, bone remodeling, bone cells and intracellular signaling pathways. The majority of the current therapies for osteoporosis are anti-resorptive.

Ginseng, the root of Panax ginseng CA Meyer (Araliaceae), has been used as a traditional Chinese medicine for more than two thousand years in Asia. In human and animals, ginseng and its active ingredients show widely beneficial effects including improving immune function (2), preventing cancer (3), enhancing sexual function (4), and inhibiting adipocyte differentiation (5). However, until now, only a few studies have been conducted to assess the anti-osteoporosis effect of ginseng, and very little is known about the principal constituents responsible for its anti-osteoporosis effect. Recently, it has been documented that the ginsenoside Rh2(R) significantly depresses osteoclast formation and shows a stronger inhibitory effect on osteoclast formation than Rh2(S) (6).

As part of our search for biologically active anti-osteoporotic agents that 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 the protein kinase D (PKD) and AMP-activated protein kinase (AMPK) signaling pathways.

Materials and methods

Materials. Rh2(S) was obtained from the EMBO Institute (Seoul, Korea), and dissolved in 0.1% DMSO. Antibodies against AMPK, phospho-AMPK, acetyl Co-A carboxylase (ACC), phospho-ACC, PKD and phospho-PKD were purchased from Cell Signaling Technology (Beverly, MA, USA), and the antibody against actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Ara-A (AMPK inhibitor) and Go6976 (PKCµ/PKD inhibitor) were purchased from Calbiochem (Darmstadt, Germany). The Bradford protein
assay, RNA and protein extraction, cDNA synthesis and ECL kits were supplied by Intron Biotechnology, Inc. (Beverly, MA, USA). Other reagents and chemicals were of analytical grade.

Cell culture and differentiation. MC3T3-E1, a clonal osteoblastic cell line isolated from the calvariae of a late-stage mouse embryo (7), was obtained from the Riken Cell Bank (Ibaragi, Japan). Cells were cultured in α-MEM (Gibco-BRL, Grand Island, NY, USA) with 10% FBS, 20 mM HEPES and 1% penicillin-streptomycin in an atmosphere of 95% air and 5% CO2. To induce differentiation, cells were seeded onto a 6- or 12-well culture dish and allowed to grow to confluence. At confluence (Day 0), cells were transferred to α-MEM with 10% FBS, 1% penicillin-streptomycin, 10 mM β-glycerophosphate and 100 µg/ml ascorbic acid, and cultured for an additional 7-14 days.

Cell proliferation assay. In a 96-well plate, 1x10³ cells/well were incubated in the presence or absence of Rh2(S). On the third day, 100 µl of MTS solution was added to each well and incubated for 30 min, and the absorbance at 550 nm was measured using a microplate reader.

Alkaline phosphatase (ALP) assay. At the end of the treatment, cells were washed twice with PBS, scraped into 500 µl of 10 mM Tris-HCl (pH 7.6) buffer containing 0.1% Triton X-100 on ice and sonicated. The protein concentration was determined using the Bradford protein assay. ALP activity was examined by a method modified from McCarthy et al (8). In brief, the assay mixtures contained 0.1 M 2-amino-2-methyl-1-propanol, 1 mM MgCl2, 8 mM p-nitrophenyl phosphate disodium and assay mixtures contained 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propanol, 8 mM p-nitrophenyl phosphate disodium and 0.1 M 2-amino-2-methyl-1-propano
Effects of Rh2(S) on osteogenic gene expression. To determine the mechanism underlying the promotion of mineralization by Rh2(S), gene expression profiles of osteogenic markers, such as Runx2, ALP, OCN, OPN, Osx and Col-I, were investigated on Day 3, 7 and 14 in the MC3T3-E1 cells. Rh2(S) significantly increased the mRNA expression levels of ALP, OCN, OPN, Osx and Col-I in a time- and concentration-dependent manner (Fig. 2). The highest level of ALP mRNA expression was observed on Day 3 of culture and then abated, whereas OCN, OPN, Osx and Col-I mRNA were gradually up-regulated until Day 14.

Rh2(S) stimulates differentiation and mineralization of MC3T3-E1 cells via PKD activation. Next, we investigated whether PKD is associated with Rh2(S)-induced differentiation and mineralization of MC3T3-E1 cells. Rh2(S) markedly stimulated the phosphorylation of PKD in a time- and concentration-dependent manner, whereas the expression of PKD was not affected (Fig. 3A and B). To examine whether Rh2(S)-induced differentiation could result from PKD activation, a pharmacological approach using Go6976, a PKCµ/PKD inhibitor, was explored. Pre-treatment of MC3T3-E1 cells with Go6976 significantly blocked PKD phosphorylation in a concentration-dependent manner (Fig. 3C). In addition, Rh2(S)-induced stimulation of osteogenic gene expression, ALP activity and mineralization were all attenuated in the presence of Go6976 (Fig. 3D-I). These results suggest that PKD works as a mediator for Rh2(S)-induced differentiation and mineralization of osteoblastic MC3T3-E1 cells.

Rh2(S) induces differentiation and mineralization of MC3T3-E1 cells via the PKD-AMPK pathways. We also examined whether Rh2(S) activates AMPK in MC3T3-E1 cells. Rh2(S) markedly phosphorylated AMPK and ACC (an immediate substrate of AMPK) in a time- and concentration-dependent manner (Fig. 4A and B). These results were confirmed by using Ara-A, a specific inhibitor of AMPK (Fig. 4C). To examine whether AMPK activation by Rh2(S) is associated with differentiation and mineralization of MC3T3-E1 cells, ALP activity and mineralization (quantitated by Alizarin Red staining) were determined in the presence of Ara-A as indicated. Ara-A dramatically suppressed ALP activity and mineralization in concentration-dependent manner (Fig. 4D). Finally, we wanted to know whether there is any cross-talk between PKD and AMPK; both were shown to be involved in Rh2(S)-induced differentiation and mineralization of MC3T3-E1 cells. To test this hypothesis, MC3T3-E1 cells were pre-treated with 10 or 20 µM of Go6976, and the protein expression levels of pAMPK and AMPK were determined. Go6976 significantly attenuated the phosphorylation of AMPK and ACC in concentration-dependent manners, suggesting that Rh2(S) could induce differentiation and mineralization of osteoblastic MC3T3-E1 cells via activating PKD and AMPK signaling pathways (Fig. 4E).
Figure 2. Effect of Rh2(S) on osteogenic gene expression in MC3T3-E1 cells. Cells were cultured in osteogenic medium containing 40 µM Rh2(S) for the indicated times (A-F) or cultured in osteogenic medium containing the indicated concentrations of Rh2(S) for 7 days (G-L). The levels of gene expression were analyzed by real-time PCR. Each value represents the mean ± SEM fold increase over control values (n>6). *P<0.05 and **P<0.01.
Ginsenoside Rh2(S), isolated from red ginseng, is a steroidal saponin that belongs to the protopanaxadiol saponins. Although ginseng generally shows various nutritional effects (12), this compound mostly exhibits beneficial impacts in cancer prevention (13,14) and metabolic diseases (5,15,16). Native ginseng contains trace amounts of ginsenoside Rh2(S) (about 0.01%). However, previous studies have demonstrated that some protopanaxadiol-type ginsenosides, such as Rb1, Rb2, Rc, Rd and Rg3, are metabolized to Rh2(S) by human intestinal bacteria (17). These findings suggest that Rh2(S) may contribute to ginseng’s beneficial effects. Recently, Liu et al (6) reported that Rh2(R) and Rh2(S) both significantly depress osteoclast formation, and that Rh2(R) shows a stronger inhibitory effect on osteoclast formation than Rh2(S). However, the effect of Rh2(S) on the differentiation and mineralization of osteoblast cells has not been reported. While searching for anti-osteoporotic agents that enhance the differentiation and mineralization of osteoblastic MC3T3-E1 cells, ginsenoside Rh2(S) was found to be one of the most active ginsenosides. Here, we demonstrated that Rh2(S) stimulates the differentiation and mineralization of osteoblastic MC3T3-E1 cells via PKD and AMPK signaling pathways.

Bone matrix maturation is largely regulated by a series of bone matrix proteins in osteoblasts, such as ALP (a marker of maturation and mineralization), Runx2 (a key transcription factor that plays an essential role in osteoblastic differentiation and directly regulates bone marker genes). In the present study, we found that Rh2(S) does not...
affect the expression of Runx2, whereas Rh2(S) up-regulates the transcription of Osx, a downstream target for Runx2. We speculate that Rh2(S) may regulate the transactivity of Runx2 by modulating its phosphorylation, but not its gene expression (Fig. 2).

Protein kinase C (PKC) and/or PKD have been known to be associated with osteoblastic differentiation (18-20). To investigate whether PKC or PKD mediate the Rh2(S)-induced differentiation and mineralization of osteoblastic MC3T3-E1 cells, two different staurosporine-derived inhibitors were utilized: Go6983 for PKCα, βI, βII, δ and γ and Go6976 for PKCα, βI and PKCµ/PKD. In our previous study, Go6983 did not show any effect on Rh2(S)-induced differentiation and mineralization (data not shown), whereas Go6976 completely reversed these responses (Fig. 3I). These results suggest that PKC-independent activation of PKD is involved in Rh2(S)-induced activation of osteoblastic cell differentiation.

AMPK has emerged over the last decade as a key sensing mechanism in the regulation of cellular energy homeostasis (21). AMPK is expressed ubiquitously, but its function and regulation in bone have been poorly investigated. It was recently demonstrated that the AMPK activator AICAR stimulates proliferation, differentiation and mineralization of osteoblastic MC3T3-E1 cells (22). AMPK is also involved in the mechanism of action of two anti-diabetic drugs, metformin and rosiglitazone, and both drugs affect bone cell function in vitro (23,24). Interestingly, recent studies have demonstrated a link between glucose metabolism and bone homeostasis (25,26). Hwang et al (5) demonstrated that the anti-obesity effect of Rh2(S) is associated with the activation of the AMPK signaling pathway in 3T3-L1 adipocytes. Our results clearly demonstrate that Rh2(S) phosphorylates AMPK in a time- and concentration-dependent manner, and the stimulation of osteoblast differentiation and mineralization is mediated by AMPK activation (Fig. 4A-D). These results were confirmed using an AMPK inhibitor, Ara-A. In addition, when the cells were pretreated with Go6976, Rh2(S)-induced activation of AMPK was significantly blocked, suggesting that PKD might be an upstream regulator for Rh2(S)-induced stimulation of AMPK, and PKD and AMPK signaling pathways may play roles in osteoblastic MC3T3-E1 cell metabolism.

In summary, we found that the ginsenoside Rh2(S) induces the differentiation and mineralization of osteoblastic MC3T3-E1 cells via activation of PKD and AMPK signaling...
pathways. This active component found primarily in red ginseng might be beneficial in the treatment of osteoporosis by promoting bone formation.

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