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

DO YEON KIM[1*], KI HO PARK[2*], MI SONG JUNG[1], BO HUANG[1,3], HAI-DAN YUAN[1], HAI-YAN QUAN[1,3] and SUNG HYUN CHUNG[1,3]

[1]Department of Pharmacology and Clinical Pharmacy, College of Pharmacy, [2]Department of Orthodontics, School of Dentistry, [3]Department 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 (PKC$\mu$/PKD inhibitor) were purchased from Calbiochem (Darmstadt, Germany). The Bradford protein

*Correspondence to:* Professor Sung Hyun Chung, Department of Pharmacology and Clinical Pharmacy, College of Pharmacy, Kyung Hee University, 1 Hoegi-dong, Dongdaemun-ku, Seoul 130-701, Republic of Korea
E-mail: suchung@khu.ac.kr

*Contributed equally

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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% $CO_2$ at 37˚C. 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, $1x10^3$ 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 $MgCl_2$, 8 mM p-nitrophenyl phosphate disodium and cell lysate. After 4 min of incubation at 37˚C, the reaction was stopped with 0.1 N NaOH, and the absorbance was read at 405 nm. A standard curve was drawn with p-nitrophenol, and each value was normalized to the protein concentration.

*Mineralization analysis*. Mineralization of MC3T3-E1 cells was determined in 24-well plates using von Kossa or Alizarin Red staining. The cells were fixed with 95% ethanol and stained with $AgNO_3$ by the von Kossa method to detect phosphate deposits in bone nodules (9). On the same day, the other set of plates were fixed with ice-cold 70% ethanol and stained with Alizarin Red to detect calcification. For quantification, cells stained with Alizarin Red (n>3) were destained with ethylpyridium chloride and transferred to a 96-well plate, and the absorbance was measured at 550 nm using a microplate reader, as previously described (10).

*Real-time PCR*. Type-I collagen (Col-I), osteocalcin (OCN), osteopontin (OPN), ALP, osterix (Osx) and runt-related transcription factor 2 (Runx2) mRNA levels were measured by a LightCycler Real-Time PCR System (Roche Applied Science, Indianapolis, IN, USA) using the SYBR Premix Ex Taq™ reagent. mRNA levels were normalized to the house keeping gene, 36B4 (11). Total-RNA was isolated using an Easy-BLUE Total-RNA extraction kit according to the manufacturer's instructions. Total-RNA (2 μg) was used for the synthesis of single-stranded cDNA. The primer sequences used were as follows: mouse Runx2 forward, 5'-TTCTCCAACCCACGAATGCACTA-3' and reverse, 5'-CATGGTTGACGAATTTCAATATGG-3'; mouse ALP forward, 5'-GACTGGTACTCGGATAACGAGA-3' and reverse, 5'-CTCATGATGTCCGTGGTCAATC-3'; mouse Osx forward, 5'-ACAGCTCGTCTGACTGCCTGCCTA and reverse, 5'-AGACATCTTGGGGTAGGACATGC-3'; mouse OCN forward, 5'-GCAGCTTGGTGCACACCTAG-3' and reverse, 5'-GGAGCTGCTGTGACATCCAT-3'; mouse OPN forward, 5'-GACGAATCTCACCATTCGGATGA-3' and reverse, 5'-ATGAACTCTCTAATTCATGAGAA-5'; mouse Col-I forward, 5'-ACCTCCCAGTGGCGGT TATGAC-3' and reverse, 5'-AGTTCTTCTGAGGCACAGACGG-3'; mouse 36B4 forward, 5'-AAGCGCGTCCTGGCATTGTCT-3' and reverse, 5'-CCGCAGGGGCAGCAGTGGT-3'. Real-time PCR was performed in a 25 μl of reaction mixture containing 1 μl cDNA and primers using the ABI PRISM 7000 (AB Applied Biosystems, Inc., CA, USA). The double-stranded DNA-specific dye, SYBR-Green I, was incorporated into the PCR buffer provided by the QuantiTect SYBR-Green PCR kit (Qiagen, Valencia, CA, USA) to quantitate the PCR products. PCR was performed at 95˚C for 30 sec, followed by 50˚C (Runx2, 36B4), 54˚C (ALP) or 58˚C (OCN, OPN, Osx, Col-I) for 30 sec, and 72˚C for 1 min. The last cycle was followed by a final extension step at 72˚C for 10 min.

*Western blot analysis*. Cells were rinsed with ice-cold PBS, scraped on ice and processed using a protein extraction kit. Nuclei and cell debris were removed by centrifugation (15,000 rpm for 20 min), and the supernatant was used for immunoblotting. Protein concentrations of cell lysate were measured using the Bradford protein assay kit. Equal amounts of protein (50 μg/lane) were fractionated by electrophoresis on an 8% SDS-PAGE gel, transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA), probed for binding to the respective antibodies, such as pAMPK, AMPK, pACC, ACC, pPKD, PKD and actin, and visualized using an ECL kit.

*Statistical analysis*. Results are representative of at least three independent experiments and expressed as the mean ± SEM. Comparison between the control and treatment groups was made by ANOVA variance analysis, and statistical significance was analyzed by the Tukey's test. Differences of $P<0.05$ were considered to be statistically significant.

## Results

*Effects of Rh2(S) on differentiation and mineralization of MC3T3-El cells*. First, we investigated the effect of Rh2(S) on the proliferation of MC3T3-E1 cells by the MTS assay. Growth profiles observed on the Day 2 of culture in the presence of the indicated concentrations of Rh2(S) were similar to that of the control, suggesting that Rh2(S) did not show any effect on cell proliferation (Fig. 1A). To ascertain the anabolic activity of Rh2(S) in bone metabolism, its effects on ALP activity and mineralization (determined by Alizarin Red staining) were evaluated. Rh2(S) significantly increased ALP activity and mineralization in a concentration-dependent manner (Fig. 1B). Treatment of MC3T3-E1 cells with 40 μM Rh2(S) markedly increased ALP activity and mineralization by 2.3- and 3.4-fold, respectively. Von Kossa staining also showed that phosphate deposition in MC3T3-E1 cells was augmented in a concentration dependent manner on Day 14 (Fig. 1C).

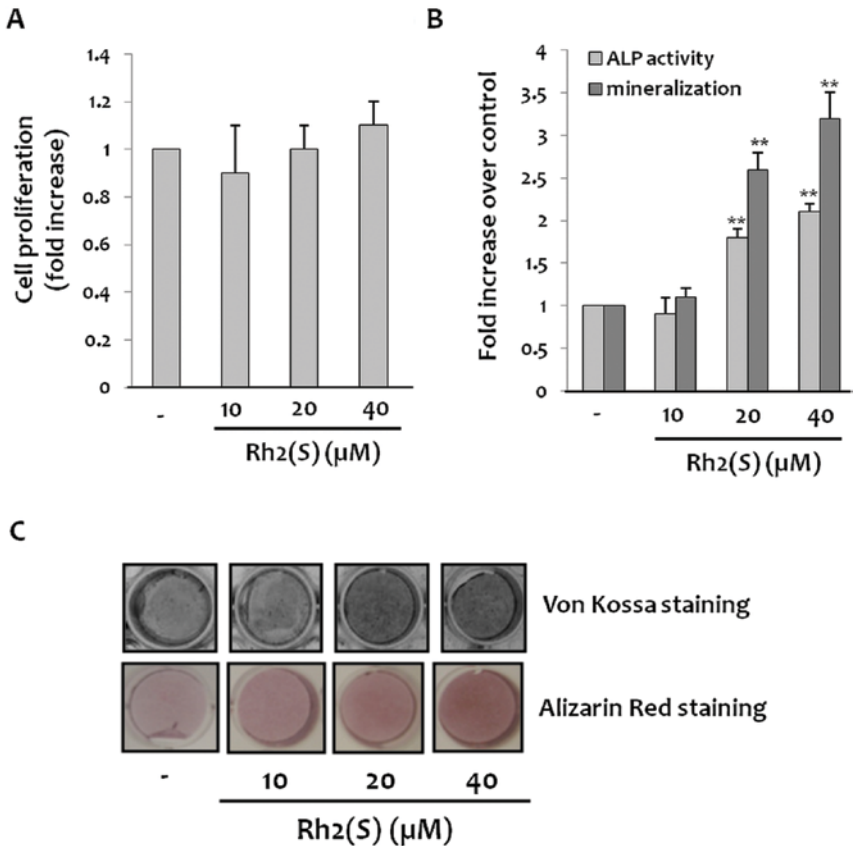


Figure 1. Effect of Rh2(S) on proliferation and differentiation of MC3T3-E1 cells. (A) Cells were incubated with different concentrations of Rh2(S) for 2 days. Cell proliferation was determined by the MTS assay. (B) Cells were cultured in osteogenic medium with or without Rh2(S) as indicated for 7 (ALP activity) or 14 (mineralization) days. (C) Alizarin Red and Von Kossa stainings were performed to visualize mineralization of MC3T3-E1 cells cultured for 14 days with Rh2(S) as indicated in the osteogenic medium. Each value represents the mean ± SEM fold increase over control values (n>6). $^{**}$P<0.01.

*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-El 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 PKC$\mu$/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 $\mu$M of G06976, 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).

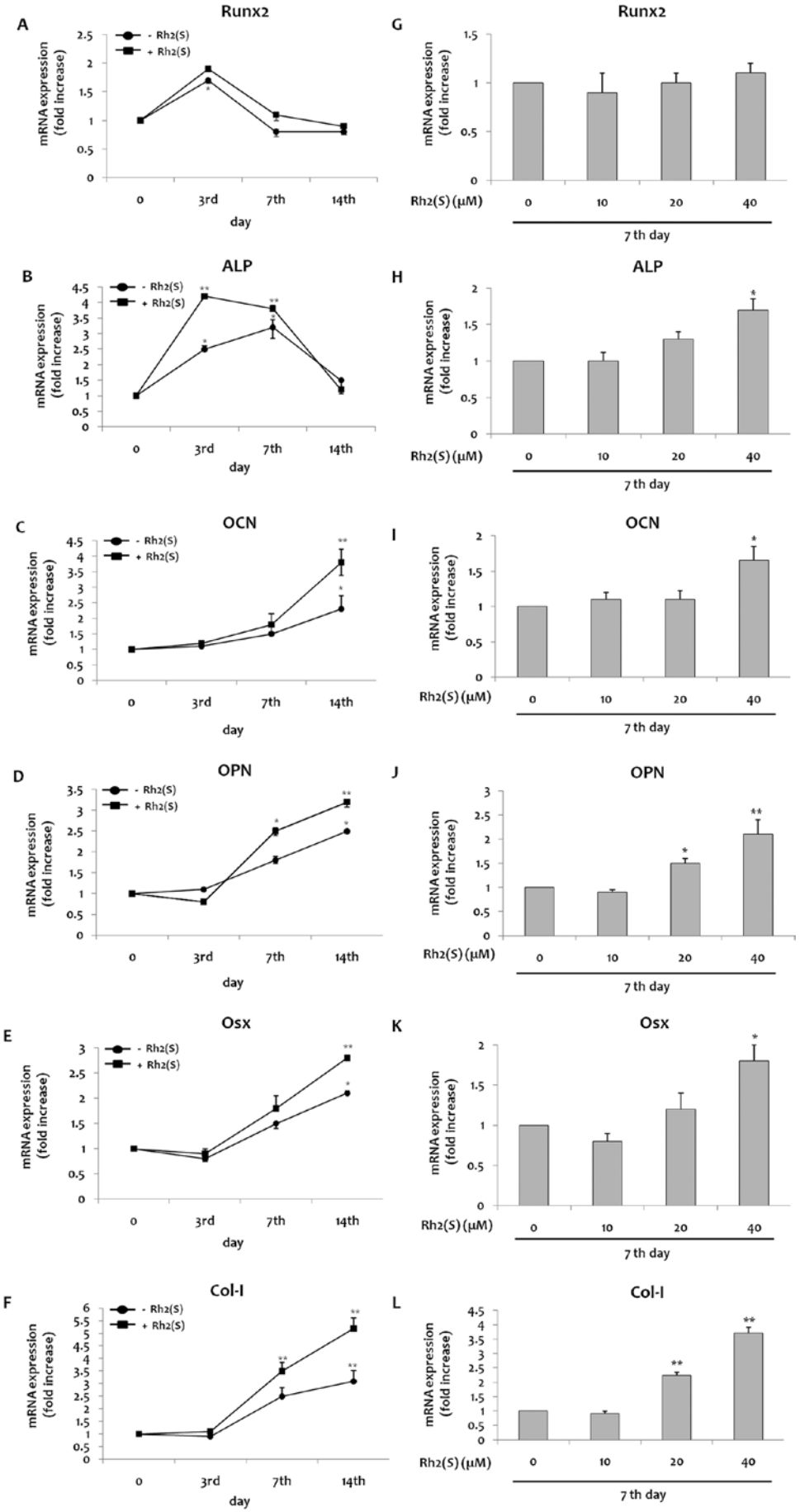


Figure 2. Effect of Rh2(S) on osteogenic gene expression in MC3T3-E1 cells. Cells were cultured in osteogenic medium containing 40 $\mu$M Rh2(S) for the indicated times (A-F) or cultured in osteogenic medium containing the indicated concentrations of Rh2(S) for 7 days (G-L). The levels of gene expression 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.

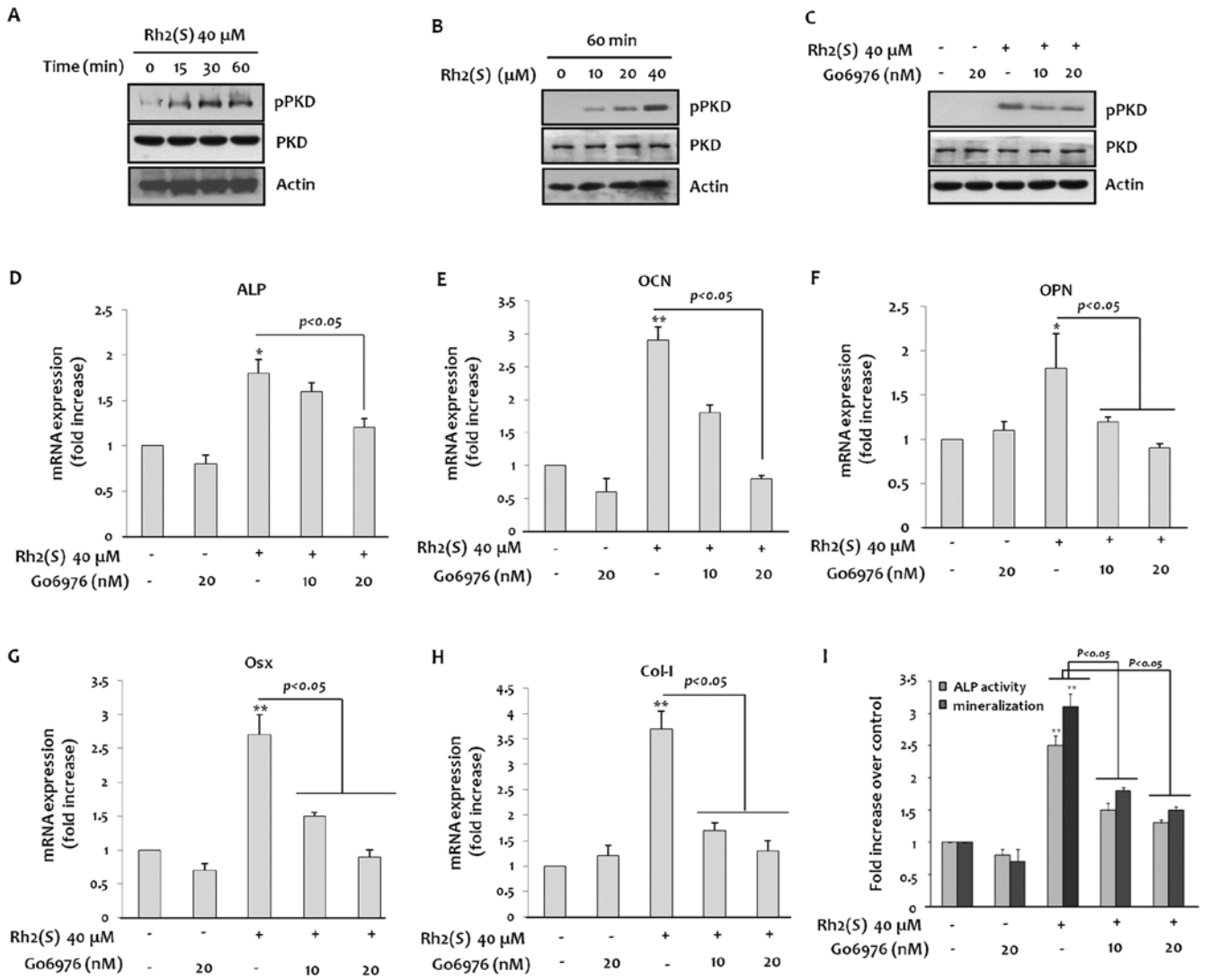


Figure 3. Role of PKD in Rh2(S)-induced osteoblast differentiation. (A and B) Cells were starved in serum-free medium for 2 h and treated with 40 $\mu$M Rh2(S) for up to 60 min or treated with 10 to 40 $\mu$M Rh2(S) for 60 min. (C) Cells were pre-treated with Go6976 as indicated or with vehicle for 2 h and exposed to 40 $\mu$M Rh2(S) for 1 h. (D-I) Cells were incubated in the osteogenic medium containing 40 $\mu$M Rh2(S) in the absence or presence of Go6976 until the 14th day. ALP activity and gene expression levels were analyzed on Day 7, and the mineralized nodules were stained with Alizarin Red on Day 14. Quantification of Alizarin Red staining was performed by extraction with ethylpyridium chloride. Each value represents the mean ± SEM fold increase over control values (n>6). $^{*}$P<0.05 and $^{**}$P<0.01.

## Discussion

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), and OPN, OCN, Osx and Col-I (markers of terminal differentiation). Rh2(S) stimulated ALP activity and up-regulated osteogenic genes, suggesting that Rh2(S) could affect the cell differentiation process at various levels, from early to terminal stages (Figs. 1B and 2C). Runx2 is 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

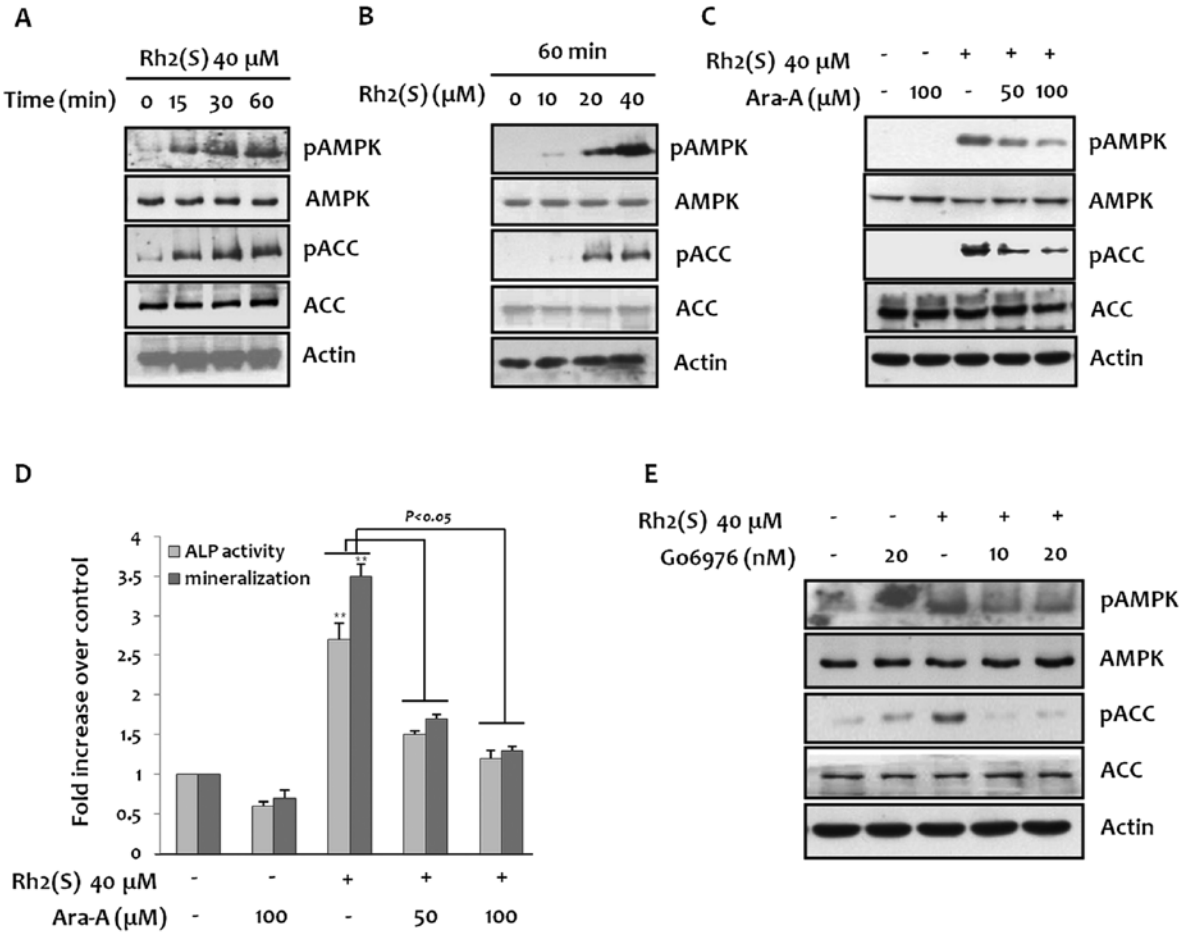


Figure 4. Effect of Rh(S) on AMPK activation in MC3T3-E1 cells via PKD signaling. (A and B) Cells were starved in serum-free medium for 2 h and treated with 40 $\mu$M Rh2(S) for up to 60 min or treated with 10 to 40 $\mu$M Rh2(S) for 60 min. The phosphorylation levels of AMPK and ACC were analyzed by Western blot analysis. (C) Cells were pretreated with Ara-A as indicated for 2 h and exposed to 40 $\mu$M Rh2(S) for 1 h. (D) Cells were incubated in the osteogenic medium containing Rh2(S) in the absence or presence of Ara-A until the 14th day. ALP activity was analyzed on the 7th day, and the mineralized nodules were stained by Alizarin Red on Day 14. Quantification of Alizarin Red staining was performed by extraction with ethylpyridium chloride. (E) Cells were pretreated with Go6976 as indicated for 2 h and exposed to 40 $\mu$M Rh2(S) for 1 h. The phosphorylation levels of AMPK and ACC were analyzed by Western blot analysis. Each value is the mean ± SEM fold increase over control values (n>6). $^{*}$P<0.05 and $^{**}$P<0.01.

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 PKC$\mu$/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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