Resveratrol amplifies BMP-4-stimulated osteoprotegerin synthesis via p38 MAP kinase in osteoblasts

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Abstract. Resveratrol is a naturally occurring polyphenol that possesses health-related properties, and is predominantly found in grapes and berries. Bone morphogenetic protein-4 (BMP-4) stimulates osteocalcin synthesis via p38 mitogen-activated protein (MAP) kinase in osteoblast-like MC3T3-E1 cells. The present study aimed to investigate the effects of resveratrol on BMP-4-induced osteoprotegerin (OPG) synthesis in MC3T3-E1 cells. Resveratrol alone had no effect on OPG expression levels, but significantly enhanced BMP-4-induced OPG release. In addition, resveratrol markedly amplified the mRNA expression levels of BMP-4-induced OPG. SB203580 is an inhibitor of p38 MAP kinase, which was shown to suppress BMP-4-stimulated OPG release. BMP-4-induced phosphorylation of p38 MAP kinase was also enhanced by resveratrol. Furthermore, SB203580 significantly reduced the resveratrol-induced amplification of BMP-4-stimulated OPG release. These results suggested that resveratrol was able to upregulate BMP-4-stimulated OPG synthesis via the amplification of p38 MAP kinase activity in osteoblasts.

Introduction

Bone metabolism is predominantly regulated by two types of functional cells: Osteoblasts and osteoclasts, which are responsible for bone formation and resorption, respectively (1). The formation and remodeling of bone structures result from the combined action of osteoblasts and osteoclasts. Bone resorption and formation are highly regulated in order to maintain adequate bone mass. Osteoblasts also have a role in the regulation of bone resorption via receptor activator of nuclear factor-κB (RANK) ligand (RANKL) expression, in response to bone resorptive stimuli (2). The binding of RANKL to RANK, which is located at the cell surface of osteoclastic precursors and mature osteoclasts, stimulates osteoclastic differentiation and activation (2). Metabolic bone diseases such as osteoporosis are caused by disordered bone remodeling. Numerous humoral factors are crucial to the bone remodeling process, including prostaglandins such as prostaglandin E2, and cytokines such as interleukin-1 (3).

Osteoprotegerin (OPG) is a secreted protein synthesized by osteoblasts, which inhibits osteoclastic differentiation and activation (4). OPG and RANK belong to the tumor necrosis factor receptor family. OPG acts as a decoy receptor by binding RANKL, which prevents it from binding to RANK, resulting in the suppression of bone resorption (4). RANKL knock-out mice have been shown to suffer from severe osteopetrosis (5). The RANK/RANKL/OPG axis is currently recognized as a major regulatory system for functional osteoclast formation (6).

Bone morphogenetic proteins (BMPs), including transforming growth factor-β (TGF-β) and activin, are multifunctional cytokines that belong to the TGF-β superfamily (7). BMPs promote bone formation via stimulation of osteoblastic proliferation and differentiation (8). BMP intracellular signaling occurs predominantly via the Smad (Smad 1/5/8)-dependent signaling pathway (5). In addition, previous studies have suggested that Smad-independent signaling, such as mitogen-activated protein (MAP) kinase signaling, also participates in BMP signaling (9,10). A previous study demonstrated that BMP-4 stimulates osteocalcin synthesis in osteoblast-like MC3T3-E1 cells, and that osteocalcin synthesis is positively regulated by p38 MAP kinase (11). In addition, BMP-4 was shown to stimulate the release of vascular endothelial growth factor (VEGF) via p38 MAP kinase in MC3T3-E1 cells (12). BMP also stimulates OPG production in osteoblasts (13). However, the precise mechanism underlying BMP-4-induced OPG synthesis in osteoblasts remains to be elucidated.

It is widely accepted that polyphenolic compounds in foods such as fruits and vegetables are beneficial to humans. Among them, flavonoids exhibit antioxidative, anti-inflammatory and anti-carcinogenic effects (14,15). Resveratrol is a

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naturally occurring polyphenolic flavonoid present in grapes and berries, which has been shown to increase murine life span (16). In addition, the observed low mortality rates due to coronary heart disease in France may be associated with the consumption of wine, which is known to contain abundant resveratrol (17). The effects of resveratrol are mediated by the longevity gene SIRT1, which improves the functioning of cells and organs by activating the NAD^+-dependent histone deacetylase (16). NAD^+ is a coenzyme of oxidoreductase synthesized as a precursor of nicotinamide, which has an important role in energy acquisition. Regarding the effects of resveratrol on osteoblasts, a previous study reported that resveratrol was able to stimulate osteoblastic differentiation (18). However, the precise mechanism underlying the effects of resveratrol on bone metabolism remains to be elucidated.

The present study aimed to investigate the effects of resveratrol on BMP-4-stimulated OPG synthesis in osteoblast-like MC3T3-E1 cells.

Materials and methods

Materials. Resveratrol and SB203580 were purchased from EMD Millipore (Billerica, MA, USA). BMP-4 and mouse OPG ELISA kits were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Phospho specific p38 MAP kinase (cat. no. 4511S), p38 MAP kinase antibodies (cat. no. 9212) and anti-rabbit IgG, horseradish peroxidase (HRP)-linked antibodies (cat. no. 7074) were obtained from Cell Signaling Technology (Danvers, MA, USA). The Enhanced Chemiluminescence (ECL) Western Blotting Detection system was obtained from GE Healthcare Life Sciences (Chalfont, UK). α-Minimum Essential medium (α-MEM) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Gibco Life Technologies (Grand Island, NY, USA). Polyvinylidene fluoride membranes were obtained from Bio-Rad Laboratories, Inc. (Berkeley, CA, USA). Resveratrol and SB203580 were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect the OPG assay or western blot analysis.

Cell culture. Cloned osteoblast like MC3T3 E1 cells were generously provided by Dr M. Kumegawa (Meikai University, Sakado, Japan). The cells were derived from newborn mouse calvaria (19) and were maintained as previously described (20). Briefly, the cells were cultured in α-MEM supplemented with 10% FBS at 37˚C in a humidified atmosphere containing 5% CO_2. The cells were subsequently seeded at a density of $5x10^4$ cells/dish onto 35 mm diameter dishes, or at a density of $2x10^5$ cells/dish onto 90 mm diameter dishes in α-MEM supplemented with 10% FBS. Following a period of five days, the medium was exchanged for α-MEM supplemented with 0.3% FBS. The cells were incubated for 48 h prior to further experimentation.

OPG assay. The cultured cells were treated with 10, 30, 50 or 70 μM resveratrol or 10, 20 or 30 μM SB203580 for 60 min, prior to being stimulated with either 70 ng/ml of BMP-4 or vehicle in 1 ml α-MEM supplemented with 0.3% FBS, and incubated for 48 h. The vehicle was a solvent, containing phosphate-buffered saline (PBS) supplemented with 0.1% bovine serum albumin, in which BMP-4 was dissolved. The conditioned medium was subsequently collected, and the OPG concentration was measured using the mouse OPG ELISA kit, according to the manufacturer's instructions. The absorbance was determined using an ELISA plate reader (Multiskan JX; Thermo Labsystems, Inc., Franklin, MA, USA).

Western blot analysis. The cultured cells were treated with 10, 30 or 50 μM resveratrol for 60 min, prior to being stimulated with either 70 ng/ml BMP-4 or vehicle in 1 ml α-MEM supplemented with 0.3% FBS for 2 h. The cells were then washed twice with PBS prior to being lysed. Adherent cells were scraped off the dish using a plastic cell scraper, then the cell suspension was gently transferred into a microcentrifuge tube in a lysis buffer containing 62.5 mM Tris/HCl (pH 6.8), 2% SDS, 50 mM dithiothreitol, and 10% glycerol, and then sonicated with 20 short burst of 1 sec. SDS-PAGE was performed according to the Laemmli method (21) in 10% polyacrylamide gels. A western blot analysis was performed as previously described (22). The membranes were blocked with 5% fat-free dry milk in Tris-buffered saline with Tween (TBST; 20 mM Tris/HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20) for 1 h prior to incubation with the primary antibodies. The membranes were then incubated with the primary antibodies targeting phospho specific p38 MAP kinase and p38 MAP kinase, all at a dilution of 1:1,000 in 5% milk in TBST overnight at 4˚C, using goat peroxidase-labeled antibodies and anti-rabbit IgG HRP-linked secondary antibody (cat. no. 7074) at a dilution of 1:1,000 in 5% milk in TBST for 1 h at room temperature (Cell Signaling Technology). The peroxidase activity on the polyvinylidene fluoride membrane (Bio-Rad Laboratories, Inc.) was visualized on an X-ray film using the ECL Western Blotting Detection system.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The cultured cells were treated with either 50 μM resveratrol or vehicle for 60 min, prior to being stimulated with either 70 ng/ml BMP-4 or vehicle in α-MEM supplemented with 0.3% FBS for 3 h. Total RNA was isolated and reverse transcribed into cDNA using TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and Omniscript Reverse Transcriptase kit (Qiagen Inc., Valencia, CA, USA). RT-qPCR was performed in capillaries using a Light Cycler 1.2 system and the Fast Start DNA Master SYBR Green I kit (Roche Diagnostics, Basel, Switzerland). The sense and antisense primers (primer set ID: MA026526) for mouse OPG and GAPDH mRNA were purchased from Takara Bio, Inc. (Otsu, Japan). The primer sequences were as follows: Forward 5'-CAATGG CTGGCTTGGTTTCATAG-3' and reverse 5'-CTGAACCAG ACATGACAGCTGGA-3'. The sense and antisense primers for mouse GAPDH mRNA were synthesized based on the study of Simpson *et al* (23) and obtained from Sigma-Aldrich. The primer sequences were as follows: Forward 5'-AACGACCCCTTCATT GAC-3' and reverse 5'-TCCACGACATACTCAGCAC-3'. The reaction mixtures were incubated at 95˚C for 10 min, followed by 40 cycles at 60˚C for 5 sec and 72˚C for 7 sec. The amplified products were determined by melting curve analysis and 1% agarose gel electrophoresis. The mRNA expression levels of OPG were normalized to those of GAPDH.

Densitometric analysis. A densitometric analysis was performed using ImageJ 1.32 (National Institutes of Health, Bethesda, MA, USA) image analysis software program. The background-subtracted signal intensity of each phosphorylation signal was normalized to the respective total protein signal and plotted as the fold increase, as compared with non-stimulated control cells.

Statistical analysis. Differences between the mean values for individual groups were assessed with a one-way analysis of variance, followed by a Bonferroni analysis in order to carry out multiple comparisons between pairs. Microsoft Excel 2010 (Redmond, WA, USA) was used for statistical analysis. $P<0.05$ was considered to indicate a statistically significant difference. All data are presented as the mean ± standard error of the mean, and all experiments were performed in triplicate from three independent cell preparations.

Results

Effects of resveratrol on BMP-4-stimulated OPG release in MC3T3-E1 cells. BMP-4 has previously been shown to stimulate the synthesis of osteocalcin and VEGF in osteoblast-like MC3T3-E1 cells (11,12). In addition, BMP-2 induces the synthesis of OPG in human osteoblasts (13). The present study investigated whether BMP-4 stimulated OPG synthesis in MC3T3-E1 cells. The results indicated that BMP-4 significantly increased the release of OPG (Fig. 1). The present study also investigated the effects of resveratrol on BMP-4-stimulated OPG release. The results suggested that although resveratrol alone had little effect on the synthesis of OPG, it significantly enhanced BMP-4-stimulated OPG release in a dose-dependent manner (10-70 μM) in MC3T3-E1 cells (Fig. 1). The maximum effect of resveratrol was observed at 70 μM, which caused a ~230% increase in the release of OPG.

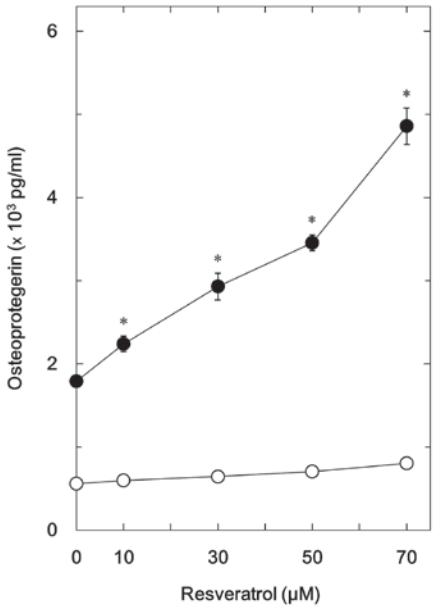

Figure 1. Effects of resveratrol on bone morphogenetic protein (BMP)-4-stimulated osteoprotegerin (OPG) release in MC3T3-E1 osteoblastic-like cells. The cultured cells were treated with various doses of resveratrol for 60 min, prior to being stimulated with either 70 ng/ml BMP-4 (black) or vehicle (white) for 48 h. The OPG concentrations of the culture medium were determined by ELISA. Each value is presented as the mean ± standard error of the mean. The experiments were performed in triplicate from three independent cell preparations. *P<0.05, vs. treatment with BMP-4 alone.

Effects of resveratrol on the BMP-4-induced mRNA expression of OPG in MC3T3-E1 cells. The results of the RT-qPCR demonstrated that BMP-4 induced the mRNA expression of OPG in osteoblast-like MC3T3-E1 cells (Fig. 2). In order to investigate whether the amplifying effects of resveratrol on BMP-4-stimulated OPG release were mediated through transcription, the effects of resveratrol on BMP-4-induced OPG mRNA expression were also examined. Resveratrol alone failed to affect the mRNA expression levels of OPG, but markedly enhanced BMP-4-induced OPG mRNA expression (Fig. 2). These findings were concordant with the observed increase in BMP-4-stimulated OPG release following resveratrol treatment.

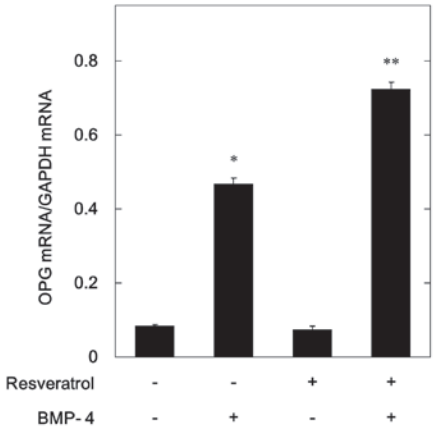

Figure 2. Effects of resveratrol on bone morphogenetic protein (BMP)-4-induced osteoprotegerin (OPG) mRNA expression in MC3T3-E1 osteoblastic-like cells. The cultured cells were treated with either 50 μM resveratrol or vehicle for 60 min, prior to being stimulated with either 70 ng/ml BMP-4 or vehicle for 3 h. The total RNA was then isolated and reverse transcribed into cDNA. The mRNA expression levels of OPG and GAPDH were quantified by reverse trancription-quantitative polymerase chain reaction. The mRNA expression levels of OPG were normalized to those of GAPDH. Each value is presented as the mean ± standard error of the mean. The experiments were performed in triplicate from three independent cell preparations. *P<0.05, vs. the control. **P<0.05, vs. treatment with BMP-4 alone.

Effects of SB203580 on BMP-4-stimulated OPG release in MC3T3-E1 cells. Results from previous studies have suggested that Smad-independent signaling, such as MAP kinase signaling, mediates the effects of BMPs (9,10). p38 MAP kinase has previously been reported to function in BMP-4-stimulated osteocalcin synthesis as a positive regulator in osteoblast-like MC3T3-E1 cells (11). p38 MAP kinase has also been shown to upregulate BMP-4-stimulated VEGF synthesis in MC3T3-E1 cells (12). In order to determine whether p38 MAP kinase is involved in BMP-4-induced OPG synthesis in MC3T3-E1 cells, the effects of SB203580, an inhibitor of p38 MAP kinase (24), were examined on BMP-4-stimulated OPG release. Treatment with SB203580 alone had little effect on OPG release, but significantly reduced BMP-4-stimulated OPG release in a dose-dependent manner (10-30 μM) (Fig. 3).

Table I. Effects of SB203580 on the resveratrol enhancement of BMP-4-stimulated OPG release in MC3T3-E1 osteoblastic-like cells.

SB203580	Resveratrol	BMP-4	OPG (pg/ml)
-	-	-	576±17
-	-	+	2,268±46[a]
-	+	-	632±14
-	+	+	4,610±265[b]
+	-	-	443±31
+	-	+	948±16[b]
+	+	-	457±20
+	+	+	1,522±41[c]

The cultured cells were treated with either 10 μM SB203580 or vehicle for 60 min, prior to being incubated with either 50 μM resveratrol or vehicle for 60 min. The cells were subsequently stimulated with either 50 ng/ml bone morphogenetic protein (BMP)-4 or vehicle for 48 h. The osteoprotegerin (OPG) concentrations in the culture medium were determined by ELISA. Each value is presented as the mean ± standard error of the mean. The experiments were performed in triplicate from three independent cell preparations. [a]P<0.05, vs. the control. [b]P<0.05, vs. treatment with BMP-4 alone. [c]P<0.05, vs. treatment with BMP-4 and resveratrol.

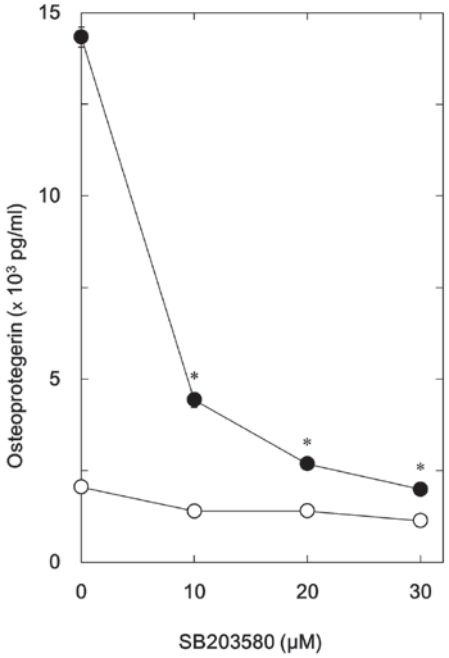

Figure 3. Effects of SB203580 on bone morphogenetic protein (BMP)-4-stimulated osteoprotegerin (OPG) release in MC3T3-E1 osteoblastic-like cells. The cultured cells were treated with various doses of SB203580 for 60 min, prior to being stimulated with either 70 ng/ml BMP-4 (black) or vehicle (white) for 48 h. The OPG concentrations in the culture medium were determined by ELISA. Each value is presented as the mean ± standard error of the mean. All experiments were performed in triplicate from three independent cell preparations. *P<0.05, vs. treatment with BMP-4 alone.

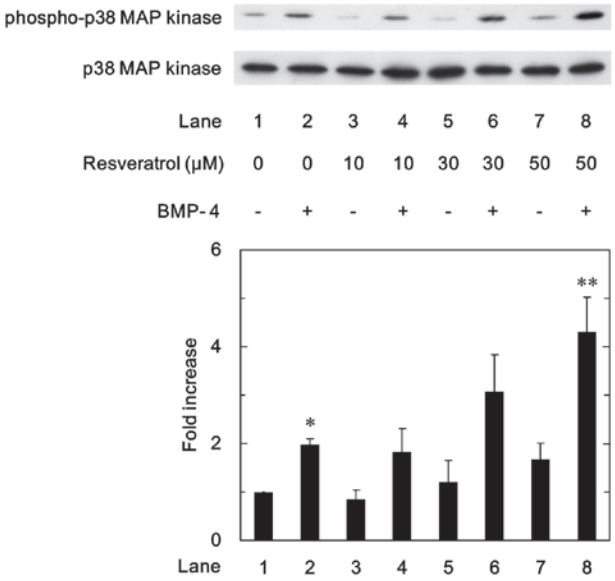

Figure 4. Effects of resveratrol on the bone morphogenetic protein (BMP)-4-induced phosphorylation of p38 mitogen-activated protein (MAP) kinase in MC3T3-E1 osteoblast-like cells. The cultured cells were treated with various doses of resveratrol for 60 min, prior to being stimulated with either 70 ng/ml BMP-4 or vehicle for 2 h. The cell extracts were then subjected to SDS-PAGE and western blotting, using antibodies targeting phospho-specific p38 MAP kinase or p38 MAP kinase. The histogram shows the quantitative representation of the levels of BMP-4-induced phosphorylation obtained from densitometric analyses. Each value is presented as the mean ± standard error of the mean. All experiments were performed in triplicate from three independent cell preparations. *P<0.05, vs. the control. **P<0.05, vs. treatment with BMP-4 alone.

Effects of resveratrol on BMP-4-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. In order to clarify whether the enhancing effects of resveratrol on BMP-4-stimulated OPG synthesis were mediated by the modulation of p38 MAP kinase activation in osteoblast-like MC3T3-E1 cells, the effects of resveratrol on BMP-4-induced phosphorylation of p38 MAP kinase were examined. The results indicated that resveratrol significantly enhanced BMP-4-induced phosphorylation of p38 MAP kinase in a dose-dependent manner (10-50 μM) (Fig. 4). Conversely, a previous study demonstrated that resveratrol did not affect BMP-4-induced phosphorylation of Smad 1/5/8 in MC3T3-E1 cells (25).

Effects of SB203580 on resveratrol enhancement of BMP-4-stimulated OPG release in MC3T3-E1 cells. The present study examined the effects of SB203580 on

resveratrol enhancement of BMP-4-stimulated OPG release in MC3T3-E1 cells. The results indicated that SB203580 significantly suppressed the resveratrol-induced amplification of BMP-4-stimulated OPG release (Table I). SB203580 caused a ~70% decrease in the effects of resveratrol-enhanced BMP-4.

Discussion

The results of the present study demonstrated that resveratrol significantly enhanced BMP-4-stimulated OPG release in osteoblast-like MC3T3-E1 cells. In addition, resveratrol was able to amplify BMP-4-induced mRNA expression levels of OPG in MC3T3-E1 cells. Therefore, it is likely that the enhancing effects of resveratrol on BMP-4-stimulated OPG release are mediated through transcriptional events. To the best of our knowledge, this is the first study to report the resveratrol enhancement of BMP-4-stimulated OPG synthesis in osteoblasts.

Based on these results, the mechanism underlying resveratrol enhancement of BMP-4-induced OPG synthesis was investigated in the osteoblast-like cells. Previous studies have demonstrated that the effects of BMPs are exerted through the intracellular signaling of both Smad-dependent, and Smad-independent pathways such as MAP kinase signaling (7,9,10). In Smad-dependent signaling, resveratrol previously failed to affect BMP-4-induced phosphorylation of Smad 1/5/8 in osteoblast-like MC3T3-E1 cells (25). These results suggested that it is unlikely that the resveratrol enhancement of BMP-4-induced OPG synthesis is mediated through the Smad-dependent pathway in osteoblast-like MC3T3-E1 cells. Previous studies have demonstrated that p38 MAP kinase acts as a positive regulator in the BMP-4-stimulated synthesis of both osteocalcin and VEGF in osteoblast-like MC3T3-E1 cells (11,12). In addition, a previous study examined the effects of SB203580 on BMP-4-stimulated OPG synthesis in MC3T3-E1 cells, and demonstrated that SB203580 significantly reduced BMP-4-stimulated OPG release (24). The results of the present study suggested that p38 MAP kinase was also involved in BMP-4-stimulated OPG synthesis in osteoblast-like MC3T3-E1 cells. In addition, resveratrol was shown to markedly enhance BMP-4-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. Furthermore, SB203580 significantly suppressed the resveratrol enhancement of BMP-4-stimulated OPG release. These results indicated that the resveratrol amplification of BMP-4-stimulated OPG synthesis may be mediated through the upregulation of p38 MAP kinase activity in osteoblast-like MC3T3-E1 cells.

Resveratrol is a naturally occurring polyphenol that is abundantly present in grapes and berries, and exhibits numerous beneficial effects on human health, such as anti-oxidation, anti-aging, and reduction of stress (26,27). BMP-4 increases bone formation and is an important regulator of fracture healing (8). OPG is a decoy receptor of RANKL, which blocks RANK/RANKL interaction thereby preventing osteoclast differentiation and activation (4). The results of the present study suggested that the resveratrol enhancement of BMP-4-induced OPG synthesis led to an increase in bone formation, and a suppression of bone resorption. Therefore, resveratrol may prove useful in the treatment of skeletal conditions via modulation of osteoblast function. Further investigation is required in order to clarify the mechanism underlying resveratrol amplification of OPG synthesis in osteoblasts.

In conclusion, the results of the present study suggested that resveratrol was able to upregulate BMP-4-stimulated OPG synthesis via amplification of p38 MAP kinase activity in osteoblasts.

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