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Regulation by resveratrol of prostaglandin E_2-stimulated osteoprotegerin synthesis in osteoblasts

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Abstract. Resveratrol is a natural polyphenol found in red grape skins, berries and red wine. Accumulating evidence suggests that resveratrol has various beneficial effects on the human body. In the present study, we investigated the effects of prostaglandin E_2 (PGE_2) on osteoprotegerin (OPG) synthesis and the effects of resveratrol on OPG synthesis in osteoblast-like MC3T3-E1 cells. PGE_2 significantly stimulated both the release of OPG and the mRNA expression levels of OPG, as shown by OPG assay and real-time RT-PCR, respectively. Resveratrol markedly suppressed the release and the mRNA levels of OPG induced by PGE_2. On the contrary, SRT1720, an activator of sirtuin 1 (SIRT1), hardly affected the PGE_2-induced release of OPG. PD98059 [a specific inhibitor of the upstream kinase that activates p44/p42 mitogen-activated protein (MAP) kinase], SB203580 (a specific inhibitor of p38 MAP kinase) and SP600125 [a specific inhibitor of stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK)], reduced the PGE_2-induced release of OPG. Resveratrol attenuated the PGE_2-induced phosphorylation of p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK. However, SRT1720 failed to affect the phosphorylation of p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK induced by PGE_2. These results strongly suggest that resveratrol reduces PGE_2-stimulated OPG synthesis through the inhibition of p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK in osteoblasts, and that these suppressive effects are independent of the activation of SIRT1.

Introduction

Osteoblasts and osteoclasts are functional cells, which are responsible for bone formation and bone resorption, respectively (1). These two types of cells are major regulators of bone metabolism. Bone remodeling is essential for maintaining the structure and strength of skeletal tissue and is caused by the coupling processes of osteoclastic bone resorption and osteoblastic bone formation. It is generally recognized that numerous humoral factors, including prostaglandins (PGs) and cytokines play important roles in the process of bone remodeling (2). Osteoblasts possess the receptors of a number of bone resorptive factors, such as parathyroid hormone, which promotes the formation of functional osteoclasts through the upregulation of receptor activator of nuclear factor-κB (RANK) ligand (RANKL) (1,2). Therefore, osteoblasts are considered to play pivotal roles in the regulation of not only bone formation, but also bone resorption. Osteoprotegerin (OPG) is a glycoprotein, belonging to the tumor necrosis factor receptor superfamily (3). OPG, which is produced and secreted from osteoblasts, binds to RANKL as a decoy receptor and inhibits the binding of RANKL to RANK, an essential step of osteoclastogenesis (development of osteoclast-precursor cells into mature osteoclasts) (3,4). Thus, it is currently recognized that the RANK/RANKL/OPG axis is an important regulatory system for functional osteoclast formation (5).

It has been firmly established that PGs act as autocrine and paracrine regulators for osteoblasts and play essential roles in the regulation of bone metabolism (6,7). Among these, prostaglandin E_2 (PGE_2) is a potent stimulator of bone resorption as it enhances osteoclast formation (8). As regards the intracellular signaling of PGE_2 in osteoblasts, we have previously demonstrated that PGE_2 stimulates interleukin-6 (IL-6) synthesis through Ca^{2+} mobilization and cAMP production in osteoblast-like MC3T3-E1 cells (9). In addition, we have previously demonstrated that PGE_2 stimulates the induction of heat shock protein 27 (HSP27) through Ca^{2+} mobilization and protein kinase C (PKC)-dependent activation of both p44/p42 mitogen-activated protein (MAP) kinase and p38 MAP kinase in MC3T3-E1 cells (10). However, the effects of PGE_2 on the synthesis of OPG in osteoblasts and the mechanisms involved have not yet been elucidated.

Resveratrol, which is a natural polyphenol abundantly found in red grapes and berries, is recognized as possessing antioxidant properties that exert various beneficial effects on human health (11). It has been reported that resveratrol increases the life span of lower organisms by activating sirtuin 1 (SIRT1), a

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nicotinamide adenine dinucleotide (NAD^+)-dependent class III deacetylase (12). The various beneficial effects of resveratrol are considered to be mediated through the activation of SIRT1 in mammalians as well (13). As regards bone cells, we have recently reported that resveratrol suppresses the synthesis of vascular endothelial growth factor (VEGF) stimulated by bone morphogenetic protein-4 (BMP-4) in a SIRT1 activation-dependent manner in osteoblast-like MC3T3-E1 cells (14). However, the mechanisms underlying the effects of resveratrol on bone metabolism remain to be elucidated.

In the present study, we investigated the mechanisms of PGE_2-induced OPG synthesis and the effects of resveratrol on OPG synthesis by PGE_2 in osteoblast-like MC3T3-E1 cells. We demonstrate that resveratrol suppresses the PGE_2-stimulated OPG synthesis by inhibiting p44/p42 MAP kinase, p38 MAP kinase and stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK) in osteoblasts and that the inhibitory effects are independent of the activation of SIRT1.

Materials and methods

Materials. Resveratrol, SRT1720, PD98059, SB203580 and SP600125 were obtained from Calbiochem-Novabiochem Co. (La Jolla, CA, USA). PGE_2 was obtained from Sigma Chemical Co. (St. Louis, MO, USA). A mouse OPG enzyme-linked immunosorbent assay (ELISA) kit was obtained from R&D Systems, Inc. (Minneapolis, MN, USA). Phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies and SAPK/JNK antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). An ECL Western Blotting Detection system was obtained from GE Healthcare (Buckinghamshire, UK). Other materials and chemicals were obtained from commercial sources. PGE_2 was dissolved in ethanol. Resveratrol, SRT1720, PD98059, SB203580 and SP600125 were dissolved in dimethyl sulfoxide. The maximum concentration of ethanol or dimethyl sulfoxide was 0.1%, which did not affect either the assay for OPG or the detection of the protein level using western blot analysis.

Cell culture. Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria (15) were generously provided by Dr M. Kumegawa (Meikai University, Sakado, Japan) and were maintained as previously described (16). Briefly, the cells were cultured in α-minimum essential medium (α-MEM) containing 10% fetal bovine serum (FBS) at 37˚C in a humidified atmosphere of 5% CO_2/95% air. The cells were seeded into 35-mm diameter dishes ($5x10^4$ cells/dish) or 90-mm diameter dishes ($2x10^5$ cells/dish) in α-MEM containing 10% FBS. After 5 days, the medium was exchanged for α-MEM containing 0.3% FBS. The cells were used for experiments after 48 h.

Assay for OPG. The cultured cells were pre-treated with various concentrations of resveratrol, SRT1720, PD98059, SB203580 or SP600125 for 60 min, and then stimulated by 10 μM of PGE_2 or the vehicle [mast cell medium (MCM); pH 7.4, 150 mM NaCl, 5 mM KCl, 5.5 mM glucose, 0.8 mM MgSO4, 1 mM CaCl2, 5 mM HEPES, 0.1% bovine serum albumin] in 1 ml of α-MEM containing 0.3% FBS for the indicated periods of time. The conditioned medium was collected at the end of the incubation period, and the OPG concentration was then measured using the OPG ELISA kit according to the manufacturer's instructions.

Real-time RT-PCR. The cultured cells were pre-treated with 50 μM of resveratrol or the vehicle for 60 min and were then stimulated with 10 μM of PGE_2 or the vehicle in α-MEM containing 0.3% FBS for 3 h. Total RNA was isolated and reverse transcribed into complementary DNA (cDNA) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and the Omniscript Reverse Transcriptase kit (Qiagen Inc., Valencia, CA, USA). Real-time RT-PCR was performed using LightCycler® Capillaries and FastStart DNA Master SYBR-Green I provided with the kit (Roche Diagnostics, Basel, Switzerland). Sense and antisense primers for mouse OPG mRNA or GAPDH mRNA were purchased from Takara Bio Inc. (Tokyo, Japan) (primer set ID: MA026526). The amplified products were determined using a melting curve analysis and agarose electrophoresis. The OPG mRNA levels were normalized to those of GAPDH mRNA.

Western blot analysis. The cultured cells were pre-treated with various concentrations of resveratrol or SRT1720 for 60 min and were then stimulated with PGE_2 or the vehicle in α-MEM containing 0.3% FBS for the indicated periods of time. The cells were washed twice with phosphate-buffered saline and then lysed, homogenized and sonicated in lysis buffer containing 62.5 mM Tris/HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol. SDS-polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Laemmli (17) in 10% polyacrylamide gels. The protein was fractionated and transferred onto Immun-Blot® polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% fat-free dry milk in Tris-buffered saline with Tween-20 (TBST; 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20) for 2 h prior to incubation with primary antibodies. Western blot analysis was performed as previously described (18) using phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies or SAPK/JNK antibodies as primary antibodies, with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as secondary antibodies. The primary and secondary antibodies were diluted at 1:1,000 with 5% fat-free dry milk in TBST. The peroxidase activity on the PVDF membranes was visualized on X-ray film using the ECL Western Blotting Detection system.

Densitometric analysis. Densitometric analysis was performed using a scanner and image analysis software (ImageJ software version 1.47). The phosphorylated protein levels were calculated as follows: the background-subtracted signal intensity of each phosphorylation signal was respectively normalized to the total protein signal and plotted as an absolute value.

Statistical analysis. All the data were analyzed by ANOVA followed by the Bonferroni method for multiple comparisons between pairs, and a value of $P<0.05$ was considered to indicate a statistically significant difference. All data are presented as the means ± standard error of the mean (SEM) of triplicate determinations obtained from 3 independent cell preparations.

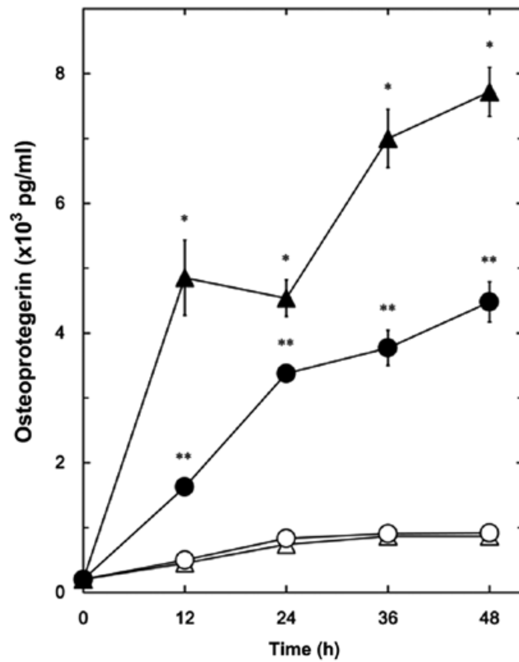

Figure 1. Effects of resveratrol on the prostaglandin E_2 (PGE_2)-stimulated release of osteoprotegerin (OPG) in MC3T3-E1 cells. The cultured cells were pre-treated with 50 μM of resveratrol (● and ○) or the vehicle (▲ and △) for 60 min, and then stimulated with 10 μM of PGE_2 (● and ▲) or the vehicle (○ and △) for the indicated periods of time. OPG concentrations in the culture medium were determined by ELISA. Each value represents the mean ± SEM of triplicate determinations from 3 independent cell preparations. *P<0.05, compared to the value of the control (vehicle). **P<0.05, compared to the value of stimulation with PGE_2 alone.

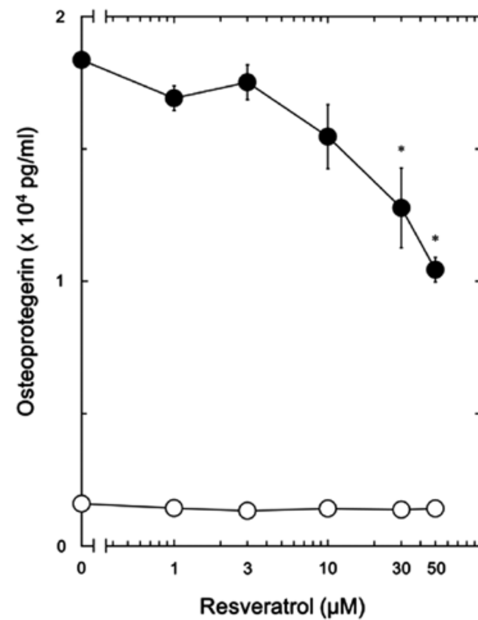

Figure 2. Effects of resveratrol on the prostaglandin E_2 (PGE_2)-stimulated release of osteoprotegerin (OPG) in MC3T3-E1 cells. The cultured cells were pre-treated with various concentrations of resveratrol for 60 min and then stimulated with 10 μM of PGE_2 (●) or the vehicle (○) for 48 h. OPG concentrations in the culture medium were determined by ELISA. Each value represents the mean ± SEM of triplicate determinations from 3 independent cell preparations. *P<0.05, compared to the value of stimulation with PGE_2 alone.

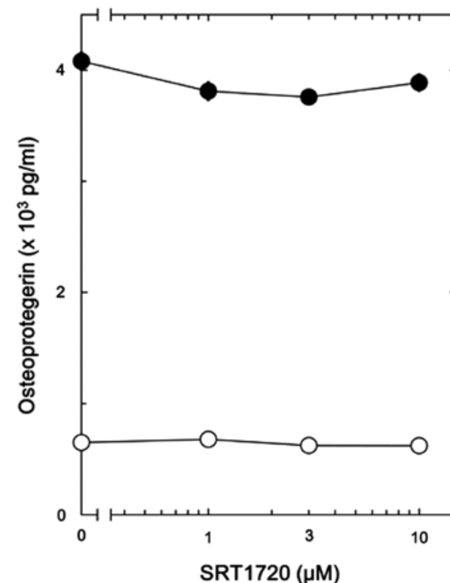

Figure 3. Effects of SRT1720 on the prostaglandin E_2 (PGE_2)-stimulated release of osteoprotegerin (OPG) in MC3T3-E1 cells. The cultured cells were pre-treated with various concentrations of SRT1720 for 60 min and then stimulated with 10 μM of PGE_2 (●) or the vehicle (○) for 48 h. OPG concentrations in the culture medium were determined by ELISA. Each value represents the mean ± SEM of triplicate determinations from 3 independent cell preparations.

Results

Effects of resveratrol on the PGE_2-stimulated release of OPG in MC3T3-E1 cells. First we examined whether PGE_2 stimulates the release of OPG in osteoblast-like MC3T3-E1 cells. PGE_2 significantly promoted the release of OPG in a time-dependent manner up to 48 h (Fig. 1). We then investigated the effects of resveratrol on the PGE_2-stimulated release of OPG in these cells. Resveratrol, which alone did not affect the OPG levels compared with the vehicle, significantly suppressed the PGE_2-stimulated release of OPG (Fig. 1). The inhibitory effects of resveratrol were dose-dependent in the dose range between 1 and 50 μM (Fig. 2). The most prominent effects of resveratrol were observed at the dose of 50 μM, which inhibited the effects of PGE_2 by approximately 50%.

Effects of SRT1720 on the PGE_2-stimulated release of OPG in MC3T3-E1 cells. SRT1720 is known as an activator of SIRT1 with a potency 1,000-fold greater than that of resveratrol (19). To investigate whether the effects of resveratrol on the PGE_2-stimulated release of OPG are mediated by the activation of SIRT1, we examined the effects of SRT1720 on the PGE_2-stimulated release of OPG in the osteoblast-like MC3T3-E1 cells. SRT1720 failed to affect the PGE_2-stimulated release of OPG from these cells (Fig. 3).

Effects of resveratrol on the PGE_2-induced mRNA expression of OPG in MC3T3-E1 cells. In order to elucidate whether the suppressive effects of resveratrol on the PGE_2-stimulated release of OPG are mediated through transcriptional events, we further examined the effects of resveratrol on the PGE_2-induced mRNA expression of OPG by real-time RT-PCR. Resveratrol, which on its own exerted minimal effects on the

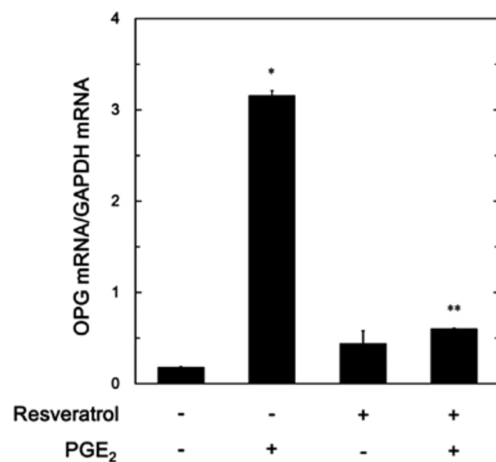

Figure 4. Effects of resveratrol on the prostaglandin E_2 (PGE_2)-induced mRNA expression of osteoprotegerin (OPG) in MC3T3-E1 cells. The cultured cells were pre-treated with 50 μM of resveratrol or the vehicle for 60 min and then stimulated with 10 μM of PGE_2 or the vehicle for 3 h. The respective total RNA was then isolated and quantified by real-time RT-PCR. Each value represents the mean ± SEM of triplicate determinations from 3 independent cell preparations. [*]P<0.05 compared to the value of the control (vehicle). [**]P<0.05 compared to the value of stimulation with PGE_2 alone.

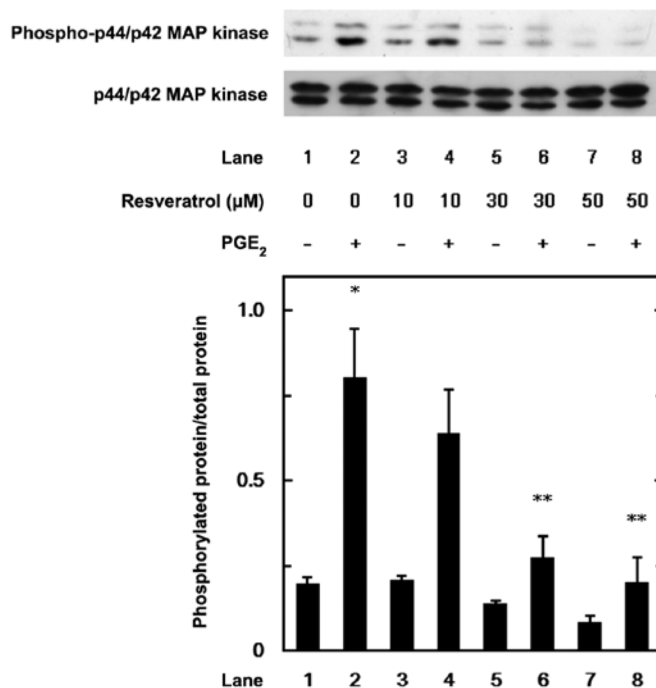

Figure 5. Effects of resveratrol on the prostaglandin E_2 (PGE_2)-induced phosphorylation of p44/p42 mitogen-activated protein (MAP) kinase in MC3T3-E1 cells. The cultured cells were pre-treated with various concentrations of resveratrol for 60 min and then stimulated with 10 μM of PGE_2 or the vehicle for 10 min. The cell extracts were then subjected to SDS-PAGE with subsequent western blot analysis with antibodies against phospho-specific p44/p42 MAP kinase or p44/p42 MAP kinase. The histogram shows the quantitative representation of the levels of PGE_2-induced phosphorylation obtained from a laser densitometric analysis of 3 independent experiments. Each value represents the mean ± SEM of triplicate determinations. [*]P<0.05, compared to the value of the control (vehicle). [**]P<0.05 compared to the value of stimulation with PGE_2 alone.

mRNA levels of OPG, markedly decreased the PGE_2-induced mRNA expression levels of OPG (Fig. 4).

Table I. Effects of PD98059, SB203580 or SP600125 on the PGE_2-stimulated release of OPG in MC3T3-E1 cells.

Inhibitors	PGE_2	OPG (pg/ml)
-	-	1,128±28
-	+	11,424±857[a]
PD98059	-	1,310±106
PD98059	+	7,018±510[b]
SB203580	-	982±1
SB203580	+	5,809±65[b]
SP600125	-	1,176±97
SP600125	+	6,508±827[b]

The cultured cells were pre-treated with 50 μM of PD98059, 3 μM of SB203580, 10 μM of SP600125 or the vehicle for 60 min, and then stimulated with 10 μM of PGE_2 or the vehicle for 48 h, followed by the measurement of OPG levels in the respective medium. Each value represents the mean ± SEM of triplicate determinations from 3 independent cell preparations. [a]P<0.05, compared to the value of the control (vehicle). [b]P<0.05, compared to the value of stimulation with PGE_2 alone. PGE_2, prostaglandin E_2; OPG, osteoprotegerin.

Effects of PD98059, SB203580 or SP600125 on the PGE_2-stimulated release of OPG in MC3T3-E1 cells. It is firmly established that the major MAP kinase superfamily, including p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK, is a central element used by mammalian cells to transduce various messages of extracellular stimuli (20). As regards PGE_2 intracellular signaling in osteoblasts, we have previously reported that p44/p42 MAP kinase and p38 MAP kinase are involved in the HSP27 induction by PGE_2 in osteoblast-like MC3T3-E1 cells (10). In order to clarify whether p44/p42 MAP kinase, p38 MAP kinase or SAPK/JNK are involved in the PGE_2-stimulated OPG synthesis in osteoblast-like MC3T3-E1 cells, we examined the effects of PD98059, a specific inhibitor of the upstream kinase activating p44/p42 MAP kinase (21), SB203580, a specific inhibitor of p38 MAP kinase (22) or SP600125, a specific inhibitor of SAPK/JNK (23), on the release of OPG stimulated by PGE_2. PD98059, SB203580 and SP600125, which alone had little effect on the release of OPG, markedly reduced the PGE_2-stimulated release of OPG in these cells (Table I).

Effects of resveratrol or SRT1720 on the PGE_2-induced phosphorylation of p44/p42 MAP kinase, p38 MAP kinase or SAPK/JNK in MC3T3-E1 cells. To investigate whether resveratrol affects the PGE_2-stimulated OPG synthesis through the activation of p44/p42 MAP kinase, p38 MAP kinase or SAPK/JNK in osteoblast-like MC3T3-E1 cells, we examined the effects of resveratrol on the PGE_2-induced phosphorylation of p44/p42 MAP kinase, p38 MAP kinase or SAPK/JNK. Resveratrol suppressed the PGE_2-induced phosphorylation of p44/p42 MAP kinase (Fig. 5), p38 MAP kinase (Fig. 6) and SAPK/JNK (Fig. 7) in a dose-dependent manner at the dose range between 10 and 50 μM. We further examined the effects of SRT1720 on the PGE_2-induced phosphorylation of p44/

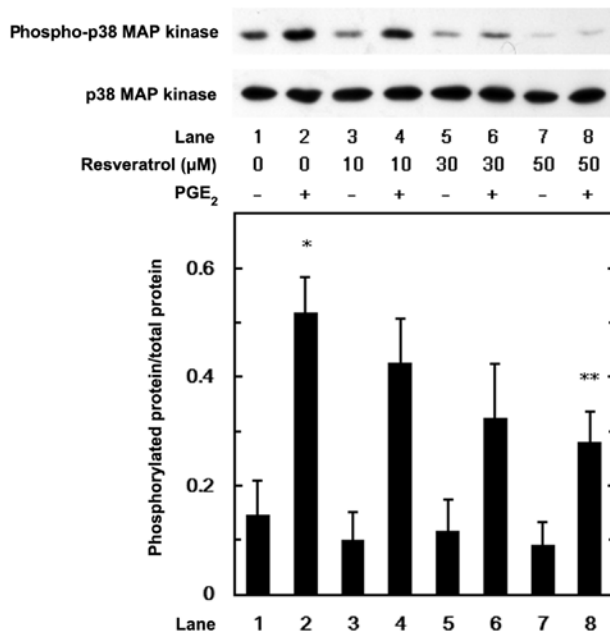

Figure 6. Effects of resveratrol on the prostaglandin E_2 (PGE_2)-induced phosphorylation of p38 mitogen-activated protein (MAP) kinase in MC3T3-E1 cells. The cultured cells were pre-treated with various concentrations of resveratrol for 60 min and then stimulated with 10 μM of PGE_2 or the vehicle for 3 min. The cell extracts were then subjected to SDS-PAGE with subsequent western blot analysis with antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase. The histogram shows the quantitative representation of the levels of PGE_2-induced phosphorylation obtained from a laser densitometric analysis of 3 independent experiments. Each value represents the mean ± SEM of triplicate determinations. *P<0.05, compared to the value of the control (vehicle). **P<0.05, compared to the value of stimulation with PGE_2 alone.

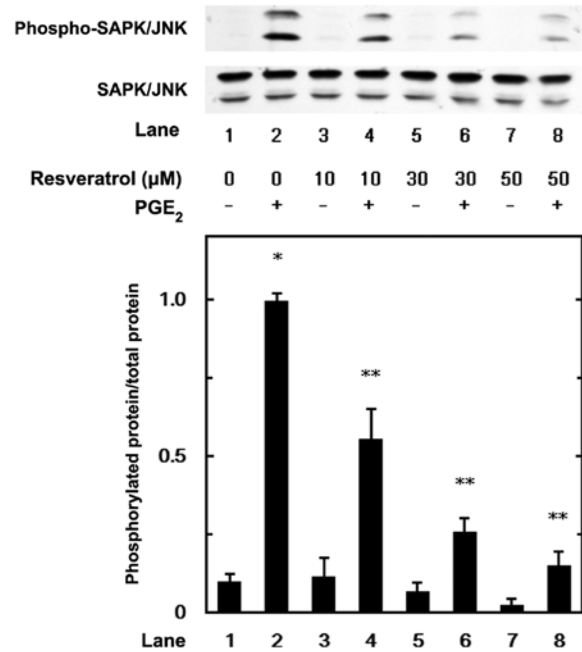

Figure 7. Effects of resveratrol on the prostaglandin E_2 (PGE_2)-induced phosphorylation of stress-activated protein kinase/*c-Jun* N-terminal kinase (SAPK/JNK) in MC3T3-E1 cells. The cultured cells were pre-treated with various concentrations of resveratrol for 60 min and then stimulated with 10 μM of PGE_2 or the vehicle for 20 min. The cell extracts were then subjected to SDS-PAGE with subsequent western blot analysis with antibodies against phospho-specific SAPK/JNK or SAPK/JNK. The histogram shows the quantitative representation of the levels of PGE_2-induced phosphorylation obtained from a laser densitometric analysis of 3 independent experiments. Each value represents the mean ± SEM of triplicate determinations. *P<0.05, compared to the value of the control (vehicle). **P<0.05, compared to the value of stimulation with PGE_2 alone.

p42 MAP kinase, p38 MAP kinase or SAPK/JNK. However, SRT1720 hardly affected the PGE_2-induced phosphorylation of p44/p42 MAP kinase, p38 MAP kinase or SAPK/JNK (Fig. 8).

Discussion

In the present study, we demonstrated that PGE_2 significantly stimulated the release of OPG in osteoblast-like MC3T3-E1 cells and that resveratrol markedly suppressed the PGE_2-stimulated release of OPG. Additionally, we demonstrated that PGE_2 upregulated the mRNA expression levels of OPG, and that resveratrol reduced the mRNA expression of OPG induced by PGE_2 in these cells. Therefore, these findings suggest that the suppressive effects of resveratrol on the PGE_2-stimulated release of OPG are mediated through transcriptional events in MC3T3-E1 cells. Thus, we further investigated the exact mechanisms behind the inhibitory effects of resveratrol on the PGE_2-stimulated OPG synthesis in osteoblasts.

The three major MAP kinases, p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK are recognized as central elements used by mammalian cells to transduce diverse messages (24) and play central roles in a variety of cellular functions, including proliferation, differentiation and survival (20). As regards the intracellular signaling of PGE_2 in osteoblasts, we have previously demonstrated that PGE_2 induces the activation of p44/p42 MAP kinase and p38 MAP kinase in osteoblast-like MC3T3-E1 cells, and that PGE_2 stimulates the induction of HSP27 through the PKC-dependent activation of both p44/p42 MAP kinase and p38 MAP kinase in MC3T3-E1 cells (10). In the present study, we found that PGE_2 stimulated the phosphorylation of SAPK/JNK in the MC3T3-E1 cells in a time-dependent manner and the most prominent effects of PGE_2 were observed at 20 min following stimulation (data not shown). It is generally established that MAP kinases are activated by the phosphorylation of threonine and tyrosine residues by dual-specificity MAP kinase kinase (24). Therefore, our findings suggest that PGE_2 stimulates the activation of SAPK/JNK in addition to that of p44/p42 MAP kinase and p38 MAP kinase in osteoblast-like MC3T3-E1 cells. Furthermore, we demonstrated that PD98059, a specific inhibitor of the upstream kinase activating p44/p42 MAP kinase (21), SB203580, a specific inhibitor of p38 MAP kinase (22) and SP600125, a specific inhibitor of SAPK/JNK (23) markedly reduced the PGE_2-stimulated release of OPG, suggesting that three major MAP kinases, namely the p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK function as positive regulators in the PGE_2-stimulated OPG synthesis in these cells. In addition, we demonstrated that resveratrol markedly suppressed the phosphorylation of p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK induced by PGE_2 in the MC3T3-E1 cells. Taking our findings into account, it is likely that resveratrol inhibits the PGE_2-induced OPG synthesis in osteoblast-like cells, and

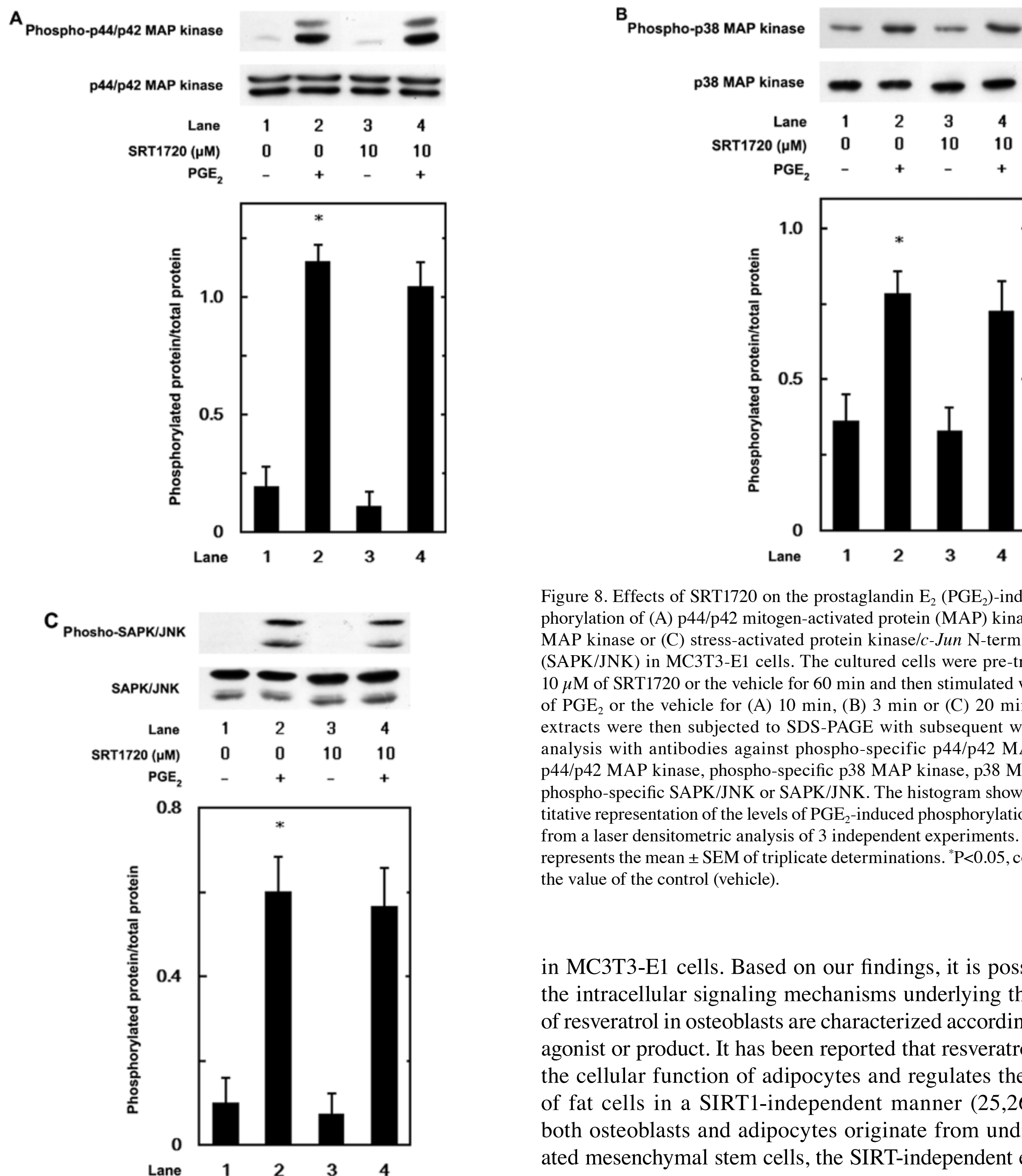

Figure 8. Effects of SRT1720 on the prostaglandin E_2 (PGE_2)-induced phosphorylation of (A) p44/p42 mitogen-activated protein (MAP) kinase, (B) p38 MAP kinase or (C) stress-activated protein kinase/*c-Jun* N-terminal kinase (SAPK/JNK) in MC3T3-E1 cells. The cultured cells were pre-treated with 10 μM of SRT1720 or the vehicle for 60 min and then stimulated with 10 μM of PGE_2 or the vehicle for (A) 10 min, (B) 3 min or (C) 20 min. The cell extracts were then subjected to SDS-PAGE with subsequent western blot analysis with antibodies against phospho-specific p44/p42 MAP kinase, p44/p42 MAP kinase, phospho-specific p38 MAP kinase, p38 MAP kinase, phospho-specific SAPK/JNK or SAPK/JNK. The histogram shows the quantitative representation of the levels of PGE_2-induced phosphorylation obtained from a laser densitometric analysis of 3 independent experiments. Each value represents the mean ± SEM of triplicate determinations. *P<0.05, compared to the value of the control (vehicle).

that the suppressive effects of resveratrol are exerted at a point upstream of three MAP kinases.

Evidence is accumulating that the beneficial effects of resveratrol are mediated through SIRT1 activation (13). On the other hand, we demonstrated that SRT1720, a potent activator of SIRT1 (19), exerted minimal effects on the PGE_2-stimulated release of OPG and on the phosphorylation of the three MAP kinases induced by PGE_2 in osteoblast-like MC3T3-E1 cells. Therefore, it seems unlikely that the inhibitory effects of resveratrol on the PGE_2-induced events shown in our study are SIRT1-dependent in these cells. In a previous study of ours (14), we demonstrated that resveratrol significantly suppressed the BMP-4-induced VEGF synthesis, and that the effects were mediated at least in part by the activation of SIRT1 in MC3T3-E1 cells. Based on our findings, it is possible that the intracellular signaling mechanisms underlying the effects of resveratrol in osteoblasts are characterized according to each agonist or product. It has been reported that resveratrol affects the cellular function of adipocytes and regulates the number of fat cells in a SIRT1-independent manner (25,26). Since both osteoblasts and adipocytes originate from undifferentiated mesenchymal stem cells, the SIRT-independent effects of resveratrol observed in osteoblasts may be due to these same stem cells.

It is recognized that the upregulation of RANKL is an essential step for the action of bone resorptive agents, including PGE_2 and promoting osteoclast formation (8). Although OPG plays a suppressive role in osteoclastogenesis as a decoy receptor of RANKL, it seems that the OPG suppression of the acceleration of bone resorption may be the initial signal to promote the bone remodeling required for maintaining the quality of bone. The sophisticated regulation of bone remodeling is important to maintain the quantity and quality of bone, and proper osteoclastic bone resorption is essential to bone turnover that involves the removal of old fragile bone and its replacement. Therefore, the effects of resveratrol shown in our study may provide a new aspect of the regulatory mechanisms of bone metabolism. Further studies are required to clarify the exact mechanisms behind the effects of resveratrol on osteoblasts.

In conclusion, our results strongly suggest that resveratrol reduces the PGE_2-stimulated OPG synthesis through the inhibition of p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK in osteoblasts, and that these suppressive effects are not mediated through the activation of SIRT1.

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