MC3T3-E1 cells, we examined the effect of resveratrol on the BMP-4-induced phosphorylation of p70 S6 kinase. Resveratrol significantly attenuated the phosphorylation of p70 S6 kinase induced by BMP-4 in a dose-dependent manner between 10 and 50 μM (Fig. 5A). Furthermore, SRT1720, which alone barely affected the phosphorylation of p70 S6 kinase, also suppressed the p70 S6 kinase phosphorylation in these cells (Fig. 5B).

Discussion

In the present study, we demonstrated that resveratrol, a polyphenolic flavonoid enriched in the skins of red grapes or red wine (12,13), significantly suppressed BMP-4-stimulated VEGF release in osteoblast-like MC3T3-E1 cells. Resveratrol reportedly functions at least in part via activation of SIRT1, which is known as one of the longevity genes (21). We also found that SRT1720 suppressed BMP-4-stimulated VEGF release in these cells. Therefore, the suppressive effect of resveratrol on BMP-4-induced VEGF synthesis in osteoblasts appears to be a SIRT1-dependent event. In addition, we demonstrated that both resveratrol and SRT1720 markedly decreased the BMP-4-induced expression levels of VEGF mRNA. Based on our findings, it is probable that the inhibitory effect of resveratrol on BMP-4-induced VEGF release is mediated through transcriptional events. To the best of our knowledge, this is the first report to demonstrate the suppression of VEGF synthesis by resveratrol in osteoblasts.

It is well known that Smad proteins are central mediators of the intracellular signaling system of the TGF-β superfamily such as TGF-β and BMPs (23). Regarding BMP signaling, BMPs employ the activation of Smad1/5/8 as receptor-regulated Smads (23). Thus, we investigated whether Smad1/5/8 are involved in the inhibitory effects of resveratrol or SRT1720 on BMP-4-stimulated VEGF synthesis in osteoblast-like MC3T3-E1 cells. However, neither resveratrol nor SRT1720 affected the BMP-4-induced phosphorylation levels of Smad1/5/8. Therefore, it seems unlikely that the suppressive effect of resveratrol on VEGF synthesis stimulated by BMP-4 is due to the modulation of Smad1/5/8-mediating signaling. Moreover, accumulating evidence indicates that the TGF-β superfamily exerts their effects on a variety of biological functions via Smad-independent signaling (24). We previously reported that activation of p70 S6 kinase positively regulates BMP-4-stimulated VEGF synthesis in osteoblast-like MC3T3-E1 cells (8). Thus, we next investigated whether resveratrol or SRT1720 affects the activation of p70 S6 kinase upregulated by BMP-4 in MC3T3-E1 cells. We found that the phosphorylation levels of p70 S6 kinase induced by BMP-4 were significantly attenuated by both resveratrol and SRT1720. Based on our findings, it is likely that the suppression of BMP-4-stimulated VEGF synthesis by resveratrol through SIRT-1 activation is mediated by the modulation of p70 S6 kinase in osteoblast-like MC3T3-E1 cells.

Resveratrol is a natural polyphenol abundantly found in grape skins and red wine and shows numerous favorable effects on the health of humans through antioxidation, anti-aging and anti-stress (12,13). It has been reported that resveratrol prevents various types of cancers such as colon carcinoma, and attenuates the progression of Alzheimer's disease, in addition to protection against obesity and its associated diseases (25-27). Recent studies have also linked resveratrol to a prolonged lifespan in humans and other species (28,29). It has been shown that BMP signaling is required for both bone development and angiogenesis (30). During bone development and fracture healing, BMPs not only increase bone formation, but also enhance angiogenesis through regulation of the expression of VEGF. Moreover, modifications of VEGF expression were reportedly observed in osteoporosis *in vivo* (7). Both the reduction in VEGF expression in the tibial metaphysis and the contrasting increases in VEGF expression related to vascularization in the periosteum have been recognized in osteoporotic rat models (7), suggesting the complicated mechanism of the pathogenesis. Thus, our present findings, clearly demonstrating the reduction of BMP-4-induced VEGF synthesis by resveratrol in osteoblasts-like MC3T3-E1 cells, provides novel insight underlying the favorable effects of polyphenols on the health of humans particularly on elder individuals. Further investigation is necessary to clarify the detailed mechanisms of resveratrol underlying the VEGF synthesis in osteoblasts.

In conclusion, our findings strongly suggest that resveratrol attenuates BMP-4-stimulated VEGF synthesis through suppression of the activation of p70 S6 kinase in osteoblasts, and that the inhibitory effect is at least in part mediated by SIRT1 activation.

Acknowledgements

We are grateful to Yumiko Kurokawa for her skillful technical assistance. This investigation was supported in part by a Grant-in-Aid for Scientific Research (19591042) from the Ministry of Education, Science, Sports and Culture of Japan, the Foundation for Growth Science, and the Research Funding for Longevity Sciences (22-4, 23-9) from the National Center for Geriatrics and Gerontology (NCGG), Japan.

References

1. Karsenty G and Wagner EF: Reaching a genetic and molecular understanding of skeletal development. Dev Cell 2: 389-406, 2002.
2. Boyce BF and Xing L: Functions of RANKL/RANK/OPG in bone modeling and remodeling. Arch Biochem Biophys 473: 139-146, 2008.
3. Parfitt AM: Targeted and nontargeted bone remodeling: relationship to basic multicellular unit origination and progression. Bone 30: 5-7, 2002.
4. Miyazono K, Maeda S and Imamura T: BMP receptor signaling: transcriptional targets, regulation of signals, and signaling cross-talk. Cytokine Growth Factor Rev 16: 251-263, 2005.
5. Krause C, de Gorter DJJ, Karperien M and ten Dijke P: Signal transduction cascades controlling osteoblast differentiation. In: Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism. Rosen CJ (ed). 7th edition. American Society for Bone and Mineral Research, Washington, pp10-16, 2008.
6. Yamaguchi K, Nagai S, Ninomiya-Tsuji J, Nishita M, Tamai K, Irie K, Ueno N, Nishida E, Shibuya H and Matsumoto K: XIAP, a cellular member of the inhibitor of apoptosis protein family, links the receptors to TAB1-TAK1 in the BMP signaling pathway. EMBO J 18: 179-187, 1999.
7. Clarkin CE and Gerstenfeld LC: VEGF and bone cell signalling: an essential vessel for communication? Cell Biochem Funct 31: 1-11, 2013.
8. Kozawa O, Matsuno H and Uematsu T: Involvement of p70 S6 kinase in bone morphogenetic protein signaling: vascular endothelial growth factor synthesis by bone morphogenetic protein-4 in osteoblasts. J Cell Biochem 81: 430-436, 2001.

9. Tokuda H, Hatakeyama D, Shibata T, Akamatsu S, Oiso Y and Kozawa O: p38 MAP kinase regulates BMP-4-stimulated VEGF synthesis via p70 S6 kinase in osteoblasts. Am J Physiol Endcrinol Metab 284: E1202-E1209, 2003.
10. Jankun J, Selman SH, Swiercz R and Skrzypczak-Jankun E: Why drinking green tea could prevent cancer. Nature 387: 561, 1997.
11. Harborne JB and Williams CA: Advances in flavonoid research since 1992. Phytochemistry 55: 481-504, 2000.
12. Blander G and Guarente L: The Sir2 family of protein deacetylases. Annu Rev Biochem 73: 417-435, 2004.
13. Koo SH and Montminy M: In vino veritas: a tale of two sirt1s? Cell 127: 1091-1093, 2006.
14. Baur JA, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A, Prabhu VV, Allard JS, Lopez-Lluch G, Lewis K, Pistell PJ, Poosala S, Becker KG, Boss O, Gwinn D, Wang M, Ramaswamy S, Fishbein KW, Spencer RG, Lakatta EG, Le Couteur D, Shaw RJ, Navas P, Puigserver P, Ingram DK, de Cabo R and Sinclair DA: Resveratrol improves health and survival of mice on a high-calorie diet. Nature 444: 337-342, 2006.
15. Mizutani K, Ikeda K, Kawai Y and Yamori Y: Resveratrol stimulates the proliferation and differentiation of osteoblastic MC3T3-E1 cells. Biochem Biophys Res Commun 253: 859-863, 1998.
16. Kupisiewicz K, Boissy P, Abdallah BM, Hansen FD, Erben RG, Savouret JF, Søe K, Andersen TL, Plesner T and Delaisse JM: Potential of resveratrol analogues as antagonists of osteoclasts and promoters of osteoblasts. Calcif Tissue Int 87: 437-449, 2010.
17. Sudo H, Kodama HA, Amagai Y, Yamamoto S and Kasai S: In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J Cell Biol 96: 191-198, 1983.
18. Kozawa O, Tokuda H, Miwa M, Kotoyori J and Oiso Y: Cross-talk regulation between cyclic AMP production and phosphoinositide hydrolysis induced by prostaglandin E_2 in osteoblast-like cells. Exp Cell Res 198: 130-134, 1992.
19. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685, 1970.
20. Kato K, Ito H, Hasegawa K, Inaguma Y, Kozawa O and Asano T: Modulation of the stress-induced synthesis of hsp27 and αB-crystallin by cyclic AMP in C6 rat glioma cells. J Neurochem 66: 946-950, 1996.
21. Bradamante S, Barenghi L and Villa A: Cardiovascular protective effects of resveratrol. Cardiovasc Drug Rev 22: 169-188, 2004.
22. Milne JC, Lambert PD, Schenk S, Carney DP, Smith JJ, Gagne DJ, Jin L, Boss O, Perni RB, Vu CB, Bemis JE, Xie R, Disch JS, Ng PY, Nunes JJ, Lynch AV, Yang H, Galonek H, Israelian K, Choy W, Iffland A, Lavu S, Medvedik O, Sinclair DA, Olefsky JM, Jirousek MR, Elliott PJ and Westphal CH: Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes. Nature 450: 712-716, 2007.
23. Miyazawa K, Shinozaki M, Hara T, Furuya T and Miyazono K: Two major Smad pathways in TGF-β superfamily signalling. Genes Cells 7: 1191-1204, 2002.
24. Moustakas A and Heldin CH: Non-Smad TGF-beta signals. J Cell Sci 118: 3573-3584, 2005.
25. Kundu JK and Surh YJ: Cancer chemopreventive and therapeutic potential of resveratrol: mechanistic perspectives. Cancer Lett 269: 243-261, 2008.
26. Ramassamy C: Emerging role of polyphenolic compounds in the treatment of neurodegenerative diseases: a review of their intracellular targets. Eur J Pharmacol 545: 51-64, 2006.
27. Li F, Gong Q, Dong H and Shi J: Resveratrol, a neuroprotective supplement for Alzheimer's disease. Curr Pharm Des 18: 27-33, 2012.
28. Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, Zipkin RE, Chung P, Kisielewski A, Zhang LL, Scherer B and Sinclair DA: Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. Nature 425: 191-196, 2003.
29. Wood JG, Rogina B, Lavu S, Howitz K, Helfand SL, Tatar M and Sinclair D: Sirtuin activators mimic caloric restriction and delay ageing in metazoans. Nature 430: 686-689, 2004.
30. Zhang F, Qiu T, Wu X, Wan C, Shi W, Wang Y, Chen JG, Wan M, Clemens TL and Cao X: Sustained BMP signaling in osteoblasts stimulates bone formation by promoting angiogenesis and osteoblast differentiation. J Bone Miner Res 24: 1224-1233, 2009.

Prostaglandins, Leukotrienes and Essential Fatty Acids 91 (2014) 73–80

Contents lists available at ScienceDirect

Prostaglandins, Leukotrienes and Essential Fatty Acids

journal homepage: www.elsevier.com/locate/plefa

Suppression by resveratrol of prostaglandin D_2-stimulated osteoprotegerin synthesis in osteoblasts

Gen Kuroyanagi [a,b], Jun Mizutani [a], Akira Kondo [a,b], Naohiro Yamamoto [a,b], Rie Matsushima-Nishiwaki [b], Takanobu Otsuka [a], Osamu Kozawa [b], Haruhiko Tokuda [b,c,*]

[a] Department of Orthopedic Surgery, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601, Japan
[b] Department of Pharmacology, Gifu University Graduate School of Medicine, Gifu 501-1194, Japan
[c] Department of Clinical Laboratory, National Center for Geriatrics and Gerontology, Obu, Aichi 474-8511, Japan

ARTICLE INFO

Article history:
Received 30 October 2013
Received in revised form
7 April 2014
Accepted 17 April 2014

Keywords:
Resveratrol
PGD_2
Osteoprotegerin
Osteoblast

ABSTRACT

Resveratrol, a natural polyphenol with health-related properties mainly existing in grape skins and red wine, possesses beneficial effects on human being. We have previously reported that prostaglandin D_2 (PGD_2) stimulates heat shock protein 27 (HSP27) induction via activation of p44/p42 mitogen-activated protein (MAP) kinase, p38 MAP kinase and stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK) in osteoblast-like MC3T3-E1 cells. In the present study, we investigated the mechanism behind the effect of PGD_2 on osteoprotegerin (OPG) synthesis and the effect of resveratrol on the OPG synthesis in MC3T3-E1 cells. PGD_2 significantly stimulated both the OPG release and the expression levels of OPG mRNA. Resveratrol and SRT1720, an activator of SIRT1, markedly suppressed the PGD_2-induced OPG release and the mRNA levels of OPG. PD98059, a specific MEK inhibitor, SB203580, a specific p38 MAP kinase inhibitor, and SP600125, a specific SAPK/JNK inhibitor suppressed the PGD_2-stimulated OPG release. PGD_2-induced phosphorylation of p38 MAP kinase and SAPK/JNK was attenuated by resveratrol or SRT1720. However, resveratrol or SRT1720 failed to affect the phosphorylation of myosin phosphatase-targeting subunit-1 (MYPT-1), a downstream substrate of Rho-kinase and p44/p42 MAP kinase. These results strongly suggest that resveratrol suppresses PGD_2-stimulated OPG synthesis through inhibiting p38 MAP kinase and SAPK/JNK in osteoblasts, and that the suppressive effect is exerted at the point downstream of Rho-kinase but upstream of p38 MAP kinase or SAPK/JNK.

1. Introduction

Bone metabolism is regulated mainly by two functional cells, osteoblasts and osteoclasts responsible for bone formation and bone resorption, respectively [1]. The formation of bone structures and bone remodeling result from the coupling process of osteoblasts and osteoclasts. The disorder of bone remodeling causes metabolic bone diseases such as osteoporosis. In proceeding of bone remodeling, it is generally recognized that numerous humoral factors including prostaglandins and cytokines play pivotal roles [2].

Osteoprotegerin (OPG), a glycoprotein belonging to the tumor necrosis factor receptor superfamily, has been well recognized to possess inhibitory effects on osteoclast activation along with receptor activator of nuclear factor κB (RANK). In response to numerous hormones, cytokines and prostaglandins, osteoblastic cells are well known to generate RANK ligand (RANKL) and OPG [3]. OPG binds to RANKL as a decoy receptor and inhibits the binding of RANK to RANKL, an essential step of osteoclastogenesis for osteoclast-precursor cells derived from macrophages [4]. It has been reported that RANKL-knock out mice are suffered from severe osteopetrosis, suggesting that RANKL is a central regulator of osteoclastogenesis [5]. It is currently recognized that the RANK/RANKL/OPG axis is an important regulatory system for osteoclasts.

It is firmly established that prostaglandins act as local factors, autacoids, in osteoblasts. Among them, prostaglandin D_2 (PGD_2) is recognized to be implicated in the control of osteoclast function and bone anabolism [6]. It has been shown that PGD_2 stimulates collagen synthesis during calcification of osteoblasts [7]. In addition, PGD_2 produced by osteoblasts reportedly modulates expression of OPG and RANKL in osteoblasts [8]. In our previous study [9], we have shown that PGD_2 stimulates interleukin-6 (IL-6) synthesis via Ca^{2+} mobilization in osteoblast-like MC3T3-E1 cells. We also reported the involvement of Rho-kinase in PGD_2-induced IL-6 synthesis in these cells [10]. In addition, we have demonstrated that PGD_2 stimulates the induction of heat shock protein 27 (HSP27) via three major mitogen-activated protein (MAP)

* Corresponding author at: Department of Clinical Laboratory, National Center for Geriatrics and Gerontology, Obu, Aichi 474-8511, Japan. Tel.: +81 562 46 2311; fax: +81 562 46 8396.
E-mail address: tokuda@ncgg.go.jp (H. Tokuda).

http://dx.doi.org/10.1016/j.plefa.2014.04.003
0952-3278/

kinases, p44/p42 MAP kinase, p38 MAP kinase and stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK) and that Rho-kinase functions at a point upstream of both p38 MAP kinase and SAPK/JNK in osteoblast-like MC3T3-E1 cells [11,12].

It is generally known that polyphenolic compounds in foods such as vegetables and fruits have beneficial properties for human beings. Among them, flavonoids show antioxidative, antiinflammatory and antitumor effects [13,14]. Resveratrol, a natural polyphenolic flavonoid enriched in the skins of red grapes and red wine, has been shown to increase life span in lower organisms by activating the nicotinamide adenine dinucleotide (NAD^+)-dependent histone deacetylase SIRT1 [15]. NAD^+ is biosynthesized in our body as a precursor of nicotinamide, and has an important role for energy acquisition as a coenzyme of oxidoreductase. SIRT1 is identified as a transcriptional silencer in yeast and modulates a number of transcriptional regulators in mammals [16]. It has been shown that there is low mortality from coronary heart disease in France as wine consumption contains high amount of abundant resveratrol [17]. It has been reported that post-menopausal women who preferentially consume wine have a lower risk of hip fracture compared to non-drinkers, past drinkers and those with other alcohol preferences [18]. However, the exact mechanism underlying resveratrol-effects on bone metabolism remains to be elucidated.

In the present study, we investigated the mechanism of OPG synthesis induced by PGD_2 and the effect of resveratrol on the OPG synthesis in osteoblast-like MC3T3-E1 cells. We herein demonstrate that resveratrol suppresses PGD_2-stimulated OPG synthesis through inhibiting p38 MAP kinase and SAPK/JNK in osteoblasts, and that the suppressing effect is exerted at the point downstream of Rho-kinase but upstream of p38 MAP kinase or SAPK/JNK.

2. Materials and methods

2.1. Materials

Resveratrol, SRT1720, PD98059, SB203580 and SP600125 were obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). PGD_2 was obtained from Sigma Chemical Co. (St. Louis, MO). A mouse OPG enzyme-linked immunosorbent assay (ELISA) kit was obtained from R&D Systems, Inc. (Minneapolis, MN). 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, SAPK/JNK antibodies, phospho-specific myosin phosphatase targeting subunit-1 (MYPT-1) antibodies and MYPT-1 antibodies were obtained from Cell Signaling, Inc. (Beverly, MA). An ECL Western blotting detection system was obtained from GE Healthcare (Buckinghamshire, UK). Other materials and chemicals were obtained from commercial sources. PGD_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.

2.2. Cell culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [19] were maintained as described previously [20]. 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 (5×10^4 cells/dish) or 90-mm diameter dishes (2×10^5 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.

2.3. Assay for OPG

The cultured cells were pretreated with various doses of resveratrol, SRT1720, PD98059, SB203580 or SP600125 for 60 min, and then stimulated by 10 μM of PGD_2 or vehicle in 1 ml of α-MEM containing 0.3% FBS for the indicated periods. The conditioned medium was collected at the end of incubation, and the OPG concentration was then measured using the OPG ELISA kit according to the manufacturer's protocol.

2.4. Real-time RT-PCR

The cultured cells were pretreated with 50 μM of resveratrol or vehicle for 60 min, and then stimulated by 10 μM of PGD_2 or vehicle in α-MEM containing 0.3% FBS for 3 h. Total RNA was isolated and transcribed into complementary DNA using Trizol regand (Invitrogen Corp., Carlsbad, CA) and Omniscript Reverse Transcriptase kit (QIAGEN Inc., Valencia, CA), respectively. Real-time RT-PCR was performed using a Light Cycler system in capillaries and Fast Start 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.

2.5. Western blot analysis

The cultured cells were pretreated with various doses of resveratrol or SRT1720 for 60 min, and then stimulated by PGD_2 in α-MEM containing 0.3% FBS for the indicated periods. The cells were washed twice with phosphate-buffered saline and then lysed, homogenized and sonicated in a 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 by the method of Laemmli [21] in 10% polyacrylamide gels. The protein was fractionated and transferred onto an Immun-Blot PVDF Membrane (Bio-Rad, Hercules, CA). The membranes were blocked with 5% fat-free dry milk in Tris-buffered saline-Tween (TBS-T; 20 mM Tris–HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) for 2 h before incubation with primary antibodies. A Western blot analysis was performed as previously described [22] 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, SAPK/JNK antibodies, phospho-specific MYPT-1 antibodies or MYPT-1 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 1:1000 with 5% fat-free dry milk in TBS-T. The peroxidase activity on the PVDF sheet was visualized on X-ray film by means of the ECL Western blotting detection system.

2.6. Determination of densitometric analysis

Densitometric analysis was performed using scanner and image analysis software (image J version 1.32). The phosphorylated protein levels were calculated as follows: the back ground-subtracted signal intensity of each phosphorylation signal was normalized to the respective total protein signal and plotted as the fold increase in comparison to control cells without stimulation.

2.7. Statistical analysis

The data were analyzed by ANOVA followed by the Bonferroni method for multiple comparisons between pairs, and $p < 0.05$ was considered to be statistically significant. All data are presented as the mean ± S.E.M. of triplicate determinations from three independent cell preparations.

3. Results

3.1. Effect of resveratrol on the PGD_2-stimulated OPG release in MC3T3-E1 cells

We first investigated whether PGD_2 stimulates OPG synthesis or not in osteoblast-like MC3T3-E1 cells. PGD_2 significantly stimulated the release of OPG in a time-dependent manner up to 12 h (Fig. 1). We next examined the effect of resveratrol on the PGD_2-stimulated OPG release in these cells. Resveratrol, which alone had a little effect on the OPG levels, significantly suppressed the PGD_2-stimulated OPG release in a dose-dependent manner in the range between 1 and 50 μM (Fig. 2). The maximum effect of resveratrol was observed at 50 μM, which caused an approximate 50% decrease in the PGD_2-effect.

3.2. Effect of SRT1720 on the PGD_2-stimulated OPG release in MC3T3-E1 cells

SRT1720 is known to be an activator of SIRT1 as well as resveratrol [23]. Thus, we examined the effect of SRT1720 on the PGD_2-stimulated OPG release in MC3T3-E1 cells. SRT1720 significantly reduced the PGD_2-stimulated OPG release (Table 1). The effect of SRT1720 at 10 μM caused an approximate 40% decrease in the PGD_2-effect.

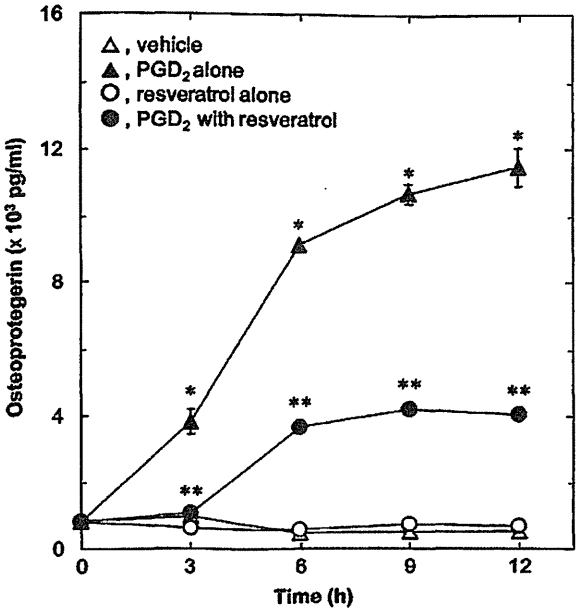

Fig. 1. Effect of resveratrol on the PGD_2-stimulated OPG release in MC3T3-E1 cells. The cultured cells were pretreated with 50 μM of resveratrol or vehicle for 60 min, and then stimulated by 10 μM of PGD_2 or vehicle for the indicated periods. OPG concentrations of the culture mediums were determined by ELISA. Each value represents the mean ± S.E.M. of triplicate determinations from three independent cell preparations. △, vehicle; ▲, PGD_2 alone; ○, resveratrol alone; ●, PGD_2 with resveratrol. *$p < 0.05$, compared to the value of control. **$p < 0.05$, compared to the value of PGD_2 alone.

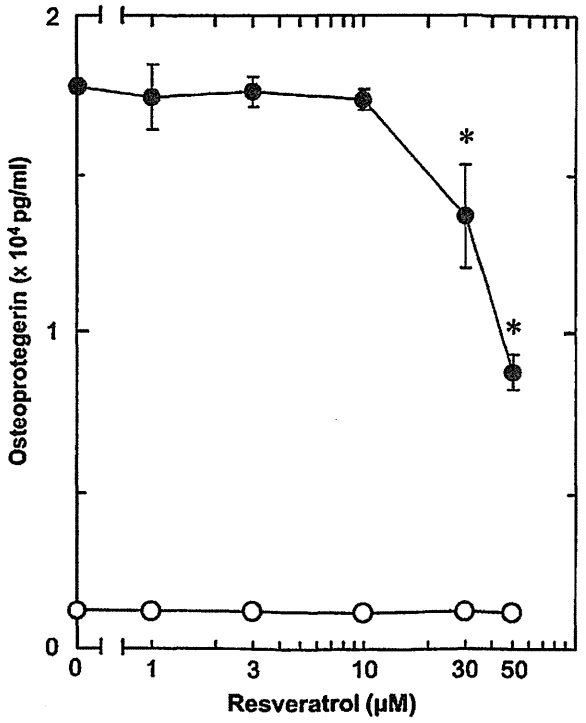

Fig. 2. Effect of resveratrol on the PGD_2-stimulated OPG release in MC3T3-E1 cells. The cultured cells were pretreated with various doses of resveratrol for 60 min, and then stimulated by 10 μM of PGD_2 (●) or vehicle (○) for 12 h. OPG concentrations of the culture mediums were determined by ELISA. Each value represents the mean ± S.E.M. of triplicate determinations from three independent cell preparations. *$p < 0.05$, compared to the value of PGD_2 alone.

3.3. Effect of resveratrol or SRT1720 on the PGD_2-induced expression levels of OPG mRNA in MC3T3-E1 cells

In order to investigate whether the suppressing effects of resveratrol or SRT1720 on PGD_2-stimulated OPG release are meditated through transcriptional events or not, we further examined the effect of resveratrol or SRT1720 on the PGD_2-induced OPG mRNA expression by real-time RT-PCR. Resveratrol, which alone had a little effect on the mRNA levels of OPG, significantly suppressed the PGD_2-induced OPG mRNA expression (Fig. 3A). In addition, SRT1720 significantly reduced OPG mRNA expression levels induced by PGD_2 (Fig. 3B).

3.4. Effects of PD98059, SB203580 and SP600125 on the PGD_2-stimulated OPG release in MC3T3-E1 cells

It is firmly established that major MAP kinases such as p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK among the MAP kinase superfamily are central elements used by mammalian cells to transduce the various messages of extracellular stimuli [24]. As for PGD_2 signaling in osteoblasts, we have previously reported that these three MAP kinases are involved in the HSP27 induction by PGD_2 in osteoblast-like MC3T3-E1 cells [11]. In order to elucidate whether p44/p42 MAP kinase, p38 MAP kinase or SAPK/JNK in MC3T3-E1 cells are involved in the PGD_2-stimulated OPG synthesis, we examined the effects of PD98059, a specific inhibitor of the upstream kinase that activates p44/p42 MAP kinase [25], SB203580, a specific inhibitor of p38 MAP kinase [26] or SP600125, a specific inhibitor of SAPK/JNK [27], on the OPG release stimulated by PGD_2. PD98059, SB203580 and SP600125, which by themselves had a little effect on the OPG release, significantly reduced the PGD_2-stimulated OPG release (Table 2).

Table 1
Effect of SRT1720 on the PGD_2-stimulated OPG release in MC3T3-E1 cells.

SRT1720	PGD_2	OPG (pg/ml)
−	−	849 ± 16
−	+	16,765 ± 1125*
+	−	741 ± 33
+	+	9336 ± 314**

The cultured cells were pretreated with 10 μM of SRT1720 or vehicle for 60 min, and then stimulated by 10 μM of PGD_2 or vehicle for 12 h. OPG concentrations of the culture mediums were determined by ELISA. Each value represents the mean ± S.E.M. of triplicate determinations from three independent cell preparations. *$p < 0.05$, compared to the value of control. **$p < 0.05$, compared to the value of PGD_2 alone.

3.5. Effect of resveratrol on the PGD_2-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells

In order to clarify whether the suppressive effect of resveratrol on the PGD_2-stimulated OPG synthesis is mediated by the modulation of p44/p42 MAP kinase activation in MC3T3-E1 cells, we examined the effect of resveratrol on the PGD_2-induced phosphorylation of p44/p42 MAP kinase. However, resveratrol did not affect the PGD_2-induced phosphorylation of p44/p42 MAP kinase in the range between 10 and 50 μM (Fig. 4).

3.6. Effects of resveratrol on the PGD_2-induced phosphorylation of p38 MAP kinase and SAPK/JNK in MC3T3-E1 cells

To investigate whether the resveratrol-effect on the PGD_2-stimulated OPG synthesis is mediated through p38 MAP kinase and/or SAPK/JNK activation in MC3T3-E1 cells, we next examined the effects of resveratrol on the PGD_2-induced phosphorylation of p38 MAP kinase or SAPK/JNK. Resveratrol markedly suppressed the PGD_2-induced phosphorylation of both p38 MAP kinase and SAPK/JNK in a dose-dependent manner in the range between 10 and 50 μM (Fig. 5).

3.7. Effect of resveratrol on the PGD_2-induced phosphorylation of MYPT-1 in MC3T3-E1 cells

We have previously reported that Rho-kinase positively regulates PGD_2-stimulated HSP27 induction at a point upstream from both p38 MAP kinase and SAPK/JNK in osteoblast-like MC3T3-E1 cells [12]. It is well recognized that MYPT-1, a component of myosin phosphatase, is a downstream substrate of Rho-kinase [28,29]. In order to elucidate whether Rho-kinase activation is involved in the inhibitory effect of resveratrol on the PGD_2-stimulated OPG synthesis in MC3T3-E1 cells, we examined the effect of resveratrol on the phosphorylation of MYPT-1 induced by PGD_2. However, we found that resveratrol failed to affect the PGD_2-induced phosphorylation of MYPT-1 (Fig. 6).

3.8. Effects of SRT1720 on the PGD_2-stimulated phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, SAPK/JNK or MYPT-1 in MC3T3-E1 cells

To furthermore clarify whether the inhibitory effect of resveratrol on the PGD_2-stimulated OPG synthesis is mediated through SIRT1 activation in MC3T3-E1 cells, we examined the effects of SRT1720 on the PGD_2-induced phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, SAPK/JNK or MYPT-1. SRT1720 significantly suppressed the PGD_2-induced phosphorylation of p38 MAP kinase and SAPK/JNK (Fig. 7). On the contrary, SRT1720 hardly affected the PGD_2-induced phosphorylation of p44/p42 MAP kinase or MYPT-1 (Fig. 7). Thus, our findings suggest that

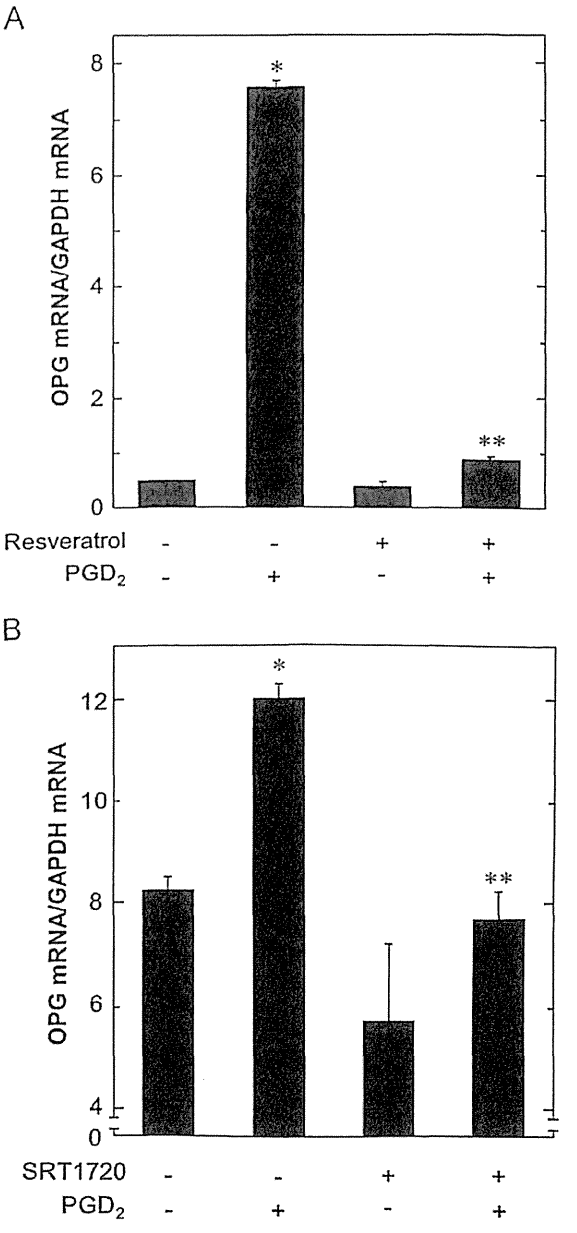

Fig. 3. Effects of resveratrol (A) or SRT1720 (B) on the PGD_2-induced OPG mRNA expression in MC3T3-E1 cells. The cultured cells were pretreated with 50 μM of resveratrol, 10 μM of SRT1720 or vehicle for 60 min, and then stimulated by 10 μM of PGD_2 or vehicle for 3 h. The respective total RNA were then isolated and transcribed into cDNA. The expressions of OPG mRNA and GAPDH mRNA were quantified by real-time RT-PCR. The OPG mRNA levels were normalized to those of GAPDH mRNA. Each value represents the mean ± S.E.M. of triplicate determinations from three independent cell preparations. *$p < 0.05$ compared to the value of control. **$p < 0.05$ compared to the value of PGD_2 alone.

SRT1720 mimics the effects of resveratrol on the PGD_2-induced phosphorylation of three MAP kinases and MYPT-1.

4. Discussion

In the present study, we demonstrated that resveratrol significantly suppressed the PGD_2-stimulated OPG release in osteoblast-like MC3T3-E1 cells. In addition, we showed that PGD_2 increased the levels of OPG mRNA, and resveratrol reduced

Table 2
Effects of PD98059, SB203580 or SP600125 on the PGD_2-stimulated OPG release in MC3T3-E1 cells.

Inhibitors	PGD_2	OPG (pg/ml)
–	–	791 ± 13
–	+	4382 ± 160*
PD98059	–	756 ± 62
PD98059	+	2695 ± 120**
SB203580	–	468 ± 34
SB203580	+	800 ± 27**
SP600125	–	989 ± 38
SP600125	+	3471 ± 92**

The cultured cells were pretreated with 50 μM of PD98059, 30 μM of SB203580, and 10 μM of SP600125 or vehicle for 60 min, and then stimulated by 10 μM of PGD_2 or vehicle for 12 h. OPG concentrations of the culture mediums were determined by ELISA. Each value represents the mean ± S.E.M. of triplicate determinations from three independent cell preparations. *$p < 0.05$, compared to the value of control. **$p < 0.05$, compared to the value of PGD_2 alone.

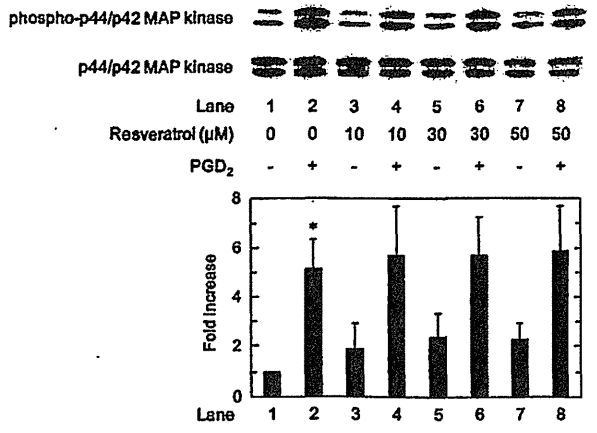

Fig. 4. Effect of resveratrol on the PGD_2-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of resveratrol for 60 min, and then stimulated by 10 μM of PGD_2 or vehicle for 20 min. The cell extracts were then subjected to SDS-PAGE with subsequent Western blot analysis with antibodies against phospho-specific p44/p42 MAP kinase or p44/p42 MAP kinase. The histogram shows quantitative representation of the levels of PGD_2-induced phosphorylation obtained from a laser densitometric analysis of three independent experiments. Each value represents the mean ± S.E.M. of triplicate determinations. *$p < 0.05$, compared to the value of control.

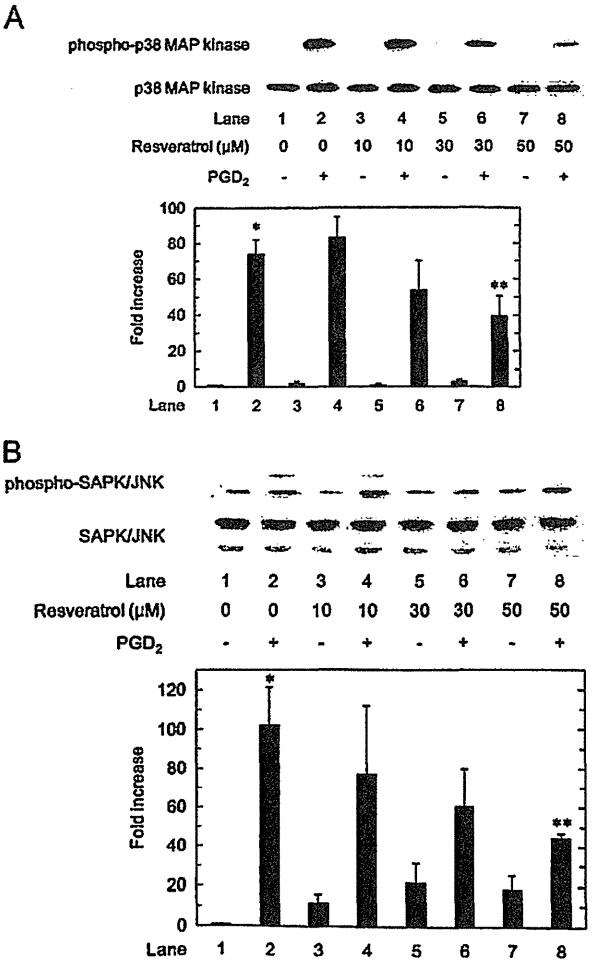

Fig. 5. Effects of resveratrol on the PGD_2-induced phosphorylation of p38 MAP kinase (A) or SAPK/JNK (B) in MC3T3-E1 cells. The cultured cells were pretreated with various doses of resveratrol for 60 min, and then stimulated by 10 μM of PGD_2 or vehicle for 10 min. The cell extracts were then subjected to SDS-PAGE with subsequent Western blot analysis with antibodies against phospho-specific p38 MAP kinase, p38 MAP kinase, phospho-specific SAPK/JNK or SAPK/JNK. The histogram shows quantitative representation of the levels of PGD_2-induced phosphorylation obtained from a laser densitometric analysis of three independent experiments. Each value represents the mean ± S.E.M. of triplicate determinations. *$p < 0.05$, compared to the value of control. **$p < 0.05$, compared to the value of PGD_2 alone.

the expression levels of OPG mRNA up-regulated by PGD_2 in these cells. Therefore, our findings suggest that the suppressing effect of resveratrol on PGD_2-stimulated OPG release was exerted at a point upstream of transcriptional level on osteoblast-like MC3T3-E1 cells. This is probably the first report which clearly shows the suppression by resveratrol of PGD_2-stimulated OPG synthesis in osteoblasts. In addition, we found that SRT1720, known as an activator of SIRT1 with potencies 1000-fold greater than resveratrol [23], inhibited the PGD_2-stimulated OPG release and the OPG mRNA expression levels. Based on our findings, it is most likely that the inhibitory effect of resveratrol on the PGD_2-induced OPG synthesis is mediated at least in part by the activation of SIRT1 in osteoblast-like MC3T3-E1 cells.

We next investigated the mechanism underlying PGD_2-induced OPG synthesis and the suppressive effect of resveratrol in osteoblasts. We have previously reported that PGD_2 stimulates the induction of HSP27 via three major MAP kinases, p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK in osteoblast-like MC3T3-E1cells, and that Rho-kinase positively regulates PGD_2-stimulated HSP27 induction via activation of both p38 MAP kinase and SAPK/JNK [11,12]. We also demonstrated that PGD_2 activates Rho-kinase in MC3T3-E1 cells, resulting in the regulation of IL-6 synthesis via activation of p38 MAP kinase but not p44/p42 MAP kinase [10]. In the present study, we examined the effects of three MAP kinases inhibitors, PD98059 [25], SB203580 [26] and SP600125 [27], on the PGD_2-stimulated OPG synthesis in MC3T3-E1 cells, and showed that these inhibitors significantly reduced the PGD_2-stimulated OPG release. These results suggest that p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK function as positive regulators in the PGD_2-stimulated OPG synthesis in osteoblast-like MC3T3-E1 cells. In addition, resveratrol attenuated the PGD_2-induced phosphorylation of p38 MAP kinase and SAPK/JNK without affecting the phosphorylation of p44/p42 MAP kinase. Based on our findings, it is probable that the suppressing effect of resveratrol on the PGD_2-stimulated OPG synthesis is mediated through inhibiting p38 MAP kinase and SAPK/JNK in osteoblast-like MC3T3-E1 cells. Furthermore, we demonstrated that resveratrol hardly affected the PGD_2-induced phosphorylation of MYPT-1,

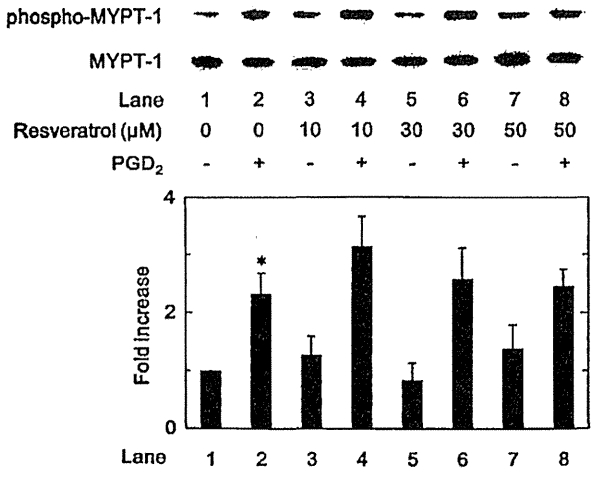

Fig. 6. Effect of resveratrol on the PGD_2-induced phosphorylation of MYPT-1 in MC3T3-E1 cells. The cultured cells were pretreated with various doses of resveratrol for 60 min, and then stimulated by 10 μM of PGD_2 or vehicle for 2 min. The cell extracts were then subjected to SDS-PAGE with subsequent Western blot analysis with antibodies against phospho-specific MYPT-1 or MYPT-1. The histogram shows quantitative representation of the levels of PGD_2-induced phosphorylation obtained from a laser densitometric analysis of three independent experiments. Each value represents the mean ± S.E.M. of triplicate determinations. $^*p < 0.05$, compared to the value of control.

suggesting that resveratrol could not affect PGD_2-induced activation of Rho-kinase, an upstream regulator of p38 MAP kinase and SAPK/JNK in the PGD_2 signaling in these cells. Taking our findings into account, it is most likely that resveratrol functions at a point downstream of Rho-kinase but upstream of p38 MAP kinase or SAPK/JNK in osteoblast-like MC3T3-E1 cells. We additionally showed that SRT1720 mimicked the suppressive effects of resveratrol on the PGD_2-induced phosphorylation of p38 MAP kinase and SAPK/JNK without affecting the MYPT-1 phosphorylation. Therefore, our results suggest that the effect of resveratrol on the PGD_2-stimulated OPG synthesis is mediated at least in part through activation of SIRT1 in osteoblast-like MC3T3-E1 cells. The possible mechanism of resveratrol in PGD_2-stimulated OPG synthesis in osteoblasts is shown in Fig. 8.

It has been reported that PGD_2 has a stimulatory effect on osteoblast calcification [30]. In human osteoblasts, PGD_2 reportedly functions as an autacoid, and decreases OPG production, showing inconsistency with our findings [8]. The discrepancy may be due to the differences of species. Although OPG plays a role in suppression of osteoclastogenesis as a decoy receptor of RANKL [3], it is possible that the suppression of OPG synthesis promotes proper bone remodeling required for maintaining the quality of bone. Adequate regulation of bone remodeling is important to maintain the quantity of bone and the quality, and proper osteoclastic bone resorption is essential to remove old fragile

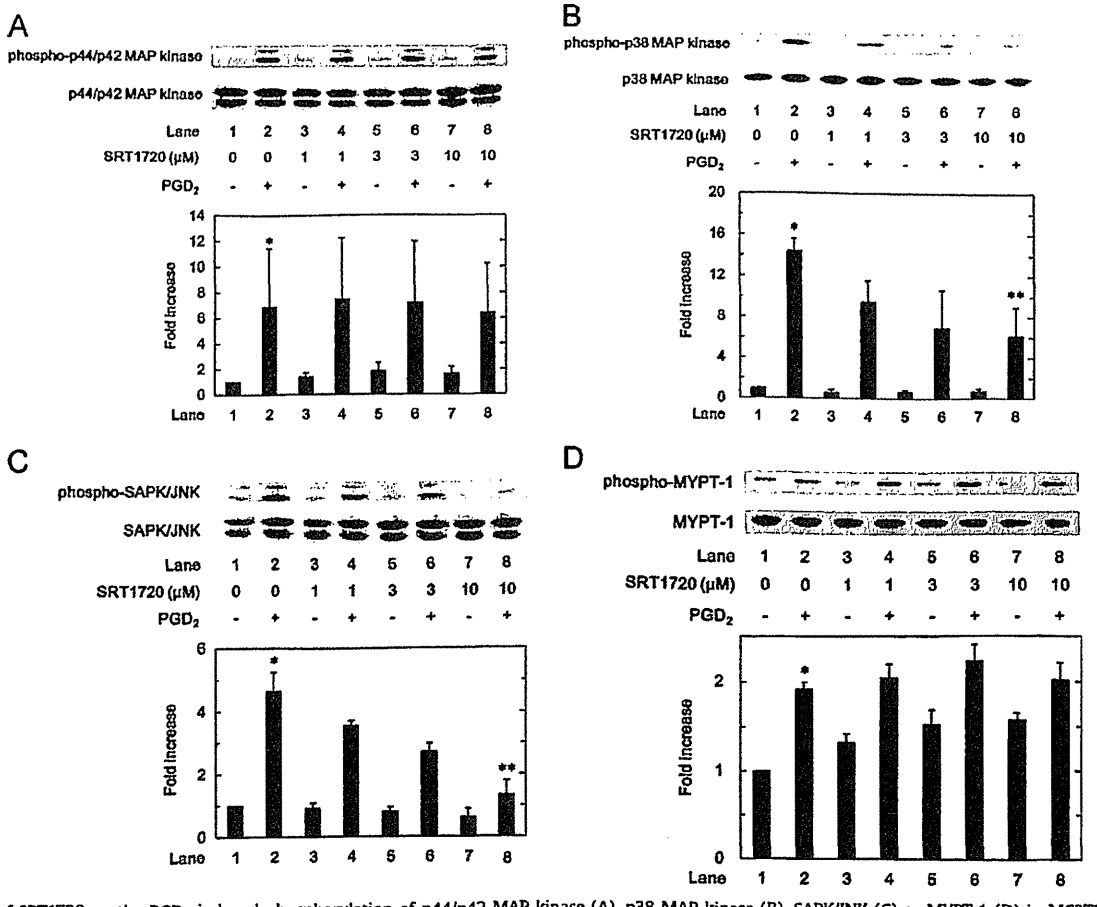

Fig. 7. Effects of SRT1720 on the PGD_2-induced phosphorylation of p44/p42 MAP kinase (A), p38 MAP kinase (B), SAPK/JNK (C) or MYPT-1 (D) in MC3T3-E1 cells. The cultured cells were pretreated with various doses of resveratrol for 60 min, and then stimulated by 10 μM of PGD_2 or vehicle for 20 min (A), 10 min (B, C) or 2 min (D). 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, SAPK/JNK, phospho-specific MYPT-1 or MYPT-1. The histogram shows quantitative representation of the levels of PGD_2-induced phosphorylation obtained from a laser densitometric analysis of three independent experiments. Each value represents the mean ± S.E.M. of triplicate determinations. $^*p < 0.05$, compared to the value of control. $^{**}p < 0.05$, compared to the value of PGD_2 alone.

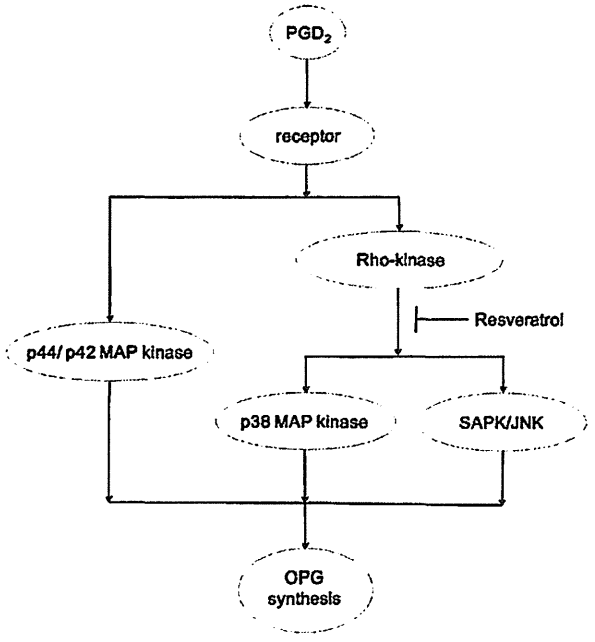

Fig. 8. Schematic illustration of the regulatory mechanism of PGD_2-induced OPG synthesis and the inhibitory effect of resveratrol in osteoblast-like MC3T3-E1 cells. PGD_2, prostaglandin D_2; MAP kinase, mitogen-activated protein kinase; SAPK/JNK, stress-activated protein kinase/*c-Jun* N-terminal kinase; OPG, osteoprotegerin.

skeleton and its renewal. Based on our findings, it is probable that resveratrol could affect the bone remodeling as an activator through the suppression of OPG production in osteoblasts. It has been reported that the plasma concentration of resveratrol reached the peak about 2 μM at 1 h after an oral dose of 25 mg of resveratrol in human subjects [31]. It has also been reported that the concentration of PGD_2 measured in cerebrospinal fluid collected from cisterna magna of conscious rats is about 2 nM to 5 nM [32]. The tissue levels of resveratrol or plasma levels of PGD_2 have not yet been reported as far as we know. We herein showed that the inhibitory effect of resveratrol on the PGD_2 (10 μM)-stimulated OPG release was significantly observed over the range 30–50 μM. The concentrations of resveratrol and PGD_2 that we used in this study exceed in vivo values. Therefore, it is likely that the need for high concentrations of resveratrol and PGD_2 in vitro is due to its condition as compared with in vivo conditions. Further investigation would be necessary to clarify the detailed mechanism of resveratrol underlying the OPG synthesis in osteoblasts.

Taken together, our results strongly suggest that resveratrol suppresses PGD_2-stimulated OPG synthesis through inhibiting p38 MAP kinase and SAPK/JNK in osteoblasts, and that the suppressing effect of resveratrol is exerted at the point downstream of Rho-kinase but upstream of p38 MAP kinase or SAPK/JNK.

Acknowledgments

We are very grateful to Yumiko Kurokawa for her skillful technical assistance. This investigation was supported in part by Grant-in-Aid for Scientific Research (19591042) from the Ministry of Education, Science, Sports and Culture of Japan, the Foundation for Growth Science and the Research Funding for Longevity Sciences (22-4 and 23-9) from National Center for Geriatrics and Gerontology (NCGG), Japan.

References

[1] G. Karsenty, E.F. Wagner, Reaching a genetic and molecular understanding of skeletal development, Dev. Cell 2 (2002) 389–406.
[2] A.M. Parfit, Targeted and nontargeted bone remodeling: relationship to basic multice, Bone 30 (2002) 5–7.
[3] P.A. Ross, Osteoclast biology and bone resorption, in: C.J. Rosen (Ed.), Primer on the Metabolic Bone Diseases of Minerel Metabolism, 8th ed., John Wiley & Sons, Inc., Hoboken, NJ, 2013.
[4] W.S. Simonet, D.L. Lacey, C.R. Dunstan, M. Kelley, M.S. Chang, R. Lüthy, H.Q. Nguyen, S. Wooden, L. Bennett, T. Boone, G. Shimamoto, M. DeRose, R. Elliott, A. Colombero, H.L. Tan, G. Trail, J. Sullivan, E. Davy, N. Bucay, L. Renshaw-Gegg, T.M. Hughes, D. Hill, W. Pattison, P. Campbell, S. Sander, G. Van, J. Tarpley, P. Derby, R. Lee, W.J. Boyle, Osteoprotegerin: a novel secreted protein involved in the regulation of bone density, Cell 89 (1997) 309–319.
[5] A. Mizuno, T. Kanno, M. Hoshi, O. Shibata, K. Yano, N. Fujise, M. Kinosaki, K. Yamaguchi, E. Tsuda, A. Murakami, H. Yasuda, K. Higashio, Transgenic mice overexpressing soluble osteoclast differentiation factor (sODF) exhibit severe *osteoporosis*, J. Bone Miner. Metab. 20 (2002) 337–344.
[6] H. Hikiji, T. Takato, T. Shimizu, S. Ishii, The roles of prostanoids, leukotrienes, and platelet-activating factor in bone metabolism and disease, Prog. Lipid Res. 47 (2008) 107–126.
[7] Y. Tasaki, R. Takamori, Y. Koshihara, Prostaglandin D_2 metabolite stimulates collagen synthesis by human osteoblasts during calcification, Prostaglandins 41 (1991) 303–313.
[8] M.A. Gallant, R. Samadfam, J.A. Hackett, J. Antoniou, J.L. Parent, A.J. de Brum-Fernandes, Production of prostaglandin D_2 by human osteoblasts and modulation of osteoprotegerin, RANKL, and cellular migration by DP and CRTH2 receptors, J. Bone Miner. Res. 20 (2005) 672–681.
[9] H. Tokuda, O. Kozawa, A. Harada, T. Uematsu, Prostaglandin D_2 induces interleukin-6 synthesis via Ca2+ mobilization in osteoblasts: regulation by protein kinase C, Prostaglandins Leukot. Essent. Fat. Acids 61 (1999) 189–194.
[10] H. Tokuda, S. Takai, R. Matsushima-Nishiwaki, Y. Hanai, S. Adachi, C. Minamitani, J. Mizutani, T. Otsuka, O. Kozawa, Function of Rho-kinase in prostaglandin D_2-induced interleukin-6 synthesis in osteoblasts, Prostaglandins Leukot. Essent. Fat. Acids 79 (2008) 41–46.
[11] O. Kozawa, T. Otsuka, D. Hatakeyama, M. Niwa M, H. Matsuno, H. Ito H, K. Kato, N. Matsui, T. Uematsu, Mechanism of prostaglandin D_2-stimulated heat shock protein 27 induction in osteoblasts, Cell. Signal. 13 (2001) 535–541.
[12] K. Kato, H. Tokuda, H. Natsume, S. Adachi, R. Matsushima-Nishiwaki, C. Minamitani, J. Mizutani, O. Kozawa, T. Otsuka T, Rho-kinase regulates prostaglandin D_2-stimulated heat shock protein 27 induction in osteoblasts, Exp. Ther. Med. 1 (2010) 579–583.
[13] J. Jankun, S.H. Selman, R. Swiercz, E. Skrzypczak-Jankun, Why drinking green tea could prevent cancer, Nature 387 (1997) 561.
[14] J.B. Harbourne, C.A. Williams, Advances in flavonoid research since 1992, Phytochemistry 55 (2000) 481–504.
[15] J.A. Baur, K.J. Pearson, N.L. Price, H.A. Jamieson, C. Lerin, A. Kalra, V.V. Prabhu, J.S. Allard, G. Lopez-Lluch, K. Lewis, P.J. Pistell, S. Poosala, K.G. Becker, O. Boss, D. Gwinn, M. Wang, S. Ramaswamy, K.W. Fishbein, R.G. Spencer, E.G. Lakatta, D. Le Couteur, R.J. Shaw, P. Navas, P. Puigserver, D.K. Ingram, R. de Cabo, D.A. Sinclair, Resveratrol improves health and survival of mice on a high-calorie diet, Nature 444 (2006) 37–42.
[16] S.H. Koo, M. Montminy, In vino veritas: a tale of two sirt1s? Cell 127 (2006) 1091–1093.
[17] S. Renaud, M. de Lorgeril, Wine, alcohol, platelets, and the French paradox for coronary heart disease, Lancet 339 (1992) 1523–1526.
[18] J.T. Kubo, M.L. Stefanick, J. Robbins, J. Wactawski-Wende, M.R. Cullen, M. Freiberg, M. Desai, Preference for wine is associated with lower hip fracture incidence in post-menopausal women, BMC Women's Health 13 (2013) 36.
[19] H. Sudo, H. Kodama, Y. Amagai, S. Yamamoto, S. Kasai, in vivo differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria, J. Cell Biol. 96 (1983) 191–198.
[20] O. Kozawa, H. Tokuda, M. Miwa, J. Kotoyori, Y. Oiso, Cross-talk regulation between cyclic AMP production and phosphoinositide hydrolysis induced by prostaglandin E_2 in osteoblast-like cells, Exp. Cell. Res. 198 (1992) 130–134.
[21] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[22] K. Kato, H. Ito, K. Hasegawa, Y. Inaguma, O. Kozawa, T. Asano, Modulation of the stress-induced synthesis of hsp27 and αB-crystallin by cyclic AMP in C6 glioma cells, J. Neurochem. 66 (1996) 946–950.
[23] J.C. Milne, P.D. Lambert, S. Schenk, D.P. Carney, J.J. Smith, D.J. Gagne, Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes, Nature 450 (2007) 712–716.
[24] J.M. Kyriakis, J. Avruch, Mammalian *mitogen-activated protein kinase signal* transduction pathways activated by stress and inflammation, Physiol. Rev. 81 (2001) 807–869.
[25] D.R. Alessi, PD98059 is a specific inhibitor of the activation of mitogen-activated protein kinase in vitro and in vivo, J. Biol. Chem. 270 (1995) 27489–27494.
[26] A. Cuenda, J. Rouse, Y.N. Doza, R. Meier, P. Cohen, T.F. Gallagher, P.R. Young, J. C. Lee, SB203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1, FEBS Lett. 364 (1995) 229–233.
[27] B.L. Bennett, D.T. Sasaki, B.W. Murray, E.C. O'Leary, S.T. Sakata, W. Xu, J. C. Leisten, L. Motiwala, S. Peierce, Y. Satoh, S.S. Bhagwat, A.M. Manning, D.

W. Anderson, SP600125, an anthrapyrazolone inhibitor of Jun N-terminal Kinase, Proc. Natl. Acad. Sci. U.S.A 98 (2001) 13681–13686.
[28] Y. Fukata, M. Amano, K. Kaibuchi, Rho-Rho-kinase pathway in smooth muscle contraction and cytoskeletal reorganization of non-muscle cells, Trends Pharmacol. Sci. 22 (2001) 32–39.
[29] M. Ito, T. Nakano, F. Erdodi, D.J. Hartshorne, Myosin phosphatase: structure, regulation and function, Mol. Cell. Biochem. 259 (2004) 197–209.
[30] Y. Koshihara, M. Kawamura, Prostaglandin D_2 stimulates calcification of human osteoblastic cells, Biochem. Biophys. Res. Commun. 159 (1989) 1206–1212.
[31] T. Walle, F. Hsieh, M.H. DeLegge, J.E. Oatis Jr, U.K. Walle, High absorption but very low bioavailability of oral resveratrol in humans, Drug Metab. Dispos. 32 (2004) 1377–1382.
[32] A. Ram, H.P. Pandey, H. Matsumura, K. Kasahara-Orita, T. Nakajima, R. Takahata, S. Satoh, A. Terao, O. Hayaishi, CSF levels of prostaglandins, especially the level of prostaglandin D_2, are correlated with increasing propensity towards sleep in rats, Brain Res. 751 (1997) 81–89.

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

NAOHIRO YAMAMOTO[1,2], HARUHIKO TOKUDA[2,3], GEN KUROYANAGI[1,2], JUN MIZUTANI[1], RIE MATSUSHIMA-NISHIWAKI[2], AKIRA KONDO[1,2], OSAMU KOZAWA[2] and TAKANOBU OTSUKA[1]

[1]Department of Orthopedic Surgery, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601; [2]Department of Pharmacology, Gifu University Graduate School of Medicine, Gifu 501-1194; [3]Department of Clinical Laboratory, National Center for Geriatrics and Gerontology, Obu, Aichi 474-8511, Japan

Received April 23, 2014; Accepted September 8, 2014

DOI: 10.3892/ijmm.2014.1934

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

Correspondence to: Dr Haruhiko Tokuda, Department of Clinical Laboratory, National Center for Geriatrics and Gerontology, 35 Gengo, Morioka-cho, Obu, Aichi 474-8511, Japan
E-mail: tokuda@ncgg.go.jp

Key words: resveratrol, prostaglandin E_2, osteoprotegerin, osteoblast

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.

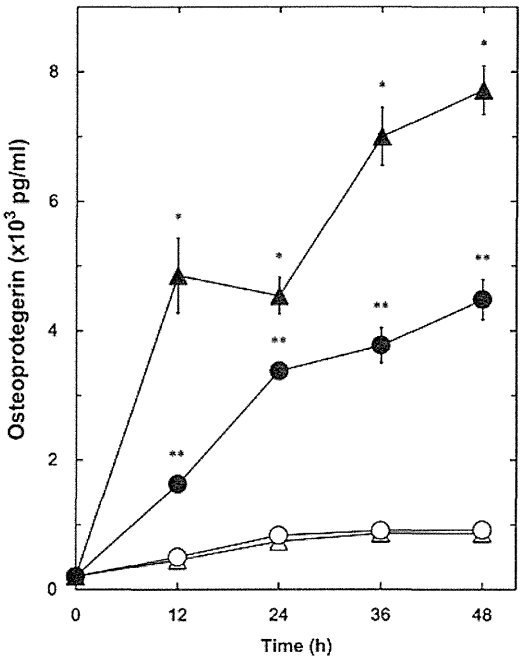

Figure 1. Effects of resveratrol on the prostaglandin E_2 (PGE_2)-stimulated release of osteoprotegerin (OPG) in MC3T3-E1 cells. The cultured cells were pre-treated with 50 μM of resveratrol (● and ○) or the vehicle (▲ and △) for 60 min, and then stimulated with 10 μM of PGE_2 (● and ▲) or the vehicle (○ 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.

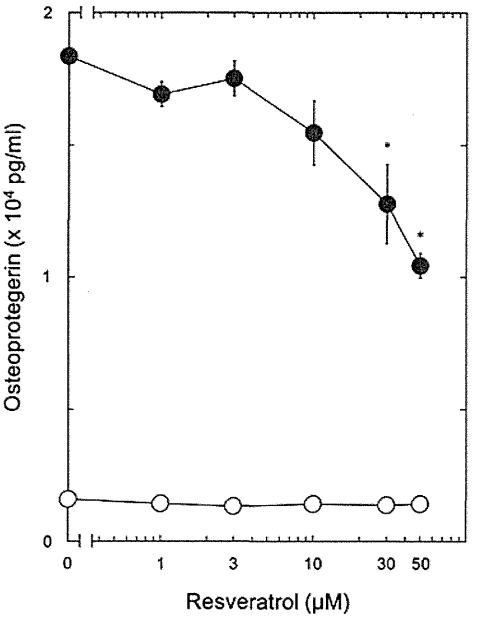

Figure 2. Effects of resveratrol on the prostaglandin E_2 (PGE_2)-stimulated release of osteoprotegerin (OPG) in MC3T3-E1 cells. The cultured cells were pre-treated with various concentrations of resveratrol for 60 min and then stimulated with 10 μM of PGE_2 (●) or the vehicle (○) for 48 h. OPG concentrations 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.

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).

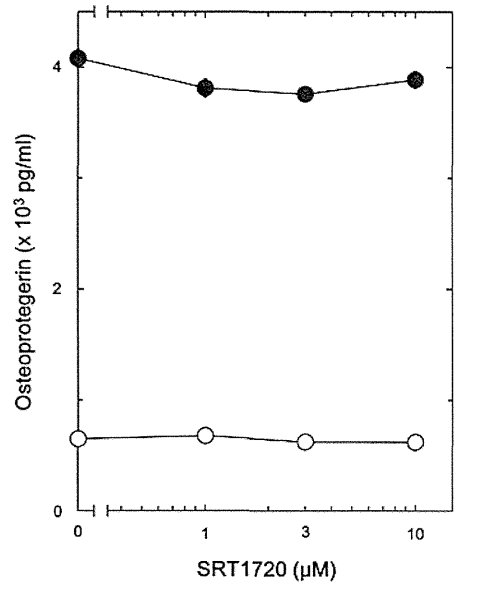

Figure 3. Effects of SRT1720 on the prostaglandin E_2 (PGE_2)-stimulated release of osteoprotegerin (OPG) in MC3T3-E1 cells. The cultured cells were pre-treated with various concentrations of SRT1720 for 60 min and then stimulated with 10 μM of PGE_2 (●) or the vehicle (○) for 48 h. OPG concentrations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

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

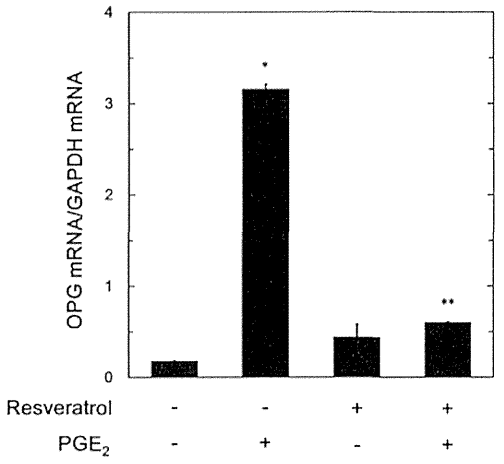

Figure 4. Effects of resveratrol on the prostaglandin E_2 (PGE_2)-induced mRNA expression of osteoprotegerin (OPG) in MC3T3-E1 cells. The cultured cells were pre-treated with 50 μM of resveratrol or the vehicle for 60 min and then stimulated with 10 μM of PGE_2 or the vehicle for 3 h. The respective 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.

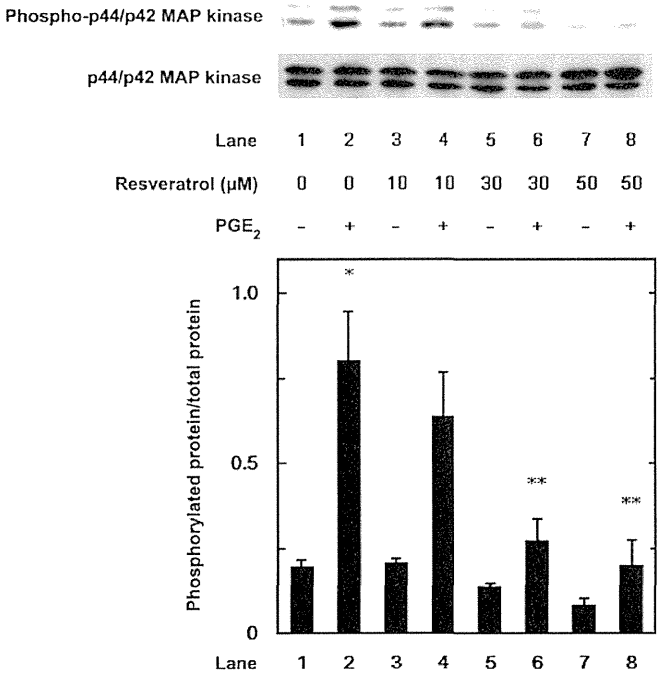

Figure 5. Effects of resveratrol on the prostaglandin E_2 (PGE_2)-induced phosphorylation of p44/p42 mitogen-activated protein (MAP) kinase in MC3T3-E1 cells. The cultured cells were pre-treated with various concentrations of resveratrol for 60 min and then stimulated with 10 μM of PGE_2 or the vehicle 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/

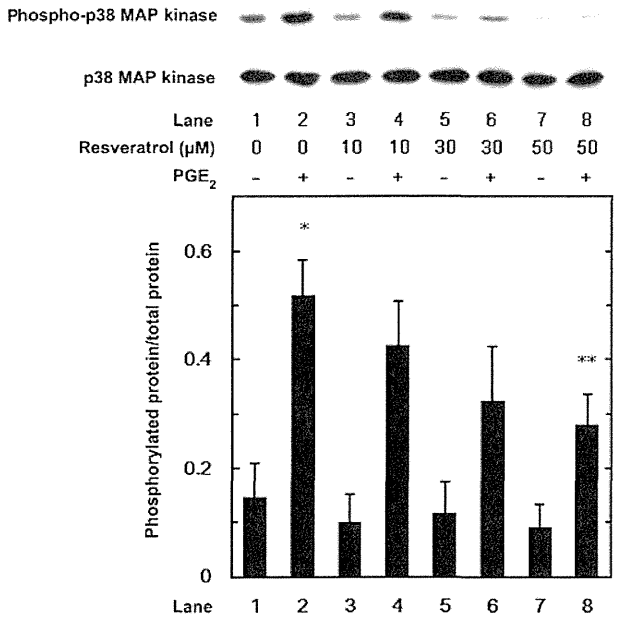

Figure 6. Effects of resveratrol on the prostaglandin E_2 (PGE_2)-induced phosphorylation of p38 mitogen-activated protein (MAP) kinase in MC3T3-E1 cells. The cultured cells were pre-treated with various concentrations of resveratrol for 60 min and then stimulated with 10 μM of PGE_2 or the vehicle for 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.

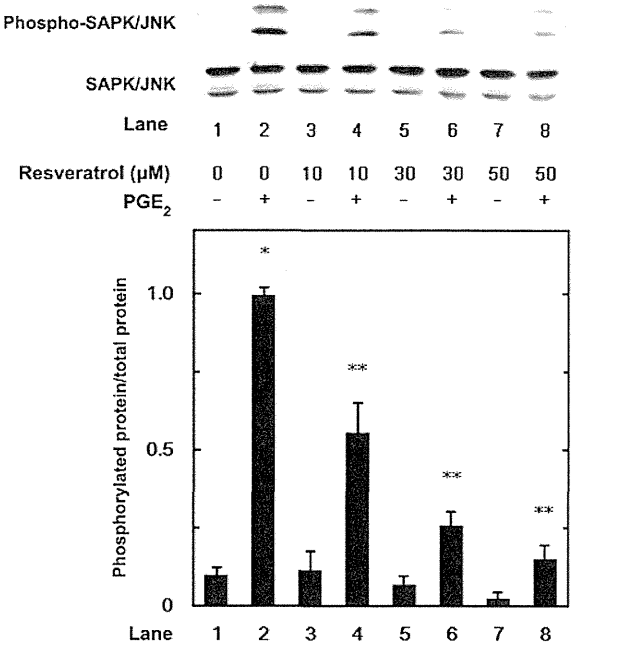

Figure 7. Effects of resveratrol on the prostaglandin E_2 (PGE_2)-induced phosphorylation of stress-activated protein kinase/*c-Jun* N-terminal kinase (SAPK/JNK) in MC3T3-E1 cells. The cultured cells were pre-treated with various concentrations of resveratrol for 60 min and then stimulated with 10 μM 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

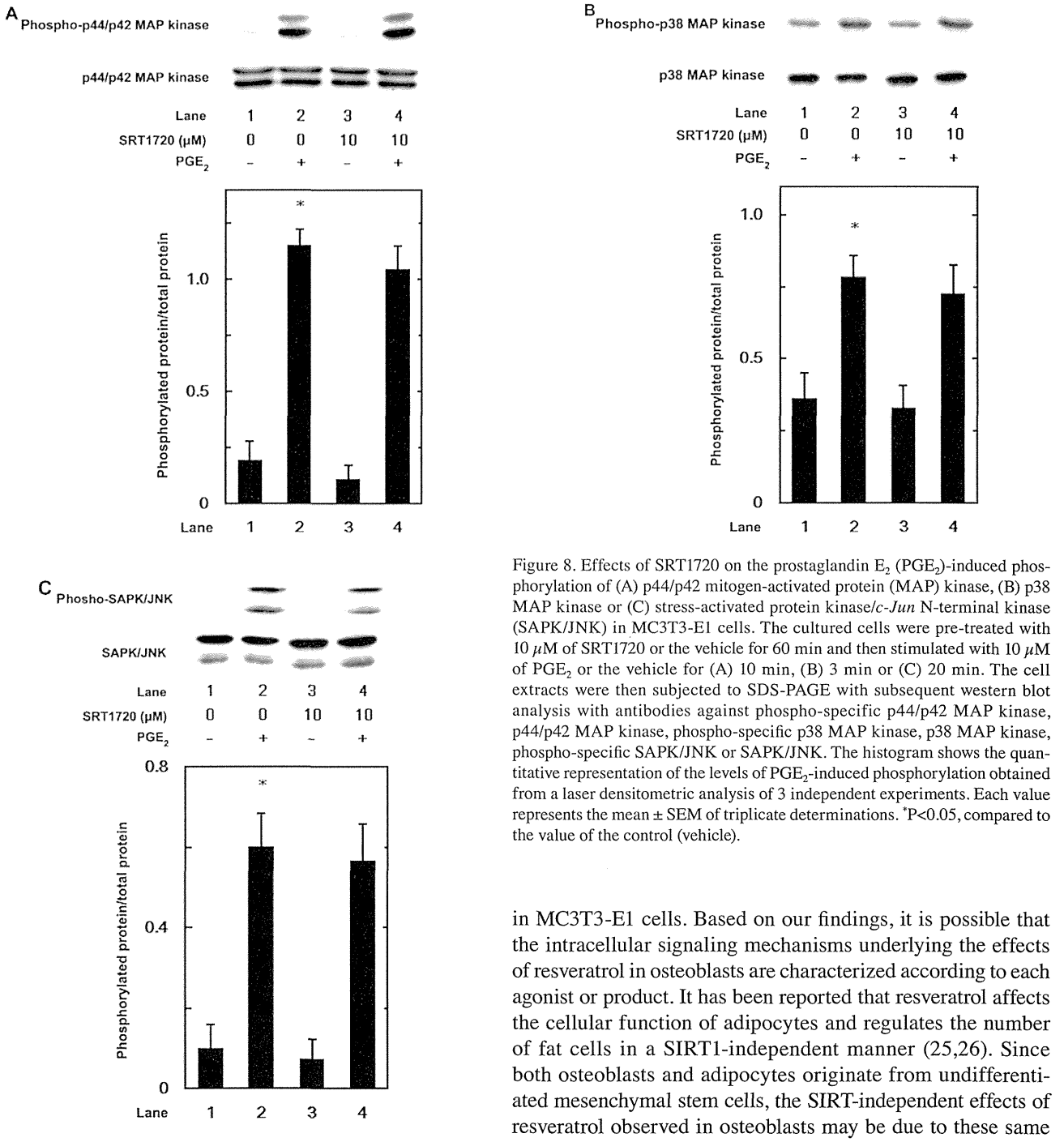

Figure 8. Effects of SRT1720 on the prostaglandin E_2 (PGE_2)-induced phosphorylation of (A) p44/p42 mitogen-activated protein (MAP) kinase, (B) p38 MAP kinase or (C) stress-activated protein kinase/*c-Jun* N-terminal kinase (SAPK/JNK) in MC3T3-E1 cells. The cultured cells were pre-treated with 10 μM 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.

Acknowledgements

We are very grateful to Yumiko Kurokawa for her skillful technical assistance. This investigation was supported in part by Grants-in-Aid for Scientific Research (19591042) from the Ministry of Education, Science, Sports and Culture of Japan, the Foundation for Growth Science, the Research Funding for Longevity Sciences (23-9, 25-4) and the National Center for Geriatrics and Gerontology (NCGG), Japan.

References

1. Karsenty G and Wagner EF: Reaching a genetic and molecular understanding of skeletal development. Dev Cell 2: 389-406, 2002.
2. Parfitt AM: Targeted and nontargeted bone remodeling: relationship to basic multicellular unit origination and progression. Bone 30: 5-7, 2002.
3. Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Lüthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G, DeRose M, Elliott R, Colombero A, Tan HL, Trail G, Sullivan J, Davy E, Bucay N, Renshaw-Gegg L, Hughes TM, Hill D, Pattison W, Campbell P, Sander S, Van G, Tarpley J, Derby P, Lee R and Boyle WJ: Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Cell 89: 309-319, 1997.
4. Udagawa N, Takahashi N, Yasuda H, Mizuno A, Itoh K, Ueno Y, Shinki T, Gillespie MT, Martin TJ, Higashio K and Suda T: Osteoprotegerin produced by osteoblasts is an important regulator in osteoclast development and function. Endocrinology 141: 3478-3484, 2000.
5. Kwan TS, Padrines M, Théoleyre S, Heymann D and Fortun Y: IL-6, RANKL, TNF-α/IL-1: interrelations in bone resorption pathophysiology. Cytokine Growth Factor Rev 15: 49-60, 2004.
6. Nijiweide PJ, Burger EH and Feyen JH: Cells of bone: proliferation, differentiation, and hormonal regulation. Physiol Rev 66: 855-886, 1986.
7. Smith WL: The eicosanoids and their biochemical mechanisms of action. Biochem J 259: 315-324, 1989.
8. Blackwell KA, Raisz LG and Pilbeam CC: Prostaglandins in bone: bad cop, good cop? Trends Endocrinol Metab 21: 294-301, 2010.
9. Kozawa O, Suzuki A, Tokuda H, Kaida T and Uematsu T: Interleukin-6 synthesis induced by prostaglandin E_2: cross-talk regulation by protein kinase C. Bone 22: 355-360, 1998.
10. Tokuda H, Kozawa O, Niwa M, Matsuno H, Kato K and Uematsu T: Mechanism of prostaglandin E_2-stimulated heat shock protein 27 induction in osteoblasts-like MC3T3-E1 cells. J Endocrinol 172: 271-281, 2002.
11. Frémont L: Biological effects of resveratrol. Life Sci 66: 663-673, 2000.
12. Baur JA, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A, Prabhu VV, Allard JS, Lopez-Lluch G, Lewis K, Pistell PJ, Poosala S, Becker KG, Boss O, Gwinn D, Wang M, Ramaswamy S, Fishbein KW, Spencer RG, Lakatta EG, Le Couteur D, Shaw RJ, Navas P, Puigserver P, Ingram DK, de Cabo R and Sinclair DA: Resveratrol improves health and survival of mice on a high-calorie diet. Nature 444: 337-342, 2006.
13. Timmers S, Auwerx J and Schrauwen P: The journey of resveratrol from yeast to human. Aging 4: 146-158, 2012.
14. Kondo A, Otsuka T, Kuroyanagi G, Yamamoto N, Matsushima-Nishiwaki R, Mizutani J, Kozawa O and Tokuda H: Resveratrol inhibits BMP-4-stimulated VEGF synthesis in osteoblasts: Suppression of S6 kinase. Int J Mol Med 33: 1013-1018, 2014.
15. Sudo H, Kodama H, Amagai Y, Yamamoto S and Kasai S: In vivo differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J Cell Biol 96: 191-198, 1983.
16. Kozawa O, Tokuda H, Miwa M, Kotoyori J and Oiso Y: Cross-talk regulation between cyclic AMP production and phosphoinositide hydrolysis induced by prostaglandin E_2 in osteoblast-like cells. Exp Cell Res 198: 130-134, 1992.
17. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685, 1970.
18. Kato K, Ito H, Hasegawa K, Inaguma Y, Kozawa O and Asano T: Modulation of the stress-induced synthesis of hsp27 and αB-crystallin by cyclic AMP in C6 glioma cells. J Neurochem 66: 946-950, 1996.
19. Milne JC, Lambert PD, Schenk S, Carney DP, Smith JJ and Gagne DJ: Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes. Nature 450: 712-716, 2007.
20. Kyriakis JM and Avruch J: Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev 81: 807-869, 2001.
21. Alessi DR: PD98059 is a specific inhibitor of the activation of mitogen-activated protein kinase in vitro and in vivo. J Biol Chem 270: 27489-27494, 1995.
22. Cuenda A, Rouse J, Doza YN, Meier R, Cohen P, Gallagher TF, Young PR and Lee JC: SB203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. FEBS Lett 364: 229-233, 1995.
23. Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Leisten JC, Motiwala L, Peierce S, Satoh Y, Bhagwat SS, Manning SM and Anderson DW: SP600125, an anthrapyrazolone inhibitor of Jun N-terminal Kinase. Proc Natl Acad Sci USA 98: 13681-13686, 2001.
24. Widmann C, Gibson S, Jarpe MB and Johnson GL: Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol Rev 79: 143-180, 1999.
25. Mader I, Wabitsch M, Debatin KM, Fischer-Posovsky P and Fulda S: Identification of a novel proapoptotic function of resveratrol in fat cells: SIRT1-independent sensitization to TRAIL-induced apoptosis. FASEB J 24: 1997-2009, 2010.
26. Wang A, Liu M, Liu X, Dong LQ, Glickman RD, Slaga TJ, Zhou Z and Liu F: Up-regulation of adiponectin by resveratrol: the essential roles of the Akt/FOXO1 and AMP-activated protein kinase signaling pathways and DsbA-L. J Biol Chem 286: 60-66, 2011.

Int. J. Mol. Sci. **2014**, 15, 17886-17900; doi:10.3390/ijms151017886

OPEN ACCESS
International Journal of
Molecular Sciences
ISSN 1422-0067
www.mdpi.com/journal/ijms

Article

Down-Regulation by Resveratrol of Basic Fibroblast Growth Factor-Stimulated Osteoprotegerin Synthesis through Suppression of Akt in Osteoblasts

Gen Kuroyanagi [1,2], Takanobu Otsuka [1], Naohiro Yamamoto [1,2], Rie Matsushima-Nishiwaki [2], Akira Nakakami [2], Jun Mizutani [1], Osamu Kozawa [2] and Haruhiko Tokuda [2,3,*]

[1] Department of Orthopedic Surgery, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601, Japan; E-Mails: kokuryugen@yahoo.co.jp (G.K.); pharma2@gifu-u.ac.jp (T.O.); artemicion2000@gmail.com (N.Y.); yakuri@gifu-u.ac.jp (J.M.)

[2] Department of Pharmacology, Gifu University Graduate School of Medicine, Gifu 501-1194, Japan; E-Mails: riemn@gifu-u.ac.jp (R.M.-N.); pharma1@gifu-u.ac.jp (A.N.); okozawa@gifu-u.ac.jp (O.K.)

[3] Department of Clinical Laboratory, National Center for Geriatrics and Gerontology, Obu, Aichi 474-8511, Japan

* Author to whom correspondence should be addressed; E-Mail: tokuda@ncgg.go.jp; Tel./Fax: +81-562-462-311.

External Editor: Xiaofeng Jia

Received: 21 August 2014; in revised form: 18 September 2014 / Accepted: 26 September 2014 / Published: 6 October 2014

Abstract: It is firmly established that resveratrol, a natural food compound abundantly found in grape skins and red wine, has beneficial properties for human health. In the present study, we investigated the effect of basic fibroblast growth factor (FGF-2) on osteoprotegerin (OPG) synthesis in osteoblast-like MC3T3-E1 cells and whether resveratrol affects the OPG synthesis. FGF-2 stimulated both the OPG release and the expression of OPG mRNA. Resveratrol significantly suppressed the FGF-2-stimulated OPG release and the mRNA levels of OPG. SRT1720, an activator of SIRT1, reduced the FGF-2-induced OPG release and the OPG mRNA expression. PD98059, an inhibitor of upstream kinase activating p44/p42 mitogen-activated protein (MAP) kinase, had little effect on the FGF-2-stimulated OPG release. On the other hand, SB203580, an inhibitor of p38 MAP kinase, SP600125, an inhibitor of stress-activated protein kinase/c-*Jun* *N*-terminal kinase (SAPK/JNK), and Akt inhibitor suppressed the OPG release induced by

FGF-2. Resveratrol failed to affect the FGF-2-induced phosphorylation of p44/p42 MAP kinase, p38 MAP kinase or SAPK/JNK. The phosphorylation of Akt induced by FGF-2 was significantly suppressed by resveratrol or SRT1720. These findings strongly suggest that resveratrol down-regulates FGF-2-stimulated OPG synthesis through the suppression of the Akt pathway in osteoblasts and that the inhibitory effect of resveratrol is mediated at least in part by SIRT1 activation.

Keywords: resveratrol; fibroblast growth factor (FGF-2); osteoprotegerin; osteoblast

1. Introduction

Bone metabolism is a sophisticated process composed of osteoblastic bone formation and osteoclastic bone resorption [1]. The resorption of preexisting bone by osteoclasts and the formation of new bone by osteoblasts are strictly coordinated to maintain adequate bone mass and strength. Disordered bone remodeling causes metabolic bone diseases, including osteoporosis and fracture healing distress. In the process of bone remodeling, it is generally recognized that a variety of humoral factors, such as prostaglandins and cytokines, play important roles [2]. Osteoprotegerin, an essential secreted protein in bone turnover, which has inhibitory effects on osteoclast activation, is a member of the tumor necrosis factor receptor family, along with receptor activator of nuclear factor-κB (RANK) [3]. Osteoprotegerin is known to be synthesized in osteoblasts and secreted, bind to RANK ligand (RANKL) as a decoy receptor and prevent RANKL from binding to RANK, resulting in suppression of bone resorption [3]. It has been shown that osteoprotegerin-knock out mice suffer from severe osteoporosis, suggesting that osteoprotegerin is a key regulator of osteoclastogenesis and bone metabolism [4]. It is currently recognized that the RANK/RANKL/osteoprotegerin axis is a major regulatory system for osteoclast formation and action [5].

Basic fibroblast growth factor (FGF-2), one of heparin-binding growth factors, is synthesized by osteoblasts and embedded in bone matrix [6,7]. It has been reported that FGF-2 has a potent stimulatory effect on bone formation [8]. During fracture repair, up-regulation of FGF-2 expression in osteoblasts is detected [9]. Therefore, it is currently recognized that FGF-2 plays a crucial role in fracture healing, bone remodeling and osteogenesis [10]. Regarding the intracellular signaling mechanism of FGF-2 in osteoblasts, we have previously demonstrated that FGF-2 stimulates the synthesis of vascular endothelial growth factor (VEGF), a specific growth factor for endothelial proliferation, through p44/p42 mitogen-activated protein (MAP) kinase and stress-activated protein kinase/c-*Jun N*-terminal kinase (SAPK/JNK) in osteoblast-like MC3T3-E1 cells [11,12]. We have also shown that FGF-2 stimulates interleukin-6 (IL-6) synthesis through p38 MAP kinase in these cells [13]. On the other hand, Akt, also called protein kinase B, has been identified as a downstream target of phosphatidylinositol 3-kinase in a variety of cells, including osteoblasts [14–17]. We have previously reported that Akt activated by FGF-2 negatively regulates the FGF-2-induced VEGF release in MC3T3-E1 cells [18]. Thus, three MAP kinases and Akt are considered to play important roles cooperatively in the FGF-2-intracellular signaling in osteoblast functions.

Polyphenolic compounds in foods, including vegetables and fruits, have beneficial properties for human beings. It is generally known that the natural food compounds possess antioxidative, anti-inflammatory and antitumor effects on various tissues and cells [19,20]. Among them, resveratrol, a polyphenol found abundantly in red grape and berries, can delay the aging process, extend lifespan and reduce the risk of numerous degenerative diseases [21,22]. The French population reportedly tends to smoke and to take saturated fatty acid in meals, but yet maintain relatively low levels of cardiovascular events, which is due to the many amounts of consumption of wine containing abundant resveratrol [23]. Regarding the health of bone, it has recently been reported that women, who preferentially consume wine, have a lower risk of hip fracture compared to non-drinkers, past drinkers and those with other alcohol preferences [24]. As for the molecular mechanism behind the effect of resveratrol, it has been demonstrated that resveratrol exerts its effects through SIRT1, which is consistent with improved cellular function and organismal health by binding to and enhancing the activity of the nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylase [25]. NAD^+ is biosynthesized in the human body as a precursor in nicotinamide and has a role important for energy acquisition as a coenzyme of oxidoreductase. However, the detailed actions of resveratrol in bone metabolism and the exact mechanism have not yet been clarified.

In the present study, we investigated the effect of FGF-2 on osteoprotegerin synthesis and whether resveratrol affects the osteoprotegerin synthesis in osteoblast-like MC3T3-E1 cells. We herein demonstrate that resveratrol suppresses FGF-2-stimulated osteoprotegerin synthesis through the down-regulation of the Akt pathway in MC3T3-E1 cells and that the effect of resveratrol is mediated at least in part via SIRT1 activation.

2. Results and Discussion

2.1. Results

2.1.1. Effect of Resveratrol on the Fibroblast Growth Factor (FGF-2)-Stimulated Osteoprotegerin Release in MC3T3-E1 Cells

We have previously demonstrated that FGF-2 stimulates the synthesis of IL-6 and VEGF in osteoblast-like MC3T3-E1 cells [11–13]. In the present study, we first investigated whether FGF-2 could stimulate osteoprotegerin synthesis or not in these cells. FGF-2 significantly stimulated the osteoprotegerin release in a time-dependent manner up to 36 h (Figure 1). The maximum effect of FGF-2 on osteoprotegerin release was observed at 36 h and decreased thereafter. We next examined the effect of resveratrol on the FGF-2-stimulated osteoprotegerin release. Resveratrol significantly suppressed the FGF-2-stimulated osteoprotegerin release (Figure 1). The maximum inhibitory effect of resveratrol on the FGF-2-induced osteoprotegerin release was observed at 48 h. The inhibitory effect of resveratrol was dose-dependent within a range between 1 and 50 μM (Figure 2). The effect of resveratrol at 50 μM caused an approximately 75% decrease compared to the osteoprotegerin levels with FGF-2 alone.