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

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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)

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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.

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