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Original Research Article

Resveratrol reduces prostaglandin E_1-stimulated osteoprotegerin synthesis in osteoblasts: Suppression of stress-activated protein kinase/c-*Jun* N-terminal kinase

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ABSTRACT

Resveratrol, a natural polyphenol mainly existing in red grapes and berries, possesses beneficial effects on human being. We have previously reported that prostaglandin E_1 (PGE_1) stimulates vascular endothelial growth factor synthesis via activation of p38 mitogen-activated protein (MAP) kinase and stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK) but not p44/p42 MAP kinase in osteoblast-like MC3T3-E1 cells. In the present study, we investigated the PGE_1-effect on osteoprotegerin (OPG) synthesis and the effect of resveratrol on the synthesis in MC3T3-E1 cells. PGE_1 induced the expression levels of OPG mRNA and stimulated the OPG release. Resveratrol significantly reduced the PGE_1-induced OPG release and the mRNA expression. SRT1720, an activator of SIRT1, suppressed the release of OPG. The protein levels of SIRT1 were not up-regulated by resveratrol with or without PGE_1. Both SB203580 and SP600125, a specific p38 MAP kinase inhibitor and a specific SAPK/JNK inhibitor, respectively, but not PD98059, a specific MEK inhibitor, reduced the PGE_1-stimulated OPG release. Resveratrol or SRT1720 failed to affect the phosphorylation of p38 MAP kinase. On the contrary, PGE_1-induced phosphorylation of SAPK/JNK was significantly attenuated by both resveratrol and SRT1720. Our results strongly suggest that resveratrol inhibits PGE_1-stimulated OPG synthesis via suppressing SAPK/JNK but not p38 MAP kinase in osteoblasts.

1. Introduction

Osteoprotegerin (OPG), a glycoprotein that belongs to the tumor necrosis factor receptor superfamily, is well recognized to possess inhibitory effects on osteoclast activation along with receptor activator of nuclear factor κB (RANK) [1]. Bone metabolism is regulated mainly by two functional cells, osteoblasts and osteoclasts responsible for bone formation and bone resorption, respectively [2]. The formation of bone structures and bone remodeling result from the sophisticated coupling process of osteoblasts and osteoclasts. The disorder of bone remodeling causes metabolic bone disorders such as osteoporosis and fracture healing distress. In proceeding of bone remodeling, it is generally recognized that numerous humoral factors including prostaglandins and cytokines play pivotal roles [3]. In response to numerous hormones, cytokines and prostaglandins, osteoblasts produce RANK ligand (RANKL) and OPG [3]. OPG binds to RANKL as a decoy receptor and inhibits the binding of RANKL to RANK, 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 the function of osteoclasts.

It is firmly established that prostaglandins act as local factors, autacoids, in bone metabolism, and play an important role in bone cell function. Among them, prostaglandin E_1 (PGE_1) is recognized as a potent bone-resorptive agent in the control of bone anabolism [6]. In our previous studies [7,8], we have shown that PGE_1 stimulates the synthesis of vascular endothelial growth factor via

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p38 mitogen-activated protein (MAP) kinase and stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK) but not p44/p42 MAP kinase in osteoblast-like MC3T3-E1 cells. However, the exact role of PGE_1 in osteoblasts remains to be elucidated.

Polyphenolic compounds in foods such as vegetables and fruits have beneficial properties for human being. Among them, flavonoids show antioxidative, antiinflammatory and anticarcinogenic effects [9,10]. The consumption of resveratrol, a natural polyphenolic flavonoid enriched in red grapes and berries, reportedly improves health and prolongs life [11]. It is well known that there is low mortality from coronary heart disease in France with many amount of wine consumption containing abundant resveratrol [12]. It has been shown that resveratrol increases life span in lower organisms by activating the nicotinamide adenine dinucleotide (NAD^+)-dependent histone deacetylase SIRT1 [13]. NAD^+ is biosynthesized in the body as a precursor of nicotinamide, and has an important role for energy acquisition as a coenzyme of oxidoreductase. SIRT1 has been identified as a transcriptional silencer in yeast and modulates the pathways downstream of caloric restriction that produces beneficial effects for mammals [11]. However, the details of resveratrol actions on bone metabolism have not yet been clarified.

In the present study, we investigated the mechanism of OPG synthesis induced by PGE_1 and the effect of resveratrol on the OPG synthesis in osteoblast-like MC3T3-E1 cells. We herein demonstrate that resveratrol suppresses PGE_1-stimulated OPG synthesis through inhibiting activation of SAPK/JNK in osteoblasts.

2. Materials and methods

2.1. Materials

Resveratrol, SRT1720, PD98059, SB203580 and SP600125 were obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). PGE_1 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 p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies, SAPK/JNK antibodies and SIRT1 antibodies were obtained from Cell Signaling, Inc. (Beverly, MA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). An ECL Western blotting detection system was obtained from GE Healthcare (Buckinghamshire, UK). Other materials and chemicals were obtained from commercial sources. PGE_1 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 that have been derived from newborn mouse calvaria [14] were maintained as previously described [15]. 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 PGE_1 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. The viability of cells stimulated by 10 μM of PGE_1 for 48 h with 1 h-pretreatment of 50 μM of resveratrol, 10 μM of SRT1720, 30 μM of SB203580 or 10 μM of SP600125 was above 97% compared to that without pretreatment by trypan blue dye exclusion test.

2.4. Real-time RT-PCR

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 PGE_1 or vehicle in α-MEM containing 0.3% FBS for 3 h. Total RNA was isolated and transcribed into complementary DNA using Trizol reagent (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 PGE_1 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 [16] 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 described previously [17] using phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies, SAPK/JNK antibodies, SIRT1 antibodies or GAPDH 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 1.45). The phosphorylated protein levels were calculated as follows: the background-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.