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Involvement of Rho-kinase in prostaglandin E_1-stimulated VEGF synthesis through stress-activated protein kinase/c-*Jun* N-terminal kinase in osteoblast-like MC3T3-E1 cells

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ABSTRACT

We have previously shown that prostaglandin E_1 (PGE_1) stimulates the synthesis of vascular endothelial growth factor (VEGF) through 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 involvement of Rho-kinase in the PGE_1-stimulated VEGF synthesis in these cells. PGE_1 induced within 3 min the phosphorylation of myosin phosphatase targeting subunit (MYPT-1), a substrate of Rho-kinase. Y27632 and fasudil, specific inhibitors of Rho-kinase, which attenuated the MYPT-1 phosphorylation, significantly suppressed the PGE_1-stimulated VEGF synthesis. Y27632 and fasudil markedly reduced the PGE_1-induced phosphorylation of SAPK/JNK without affecting the phosphorylation levels of p38 MAP kinase or p44/p42 MAP kinase. These results strongly suggest that Rho-kinase functions at a point upstream of SAPK/JNK and regulates PGE_1-stimulated VEGF synthesis in osteoblasts.

1. Introduction

Prostaglandins function as autacoids in bone metabolism and play a crucial role in bone cell function [1,2]. It is well recognized that the bone metabolism is regulated mainly by two functional cells, osteoblasts and osteoclasts, the former being responsible for bone formation and the latter for bone resorption, respectively [1]. Among them, prostaglandin (PG) E_1 and PGE_2 are recognized as potent bone resorptive agents [1,2]. Based on pharmacological studies, PGE receptors have been classified into four subtypes: EP_1 receptor, which induces phosphatidylinositol (PI) hydrolysis; EP_2 receptor and EP_4 receptor, which increase cAMP levels and EP_3 receptor, which decreases the cAMP concentrations [2,3]. In our previous studies regarding about the effects of PGE_1 and PGE_2 on osteoblasts, we have shown that PGE_2 induces the activation of EP_1 receptor and EP_2 receptor, resulting in both PI hydrolysis and cAMP increase [4]. On the other hand, PGE_1 induces cAMP production but not phosphatidylinositol hydrolysis in osteoblast-like MC3T3-E1 cells [5]. Our findings made us to speculate that the effect of PGE_1 on osteoblasts is exerted mainly via EP_2 receptor. However, the exact role of PGE_1 in osteoblasts remains to be clarified.

It is well known that vascular endothelial growth factor (VEGF) is a specific growth factor of vascular endothelium [6]. VEGF, produced and secreted from a variety of cell types, increases capillary permeability and stimulates proliferation of endothelial cells [6]. During bone remodeling, the microvasculature is provided by capillary endothelial cells, and osteoblasts and osteoprogenitor cells, which proliferate locally and differentiate into osteoblasts, migrate into the resorption lacuna. Thus, it is generally recognized that the activities of osteoblasts, osteoclasts, and capillary endothelial cells are closely coordinated and regulate bone metabolism [7]. These functional cells are considered to influence one another via humoral factors as well as by direct cell-to-cell contact. With regard to bone metabolism, it has been reported that an inactivation of VEGF causes complete suppression of blood vessel invasion concomitant with impaired trabecular bone formation and expansion of hypertrophic chondrocyte zone in mouse tibial epiphyseal growth plate [8]. Accumulating evidence suggests that osteoblasts among bone cells synthesize and secrete VEGF in response to various physiological agents such as insulin-like growth factor-I and bone morphogenetic protein [8]. In our previous studies [9,10], we have reported that PGE_1 stimulates VEGF synthesis in osteoblast-like MC3T3-E1 cells, and that VEGF synthesis is regulated by p38

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mitogen-activated protein (MAP) kinase and stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK) but not p44/p42 MAP kinase among the MAP kinase superfamily [11]. Based on these findings, VEGF secreted from osteoblasts may couple angiogenesis to bone formation by adjusting the angiogenic response to osteoblastic activity [8]. However, the exact mechanism behind VEGF synthesis in osteoblasts and its release from these cells is not precisely elucidated.

Rho and the down-stream effector, Rho-associated kinase (Rho-kinase) play crucial roles in a variety of cellular functions such as smooth muscle contraction and cell motility [12–14]. As for osteoblasts, it has been demonstrated that Rho and p38 MAP kinase are involved in the endothelin-1-induced expression of prostaglandin endoperoxide G/H synthase mRNA in osteoblasts [15]. In addition, it has been shown that the Rho/Rho-kinase pathway stimulates osteoblast proliferation whereas it inhibits osteoblast differentiation [16]. In a previous study [17], we have reported that Rho-kinase functions as a positive regulator in endothelin-1-induced synthesis of interleukin-6, a potent bone resorptive agent, in osteoblast-like MC3T3-E1 cells. However, the exact role of Rho-kinase in osteoblasts has not yet been fully clarified.

In the present study, we investigated whether Rho-kinase is involved in the PGE_1-stimulated VEGF synthesis in osteoblast-like MC3T3-E1 cells. We here show that Rho-kinase functions at a point upstream of SAPK/JNK in PGE_1-stimulated VEGF synthesis in these cells.

2. Materials and methods

2.1. Materials

PGE_1 and hydroxyfasudil (fasudil) were purchased from Sigma (St. Louis, MO). Mouse VEGF enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D Systems, Inc. (Minneapolis, MN). Y27632 was obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). Anti-phospho-specific MYPT-1 (Thr 850) antibodies were purchased from Upstate (Lake Placid, NY). Anti-MYPT-1 antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against phospho-specific p38 MAP kinase, p38 MAP kinase, phospho-specific SAPK/JNK, SAPK/JNK, phospho-specific p44/p42 MAP kinase and p44/p42 MAP kinase were purchased from Cell Signaling, Inc. (Beverly, MA). Y27632 and fasudil were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect the assay for VEGF or Western blot analysis.

2.2. Cell culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [18] were maintained as previously described [19]. Briefly, the cells were cultured in α-minimum essential medium (α-MEM) containing 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The cells were seeded into 35-mm (5×10^4/dish) or 90-mm (25×10^4/dish) diameter dishes in α-MEM containing 10% FCS. After 5 days, the medium was exchanged for α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h.

2.3. VEGF assay

The cultured cells were stimulated by 10 μM PGE_1 in 1 ml of α-MEM containing 0.3% FCS for 48 h. When indicated, the cells were pretreated with various doses of Y27632 or fasudil for 60 min. The conditioned medium was then collected at the end of the incubation, and the VEGF concentration was measured by ELISA kit.

2.4. Western blot analysis

Western blotting analysis was performed as follows [20]. The cultured cells were pretreated with various doses of Y27632 or fasudil for 60 min, and then stimulated by 10 μM PGE_1 in α-MEM containing 0.3% FCS 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, 3% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol. The cytosolic fraction was collected as a supernatant after centrifugation at 125,000 × *g* for 10 min at 4 °C. SDS–polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli [21] in 10% polyacrylamide gel. The protein (20 μg) was fractionated and transferred onto an Immun-Blot PVDF Membrane (Bio-Rad, Hercules, CA). 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 the primary antibodies. Peroxidase-labeled antibodies raised in goat against rabbit IgG were used as second antibodies. The first and second antibodies were diluted at 1:1000 with 5% fat-free dry milk in TBS-T. Peroxidase activity on the membrane was visualized on X-ray film by means of the ECL Western blotting detection system (GE Healthcare; Buckinghamshire, UK).

2.5. Determinations

The absorbance of enzyme immunoassay samples was measured at 450 nm with EL 340 Bio Kinetic Reader (Bio-Tek Instruments, Inc., Winooski, VT). The densitometric analysis of the bands on the film was performed using Molecular Analyst/Macintosh (Bio-Rad Laboratories, Hercules, CA). The cell viability was assessed by utilizing MTT.

2.6. Statistical analysis

The data were analyzed by ANOVA followed by the Bonferroni method for multiple comparisons between pairs, and a $p < 0.05$ was considered significant. All data are presented as the mean ± S.E.M. of triplicate independent determinations. Each experiment was repeated three times with similar results.

3. Results

3.1. Effect of PGE_1 on the phosphorylation of MYPT-1 in MC3T3-E1 cells

In order to investigate whether PGE_1 activates Rho-kinase in osteoblast-like MC3T3-E1 cells, we examined the effect of PGE_1 on the phosphorylation of myosin phosphatase targeting subunit (MYPT-1), which is a component of myosin phosphatase and a well known down-stream target of Rho-kinase [13,22]. We first verified that PGE_1 significantly induced the phosphorylation of MYPT-1 in a time-dependent manner (Fig. 1A). The phosphorylation level of MYPT reached its maximum at 3 min, sustained up to 10 min, and decreased thereafter. Next we found that Y27632, a specific inhibitor of Rho-kinase [14], truly attenuated the PGE_1-induced phosphorylation levels of MYPT-1 (Fig. 1B). Additionally, fasudil, another inhibitor of Rho-kinase [14], also suppressed the phosphorylation levels of MYPT-1 stimulated by PGE_1 (Fig. 1C).

3.2. Effects of Y27632 or fasudil on the PGE_1-stimulated VEGF synthesis in MC3T3-E1 cells

We have previously shown that PGE_1 stimulates VEGF synthesis in osteoblast-like MC3T3-E1 cells [9,10]. Therefore, we next examined the effects of Rho-kinase inhibitors on the PGE_1-stimulated