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Rho-kinase limits BMP-4-stimulated osteocalcin synthesis in osteoblasts: Regulation of the p38 MAP kinase pathway

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ABSTRACT

Aim: We previously reported that bone morphogenetic protein-4 (BMP-4) stimulates the synthesis of osteocalcin via p38 mitogen-activated protein (MAP) kinase in osteoblast-like MC3T3-E1 cells, whereas p44/p42 MAP kinase plays as a negative regulator in the synthesis. In the present study, we investigated whether Rho-kinase is involved in BMP-4-stimulated osteocalcin synthesis in MC3T3-E1 cells.
Main methods: The levels of osteocalcin were measured by ELISA. The phosphorylation of each protein kinase was analyzed by Western blotting. The mRNA levels of osteocalcin were determined by real-time RT-PCR.
Key findings: BMP-4 induced the phosphorylation of myosin phosphatase targeting subunit-1 (MYPT-1), a substrate of Rho-kinase. Y27632 or fasudil, specific inhibitors of Rho-kinase, which attenuated the MYPT-1 phosphorylation, significantly amplified the BMP-4-stimulated osteocalcin synthesis in a dose-dependent manner. The osteocalcin mRNA expression levels induced by BMP-4 were enhanced by Y27632 or fasudil. BMP-4-stimulated osteocalcin release was significantly up-regulated in Rho-knocked down cells with Rho A-siRNA. Y27632 or fasudil failed to affect the BMP-4-induced phosphorylation of SMAD1 or p44/p42 MAP kinase. On the other hand, Y27632 or fasudil markedly strengthened the phosphorylation levels of p38 MAP kinase induced by BMP-4.
Significance: These results strongly suggest that Rho-kinase negatively regulates BMP-4-stimulated osteocalcin synthesis via the p38 MAP kinase pathway in osteoblasts.

Introduction

Bone metabolism is strictly regulated by osteoblasts and osteoclasts, responsible for bone formation and bone resorption, respectively (Karsenty and Wagner, 2002). These functional cells are considered to affect one another via humoral factors as well as by direct cell-to-cell interaction. Nowadays, it is generally recognized that osteoblasts play a crucial role also in the regulation of bone resorption through receptor activator of nuclear factor κB ligand (RANKL) expression in response to a variety of bone resorptive stimuli (Boyce and Xing, 2008). Osteoblasts, which are differentiated from mesenchymal progenitors, express various cell type-specific markers during the differentiation process. Since osteocalcin is synthesized specifically in osteoblasts, it is recognized as one of the markers of mature osteoblast phenotype (Hauschka et al., 1989). Osteocalcin, also known as bone Gla-protein, is modified post-translationally by vitamin K-dependent γ-carboxylation (Hauschka et al., 1989). It has been shown that osteocalcin-deficient mice reportedly display an increase of bone formation without impairing bone resorption, suggesting that osteocalcin is a determinant of moderate bone formation (Ducy et al., 1996). In addition, it has recently been proposed that un-carboxylated osteocalcin released from osteoblasts could function as a potent hormone, and influence glucose and fat metabolism through acting on pancreatic β-cells to increase insulin synthesis, adipocytes to increase adiponectin, and skeletal myocytes to increase glucose uptake (Lee and Karsenty, 2008; Rosen, 2008). It is generally known that osteocalcin synthesis is stimulated by bone morphogenetic proteins (BMPs) (Hauschka et al., 1989). BMPs are multifunctional cytokines and belong to the transforming growth factor-β (TGF-β) superfamily including TGF-β and activin (Miyazono et al., 2005). The effects of BMPs are exerted through the intracellular signaling of Smad proteins and mitogen-activated protein (MAP) kinases (Miyazono et al., 2005; Yamaguchi et al., 1999). We have previously reported that BMP-4 stimulates osteocalcin synthesis in osteoblast-like MC3T3-E1 cells, and that among the MAP kinase superfamily (Kyriakis and Avruch, 2001), the osteocalcin synthesis is positively regulated by p38 MAP kinase while it is negatively regulated by p44/p42 MAP kinase (Kozawa et al.,

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2002). However, the exact mechanism behind osteocalcin synthesis in osteoblasts has not been precisely elucidated.

It is generally known that 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 (Fukata et al., 2001; Riento and Ridley, 2003; Shimokawa and Rashid, 2007). With regard to 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 (Windischhofer et al., 2002). In addition, the activation of Rho/Rho-kinase pathway reportedly stimulates osteoblast proliferation whereas it inhibits osteoblast differentiation (Harmey et al., 2004). In a previous study (Tokuda et al., 2007), we have reported that Rho-kinase acts as a positive regulator in endothelin-1-induced synthesis of interleukin-6, a potent bone resorptive agent, in osteoblast-like MC3T3-E1 cells. With regard to the BMP-effects on bone metabolism, it has recently been shown that Rho-kinase inhibitor stimulates the BMP-2-induced ectopic bone formation in murine calvarial cells (Yoshikawa et al., 2009). 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 BMP-4-stimulated osteocalcin synthesis in osteoblast-like MC3T3-E1 cells. We here show that Rho-kinase negatively regulates BMP-4-stimulated osteocalcin synthesis via suppression of p38 MAP kinase in these cells.

Materials and methods

Materials

BMP-4 was purchased from R&D Systems, Inc. (Minneapolis, MN). Y27632 and SB203580 were obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). Hydroxyfasudil (fasudil) was purchased from Sigma (St. Louis, MO). Mouse osteocalcin enzyme-linked immunosorbent assay (ELISA) kit was obtained from Biomedical Technologies, Inc. (Stoughton, MA). Phospho-specific myosin phosphatase targeting subunit-1 (MYPT-1) antibodies, Rho A antibodies, phospho-specific SMAD1 antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific p44/p42 MAP kinase antibodies and p44/p42 MAP kinase antibodies were purchased from Cell Signaling Technology (Beverly, MA). MYPT-1 antibodies and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). ECL Western blotting detection system was purchased from GE Healthcare (Buckinghamshire, UK). Control short interfering RNA (siRNA; Silencer Negative Control no. 1 siRNA) was purchased from Ambion (Austin, TX). Rho A-siRNAs (Mm_Rhoa_2 and Mm_Rhoa_3) and Omniscript Reverse Transcriptase kit were purchased from QIAGEN (Hilden, Germany). SiLentFect was purchased from Bio-Rad (Hercules, CA). Trizol reagent was purchased from Invitrogen (Carlsbad, CA). FastStart DNA Master SYBR Green I was purchased from Roche Diagnostics (Mannheim, Germany). Other materials and chemicals were obtained from commercial sources. Y27632 and SB203580 were dissolved in dimethyl sulfoxide (DMSO). The maximum concentration of DMSO was 0.1%, which did not affect the assay for osteocalcin or Western blot analysis.

Cell culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria (Sudo et al., 1983) were maintained as previously described (Kozawa et al., 1992). Briefly, the cells were cultured in α-minimum essential medium (α-MEM) containing 10% fetal calf serum (FCS) at 37 ºC in a humidified atmosphere of 5% CO_2/95% air. The cells were seeded into 35-mm (5×10^4/dish) or 90-mm (2×10^5/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.

Osteocalcin assay

The cultured cells were stimulated by 70 ng/ml of BMP-4 in 1 ml of α-MEM containing 0.3% FCS for the indicated periods. When indicated, the cells were pretreated with SB203580 for 60 min, and then incubated with Y27632 or fasudil for 60 min. The conditioned medium was collected at the end of the incubation, and the osteocalcin concentration was measured by ELISA kit.

Western blot analysis

The cultured cells were stimulated by BMP-4 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, 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol. SDS-polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli (Laemmli, 1970) in 10% polyacrylamide gel. Western blot analysis was performed as previously described (Kato et al., 1996) by using phospho-specific MYPT-1 antibodies, MYPT-1 antibodies, GAPDH antibodies, Rho A antibodies, phospho-specific SMAD1 antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific p44/p42 MAP kinase antibodies or p44/p42 MAP kinase antibodies with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as secondary antibodies. Peroxidase activity on the PVDF sheet was visualized on X-ray film by means of the ECL Western blotting detection system.

Real-time RT-PCR

The cultured cells were pretreated with 30 μM of Y27632, 30 μM of fasudil or vehicle for 60 min, and then stimulated by 70 ng/ml of BMP-4 or vehicle in α-MEM containing 0.3% FCS for the indicated periods. Total RNA was isolated and transcribed into complementary DNA using Trizol reagent and Omniscript Reverse Transcriptase kit, respectively. Real-time RT-PCR was performed using a Light Cycler system in capillaries and FastStart DNA Master SYBR Green I provided with the kit. Sense and antisense primers were synthesized based on the reports of Zhang et al. (2008) for mouse osteocalcin and Simpson et al. (2000) for mouse GAPDH. Osteocalcin mRNA levels were normalized with those of GAPDH mRNA.

siRNA transfection

To knock down Rho A in MC3T3-E1 cells, the cells were transfected with negative control siRNA or Rho A-siRNA utilizing siLentFect according to the manufacturer's protocol. In brief, the cells (1×10^5 cells) were seeded into 35-mm diameter dishes in α-MEM containing 10% FCS and sub-cultured for 48 h. The cells were then incubated at 37 °C with 50 nM siRNA–siLentFect complexes. After 24 h, the medium was exchanged to α-MEM containing 0.3% FCS. Then, they were stimulated by BMP-4 in α-MEM containing 0.3% FCS for the indicated periods.

Determination

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 was performed using Molecular Analyst/Macintosh (Bio-Rad Laboratories, Hercules, CA). The phosphorylated protein levels were calculated and corrected by total protein as follows: the background-subtracted signal intensity of each phosphorylation signal was normalized to the respective total protein signal and