Attenuation of prostaglandin E_1-induced osteoprotegerin synthesis in osteoblasts by normoxic HIF inducers

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Abstract. Mimosine, which is a natural plant amino acid present in the *Leucaena* genus, is able to induce hypoxia-inducible factors (HIFs). Previous evidence has indicated that HIF regulates angiogenesis-osteogenesis coupling in bone metabolism, and it has previously been reported that mimosine inhibits prostaglandin (PG)$F_{2\alpha}$-induced osteoprotegerin (OPG) synthesis without affecting interleukin-6 (IL-6) production in osteoblast-like MC3T3-E1 cells. In addition, PGE_1 has been demonstrated to induce OPG synthesis via activation of p38 mitogen-activated protein (MAP) kinase and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in these cells, and PGE_1 stimulates IL-6 production via the activation of protein kinase A. In the present study, the effects of mimosine on the PGE_1-stimulated synthesis of OPG and IL-6 were investigated in osteoblast-like MC3T3-E1 cells. The concentrations of OPG and IL-6 were measured using relevant ELISA kits. OPG mRNA was measured by semi-quantitative reverse transcription polymerase chain reaction. The phosphorylation of p38 MAP kinase and SAPK/JNK was analyzed by western blotting. Mimosine significantly reduced PGE_1-induced release of OPG and OPG mRNA expression levels without affecting the release of IL-6. In addition, deferoxamine, which is also a normoxic HIF inducer, significantly inhibited PGE_1-induced OPG release and OPG mRNA expression levels; however, it had little effect on IL-6 release. Furthermore, mimosine and deferoxamine failed to affect PGE_1-stimulated phosphorylation of p38 MAP kinase or SAPK/JNK. These results strongly suggest that normoxic HIF inducers attenuate PGE_1-stimulated OPG synthesis without affecting IL-6 production in osteoblasts.

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Introduction

Bone quantity and quality is maintained by bone remodeling, a biphasic process that consists of bone resorption and formation (1). The process is dependent on two antagonistic functional cell types: Mesenchymal stem cell-derived osteoblasts and hematopoietic stem cell-derived osteoclasts, which are responsible for bone formation and resorption, respectively (1). Dysregulation of bone remodeling leads to metabolic bone disorders, including osteoporosis and perturbation of fracture healing (1). Numerous humoral factors, including cytokines, growth factors and prostaglandins (PGs), have been demonstrated to affect bone remodeling (2). Osteoprotegerin (OPG), which belongs to the tumor necrosis factor receptor superfamily, is an essential osteoblast-secretory protein and decoy receptor for the receptor activator of nuclear factor-κB ligand (RANKL) (3). OPG prevents osteoclastogenesis from blocking RANK-RANKL binding, which is an essential step of bone resorption (3). It has previously been reported that OPG-deficient mice display severe osteoporosis (4). The competitive antagonism between RANKL and OPG for RANK binding is a crucial regulatory system in bone remodeling (5).

PGs, which are lipid signaling molecules, are involved in various physiological processes (6). PGs act as autacoids in bone metabolism, and modulate bone cell function (6). Although PGs have conventionally been recognized as bone resorptive agents (7), there is accumulating evidence supporting the involvement of PGs in the process of bone formation (7,8). It has previously been reported that PGE_1 stimulates the synthesis of OPG via activation of p38 mitogen-activated protein (MAP) kinase and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in osteoblast-like MC3T3-E1 cells (9). PGE_1 has also been demonstrated to stimulate the secretion of interleukin-6 (IL-6), a multifunctional cytokine that modulates bone metabolism (10,11), via the cAMP protein kinase A pathway in osteoblast-like MC3T3-E1 cells (12).

Mimosine, which is a natural plant amino acid present in the *Leucaena* genus, is an iron chelator that suppresses

DNA replication in mammals (13). In addition, mimosine is a normoxic hypoxia-inducible factor (HIF) inducer (14). HIFs act as DNA-binding transcription factors with specific nuclear cofactors under low oxygen concentrations, and activate a series of hypoxia-associated genes in response to hypoxic environments (15). Numerous genes, including glucose transporter protein 1, erythropoietin and vascular endothelial growth factor (VEGF), have been identified as HIF target genes (14). Therefore, HIFs are recognized to be implicated in the regulation of glucose metabolism, erythropoiesis, and angiogenesis (14). It has previously been demonstrated that hypoxia enhances osteoclast-mediated bone resorption (15), suggesting that HIFs are also involved in bone metabolism. In addition, the complex HIF-1α reportedly promotes bone formation by the direct stimulation of osteoblast proliferation and induces angiogenesis, resulting in the stimulation of bone regeneration (16,17). It has previously been reported that mimosine upregulates HIF-1α protein levels and inhibits $PGF_{2\alpha}$-induced OPG synthesis without affecting IL-6 release in osteoblast-like MC3T3-E1 cells (18). However, the precise mechanism underlying the effects of HIFs on osteoblasts is yet to be elucidated.

In the present study, the effects of two normoxic HIF inducers, mimosine and deferoxamine (19) on PGE_1-stimulated synthesis of OPG and IL-6 in osteoblast-like MC3T3-E1 cells were investigated. The results strongly suggest that normoxic HIF inducers suppress PGE_1-stimulated OPG synthesis without affecting IL-6 production in osteoblasts.

Materials and methods

Materials. Mimosine, deferoxamine and PGE_1 were obtained from Sigma-Aldrich; Merck Millipore. Mouse OPG enzyme-linked immunosorbent assay (ELISA, cat. no. MOP00) and mouse IL-6 ELISA (cat. no. SM6000B) kits were obtained from R&D Systems, Inc. (Minneapolis, MN, USA). Phospho-specific p38 MAP kinase antibodies (cat. no. 4511), p38 MAP kinase antibodies (cat. no. 9212), phospho-specific SAPK/JNK antibodies (cat. no. 4671) and SAPK/JNK antibodies (cat. no. 9252) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). An enhanced chemiluminescence (ECL) western blotting detection system was obtained from GE Healthcare Life Sciences (Little Chalfont, UK). Other materials and chemicals were obtained from commercial sources. PGE_1 was dissolved in ethanol. Mimosine was dissolved in PBS supplemented with 0.01% bovine serum albumin (BSA) containing 7.5% $NaHCO_3$. Deferoxamine was dissolved in PBS supplemented with 0.01% BSA. The maximum concentration of ethanol was 0.1%, which did not affect the assay for osteoprotegerin release, IL-6 release, osteoprotegerin mRNA expression, or western blot analysis.

Cell culture. Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria (20) were maintained as previously described (21). Cells were cultured in α-minimum essential medium (α-MEM; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C in a humidified atmosphere containing 5% CO_2. 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. Medium was exchanged for α-MEM containing 0.3% FBS following 5 days of culture. Cells were used for experiments 48 h after this.

OPG and IL-6 assay. Cultured cells were pretreated with 300, 500 or 700 μM mimosine or 100, 300, 500 μM deferoxamine, or vehicle (PBS supplemented with 0.01% BSA) for 60 min at 37˚C, and then stimulated with 10 μM of PGE_1 or 50 μl of vehicle (PBS supplemented with 0.01% BSA containing 0.1% ethanol) in 1 ml of α-MEM containing 0.3% FBS for the for 48 h at 37˚C. The conditioned medium was collected at the end of incubation, and the concentrations of OPG and IL-6 were measured using OPG ELISA and IL-6 ELISA kits, respectively, according to the manufacturer's protocols.

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). Cultured cells were pretreated with 700 μM mimosine, 500 μM deferoxamine or vehicle (PBS supplemented with 0.01% BSA) for 60 min at 37˚C, and then stimulated with 10 μM of PGE_1 or vehicle (PBS supplemented with 0.01% BSA containing 0.1% ethanol) in α-MEM containing 0.3% FBS for 3 h at 37˚C. Total RNA was isolated and transcribed into cDNA using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and an Omniscript Reverse Transcriptase kit (Qiagen Inc., Valencia, CA, USA), respectively. RT-PCR was performed using a Light Cycler system with capillaries and the Fast Start DNA Master SYBR Green I provided with the kit (Roche Diagnostics, Basel, Switzerland). Sense and antisense primers for mouse OPG mRNA were purchased from Takara Bio, Inc. (Otsu, Japan; primer set ID: OPG; MA026526), while glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA primers were synthesized based on the report of Simpson *et al* (22). The 20 μl reaction mixture was incubated at 95˚C for 10 min, followed by 40 cycles at 60˚C for 5 sec and 72˚C for 7 sec. Amplified products were determined using melting curve analysis according to the system protocol. OPG mRNA levels were normalized to those of GAPDH mRNA.

Western blot analysis. Cultured cells were pretreated with 300, 500 or 700 μM of mimosine or 100, 300 or 500 μM of deferoxamine for 60 min at 37˚C, and then stimulated with 10 μM of PGE_1 or vehicle (PBS supplemented with 0.01% BSA containing 0.1% ethanol) in α-MEM containing 0.3% FBS for 10 min for p38 MAP kinase and 20 min for SAPK/JNK, respectively. The cells were washed twice with phosphate-buffered saline and then lysed, homogenized and sonicated with ultrasonic disruptor (Tomy Seiko Co., Ltd, Tokyo, Japan) at 20 kHz for 10 sec in lysis buffer containing 62.5 mM Tris/HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol. The protein concentration in each cell lysate was determined by the Pierce BCA Protein Assay kit (cat. no. 23227, Thermo Fisher Scientific, Inc.). A total of 10 μl containing 10 μg of protein was loaded to each lane. SDS-polyacrylamide gel electrophoresis (PAGE) was performed in the method of Laemmli (23) using 10% polyacrylamide gel. Protein was fractionated and transferred onto Immun-Blot polyvinylidene fluoride (PVDF)

membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked with 5% fat-free dry milk in Tris-buffered saline-Tween (TBST; 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) for 1 h at room temperature prior to incubation with primary antibodies. A western blot analysis was performed as described previously (24) using 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 goat anti-rabbit IgG antibodies being used as secondary antibodies (cat. no. 074-1506; KPL, Inc., Gaithersburg, MD, USA). Primary and secondary antibodies were diluted at 1:1,000 with 5% fat-free dry milk in TBST for overnight at room temperature for primary antibodies, and 1 h at room temperature for secondary antibodies, respectively. Peroxidase activity on the PVDF membrane was visualized on X-ray film by means of the ECL western blotting detection system.

Statistical analysis. Differences between the mean values for individual groups were assessed with one-way analysis of variance, followed by application of the Bonferroni correction for multiple comparisons between pairs. P<0.05 was considered to indicate a statistically significant difference. All data are presented as the mean ± standard error of the mean, which was determined from three independent cell preparations.

Results

Effects of mimosine or deferoxamine on PGE_1-stimulated OPG release in MC3T3-E1 cells. It has previously been reported that PGE_1 stimulates OPG synthesis in osteoblast-like MC3T3-E1 cells (9). Therefore, the effects of mimosine, an inducer of HIF (14), on PGE_1-stimulated OPG release in MC3T3-E1 cells were investigated. Mimosine treatment significantly decreased PGE_1-stimulated OPG release for up to 48 h compared with untreated cells (12 h, P=0.01; 24 h, P=0.001; 36 h, P=0.0001; 48 h, P=0.000001; Fig. 1), and the inhibitory effect was dose-dependent between 300 and 700 μM (300 μM, P=0.02; 500 μM, P=0.01; 700 μM, P=0.01 vs. untreated cells; Fig. 2A). The maximum inhibitory effect of mimosine was observed at 700 μM, which resulted in a ~90% decrease in PGE_1-stimulated OPG release compared with the untreated group (Fig. 2A). In addition, deferoxamine, another inducer of HIF-1α that exerts an angiogenic action through stimulation of the HIF-1α pathway (19), also significantly reduced PGE_1-stimulated OPG release compared with untreated cells (12 h, P=0.02; 24 h, P=0.000003; 36 h, P=0.0001; 48 h, P=0.000001; Fig. 1). The suppressive effect of deferoxamine on OPG release was dose-dependent between 100 and 500 μM (Fig. 2B). Deferoxamine at 500 mM induced a ~80% decrease in the PGE_1-stimulated OPG release (Fig. 2B).

Effects of mimosine and deferoxamine on PGE_1-stimulated IL-6 release in MC3T3-E1 cells. It has previously been demonstrated that PGE_1 upregulates the synthesis of IL-6 in osteoblast-like MC3T3-E1 cells (12). Therefore, the effects of mimosine and deferoxamine on PGE_1-stimulated IL-6 release were investigated in these cells. Treatment with up to 700 μM mimosine had little effect on PGE_1-induced IL-6 release (Fig. 3A). In addition, treatment with up to 500 μM deferoxamine failed to affect PGE_1-induced IL-6 release (Fig. 3B).

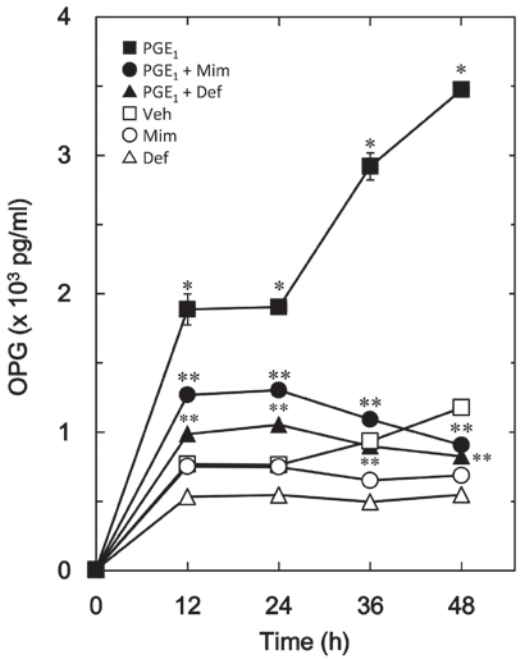

Figure 1. Effects of mimosine or deferoxamine on PGE_1-stimulated OPG release in MC3T3-E1 cells. Cultured cells were pretreated with 700 μM mimosine (●,○), 500 μM deferoxamine (▲,Δ) or vehicle (■,□) for 60 min, and then stimulated with 10 mM of PGE_1 (●,▲,■) or vehicle (○,Δ,□) for the indicated periods. Each value represents the mean ± standard error of the mean, calculated from three independent cell preparations. *P<0.05 vs. control. **P<0.05 vs. PGE_1 alone. PGE_1, prostaglandin E_1; OPG, osteoprotegerin; Mim, mimosine: Def, deferoxamine; Veh, vehicle.

Effects of mimosine and deferoxamine on PGE_1-induced OPG mRNA expression levels in MC3T3-E1 cells. To clarify whether the suppressive effects of mimosine and deferoxamine on PGE_1-stimulated OPG release were mediated via transcriptional events, the effects of mimosine and deferoxamine on PGE_1-induced mRNA expression levels of OPG were examined using semi-quantitative RT-PCR. OPG mRNA expression levels were downregulated by 700 μM mimosine and 500 μM deferoxamine compared with in untreated cells (P=0.01 and P=0.004, respectively; Fig. 4).

Effects of mimosine and deferoxamine on PGE_1-induced phosphorylation of p38 MAP kinase or SAPK/JNK in MC3T3-E1 cells. A previous study demonstrated that PGE_1 stimulates OPG synthesis via the activation of p38 MAP kinase and SAPK/JNK in osteoblast-like MC3T3-E1 cells (9). In order to clarify whether the inhibitory effects of mimosine and deferoxamine on PGE_1-stimulated OPG synthesis are exerted through the modulation of p38 MAP kinase or SAPK/JNK, the effects of mimosine and deferoxamine on PGE_1-induced phosphorylation of p38 MAP kinase or SAPK/JNK were examined in MC3T3-E1 cells. Mimosine failed to visibly affect PGE_1-induced phosphorylation of either p38 MAP kinase or SAPK/JNK (Figs. 5 and 6). Deferoxamine also demonstrated little effect on the phosphorylation of p38 MAP kinase or SAPK/JNK stimulated by PGE_1 (Figs. 5 and 6).

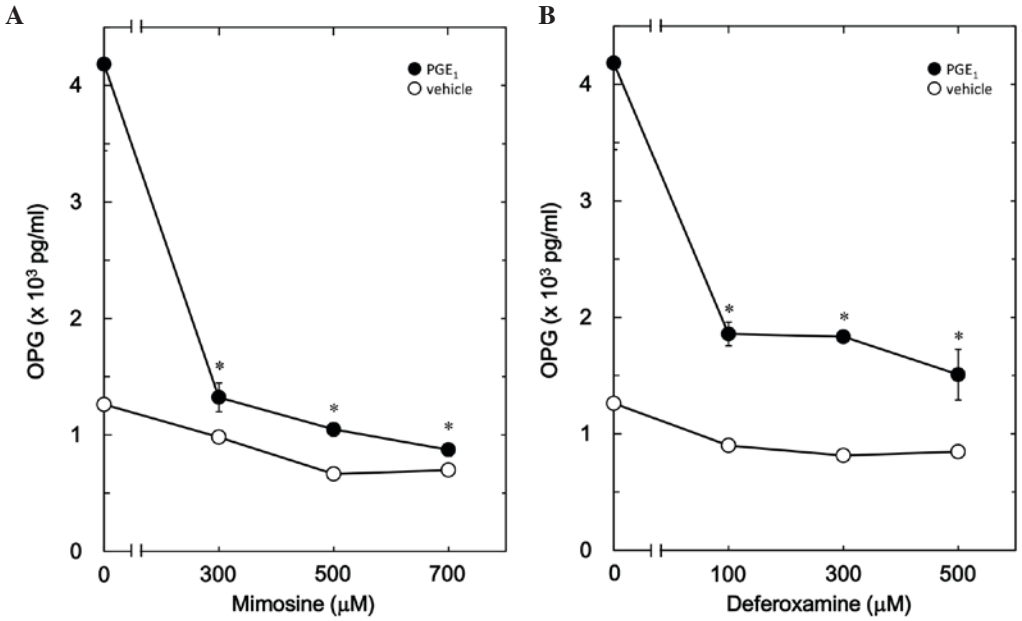

Figure 2. Effects of mimosine and deferoxamine on PGE_1-stimulated OPG release in MC3T3-E1 cells. Cultured cells were pretreated with various doses of (A) mimosine or (B) deferoxamine for 60 min, then stimulated with 10 μM PGE_1 (●) or vehicle (○) for 48 h. Each value represents the mean ± standard error of the mean, calculated from three independent cell preparations. *P<0.05 vs. PGE_1 alone. PGE_1, prostaglandin E_1; OPG, osteoprotegerin.

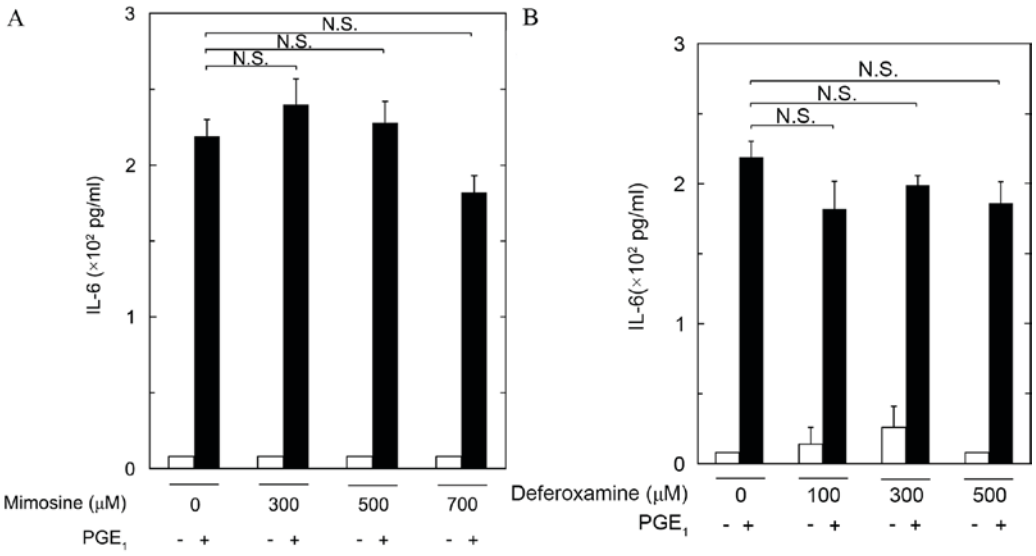

Figure 3. Effects of mimosine and deferoxamine on PGE_1-stimulated IL-6 release in MC3T3-E1 cells. Cultured cells were pretreated with various doses of (A) mimosine or (B) deferoxamine for 60 min, then stimulated with 10 μM PGE_1 or vehicle for 48 h. Each value represents the mean ± standard error of the mean, calculated from three independent cell preparations. N.S. designates no significant difference between the indicated pairs. PGE_1, prostaglandin E_1; IL-6, interleukin 6.

Discussion

The present study demonstrated that PGE_1-induced OPG synthesis was suppressed by mimosine in osteoblast-like MC3T3-E1 cells. Deferoxamine also reduced PGE_1-induced release of OPG in these cells. Mimosine is recognized as an inhibitor of DNA replication, as well as prolyl hydroxylase domain proteins, which are responsible for degrading HIF-1α (14,25). It has been established that deferoxamine, an iron chelator, exerts its angiogenic effects via stimulation of the HIF-1α pathway (19), and it has previously been demonstrated that mimosine and deferoxamine upregulate HIF-1α protein levels in osteoblast-like MC3T3-E1 cells (18). Therefore, it is possible that the inhibitory effects of mimosine and deferoxamine on PGE_1-induced OPG release are exerted via upregulation of the HIF-1α-dependent pathway in MC3T3-E1 cells. In addition, mimosine and deferoxamine significantly attenuated PGE_1-induced OPG mRNA expression levels. It seems unlikely that the suppressive effects of mimosine or deferoxamine on the PGE_1-induced release of OPG are mediated through a post-transcriptional regulatory event in these cells. Therefore, these findings suggested that

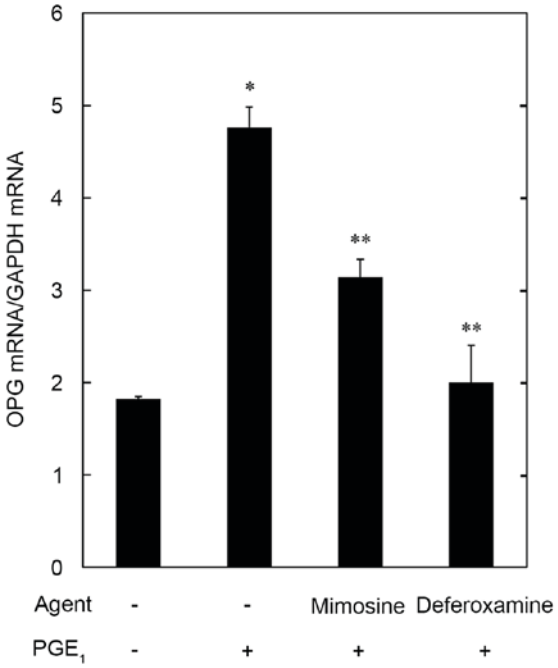

Figure 4. Effects of mimosine and deferoxamine on PGE_1-induced OPG mRNA expression levels in MC3T3-E1 cells. OPG and GAPDH mRNA expression levels were measured by semi-quantitative reverse transcription polymerase chain reaction. OPG mRNA levels were normalized to GAPDH mRNA levels. Each value represents the mean ± standard error of the mean, calculated from three independent cell preparations. *P<0.05 vs. control; **P<0.05 vs. PGE_1 alone. PGE_1, prostaglandin E_1; OPG, osteoprotegerin.

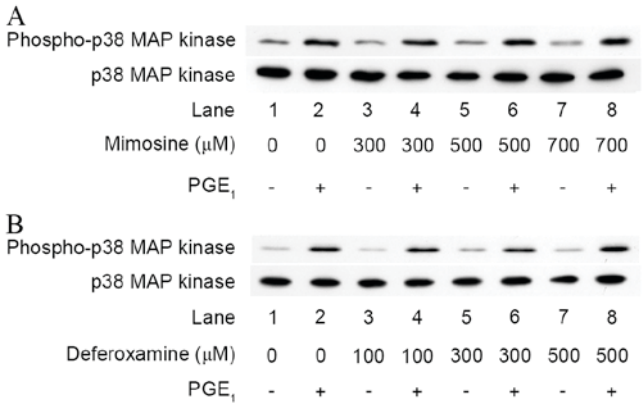

Figure 5. Effects of mimosine and deferoxamine on PGE_1-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. (A) Results of western blotting following mimosine treatment. (B) Results of western blotting following deferoxamine treatment. PGE_1, prostaglandin E_1; MAP, mitogen-activated protein.

mimosine and deferoxamine suppress the synthesis of OPG through upregulation of HIF-1α in response to these agents in osteoblast-like MC3T3-E1 cells. HIF-1 consists of HIF-1α and HIF-1β subunits (14,25), and under normoxic conditions HIF-1α is immediately degraded by the ubiquitin-proteasome system. Chemical hydroxylase inhibitors, including mimosine and deferoxamine, attenuate the process of HIF-1α degradation, resulting in stabilization (14,19,25). Taking these findings into account, it is likely that PGE_1-induced OPG synthesis is downregulated by normoxic HIF inducers in osteoblast-like MC3T3-E1 cells.

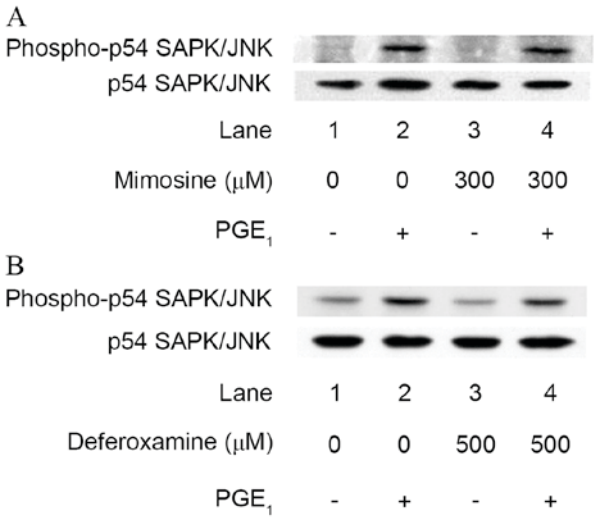

Figure 6. Effects of mimosine and deferoxamine on PGE_1-induced phosphorylation of SAPK/JNK in MC3T3-E1 cells. (A) Results of western blotting following mimosine treatment. (B) Results of western blotting following deferoxamine treatment. PGE_1, prostaglandin E_1; SAPK/JNK, stress-activated protein kinase/c-Jun N-terminal kinase.

It has previously been demonstrated that PGE_1 stimulates the secretion of IL-6 in osteoblast-like MC3T3-E1 cells (12). In the present study, however, PGE_1-induced release of IL-6 was demonstrated to be unaffected by mimosine or deferoxamine in MC3T3-E1 cells. This result indicated that the inhibitory effects of mimosine and deferoxamine on PGE_1-stimulation are OPG synthesis-specific in osteoblast-like MC3T3-E1 cells.

The MAP kinase superfamily is involved in the regulation of cell proliferation, differentiation and survival (26). It is currently established that three MAP kinases, including p38 MAP kinase, p44/p42 MAP kinase and SAPK/JNK, are the most important elements of the superfamily (27). With regards to the effects of PGE_1-intracellular signaling on OPG synthesis, it has previously been reported that p38 MAP kinase and SAPK/JNK act as positive regulators of PGE_1-induced OPG synthesis in osteoblast-like MC3T3-E1 cells, whereas p44/p42 MAP kinase does not (9). The effects of mimosine on PGE_1-stimulated activation of p38 MAP kinase and SAPK/JNK were investigated in these cells, and it was revealed that PGE_1-stimulated phosphorylation of p38 MAP kinase or SAPK/JNK was unaffected by mimosine or deferoxamine. Therefore, it seems unlikely that the suppressive effects of mimosine and deferoxamine are mediated through modulation of MAP kinase activity in MC3T3-E1 cells. In addition, it has previously been reported that mimosine reduces $PGF_{2\alpha}$-stimulated synthesis of OPG, but not IL-6, in MC3T3-E1 cells, and also fails to affect $PGF_{2\alpha}$-stimulated activation of p38 MAP kinase, p44/p42 MAP kinase and SAPK/JNK (18). It is generally recognized that the intracellular signaling of PGE_1 and $PGF_{2\alpha}$ is transduced through specific prostaglandin receptors: EP and FP, respectively (28). Therefore, it is likely that the suppressive effects of mimosine on PGE_1- or $PGF_{2\alpha}$-induced OPG synthesis are exerted at the point between the action of MAP kinases and gene transcription in osteoblast-like MC3T3-E1 cells. Further investigations are required to elucidate the exact mechanism underlying the inhibitory effects of normoxic HIF inducers on OPG synthesis in osteoblasts.

It is well established that RANKL-mediated osteoclastic bone resorption is the initial step of bone remodeling (1). OPG, which is secreted by osteoblasts, functions as a decoy receptor for RANKL and results in the regulation of bone remodeling (3). Therefore, reduction of OPG secretion may induce acceleration of bone resorption through upregulation of osteoclastic bone resorption. Correct bone remodeling is essential to ensure the removal of old, fragile bone and the renewal of the skeleton, maintaining skeletal quality and quantity. It has previously been demonstrated that mimosine induces the synthesis of VEGF, a HIF-1 target gene considered to promote bone formation by stimulating the generation of microvasculature (29) in osteoblast-like MC3T3-E1 cells (18). Taking these findings into account, the results of the present study, which demonstrated the inhibitory effects of mimosine and deferoxamine on PGE_1-stimulated OPG synthesis in osteoblasts, may provide novel insights into the hypoxic signaling pathway in bone metabolism. However, further investigation is required to understand the effects of hypoxic conditions on bone metabolism.

In conclusion, the findings of the present study strongly suggested that normoxic HIF inducers attenuate PGE_1-stimulated OPG synthesis without affecting IL-6 production in osteoblasts, providing novel insight into the regulatory mechanisms underlying bone metabolism.

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