Mimosine suppresses the $PGF_{2\alpha}$-induced synthesis of osteoprotegerin but not interleukin-6 in osteoblasts

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Abstract. Mimosine, a plant amino acid, is known to act as a normoxic inducer of hypoxia-inducible factor (HIF). Previous research has suggested that HIF plays important roles in angiogenesis-osteogenesis coupling and bone metabolism. We previously reported that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) induced osteoprotegerin synthesis through p38 mitogen-activated protein (MAP) kinase, p44/p42 MAP kinase and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in osteoblast-like MC3T3-E1 cells. We have also demonstrated that $PGF_{2\alpha}$ induced the synthesis of interleukin-6 (IL-6) via p38 MAP kinase and p44/p42 MAP kinase but not SAPK/JNK in these cells. In the present study, we investigated the effects of mimosine on the $PGF_{2\alpha}$-induced synthesis of osteoprotegerin or IL-6 in MC3T3-E1 cells. We found that deferoxamine, another inducer of HIF, as well as mimosine, upregulated the protein levels of HIF-1α. Both mimosine and deferoxamine significantly suppressed the $PGF_{2\alpha}$-induced release of osteoprotegerin, and the mRNA expression level, without markedly affecting $PGF_{2\alpha}$-induced IL-6 release. Both mimosine and deferoxamine, by themselves, induced the release of vascular endothelial growth factor. The phosphorylation of p38 MAP kinase, p44/p42 MAP kinase or SAPK/JNK induced by $PGF_{2\alpha}$ was not markedly affected by either mimosine or deferoxamine. Thus, the results of the present study strongly suggest that mimosine, a normoxic inducer of HIF, inhibits the $PGF_{2\alpha}$-induced osteoprotegerin synthesis without affecting the IL-6 synthesis in osteoblasts.

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Introduction

Bone metabolism is regulated strictly by two types of functional cells, osteoblasts and osteoclasts, which are responsible for bone formation and bone resorption, respectively (1). Bone remodeling is known to be the outcome of the coupling and fine-tuning process of osteoblastic bone formation and osteoclastic bone resorption (1). Numerous humoral factors, including cytokines and prostaglandins, have been demonstrated to participate in bone remodeling (2). Osteoprotegerin, which belongs to the tumor necrosis factor receptor family, along with receptor activator of nuclear factor-κB (RANK), is synthesized by osteoblasts and plays an inhibitory role in osteoclastic differentiation and activation (3). Osteoprotegerin, secreted by osteoblasts, binds to RANK ligand (RANKL) as a decoy receptor, and prevents RANKL from binding to RANK, resulting in the inhibition of bone resorption (3). It has been reported in previous research that RANKL knock-out mice and osteoprotegerin knock-out mice suffered from severe osteopetrosis and osteoporosis, respectively (4,5). Therefore, it has been firmly established that the RANK/RANKL/osteoprotegerin axis is a major regulatory aspect of bone remodeling (6).

It has previously been noted that prostaglandins act as autacoids in osteoblasts (7). Prostaglandins, which have previously been recognized as potent bone-resorptive agents (8), are known to play important roles also in the process of bone formation (8,9). Of the prostaglandins, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) has been shown to act as a bone remodeling mediator (9). In relation to osteoblasts, we have previously demonstrated that $PGF_{2\alpha}$ induces activation of p38 mitogen-activated protein (MAP) kinase, p44/p42 MAP kinase and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in osteoblast-like MC3T3-E1 cells, and that $PGF_{2\alpha}$-induced osteoprotegerin synthesis is mediated through these MAP kinases (10). We have also shown that $PGF_{2\alpha}$ stimulates the synthesis of interleukin-6 (IL-6), a multifunctional cytokine modulating bone metabolism (11,12), via the activation of p38 MAP kinase and p44/p42 MAP kinase but not SAPK/JNK in osteoblast-like MC3T3-E1 cells (13,14). However, the details of the effects of $PGF_{2\alpha}$ on osteoblasts still remain unclear.

Mimosine, a plant amino acid, is well known to chelate iron and inhibit mammalian DNA replication (15). Mimosine is additionally recognized as a normoxic inducer of hypoxia-inducible factor (HIF) (16). HIF is a DNA-binding transcription factor that interacts with specific nuclear cofactors under low oxygen conditions, and activates a series of hypoxia-associated genes to facilitate responses to hypoxic environments (17). A large number of target genes of HIF have been identified, e.g., erythropoietin, glucose transporter protein 1 and vascular endothelial growth factor (VEGF) (16). HIF is known to play an important role in angiogenesis, erythropoiesis and metabolism (16). Regarding HIF and bone metabolism, it has been previously demonstrated that hypoxia regulates osteoclast-mediated bone resorption (17). In osteoblasts, HIF-1α reportedly promotes bone formation by direct stimulation of osteoblast proliferation (18). Additionally, HIF-1α promotes angiogenesis and stimulates bone regeneration (19). However, the exact role of HIF in bone metabolism has not yet been clarified.

In the present study, we investigated the effect of mimosine, a normoxic inducer of HIF, on the $PGF_{2\alpha}$-induced synthesis of osteoprotegerin and IL-6, and the exact mechanism in osteoblast-like MC3T3-E1 cells. Herein, we show that that mimosine suppresses the $PGF_{2\alpha}$-induced osteoprotegerin synthesis without affecting IL-6 synthesis in these cells.

Materials and methods

Materials. Mimosine and deferoxamine were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). A mouse osteoprotegerin enzyme-linked immunosorbent assay (ELISA) kit, mouse IL-6 ELISA kit, mouse VEGF ELISA kit and $PGF_{2\alpha}$ were obtained from R&D Systems, Inc. (Minneapolis, MN, USA). HIF-1α antibodies (ab16066) were obtained from Abcam (Cambridge, UK). Phospho-specific p38 MAP kinase antibodies (#4511), p38 MAP kinase antibodies (#9212), phospho-specific p44/p42 MAP kinase antibodies (#9101), p44/p42 MAP kinase antibodies (#9102), phospho-specific SAPK/JNK antibodies (#4671) and SAPK/JNK antibodies (#9252) were all obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Actin antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). An ECL western blot detection system was obtained from GE Healthcare (Buckinghamshire, UK). Other materials and chemicals were obtained from commercial sources. $PGF_{2\alpha}$ was dissolved in ethanol. Mimosine was disolved in phosphate-buffered saline (PBS) supplemented with 0.01% bovine serum albumin (BSA) containing 7.5% $NaHCO_3$. Deferoxamine was disolved 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). 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 ($5x10^4$ cells/dish) or 90-mm diameter dishes ($2x10^5$ 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 then used for experiments after 48 h.

Assay for osteoprotegerin or IL-6. The cultured cells were pretreated with various doses of mimosine or deferoxamine for 60 min, and then stimulated with 10 μM $PGF_{2\alpha}$ or the vehicle (PBS supplemented with 0.01% BSA containing 0.1% ethanol) in 1 ml of α-MEM containing 0.3% FBS for the indicated periods of time. The conditioned medium was collected at the end of incubation, and the concentrations of osteoprotegerin or IL-6 were subsequently measured using an osteoprotegerin ELISA kit or IL-6 ELISA kit according to the manufacturer's instructions.

Assay for VEGF. The cultured cells were treated with various doses of mimosine or deferoxamine in 1 ml of α-MEM containing 0.3% FBS for 48 h. The conditioned medium was collected at the end of incubation, and the VEGF concentrations were then measured using a VEGF ELISA kit according to the manufacturer's instructions.

Reverse transcription-quantitative PCR (RT-qPCR). Cultured cells were pretreated with 700 μM mimosine, 500 μM deferoxamine or vehicle for 60 min, and then stimulated with 10 μM $PGF_{2\alpha}$ or vehicle in α-MEM containing 0.3% FBS for 3 h. Total RNA was isolated and transcribed into cDNA using TRIzol reagent (Invitrogen Co., Carlsbad, CA, USA) and an Omniscript Reverse Transcriptase kit (Qiagen, Inc., Valencia, CA, USA), respectively. RT-qPCR was performed using a LightCycler system with capillaries and the FastStart DNA Master SYBR-Green I provided with the kit (Roche Diagnostics, Basel, Switzerland). Sense and antisense primers for mouse osteoprotegerin mRNA were purchased from Takara Bio, Inc. (Tokyo, Japan) (primer set ID: OPG; MA026526) (osteoprotegerin primer sequences (5'→3'): forward, CAATGGC TGGCTTGGTTTCATAG and reverse, CTGAACCAG ACATGACAGCTGGA), whereas mouse VEGF mRNA or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA primers were synthesized based on the study of Simpson *et al* (22) (VEGF primer sequences (5'→3'): forward, TTACTGCTGTA CCTCCACC and reverse, ACAGGACGGCTTGAAGATG; and GAPDH primer sequences (5'→3') forward, AACGA CCCCTTCATTGAC and reverse, TCCACGACATACTC AGCAC. The amplified products were determined using a melting curve analysis and agarose gel electrophoresis. The mRNA levels of osteoprotegerin or VEGF were normalized to those of GAPDH mRNA, respectively.

Western blot analysis. The cultured cells were pretreated with various doses of mimosine or deferoxamine for 60 min, and then stimulated with 10 μM $PGF_{2\alpha}$ or vehicle in α-MEM containing 0.3% FBS for the indicated periods of time. 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 the method of Laemmli (23) in 10% polyacrylamide gels. The protein was fractionated and transferred onto Immun-Blot PVDF membranes (Bio-Rad, Hercules, CA, USA). The

membranes were blocked with 5% fat-free dry milk in Tris-buffered saline-Tween-20 (TBS-T; 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20) for 1 h before incubation with primary antibodies. Western blot analysis was performed as described previously (24) using HIF-1α antibodies, actin antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies or SAPK/JNK antibodies as primary antibodies, and peroxidase-labeled antibodies raised in goat anti-rabbit IgG were used as secondary antibodies (074-1506, KPL, Inc., Gaithersburg, MD, USA). The primary and secondary antibodies were diluted at 1:1,000 with 5% fat-free dried milk in TBS-T. The peroxidase activity on the PVDF membranes was visualized on X-ray film by means of the ECL western blot detection system.

Densitometric analysis. Densitometric analysis was performed using scanner and image analysis software (ImageJ version 1.48; National Institutes of Health, Bethesda, MD, USA). 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 which had not been stimulated by $PGF_{2\alpha}$ nor treated with mimosine or deferoxamine.

Statistical analysis. The data were analyzed by ANOVA followed by the Bonferroni method for multiple comparisons between pairs, and a p-value <0.05 was considered to indicate a statistically significant difference. All data are presented as the means ± SEM of triplicate determinations from three independent cell preparations.

Results

Effects of mimosine and deferoxamine on HIF-1α protein levels in MC3T3-E1 cells. Mimosine, which is an inhibitor of the prolyl hydroxylase domain proteins responsible for degrading HIF-1α, is known to act a normoxic inducer of HIF-1α (25). In the present study, we first examined the effect of mimosine on HIF-1α protein levels in osteoblast-like MC3T3-E1 cells. We noted that mimosine markedly increased the HIF-1α protein levels (Fig. 1A).

It has previously been noted that deferoxamine, an iron chelator, exerts its angiogenic effects through stimulation of the HIF-1α pathway (26), and deferoxamine is known to be another inducer of HIF-1α (26). We also found that HIF-1α protein expression levels were markedly upregulated by deferoxamine (Fig. 1B).

Effects of mimosine and deferoxamine on $PGF_{2\alpha}$-induced osteoprotegerin release in MC3T3-E1 cells. We have recently shown that $PGF_{2\alpha}$ stimulates osteoprotegerin synthesis in osteoblast-like MC3T3-E1 cells (10). Thus, in the present study we examined the effect of mimosine and deferoxamine on $PGF_{2\alpha}$-induced osteoprotegerin release in MC3T3-E1 cells. Mimosine was noted to significantly reduce osteoprotegerin release induced by $PGF_{2\alpha}$ up to 48 h (Fig. 2). The suppressive effect of mimosine on osteoprotegerin release is clearly

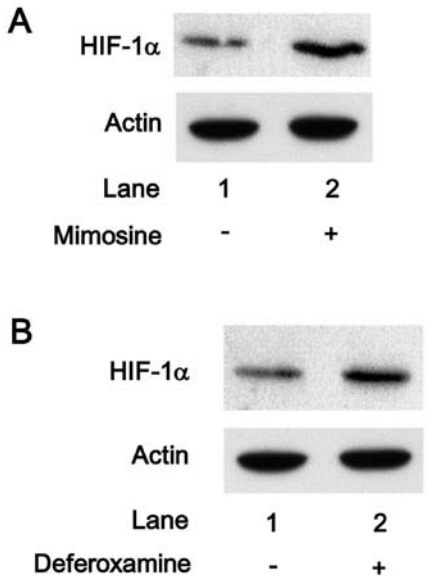

Figure 1. Effects of mimosine and deferoxamine on the hypoxia-inducible factor-1α (HIF-1α) protein levels in MC3T3-E1 cells. The cultured cells were treated with 300 μM of mimosine (A), 100 μM of deferoxamine (B) or vehicle for 3 h. The extracts were subjected toSDS-PAGE with subsequent western blot analysis with antibodies against HIF-1α or actin.

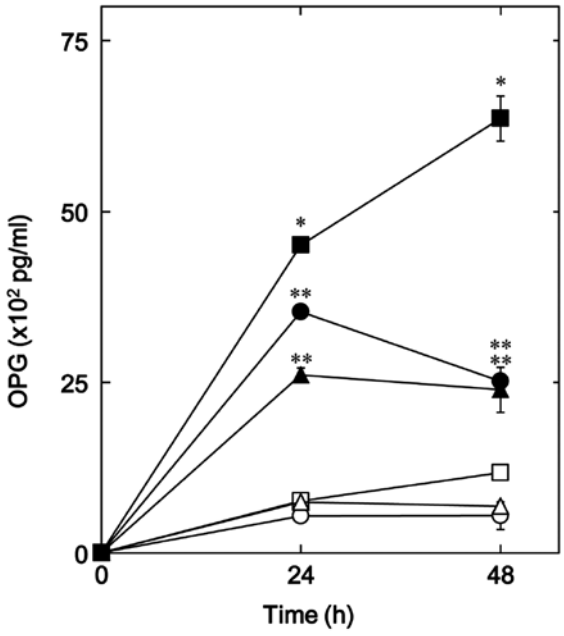

Figure 2. Effects of mimosine and deferoxamine on the prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$)-stimulated osteoprotegerin (OPG) release in MC3T3-E1 cells. The cultured cells were pretreated with 100 μM mimosine (●,○), 500 μM deferoxamine (▲,△) or vehicle (■,□) for 60 min, and then stimulated with 10 μM of $PGF_{2\alpha}$ (●,▲,■) or vehicle (○,△,□) for the indicated periods of time. OPG concentrations of the culture medium were determined by enzyme-linked immunosorbent assay (ELISA). Each value represents the means ± SEM of triplicate determinations from three independent cell preparations. *p<0.05 compared to the control (□); **p<0.05 compared to the value of cells treated with $PGF_{2\alpha}$ alone (■).

dose-dependent in the range between 300 and 700 μM (Fig. 3A); mimosine at 500 μM caused a 65% decrease in $PGF_{2\alpha}$-induced OPG release. In addition, we also noted that deferoxamine significantly decreased the release of osteoprotegerin induced by $PGF_{2\alpha}$ (Fig. 2), and the inhibitory effect was dose-dependent

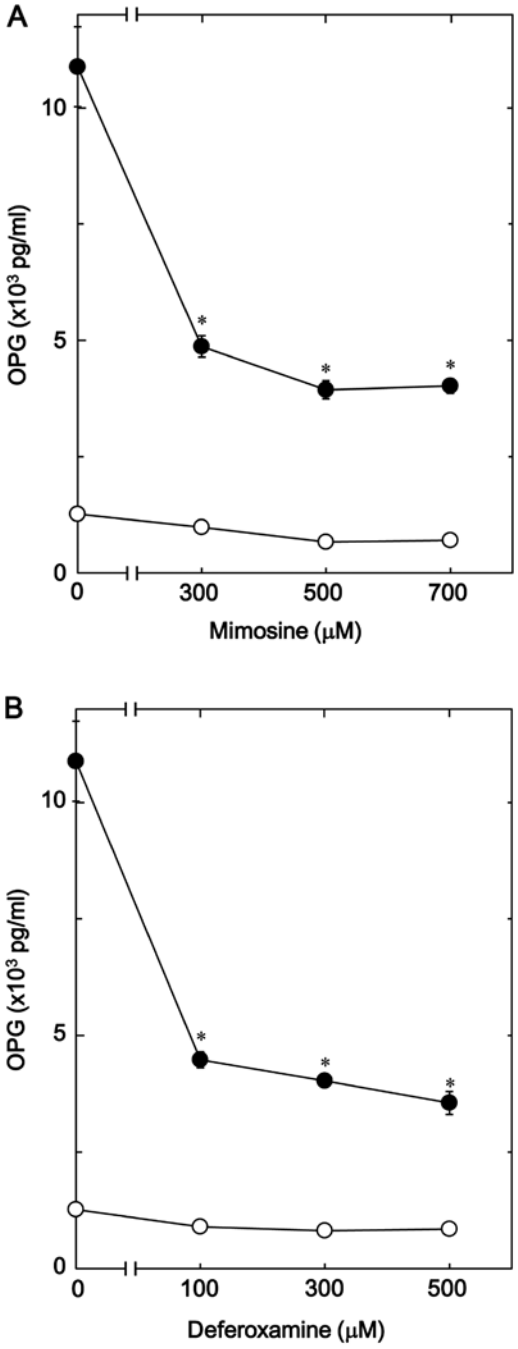

Figure 3. Dose-dependent effects of mimosine and deferoxamine on prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$)-induced osteoprotegerin (OPG) release in MC3T3-E1 cells. Cultured cells were pretreated with various doses of mimosine (A), deferoxamine (B) for 60 min, and stimulated with 10 μM $PGF_{2\alpha}$ (●) or vehicle (○) for 48 h. OPG concentrations of the culture medium were determined by enzyme-linked immunosorbent assay (ELISA). Each value represents the means ± SEM of triplicate determinations from three independent cell preparations. *p<0.05 compared to the value of cells treated with $PGF_{2\alpha}$ alone (●) and treated with 0 μM of (A) mimosine or (B) deferoxamine.

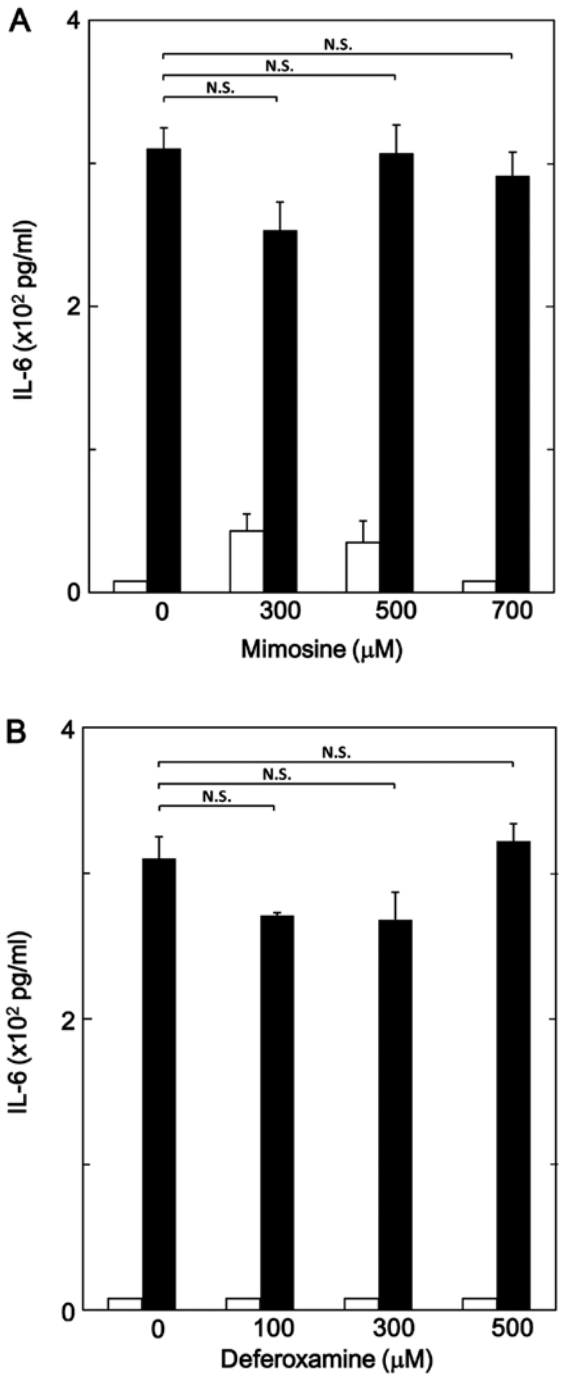

Figure 4. Effects of mimosine and deferoxamine on prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$)-induced interleukin-6 (IL-6) release in MC3T3-E1 cells. Cultured cells were pretreated with various doses of mimosine (A) or deferoxamine (B) for 60 min, and stimulated with 10 μM of $PGF_{2\alpha}$ (black columns) or vehicle (white columns) for 48 h. IL-6 concentrations of the culture medium were determined by enzyme-linked immunosorbent assay (ELISA). Each value represents the means ± SEM of triplicate determinations from three independent cell preparations. N.S., no significant difference.

in the range between 100 and 500 μM (Fig. 3B). The maximum inhibitory effect of deferoxamine in relation to OPG release was observed at 500 μM, which caused approximately an 80% decrease in $PGF_{2\alpha}$-induced OPG release.

Effects of mimosine or deferoxamine on the $PGF_{2\alpha}$-induced release of IL-6 in MC3T3-E1 cells. We have previously reported that $PGF_{2\alpha}$ stimulates IL-6 synthesis in osteoblast-like MC3T3-E1 cells (13,14). Thus, in the present study we examined the effect of mimosine or deferoxamine on the $PGF_{2\alpha}$-induced release of IL-6. It was clear that mimosine up to 700 μM failed to markedly affect the IL-6 release induced by 10 μM $PGF_{2\alpha}$ (Fig. 4A). In addition, we noted that deferoxamine up to 500 μM did not markedly effect $PGF_{2\alpha}$ (10 μM)-induced IL-6 release (Fig. 4B).

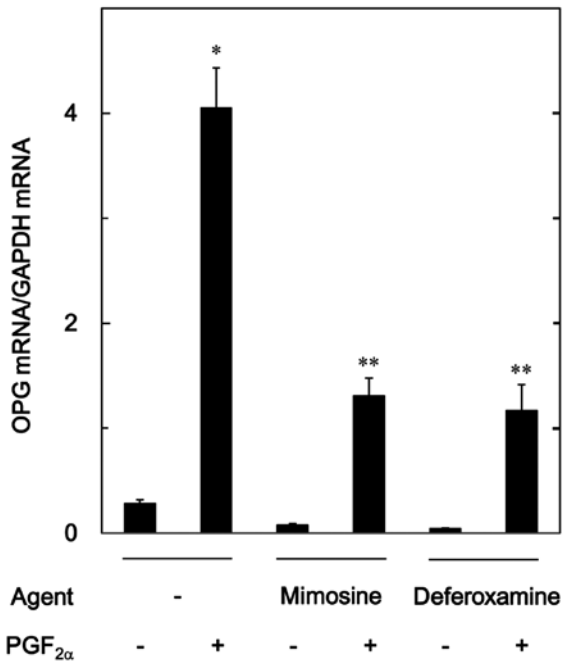

Figure 5. Effects of mimosine and deferoxamine on the prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$)-induced increase in osteoprotegerin (OPG) mRNA expression levels in MC3T3-E1 cells. Cultured cells were pretreated with 700 μM mimosine, 500 μM deferoxamine or vehicle for 60 min, and then stimulated with 10 μM $PGF_{2\alpha}$ or vehicle for 3 h. The total RNA was then isolated and transcribed into cDNA. The expressions of OPG and GAPDH mRNA were quantified by RT-qPCR. The OPG mRNA levels were normalized to those of GAPDH mRNA. Each value represents the means ± SEM of triplicate determinations from three independent cell preparations. $^{*}p<0.05$ compared to the control [Agent (-) and $PGF_{2\alpha}$ (-)]; $^{**}p<0.05$ compared to the value of cells treated with $PGF_{2\alpha}$ alone [Agent (-) and $PGF_{2\alpha}$ (+)].

Effects of mimosine or deferoxamine on $PGF_{2\alpha}$-induced osteoprotegerin mRNA expression in MC3T3-E1 cells. To investigate whether the inhibitory effect of mimosine or deferoxamine on $PGF_{2\alpha}$-induced osteoprotegerin release is mediated by transcriptional events in osteoblast-like MC3T3-E1 cells, we examined the effect of mimosine and deferoxamine on $PGF_{2\alpha}$-induced osteoprotegerin mRNA expression. Mimosine (700 μM) and deferoxamine (500 μM), which alone hardly affected the osteoprotegerin mRNA level, significantly attenuated the increase in the mRNA expression level of osteoprotegerin induced by 10 μM of $PGF_{2\alpha}$ (Fig. 5).

Effects of mimosine or deferoxamine on the release of VEGF and the expression of mRNA in MC3T3-E1 cells. It has been noted previously that HIF increases oxygen-regulated gene expression, including VEGF, and promotes angiogenesis and osteogenesis (27). Therefore, we examined whether mimosine or deferoxamine upregulates VEGF release in osteoblast-like MC3T3-E1 cells. The release of VEGF was significantly upregulated by mimosine in a dose-dependent manner in the range between 300 and 700 μM (Fig. 6A). Additionally, we noted that deferoxamine dose-dependently increased VEGF release in the range between 100 and 500 μM (Fig. 6B).

We further investigated the effects of mimosine or deferoxamine on VEGF mRNA expression in MC3T3-E1 cells. Both mimosine and deferoxamine significantly upregulated VEGF mRNA expression levels (Fig. 7).

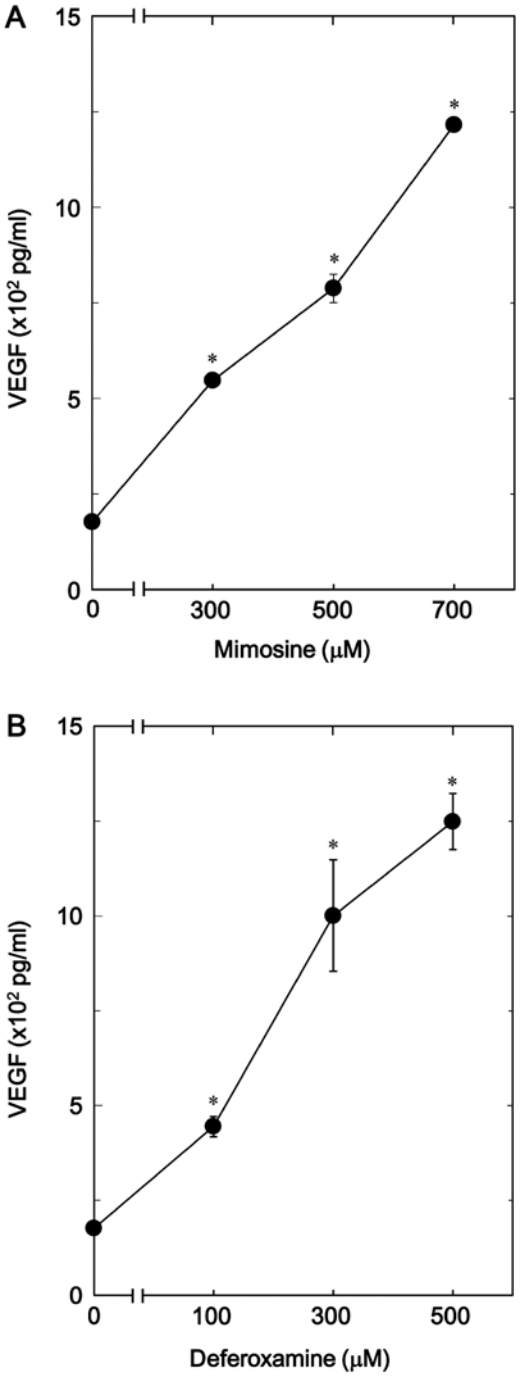

Figure 6. Effects of mimosine and deferoxamine on vascular endothelial growth factor (VEGF) release in MC3T3-E1 cells. Cultured cells were treated with various doses of mimosine (A) or deferoxamine (B) for 48 h. VEGF concentrations of the culture medium were determined by enzyme-linked immunosorbent assay (ELISA). Each value represents the means ± SEM of triplicate determinations from three independent cell preparations. $^{*}p<0.05$ compared to the value of 0 μM (A) mimosine or (B) deferoxamine.

Effects of mimosine or deferoxamine on $PGF_{2\alpha}$-induced phosphorylation of p38 MAP kinase, p44/p42 MAP kinase or SAPK/JNK in MC3T3-E1 cells. In a previous study, we have demonstrated that $PGF_{2\alpha}$-induced osteoprotegerin synthesis is mediated through activation of p38 MAP kinase, p44/p42 MAP kinase and SAPK/JNK in osteoblast-like MC3T3-E1 cells (10). Therefore, in the present study we examined whether mimosine and deferoxamine affected the $PGF_{2\alpha}$-induced

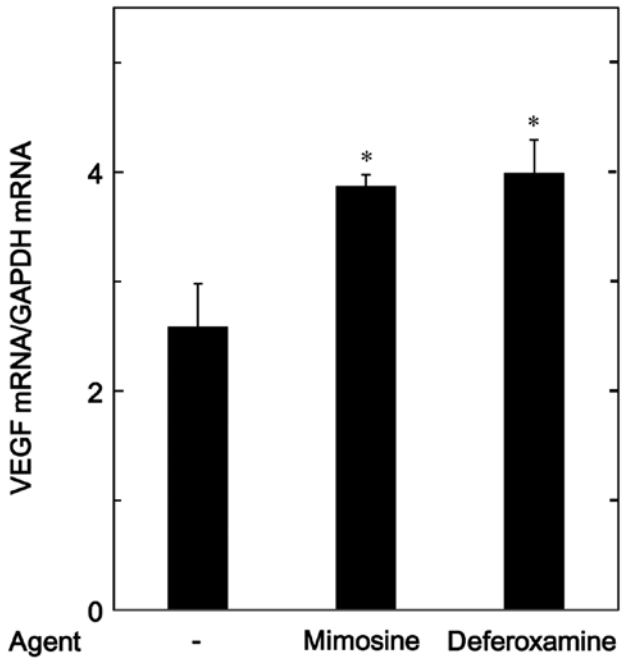

Figure 7. Effects of mimosine and deferoxamine on vascular endothelial growth factor (VEGF) mRNA expression levels in MC3T3-E1 cells. The cultured cells were treated with 100 μM mimosine, 500 μM deferoxamine or vehicle for 3 h. The expressions of VEGF mRNA and GAPDH mRNA were quantified by RT-qPCR. The VEGF mRNA levels were normalized to those of GAPDH mRNA. Each value represents the means ± SEM of triplicate determinations from three independent cell preparations. $^{*}p<0.05$ compared to the value of control [Agent (-)].

phosphorylation of p38 MAP kinase, p44/p42 MAP kinase or SAPK/JNK in these cells. We noted that the phosphorylation of p38 MAP kinase, p44/p42 MAP kinase and SAPK/JNK induced by $PGF_{2\alpha}$ was not markedly affected by mimosine, up to 700 μM (Fig. 8). It also became evident that deferoxamine exerted little effect on the $PGF_{2\alpha}$-induced phosphorylation of p38 MAP kinase, p44/p42 MAP kinase and SAPK/JNK, up to 500 μM (Fig. 9).

Discussion

In the present study, we demonstrated that mimosine significantly reduced the $PGF_{2\alpha}$-induced release of osteoprotegerin in osteoblast-like MC3T3-E1 cells. It is well known that mimosine is an inhibitor of the prolyl hydroxylase domain proteins responsible for degrading HIF-1α and also that it is an inhibitor of DNA replication (16,25). Additionally, we showed in the present study that the release of osteoprotegerin caused by $PGF_{2\alpha}$ was significantly suppressed by deferoxamine, which is another inhibitor of the prolyl hydroxylase domain proteins responsible for degrading HIF-1α, as has also been previously stated (26). We found that the protein levels of HIF-1α were considerably upregulated by both mimosine and deferoxamine in osteoblast-like MC3T3-E1 cells. Thus, it seems likely that the inhibitory effect of mimosine or deferoxamine on osteoprotegerin release is mediated through the HIF-1α-dependent pathway in MC3T3-E1 cells. In addition, we demonstrated that both mimosine and deferoxamine suppressed the $PGF_{2\alpha}$-induced osteoprotegerin mRNA expression. Therefore, our findings suggest that the suppressive effect of mimosine and deferoxamine on $PGF_{2\alpha}$-induced osteoprotegerin release is exerted at a point upstream of the transcriptional level in these cells. It is important to note that HIF-1 is a transcription factor which plays a pivotal role in the cellular response to hypoxia, and that VEGF is a target gene of HIF-1 (16). In this study, we showed that mimosine and deferoxamine by themselves induced the release and the expression of VEGF mRNA. It has been noted that the HIF-1 consists of two subunits, HIF-1α and HIF-1β, and the expressed HIF-1α is degraded immediately in normoxic cells by the ubiquitin-proteasome system, and that chemical hydroxylase inhibitors including mimosine and deferoxamine suppress the degradation of HIF-1α, resulting in its stabilization (16,25,26). Therefore, it is probable that mimosine and deferoxamine, as normoxic inducers of HIF-1α, actually stimulate VEGF synthesis in MC3T3-E1 cells. Taken together, our findings suggest that mimosine and deferoxamine suppress the $PGF_{2\alpha}$-induced synthesis of osteoprotegerin via stabilization of HIF-1α expression in osteoblast-like MC3T3-E1 cells.

In our previous studies (13,14), we reported that $PGF_{2\alpha}$ induces the synthesis of IL-6 and also osteoprotegerin in osteoblast-like MC3T3-E1 cells. IL-6 is known as a bone-resorptive cytokine, and it plays an important role in bone metabolism (6). Thus, we examined the effect of mimosine or deferoxamine on $PGF_{2\alpha}$-induced IL-6 release, and demonstrated that neither mimosine nor deferoxamine affected the release of IL-6 induced by $PGF_{2\alpha}$. Thus, it is possible that the suppressive effects of mimosine and deferoxamine on $PGF_{2\alpha}$ induction are specific to osteoprotegerin synthesis in osteoblast-like MC3T3-E1 cells.

The MAP kinase superfamily is known to play a central role in a variety of cellular functions such as proliferation, differentiation and survival (28). Three major MAP kinases, p38 MAP kinase, p44/p42 MAP kinase and SAPK/JNK, are the main elements used by cells to transfer diverse messages (29). Regarding the regulatory mechanism of $PGF_{2\alpha}$-induced osteoprotegerin synthesis in osteoblasts, we have previously reported that the activation of p38 MAP kinase, p44/p42 MAP kinase and SAPK/JNK are involved in the $PGF_{2\alpha}$-induced osteoprotegerin synthesis in osteoblast-like MC3T3-E1 cells (10). Thus, in the present study we further examined the effect of mimosine or deferoxamine on the $PGF_{2\alpha}$-induced phosphorylation of these MAP kinases in MC3T3-E1 cells. We noted that the $PGF_{2\alpha}$-induced phosphorylation of p38 MAP kinase, p44/p42 MAP kinase or SAPK/JNK was not markedly affected by mimosine or deferoxamine. Thus, it seems unlikely that the modulations of these MAP kinase activities are involved in the suppressive effect which both mimosine and deferoxamine exert on $PGF_{2\alpha}$-induced osteoprotegerin synthesis in osteoblast-like MC3T3-E1 cells. Further investigations are required in order to clarify the exact mechanism underlying the effects of mimosine and deferoxamine on osteoprotegerin synthesis in osteoblast-like MC3T3-E1 cells.

It is well known that RANKL-mediated osteoclastic bone resorption constitutes the initial step of bone remodeling (1). Osteoprotegerin produced by osteoblasts plays a crucial role in the regulation of bone remodeling as a decoy receptor of RANKL (3). However, it is also well known that osteoblasts, osteoclasts and capillary endothelial cells are closely coordinated during bone remodeling (30). VEGF, a specific growth factor of vascular endothelial cells, produced by osteoblasts,

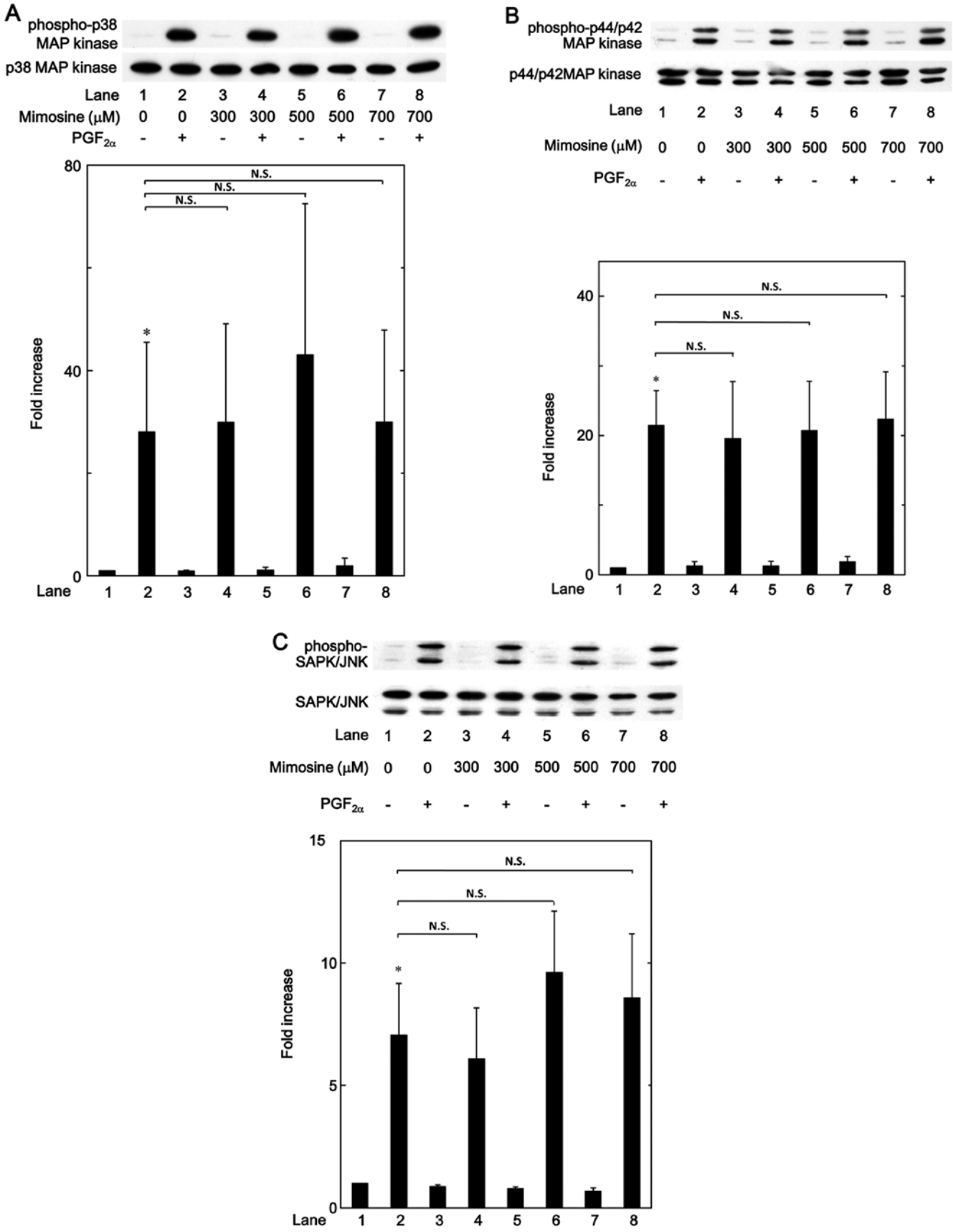

Figure 8. Effects of mimosine on prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$)-induced phosphorylation of p38 mitogen-activated protein (MAP) kinase (A), p44/p42 MAP kinase (B) or stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) (C) in MC3T3-E1 cells. The cultured cells were pretreated with various doses of mimosine for 60 min, and then stimulated with 10 μM $PGF_{2\alpha}$ or vehicle for 10 min (A) or 20 min (B and C). The cell extracts were then subjected to SDS-PAGE with subsequent western blot analysis with antibodies against phospho-specific p38 MAP kinase, p38 MAP kinase, phospho-specific p44/p42 MAP kinase, p44/p42 MAP kinase, phospho-specific SAPK/JNK or SAPK/JNK. The histogram shows quantitative representations of the levels of $PGF_{2\alpha}$-induced phosphorylation obtained from a densitometric analysis of three independent experiments. Each value represents the means ± SEM of triplicate determinations. *p<0.05 compared to the value of the control (Lane 1). N.S., no significant difference.

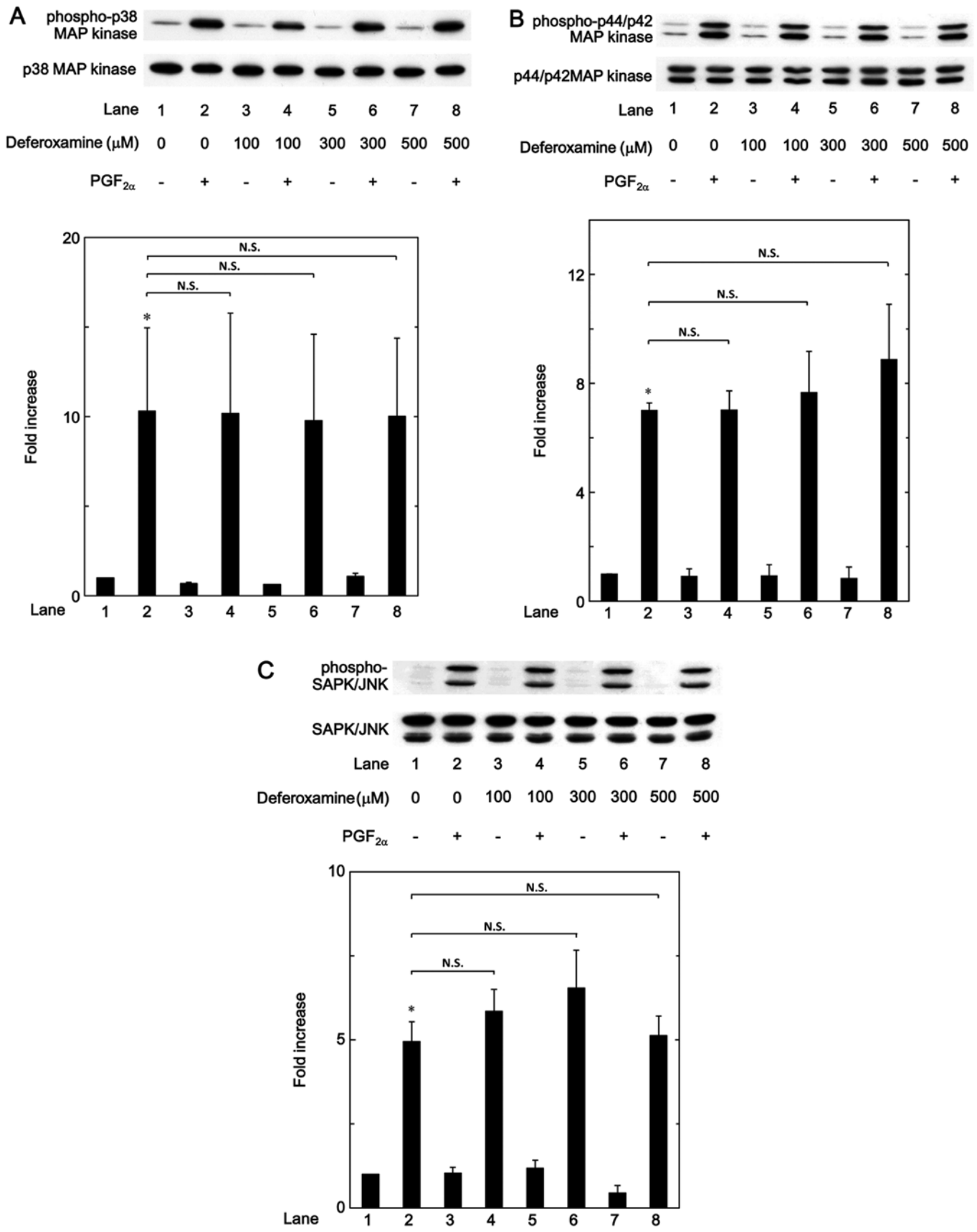

Figure 9. Effects of deferoxamine on the prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$)-induced phosphorylation of p38 mitogen-activated protein (MAP) kinase (A), p44/p42 MAP kinase (B) or stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) (C) in MC3T3-E1 cells. The cultured cells were pretreated with various doses of deferoxamine for 60 min, and then stimulated with 10 μM $PGF_{2\alpha}$ or vehicle for 10 min (A) or 20 min (B and C). The cell extracts were then subjected to SDS-PAGE with subsequent western blot analysis with antibodies against phospho-specific p38 MAP kinase, p38 MAP kinase, phospho-specific p44/p42 MAP kinase, p44/p42 MAP kinase, phospho-specific SAPK/JNK or SAPK/JNK. The histogram shows quantitative representations of the levels of $PGF_{2\alpha}$-induced phosphorylation obtained from a laser densitometric analysis of three independent experiments. Each value represents the means ± SEM of triplicate determinations. $^{*}p<0.05$ compared to the value of control (Lane 1). N.S, no significant difference.

is considered to promote bone formation by supplying microvasculature (31). To maintain the quality of bone, proper bone remodeling is essential to ensure the removal of old, fragile bone and the renewal of the skeleton. Therefore, our present findings demonstrating the inhibitory effects of mimosine and deferoxamine, which act as normoxic inducers of HIF-1α in the $PGF_{2\alpha}$-induced osteoprotegerin synthesis in osteoblasts, provide new insights regarding hypoxic conditions in bone metabolism. Further investigation is now necessary to elucidate in more detail the mechanisms of HIF in bone metabolism.

In conclusion, our findings strongly suggest that mimosine, a normoxic inducer of HIF, inhibits $PGF_{2\alpha}$-induced osteoprotegerin synthesis without affecting IL-6 synthesis in osteoblasts.

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