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Duloxetine strengthens osteoblast activation by prostaglandin E_1: Upregulation of p38 MAP kinase

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ABSTRACT

Duloxetine, a serotonin-norepinephrine reuptake inhibitor, is currently recommended as a useful medicine to chronic pain including low back pain. However, as the analogy of classical selective serotonin reuptake inhibitors, there is a concern to deteriorate osteoporosis with remaining to clarify the exact mechanism of duloxetine in bone metabolism. We have previously reported that prostaglandin E_1 (PGE_1) induces the synthesis of both osteoprotegerin (OPG) and interleukin-6 (IL-6), essential regulators of bone metabolism, in osteoblast-like MC3T3-E1 cells. Based upon them, we herein investigated the mechanism whereby the effect of duloxetine on the synthesis of OPG and IL-6 induced by PGE_1 in these cells. Duloxetine enhanced the release from MC3T3-E1 cells of both OPG and IL-6 stimulated by PGE_1. However, reboxetine, a selective and specific inhibitor of norepinephrine reuptake, failed to affect the PGE_1-induced release of OPG or IL-6. Oppositely, fluvoxamine and sertraline, agents belonging to the class of selective serotonin reuptake inhibitor, upregulated the PGE_1-stimulated release of both OPG and IL-6. Duloxetine amplified the expression of OPG mRNA and IL-6 mRNA stimulated by PGE_1. Duloxetine strengthened the PGE_1-induced p38 MAP kinase phosphorylation, which was amplified by fluvoxamine as well. SB203880, an inhibitor of p38 MAP kinase, suppressed the amplifying effects by duloxetine or fluvoxamine on the PGE_1-stimulated release of OPG and IL-6. These results strongly suggest that duloxetine could strengthen osteoblast activation by PGE_1 through the upregulation of p38 MAP kinase, leading to increasing the synthesis of OPG and IL-6.

1. Introduction

Low back pain (LBP) is one of the most common complaints for physician visits [1]. The lifetime prevalence of LBP is estimated over 80 % [2,3], and 25%–35% of patients complain the symptom LBP in Japan, a country increasing old populations [3]. According to a clinical practice guideline from American College of Physicians, in patients with LBP who had an inadequate response to non-pharmacotherapy, the treatment with nonsteroidal anti-inflammatory drugs should be considered as first line therapy [1]. Duloxetine, an inhibitor of serotonine (5-HT)-norepiniphrine reuptake (SNRI) marketed in the United States in 2004, which is FDA-approved for use in the treatment of major depressive disorder, generalized anxiety disorder, fibromyalgia, chronic musculoskeletal pain, and diabetic peripheral neuropathy [4], is recommended as a second line therapy to the chronic LBP patients of non-responder to non-pharmacotherapy, in addition to tramadol, a weak μ agonist and weak SNRI [1]. The effectiveness of duloxetine to chronic LBP has been also reported in Japan [3]. Furthermore, a clinical trial of duloxetine for the treatment and prevention of chronic musculoskeletal pain including LBP is now on going [5]. In spite of the expectation, it has been reported that the use of antidepressants including classical selective serotonin reuptake inhibitors (SSRIs) is related to an increase of fracture risk caused by osteoporosis, a major health concern of the older person [6–8]. However, those mechanisms could not be clearly shown.

Metabolic bone diseases including osteoporosis are caused due to the

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dysregulation of bone remodeling, the finely coordinated process consisted of bone resorption by osteoclasts and bone formation by osteoblasts [9]. Bone remodeling is regulated by numerous humoral agents including prostaglandins (PGs) [10]. In bone metabolism, PGs are widely known as autacoids to modulate bone cell functions [11]. The supporting evidence has been accumulated that PGs could promote not only bone resorption but also bone formation finely adapted to the bone remodeling status [12,13]. Osteoprotegerin (OPG), a member of tumor necrosis factor receptor superfamily, is an osteoblast-secreted protein and acts as a decoy receptor for receptor activator of nuclear factor-kB (RANKL) [14]. RANKL accordingly binds to RANK expressed on the osteoclast precursors leading to the maturation and the activation of osteoclastic bone resorption, therefore, the competitive antagonism between OPG and RANKL to RANK is a crucial regulatory mechanism of bone remodeling [14,15]. We have previously reported that PGE_1 among PGs stimulates OPG synthesis through the activation of p38 mitogen-activated protein (MAP) kinase and stress-activated protein kinase/c-*Jun* N-terminal kinase in osteoblast-like MC3T3-E1 cells [16]. On the other hand, interleukin-6 (IL-6), a physiologically important cytokine with the roles such as promoting B-cell differentiation and inducing acute-phase proteins [17], potentiates osteoclast formation and bone resorption in bone metabolism [18]. IL-6 is additionally considered to promote bone formation under the condition of increased bone metabolism [19]. Actually, it has been recently reported that IL-6 essentially takes part in the process of bone fracture healing [20]. We have previously reported that the secretion of IL-6 in addition to OPG is stimulated by PGE_1 in osteoblast-like MC3T3-E1 cells [21].

Regarding the effects of SNRIs on bone metabolism, it has been shown that venlafaxine could further decrease bone mineral density associated with the decrease of osteoid synthesis and increased bone resorption in rat bone tissue after orchidectomy, a model of postmenopausal osteoporosis *in vivo* [22]. Functional 5-HT2A receptors are expressed on osteoblasts including MC3T3-E1 cells [23,24]. It has been reported that fluoxetine, an SSRI, inhibits osteoblast differentiation and mineralization during fracture healing in mice whereas serotonin suppresses osteoclast differentiation via OPG secretion from osteoblasts [23,25]. In addition, it has recently shown that fluoxetine directly affects osteoprogenitor cells to apoptosis independent of serotonin concentration [26]. On the other hand, it has been reported that norepinephrine transporter is expressed on the differentiated osteoblasts [27], and that reboxetine, a potent selective norepinephrine reuptake inhibitor [28], enables to induce bone loss in mice [27]. Another recent study showed that duloxetine and fluoxetine cause an advance in osteogenic gene expression in mesenchymal stem cells [29]. Thus, the effects of duloxetine as an SNRI on the bone metabolism remain controversial, and the exact mechanisms of duloxetine underlying osteoblast function have not yet been fully understood.

On the basis of our previous findings, in the present study, we examined the mechanism whereby the effect of duloxetine on the PGE_1-stimulated release of OPG and IL-6 in osteoblast-like MC3T3-E1 cells. Our results herein show that duloxetine strengthens the synthesis of OPG and IL-6 by PGE_1 via the augmentation of p38 MAP kinase in osteoblasts.

2. Materials and methods

2.1. Materials

Duloxetine and PGE_1 were obtained from Sigma-Aldrich (Merck KGa Darmstadt, Germany). Fluvoxamine and sertraline were purchased from Tocris Bioscience (Bristol, UK). Reboxetine was obtained from Cayman Chemical (Ann Arbor, MI). Mouse OPG enzyme-linked immunosorbent assay (ELISA) kit and mouse IL-6 ELISA kit were purchased from R&D Systems, Inc, (Mineapolis, MN). SB203580 was obtained from Calbiochem-Novabiochem Corp. (San Diego, CA). Phospho-specific p38 MAP kinase antibodies (#4511) and p38 MAP kinase antibodies (#9212) were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies (sc-25778) were purchased from Santa Cruz Biotechnology, Inc. (Dalas, TX). An ECL Western blotting detection system was obtained from GE Healthcare Life Sciences (Chalfont, UK). Other materials and chemicals were obtained from commercial sources. PGE_1 was dissolved in ethanol. Duloxetine, reboxetine, fluvoxamine, sertraline and SB203580 were dissolved in DMSO. The maximum concentration of ethanol or DMSO was 0.1 %, which did not affect the assays for OPG and IL-6 release, mRNA expression or Western blot analysis.

2.2. Cell culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [30] were maintained as previously described [31]. In brief, the cells were cultured in 10 % fetal bovine serum (FBS)-containing α-minimum essential medium (α-MEM) at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were seeded into 35-mm diameter dishes (5×10^4 cells/dish) or 90-mm diameter dishes (2×10^5 cells/dish) for 5 days, sub-cultured in 0.3 % FBS-containing α-MEM for 48 h, and then used for experiments.

2.3. Assay for OPG and IL-6 release

Cultured cells were pretreated with indicated doses of duloxetine, reboxetine, fluvoxamine or sertraline for 60 min, and then stimulated with 10 μM of PGE_1 or the vehicle (PBS supplemented with 0.01 % BSA containing 0.1 % ethanol) in 0.3 % FBS-containing α-MEM for 48 h [32]. When indicated, the cells were incubated with 5 μM of SB203580 or the vehicle (PBS supplemented with 0.01 % BSA containing 0.1 % DMSO) for 60 min prior to the pretreatment above. After the series of procedure, the conditioned medium was collected, and the concentrations of OPG and IL-6 were determined using OPG ELISA and IL-6 ELISA, respectively, according to the manufacturer's protocols [32].

2.4. Real-time RT-PCR

Cultured cells were pretreated with 10 μM of duloxetine or vehicle for 60 min, and then stimulated with 10 μM of PGE_1 or vehicle for 3 h [32]. Total RNA was isolated and transcribed into cDNA using TRIzol reagent (Invitorogen; Thermo Fishcer Scientific, Inc. Carlsbad, CA) and Omniscript Reverse Transcriptase kit (Qiagen Inc., Valencia, CA), respectively [32]. 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) [32]. Sense and antisense primers for mouse OPG mRNA, mouse IL-6 mRNA and GAPDH were purchased from Takara Bio, Inc. (Otsu, Japan). The 20 μl of reaction mixture was incubated at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 1 s, annealing at 60 °C for 5 s and elongation at 72 °C for 7 s. Amplified products were determined using melting curve analysis according to the System protocol [32]. OPG mRNA levels and IL-6 mRNA levels were normalized to those of GAPDH mRNA.

2.5. Western blot analysis

Cultured cells were pretreated with 10 μM of duloxetine, 30 μM of fluvoxamine or vehicle in 0.3 % FBS-containing α-MEM for 60 min, and then stimulated by 10 μM of PGE_1 or vehicle for 10 min. The cells were washed twice with phosphate-buffered saline and then lysed, homogenized and sonicated in lysis buffer containing 62.5 mM Tris/HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10 % glycerol [32]. SDS-polyacrylamide gel electrophoresis (PAGE) under the method of Laemli [33] was performed using 10 % polyacrylamide gel [32]. Protein was fractionated and transferred onto Immun-Blot polyvinylidene fluoride (PVDF) membranes [32]. The membranes were blocked with 6% fat-free 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 [32]. A Western blot analysis was performed using phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies or GAPDH antibodies as primary antibodies with peroxidase-labeled goat anti-rabbit IgG antibodies being used as secondary antibodies (KPL, Inc., Gaitherburg, MD) [32]. Primary and secondary antibodies were diluted at 1:1000 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 [32]. Peroxidase activity on the PVDF membrane was visualized on X-ray film by means of the ECL western blotting detection system [32].

2.6. Densitometric analysis

Band densities of Western blotting were determined with a scanner and the ImageJ software program (NIH) as previously described [34]. The phosphorylated levels of p38 MAP kinase were calculated as follows: the signal intensity of each phosphorylation subtracted the background signal was normalized to the respective GAPDH intensity and plotted to indicate the fold increase compared to the control cells without stimulation [34].

2.7. Statistical analysis

Differences between the mean values for individual groups were assessed with one-way analysis of variance (ANOVA), followed by application of the Bonferroni correction for multiple comparisons between pairs [32]. $p<0.05$ was considered to indicate a statistically significant differences [32]. All data are presented as the mean ± standard error of the mean (SE), which was determined from three independent cell preparations [32].

3. Results

3.1. Effects of duloxetine on the PGE1-induced release of OPG and IL-6 in MC3T3-E1 cells

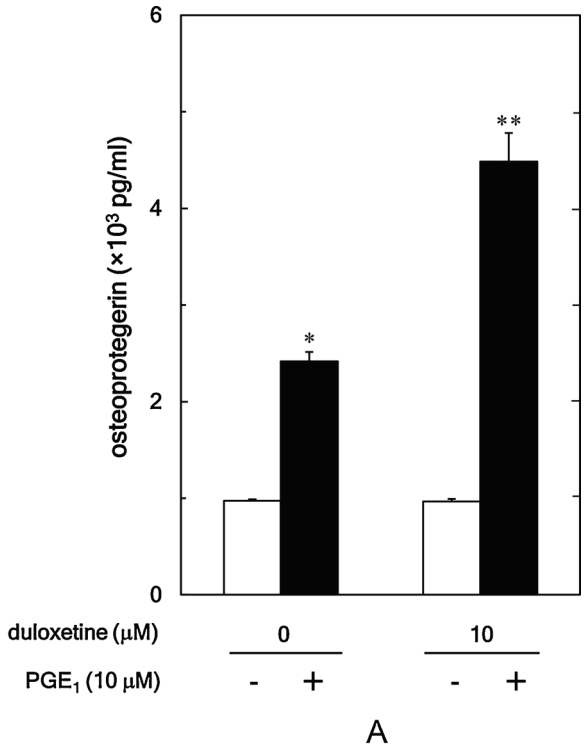
We previously reported that PGE_1 stimulates the releases of OPG and IL-6 in osteoblast-like MC3T3-E1 cells [16,21]. We first examined the effect of duloxetine on the OPG release stimulated by PGE_1 in these cells. Duloxetine (10 μM), which by itself had little effect on the OPG release, markedly enhanced the release of OPG stimulated by PGE_1 (Fig. 1A). We next examined the effect of duloxetine on the IL-6 release stimulated by PGE_1 in MC3T3-E1 cells. Duloxetine alone hardly affected the IL-6 release, but markedly strengthened the PGE_1-stimulated IL-6 release (Fig. 1B).

3.2. Effects of reboxetine on the PGE1-induced release of OPG and IL-6 in MC3T3-E1 cells

It has been reported that the transporter of norepinephrine is expressed on the differentiated osteoblasts [27]. To clarify whether the amplifying effect of duloxetine on the PGE_1-stimulated release of OPG and IL-6 is mediated through the inhibition of norepinephrine reuptake or not in osteoblast-like MC3T3-E1 cells, we investigated the effects of reboxetine, a potent selective norepinephrine reuptake inhibitor [28], on the PGE_1-stimulated OPG and IL-6 release. As a result, reboxetine in the doses up to 100 nM had no effect on the OPG release with or without PGE_1 (Fig. 2A). In addition, reboxetine, by itself did not affect the release of IL-6, failed to enhance the PGE_1-stimulated IL-6 release in the dose up to 100 nM (Fig. 2B).

3.3. Effects of fluvoxamine or sertraline on the PGE1-induced release of OPG and IL-6 in MC3T3-E1 cells

Fluvoxamine [35] and sertraline [36] are well-known as SSRIs. To elucidate whether the effects of duloxetine on the PGE_1-stimulated OPG and IL-6 release are due to the inhibition of serotonin reuptake or not in MC3T3-E1 cells, we investigated the effects of fluvoxamine or sertraline on the release of OPG and IL-6 stimulated by PGE_1. At first, we examined the effects of fluvoxamine, and found that the OPG release stimulated by PGE_1 was significantly upregulated by fluvoxamine, which alone had no



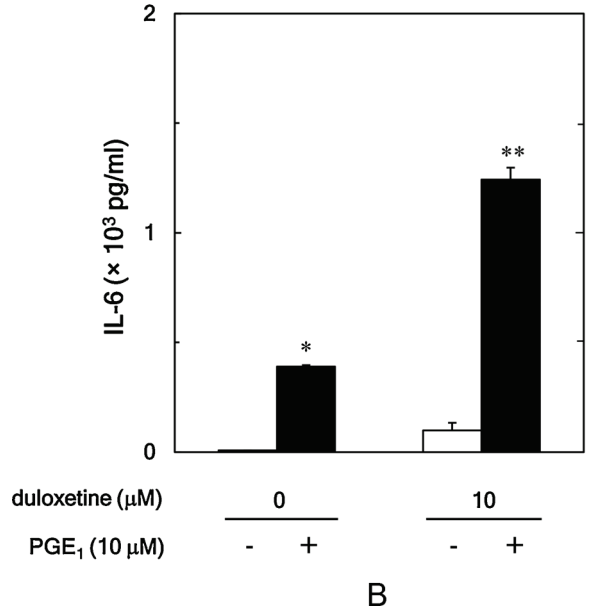


Fig. 1. Effects of duloxetine on the PGE_1-induced release of OPG and IL-6 in MC3T3-E1 cells. The cultured cells were pretreated with 10 μM of duloxetine or vehicle for 60 min, and then stimulated by 10 μM of PGE_1 (black bars) or vehicle (white bars) for 48 h. The conditioned medium was collected, and the concentrations of OPG (A) and IL-6 (B) were determined with an ELISA for each. Each value represents the mean ± SE of triplicate determinations from three independent cell preparations. *$p<0.05$ vs. control. **$p<0.05$ vs. PGE_1 alone.

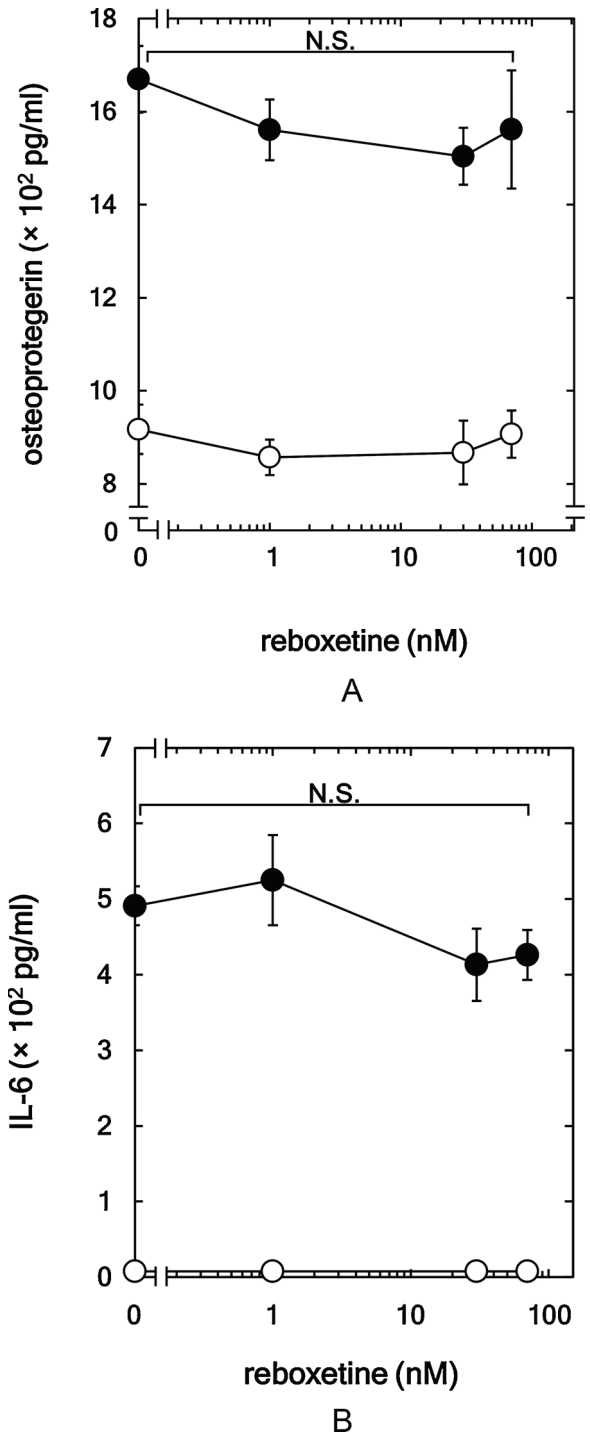


Fig. 2. Effects of reboxetine on the PGE_1-induced release of OPG and IL-6 in MC3T3-E1 cells. The cultured cells were pretreated with various doses of reboxetine or vehicle for 60 min, and then stimulated by 10 μM of PGE_1 (black circles) or vehicle (white circles) for 48 h. The conditioned medium was collected, and the concentrations of OPG (A) and IL-6 (B) were determined with an ELISA for each. Each value represents the mean ± SE of triplicate determinations from three independent cell preparations. N.S. designates no significant difference between the indicated pairs.

effect on the OPG release (Fig. 3A). Additionally, fluvoxamine that by itself failed to affect the IL-6 release, significantly strengthened the PGE_1-stimulated IL-6 release in a dose dependent manner in the range between 3 and 30 μM (Fig. 3B). We next examined the effects of sertraline, and found that sertraline significantly enhanced the effects of PGE_1 on the OPG release (Fig. 3C) and the IL-6 release (Fig. 3D).

3.4. Effects of duloxetine on the PGE1-induced expression of OPG mRNA and IL-6 mRNA in MC3T3-E1 cells

To clarify whether or not the enhancing effects of duloxetine on the release of OPG and IL-6 stimulated by PGE_1 are mediated via transcriptional events, we examined the effects of duloxetine on the expression levels of OPG mRNA and IL-6 mRNA induced by PGE_1 in osteoblast-like MC3T3-E1 cells with real-time RT-PCR. We found that duloxetine significantly upregulated the PGE_1-stimulated expression levels of OPG mRNA (Fig. 4A) and those of IL-6 mRNA (Fig. 4B).

3.5. Effects of duloxetine and fluvoxamine on the PGE_1-induced p38 MAP kinase phosphorylation in MC3T3-E1 cells

We previously reported that PGE_1 stimulates OPG synthesis at least in part through the activation of p38 MAP kinase in osteoblast-like MC3T3-E1 cells [16]. To elucidate the mechanism underlying the enhancement by duloxetine of the PGE_1-stimulated synthesis of OPG and IL-6 in MC3T3-E1 cells, we investigated the effects of duloxetine and fluvoxamine on the phosphorylation of p38 MAP kinase induced by PGE_1. At first, we confirmed that PGE_1 significantly stimulated the phosphorylation of p38 MAP kinase in these cells (Fig. 5A and B). Duloxetine, which by itself had little effect on the phosphorylation of p38 MAP kinase, significantly strengthened the PGE_1-induced phosphorylation of p38 MAP kinase (Fig. 5A). Fluvoxamine, as well as duloxetine, markedly enhanced the PGE_1-stimulated p38 MAP kinase phosphorylation, whereas fluvoxamine hardly affected the phosphorylation in itself (Fig. 5B).

3.6. Effects of SB203580 on the enhancement by duloxetine on the PGE_1-induced release of OPG and IL-6 in MC3T3-E1 cells

To clarify the involvement of p38 MAP kinase in the duloxetine-effect on the release of OPG and IL-6 stimulated by PGE_1 in osteoblast-like MC3T3-E1 cells, we examined the effects of SB203580, an inhibitor of p38 MAP kinase [37], on the release of OPG and IL-6 stimulated by PGE_1 in combination with or without duloxetine. As previously described [16], SB203580 reduced the PGE_1-stimulated OPG release (Fig. 6A). The OPG release stimulated by PGE_1 in combination with duloxetine was significantly suppressed by SB203580 almost to the level of PGE_1-effect without duloxetine (Fig. 6A). In addition, SB203580 also significantly inhibited the PGE_1-stimulated IL-6 release (Fig. 6B). The release of IL-6 stimulated by PGE_1 in combination with duloxetine was significantly suppressed by SB203580 to the level approximately half of the PGE_1-effect without duloxetine (Fig. 6B).

3.7. Effects of SB203580 on the enhancement by fluvoxamine on the PGE_1-induced release of OPG and IL-6 in MC3T3-E1 cells

We further examined the effects of SB203580 [37] on the release of OPG and IL-6 stimulated by PGE_1 in combination with or without fluvoxamine in osteoblast-like MC3T3-E1 cells. We found that the OPG release stimulated by PGE_1 in combination with fluvoxamine was significantly suppressed by SB203580 almost to the level of PGE_1-effect without fluvoxamine (Fig. 7A). In addition, the release of IL-6 stimulated by PGE_1 in combination with fluvoxamine was almost completely suppressed by SB203580 (Fig. 7B).

4. Discussion

In the present study, we investigated the effect of duloxetine, an SNRI [4] on the PGE_1-stimulated synthesis of OPG and IL-6, as important osteoblastic functions in bone remodeling, in osteoblast-like MC3T3-E1 cells. We showed that duloxetine potently strengthened the

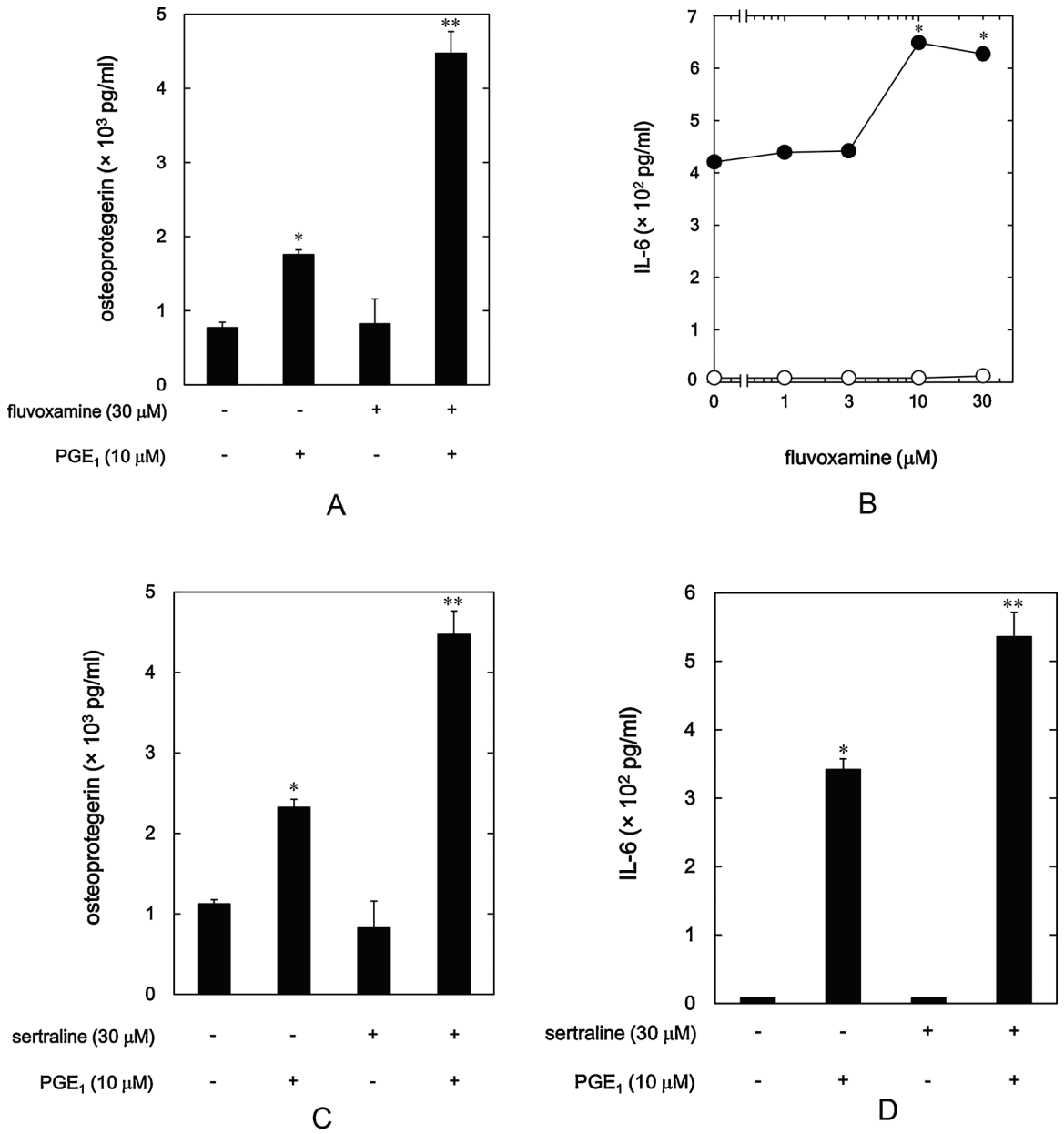


Fig. 3. Effects of fluvoxamine or sertraline on the PGE_1-induced release of OPG and IL-6 in MC3T3-E1 cells. The cultured cells were pretreated with the indicated doses of fluvoxamine or vehicle (A, B), or 30 μM of sertraline or vehicle (C, D) for 60 min, and then stimulated by 10 μM of PGE_1 (black circles in B) or vehicle (white circles in B) for 48 h. The conditioned medium was collected, and the concentrations of OPG (A, C) and IL-6 (B, D) were determined with an ELISA for each. Each value represents the mean ± SE of triplicate determinations from three independent cell preparations. *$p<0.05$ vs. control (A, C, D), **$p<0.05$ vs. PGE_1 alone (A, C, D); *$p<0.05$ vs. PGE_1 alone (B).

PGE_1-stimulated releases of OPG and IL-6 in these cells. Since the expressions of both OPG mRNA and IL-6 mRNA induced by PGE_1 were also amplified by duloxetine, the increased releases of PGE_1-stimulated OPG and IL-6 seem to be mediated through a transcriptional event. Therefore, it is most likely that duloxetine could potentiate the syntheses of OPG and IL-6 stimulated by PGE_1 in osteoblasts. To the best of our knowledge, this is probably the first report clearly demonstrating the simultaneous amplification of OPG and IL-6 syntheses by the SNRI duloxetine in osteoblasts.

We next examined the effects of a selective norepinephrine reuptake inhibitor, reboxetine [28] on the PGE_1-stimulated release of OPG and IL-6 from osteoblast-like MC3T3-E1 cells. We demonstrated that reboxetine affected neither the OPG release nor the IL-6 release stimulated by PGE_1. Thus, it seems unlikely that the inhibition of norepinephrine reuptake affects the PGE_1-stimulated releases of OPG and IL-6, although the transporter of norepinephrine is reportedly expressed on osteoblasts [27]. Since the enhancement by duloxetine of the PGE_1-stimulated releases of OPG and IL-6 is probably not mediated through the action of norepinephrine reuptake inhibition, the effects of serotonin reuptake inhibition should be selectively investigated to clarify the mechanism of duloxetine. Thus, we next examined the effect of an SSRI fluvoxamine [35] on the PGE_1-stimulated OPG and IL-6 releases from osteoblast-like MC3T3-E1 cells, and showed that fluvoxamine actually strengthened the release of OPG and IL-6 stimulated by PGE_1. Additionally, sertraline, another SSRI [36], also enhanced the PGE_1-stimulated release of OPG and IL-6 from these cells, suggesting that selective inhibition of serotonin reuptake could enhance the OPG and IL-6 releases stimulated by PGE_1 in osteoblasts. Based on our results, it is most likely that the amplifying effects of duloxetine on the PGE_1-stimulated synthesis of OPG and IL-6 is mainly due to the inhibition of serotonin

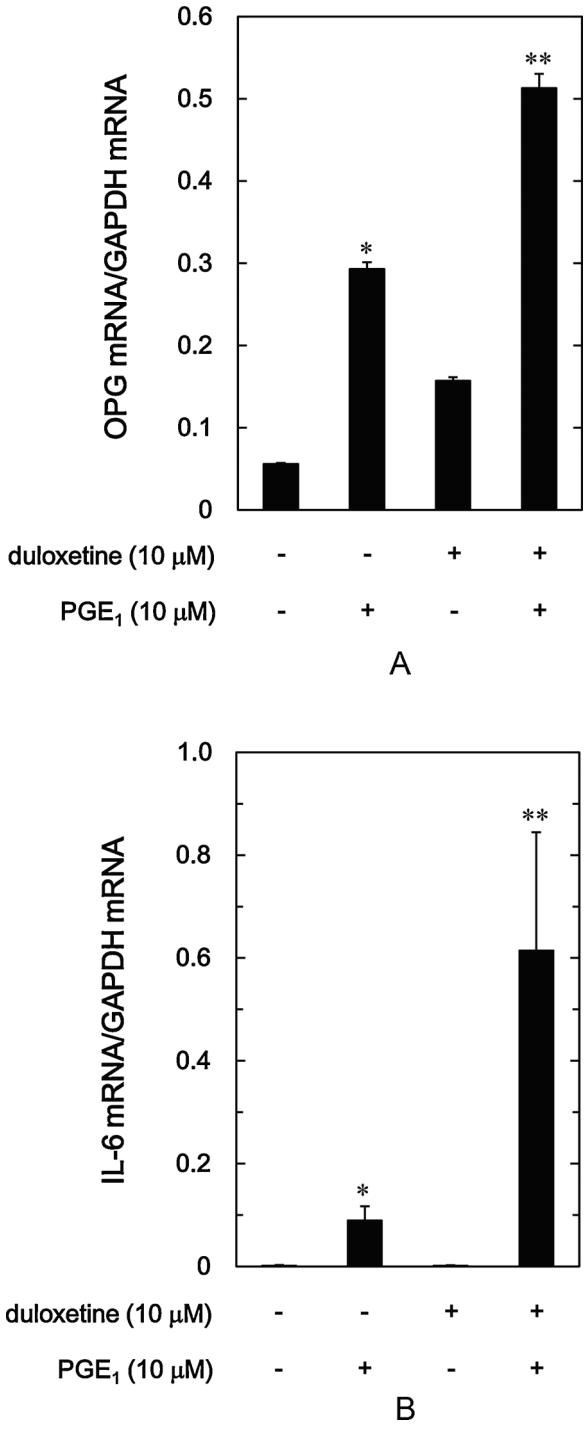


Fig. 4. Effects of duloxetine on the PGE1-induced expression of OPG mRNA (A) and IL-6 mRNA (B) in MC3T3-E1 cells. The cultured cells were pretreated with 10 μM of duloxetine or vehicle for 60 min, and then stimulated by 10 μM of PGE_1 or vehicle for 3 h. The levels of OPG mRNA, IL-6 mRNA and GAPDH mRNA were determined with real-time RT-PCR. The levels of OPG mRNA and IL-6 mRNA were adjusted to GAPDH mRNA levels. Each value represents the mean ± SE of triplicate determinations from three independent cell preparations. *$p<0.05$ vs. control. **$p<0.05$ vs. PGE_1 alone.

reuptake but not norepinephrine reuptake in osteoblasts.

On the basis of our previous findings that PGE_1 stimulates OPG release in part via the activation of p38 MAP kinase in osteoblasts, we investigated the effect of duloxetine [4] on the PGE_1-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. We showed that

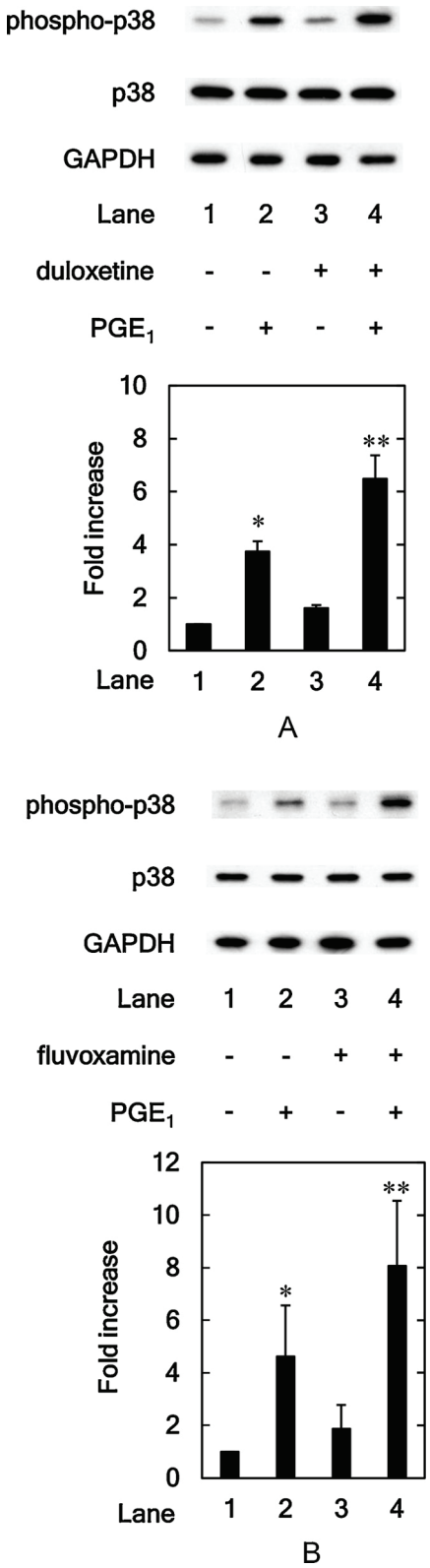
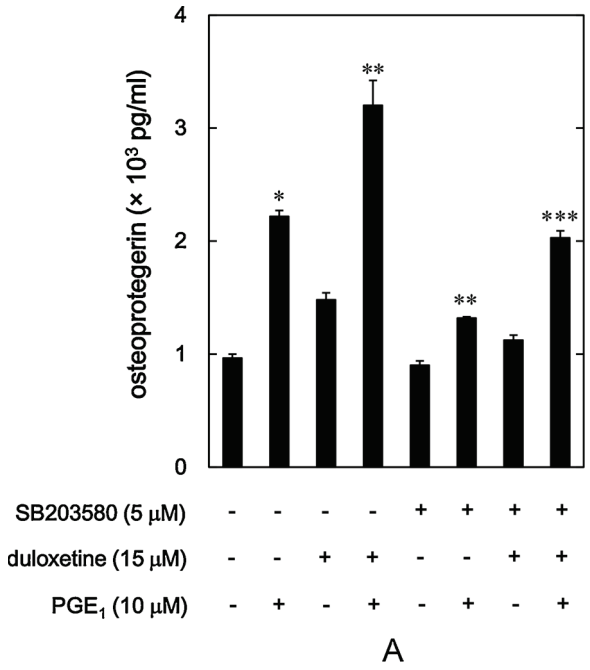


Fig. 5. Effects of duloxetine and fluvoxamine on the PGE_1-induced p38 MAP kinase phosphorylation in MC3T3-E1 cells. The cultured cells were pretreated with 3 μM of duloxetine (A), 10 μM of fluvoxamine (B) or vehicle for 60 min, and then stimulated by 10 μM of PGE_1 or vehicle for 10 min. Western blot analysis was performed using antibodies against phospho-p38 MAP kinase, p38 MAP kinase and GAPDH. The histogram shows the quantitative representation of the PGE_1-induced phosphorylation obtained a laser densitometric analysis of triplicate independent experiments. *$p<0.05$ vs. control. **$p<0.05$ vs. PGE_1 alone.



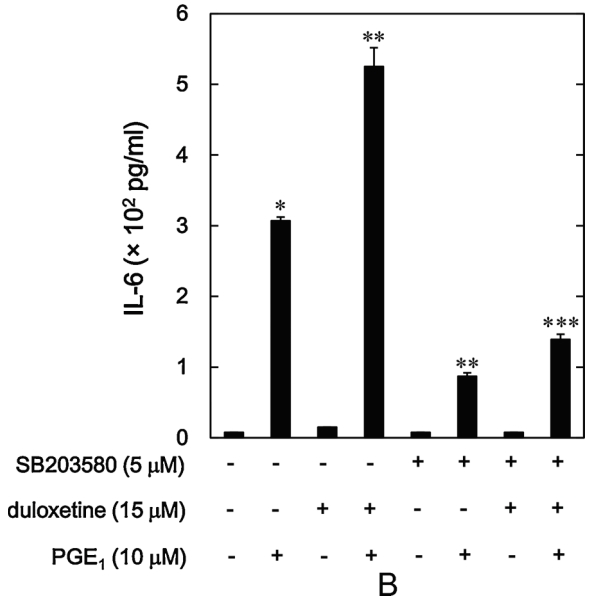
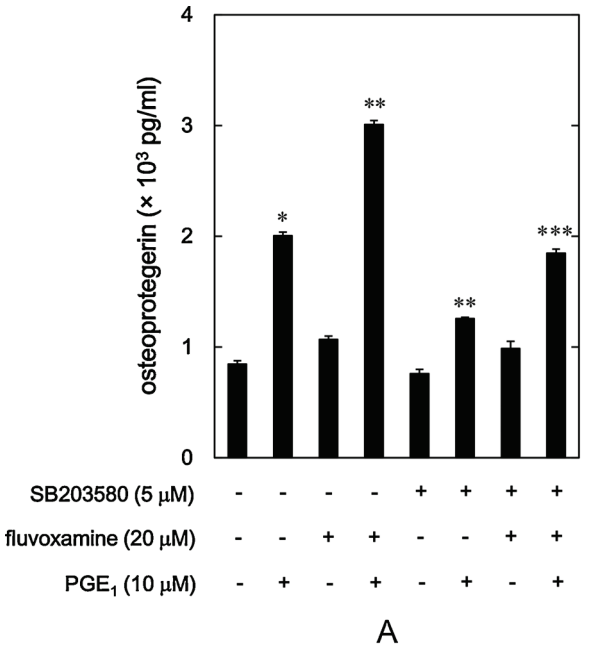


Fig. 6. Effects of SB203580 on the enhancement by duloxetine on the PGE_1-induced release of OPG and IL-6 in MC3T3-E1 cells. Before the pretreatment with 15 μM of duloxetine or vehicle for 60 min, the cells were preincubated with 5 μM of SB203580 or vehicle for 60 min. After that, the cells were stimulated by 10 μM of PGE_1 or vehicle for 48 h. The conditioned medium was collected, and the concentrations of OPG (A) and IL-6 (B) were determined with an ELISA for each. Each value represents the mean ± SE of triplicate determinations from three independent cell preparations. *$p<0.05$ vs. control. **$p<0.05$ vs. PGE1 alone. ***$p<0.05$ vs PGE1 with duloxetine.



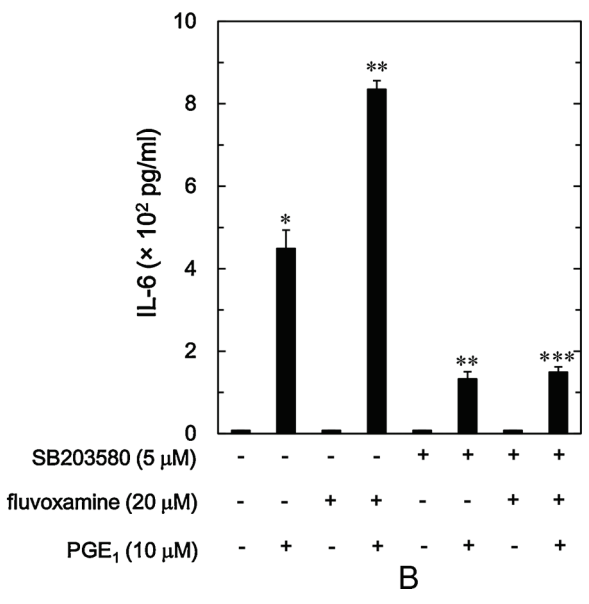


Fig. 7. Effects of SB203580 on the enhancement by fluvoxamine on the PGE_1-induced release of OPG and IL-6 in MC3T3-E1 cells. Before the pretreatment with 20 μM of fluvoxamine or vehicle for 60 min, the cells were preincubated with 5 μM of SB203580 or vehicle for 60 min. After that, the cells were stimulated by 10 μM of PGE_1 or vehicle for 48 h. The conditioned medium was collected, and the concentrations of OPG (A) and IL-6 (B) were determined with an ELISA for each. Each value represents the mean ± SE of triplicate determinations from three independent cell preparations. *$p<0.05$ vs. control. **$p<0.05$ vs. PGE1 alone. ***$p<0.05$ vs PGE1 with fluvoxamine.

duloxetine truly strengthened the p38 MAP kinase phosphorylation induced by PGE_1 in MC3T3-E1 cells. In addition, fluvoxamine, an SSRI [35], also enhanced the PGE_1-stimulated phosphorylation of p38 MAP kinase in these cells. Thus, it is probable that the amplifying effect of duloxetine on the PGE_1-induced p38 MAP kinase phosphorylation, in accordance with the release of OPG and IL-6, is due to the serotonin reuptake inhibition in MC3T3-E1 cells. Since the enhancement by duloxetine of the PGE_1-stimulated MC3T3-E1 cell function seem to be caused by the upregulation of p38 MAP kinase activation at least in part, we further examined the effects of SB203580, a p38 MAP kinase inhibitor [37], on the releases of OPG and IL-6 stimulated by PGE_1 in combination with duloxetine in osteoblast-like MC3T3-E1 cells. We demonstrated that SB203580 truly suppressed the amplification by duloxetine of the PGE_1-stimulated release of OPG and IL-6, so that duloxetine could strengthen the synthesis of OPG and IL-6 via the augmentation of p38 MAP kinase activation, at least in part in the PGE_1-stimulated osteoblasts. Furthermore, SB203580 also inhibited the enhancement by fluvoxamine of the PGE_1-stimulated release of OPG and

IL-6, suggesting the involvement of p38 MAP kinase in the amplifying effects of duloxetine on the releases in the PGE_1-stimulated cells. Taking the present findings into account as a whole, therefore, it is most likely that duloxetine could strengthen the PGE_1-stimulated synthesis of OPG and IL-6 in osteoblasts, at least in part via upregulation of the p38 MAP kinase activation through the serotonin reuptake inhibition.

OPG, a decoy receptor for RANKL, is a crucial regulator of osteoclastgenesis and osteoclastic bone resorption competitively antagonized RANK to RANKL bind [14,15]. Thus, the amplification of OPG release from osteoblasts might act to suppress the accelerated bone resorption in metabolic bone diseases including osteoporosis. In addition, IL-6 is currently recognized to promote bone formation under the increased bone metabolism such as bone fracture healing [19,20]. Thus, it is possible that the duloxetine-induced upregulation of IL-6 release from osteoblasts might lead the bone remodeling to ameliorate in cooperation with OPG release. Therefore, our present findings might decrease the physicians' concern to deterioration of osteoporosis about duloxetine use for old persons with LBP. Further investigation would be required to clarify the details about duloxetine-effect in bone metabolism.

In conclusion, the results of our present study strongly suggest that duloxetine could strengthen osteoblast activation by PGE_1 through the upregulation of p38 MAP kinase, leading to increasing the synthesis of OPG and IL-6.

Author contributions

Hiroki Iida, Kumiko Tanabe, Shinobu Yamaguchi, Haruhiko Tokuda and Osamu Kozawa conceived and designed the experiments: Junko Tachi, Woo Kim, Tomoyuki Hioki and Rie Matsushima-Nishiwaki performed the experiments; Haruhiko Tokuda, Rie Matsushima-Nishiwaki, Hiroki Iida and Osamu Kozawa analyzed the data; Junko Tachi, Haruhiko Tokuda, Kumiko Tanabe, Shinobu Yamaguchi, Takashi Onuma, Osamu Kozawa and Hiroki Iida wrote the paper.

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Declaration of Competing Interest

The authors report no declarations of interest.

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