Research article

Tramadol enhances PGF$_{2\alpha}$-stimulated osteoprotegerin synthesis in osteoblasts

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ABSTRACT

Osteoprotegerin (OPG) synthesized by osteoblasts is currently considered a crucial regulator to suppress the formation and function of osteoclasts. We previously showed that the synthesis of OPG is stimulated by prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) in the involvement of p38 mitogen-activated protein kinase (MAPK), stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK) and p44/p42 MAPK in osteoblast-like MC3T3-E1 cells. We also found that Rho-kinase is involved in the signaling of PGF$_{2\alpha}$ upstream of p38 MAPK in these cells. Tramadol is widely used to treat chronic pain, such as low back pain associated with osteoporosis. We investigated whether or not tramadol affects the OPG release induced by PGF$_{2\alpha}$ in osteoblast-like MC3T3-E1 cells. The levels of OPG in the conditioned medium were measured by an enzyme-linked immunosorbent assay. The mRNA expression of OPG was determined with real-time reverse transcription polymerase chain reaction. The phosphorylation of target protein was determined with a Western blot analysis. PGF$_{2\alpha}$ induced the release and the mRNA expression of OPG, which tramadol significantly enhanced. Morphine, a selective μ-opioid receptor (MOR) agonist, also enhanced the PGF$_{2\alpha}$-induced OPG release. In addition, naloxone, a MOR antagonist, suppressed the enhancement by tramadol or morphine of the PGF$_{2\alpha}$-induced OPG synthesis. Tramadol upregulated the phosphorylation of SAPK/JNK and p38 MAPK stimulated by PGF$_{2\alpha}$ but not that of p44/p42 MAPK or myosin phosphatase targeting protein (MYPT), a substrate of Rho-kinase. The inhibitors of both p38 MAPK and SAPK/JNK, SB203580 and SP600125, respectively, reduced the tramadol amplification of OPG release stimulated by PGF$_{2\alpha}$. The present results strongly suggest that tramadol enhances the synthesis of OPG stimulated by PGF$_{2\alpha}$ through MOR in osteoblasts, and that the amplifying effect is exerted at upstream of p38 MAPK and SAPK/JNK but downstream of Rho-kinase.

1. Introduction

Bone metabolism is predominantly performed by two types cells: osteoclasts and osteoblasts, which are responsible for bone resorption and formation, respectively (Karsenty and Wagner, 2002). To maintain adequate bone structures, the process of bone remodeling is highly orchestrated by numerous humoral factors and autacoids including prostaglandins (PGs) (Boyce et al., 2012). In addition to bone formation, osteoblasts also participate in bone resorption control through receptor activator of nuclear factor-κB (RANK) ligand (RANKL) expression responding to bone resorptive stimuli (Boyce et al., 2012). The binding of RANKL and RANK, which is expressed on the surface of osteoclastic precursors and mature osteoclasts, stimulates the differentiation and the activation of osteoclasts (Boyce et al., 2012).

Metabolic bone diseases, such as osteoporosis, result from the bone remodeling imbalance. On the other hand, osteoprotegerin (OPG), a

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member of the tumor necrosis factor receptor family, is synthesized by osteoblasts and binds RANKL as a decoy receptor to inhibit RANK-RANKL binding, resulting in the suppression of bone resorption (Simonet et al., 1997). OPG knock-out mice show severe osteoporosis (Bucay et al., 1998). Thus, OPG is currently considered a crucial regulator suppressing the formation of functional osteoclasts (Steeve et al., 2004).

PGs are lipid mediators acting as autacoids and play roles in various physiological and pathological process of bone metabolism (Hikiji et al., 2008). $PGF_{2\alpha}$ originally recognized to promote bone resorption (Raisz et al., 1990) is now understood to be involved also in bone formation, mediating the bone remodeling (Agas et al., 2013). We previously reported that $PGF_{2\alpha}$ stimulates OPG synthesis and that p38 mitogen-activated protein kinase (MAPK), p44/p42 MAPK and stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK) activation are involved in the synthesis in osteoblast-like MC3T3-E1 cells (Kuroyanagi et al., 2014). In addition, we also showed that Rho-kinase regulates $PGF_{2\alpha}$-induced interleukin-6 (IL-6) synthesis positively at p38 MAP kinase upstream in these cells (Minamitani et al., 2008) (Fujita et al., 2017).

Tramadol, a weak μ-opioid receptor (MOR) agonist with an inhibitory effect on the reuptake of serotonin and norepinephrine (Bravo et al., 2017) (Subedi et al., 2019), is widely used as an analgesic drug for the treatment of chronic pain related to cancer pain, persistent postoperative pain and back pain (Bravo et al., 2017) (Subedi et al., 2019). Among them, low back pain is a common symptom closely related to osteoporosis (Deyo and Tsui-Wu, 1987). In addition to cases of symptomatic vertebral fracture, it has been reported that patients with osteoporosis without evident vertebral fracture also suffer from low back pain known as "osteoporotic pain" (Suzuki et al., 2013) (Ohtori et al., 2010). Notably, tramadol is a useful therapeutic tool for managing low back pain, such as "osteoporotic pain" (Bravo et al., 2017). Regarding the effect of tramadol on bone metabolism, the possibility of suppression of the proliferation of human osteoblasts (Matziolis et al., 2002) and reduction of the orthodontic tooth movement in rats (Aghili and Yassaei, 2013) has been reported. However, the effects of this unique agent on bone metabolism, especially the function of osteoblasts, remains unclear.

We investigated the effect of tramadol on the release of OPG stimulated by $PGF_{2\alpha}$ in osteoblast-like MC3T3-E1 cells. We showed that tramadol enhances the $PGF_{2\alpha}$-stimulated synthesis of OPG through MOR in osteoblasts, and that the amplifying effect is exerted upstream of p38 MAP kinase and SAPK/JNK but the downstream of Rho-kinase.

2. Materials and methods

2.1. Materials

Tramadol hydrochloride, naloxone hydrochloride dihydrate, $PGF_{2\alpha}$ and hydroxyfasudil (fasudil) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Morphine hydrochloride was obtained from Takeda Pharmaceutical Co., Ltd. (Osaka, Japan). SP600125 and SB203580 were purchased from Calbiochem-Novabiochem Co. (La Jolla, CA, USA). The antibodies against phospho-specific SAPK/JNK, SAPK/JNK, phospho-specific p44/p42 MAP kinase, p44/p42 MAP kinase, phospho-specific p38 MAP kinase, and p38 MAP kinase were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). The antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and myosin phosphatase target subunit (MYPT) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). An ECL Western blotting detection system was obtained from GE Healthcare Life Sciences (Chalfont, UK). Other materials and chemicals were obtained commercially. $PGF_{2\alpha}$ was dissolved in ethanol. Tramadol, SP600125 and SB203580 were dissolved in dimethyl sulfoxide. Morphine was dissolved in mast cell medium (150 mM NaCl, 5 mM KCl, 5.5 mM glucose, 0.8 mM $MgSO_4$, 1 mM $CaCl_2$ and HEPES, pH 7.4). The maximum concentration of ethanol or dimethyl sulfoxide was 0.1%, which has no effects on the assay for

OPG or the detection of the protein level using a Western blot analysis (Kuroyanagi et al., 2014).

2.2. Cell culture

Osteoblast-like MC3T3-E1 cells, an established clonal cell line derived from newborn mouse calvariae (Sudo et al., 1983), were maintained as previously described (Kozawa et al., 1997). In brief, the cells, which were cultured in α-minimum essential medium (α-MEM) with 10% fetal bovine serum (FBS), at 37 °C under humidified conditions of 5% CO_2/95% air, were seeded into 35-mm diameter dishes (5×10^5 cells/dish) for an OPG assay and reverse transcription polymerase chain reaction (RT-PCR) or 90-mm diameter dishes (2×10^5 cells/dish) for Western blotting. After 5 days, the medium was switched to 0.3% FBS-containing α-MEM was performed, and we used the cells for experiments 48 h later (Kozawa et al., 1997).

2.3. Assay for OPG

The cells pretreated with 0, 10, 30, 100 and 200 μM of tramadol; 0, 10, 30, 100, 200 and 300 μM of morphine; 0, 200 and 300 μM of naloxone; 0, 5 and 7 μM of SB203580; 0 and 10 μM of SP600125 for 60 min were stimulated with 10 μM of $PGF_{2\alpha}$ or vehicle in α-MEM containing 0.3% FBS for the indicated periods. The conditioned medium was collected, and the levels of OPG in the medium were then measured by a mouse OPG enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's protocol (Kuroyanagi et al., 2014).

2.4. Real-time RT-PCR

The cultured cells, which has been pretreated with 300 μM of tramadol or vehicle for 60 min, were stimulated with 10 μM of $PGF_{2\alpha}$ or vehicle in α-MEM containing 0.3% FBS for 3 h. Using Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA) with Omniscrpt Reverse Transcriptase kit (QIAGEN Inc., Valencia, CA, USA), total RNA was isolated and transcribed into complementary DNA. Real-time RT-PCR using a Light Cycler system was performed in capillaries with Fast Start DNA Master SYBR Green I provided with the kit (Roche Diagnostics, Basel, Switzerland) (Kuroyanagi et al., 2014). Sense and antisense primers for both mouse OPG mRNA and GAPDH mRNA were purchased from Takara Bio Inc. (Tokyo, Japan) (Kuroyanagi et al., 2014). The amplified products were determined using a melting curve analysis. The OPG mRNA levels were respectively normalized to GAPDH mRNA levels (Kuroyanagi et al., 2014).

2.5. The western blot analysis

The cells pretreated with 0, 100, 200 and 250 μM of tramadol for 60 min were stimulated by 10 μM of $PGF_{2\alpha}$ or vehicle in α-MEM with 0.3% FBS for the indicated periods. The cells were then washed twice with phosphate-buffered saline and then lysed, sonicated and homogenized in a lysis buffer with 62.5 mM Tris/HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 50 mM of dithiothreitol and 10% glycerol. SDS-polyacrylamide gel electrophoresis (PAGE) was performed according to the Laemmli's method (Laemmli, 1970) in 10% polyacrylamide gel (Kuroyanagi et al., 2014). The protein was fractionated and then transferred onto an Immun-Blot polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA) (Kuroyanagi et al., 2014). The membranes were blocked with 5% fat-free dry milk solved in Tris-buffered saline-Tween (TBS-T; 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) for 1 h ahead of incubation with primary antibodies (Kuroyanagi et al., 2014). A western blot analysis was performed according to the method described previously (Kato et al., 2002) using phospho-specific SAPK/JNK antibodies, SAPK/JNK antibodies, phospho-specific p38 MAP kinase, p38 MAP kinase antibodies, phospho-specific MYPT antibodies and GAPDH

antibodies as primary antibodies (Kuroyanagi et al., 2014). Peroxidase-labeled antibodies raised in goat against rabbit IgG were used as secondary antibodies, and the peroxidase activity on the membrane was visualized on X-ray film using the ECL Western blotting detection system as previously described (Kuroyanagi et al., 2014).

2.6. The densitometric analysis

For densitometric analysis of the Western blots, we use a scanner and software program for image processing (image J version 1.48, NIH, Bethesda, MD) (Kuroyanagi et al., 2014). The calculation of the phosphorylated protein levels was performed with a method as follows: the background-subtracted signal intensity of the phosphorylated level of p44/p42 MAPK, p38 MAPK or SAPK/JNK was respectively normalized to the responding total protein signal, and that of MYPT-1 was normalized to the GAPDH signal. The levels were plotted as the fold increase compared to that of the control cells without stimulation (Kuroyanagi et al., 2014).

2.7. Statistical analyses

The data were analyzed by the analysis of variance method for multiple comparisons between pairs, and $p < 0.05$ was considered to be statistically significant (Kuroyanagi et al., 2014). All data are presented as the mean $\pm$ standard error of the mean of triplicate determinations from three independent cell preparations.

3. Results

3.1. Effects of tramadol on the PGF$_{2\alpha}$-stimulated OPG synthesis in MC3T3-E1 cells

We previously showed that PGF$_{2\alpha}$ stimulates OPG synthesis in osteoblast-like MC3T3-E1 cells (Kuroyanagi et al., 2014). Thus, we first examined the effect of tramadol on the PGF$_{2\alpha}$-stimulated OPG release. Tramadol, which by itself has little effect on OPG levels, significantly enhanced the PGF$_{2\alpha}$-stimulated OPG release in a dose-dependent manner between 30 and 200 μM (Figure 1A). To confirm whether or not the enhancing effects of tramadol on PGF$_{2\alpha}$-stimulated OPG release were mediated via transcriptional events, we next tested the effects of tramadol on the PGF$_{2\alpha}$-stimulated expression of OPG mRNA by real-time RT-PCR. We found that tramadol significantly enhanced the PGF$_{2\alpha}$-induced expression of OPG mRNA (Figure 1B).

3.2. Effects of morphine or naloxone on the PGF$_{2\alpha}$-stimulated OPG release in MC3T3-E1 cells

To explore whether or not the enhancing effects of tramadol on the PGF$_{2\alpha}$-stimulated OPG release were truly mediated through MOR, we examined the effects of a selective MOR agonist (morphine) (Subedi et al., 2019) on the release of OPG stimulated by PGF$_{2\alpha}$ in osteoblast-like MC3T3-E1 cells. Similar to tramadol, morphine remarkably enhanced the OPG release in the stimulation with PGF$_{2\alpha}$ (Figure 2). We next examined the effect of naloxone, an MOR antagonist (Sadée et al., 2005), on the amplification by tramadol or morphine of PGF$_{2\alpha}$-stimulated OPG release in these cells. Naloxone, which by itself did not influence the PGF$_{2\alpha}$-stimulated OPG release, significantly suppressed the tramadol-caused amplification of the PGF$_{2\alpha}$-stimulated OPG release (Figure 3A). Naloxone also markedly inhibited the amplification by morphine of the PGF$_{2\alpha}$-stimulated OPG release (Figure 3B). We presented the results using the different doses of naloxone in these experiments. The reason was that the levels of PGF$_{2\alpha}$-stimulated OPG release with or without naloxone to be similar in the different cell preparations. In addition, we used a dose of 200 μM of morphine, an intermediate dose between 100 and 300 μM, which was adequate to clearly indicate the effect of naloxone on the amplification by morphine of the PGF$_{2\alpha}$-stimulated OPG release.
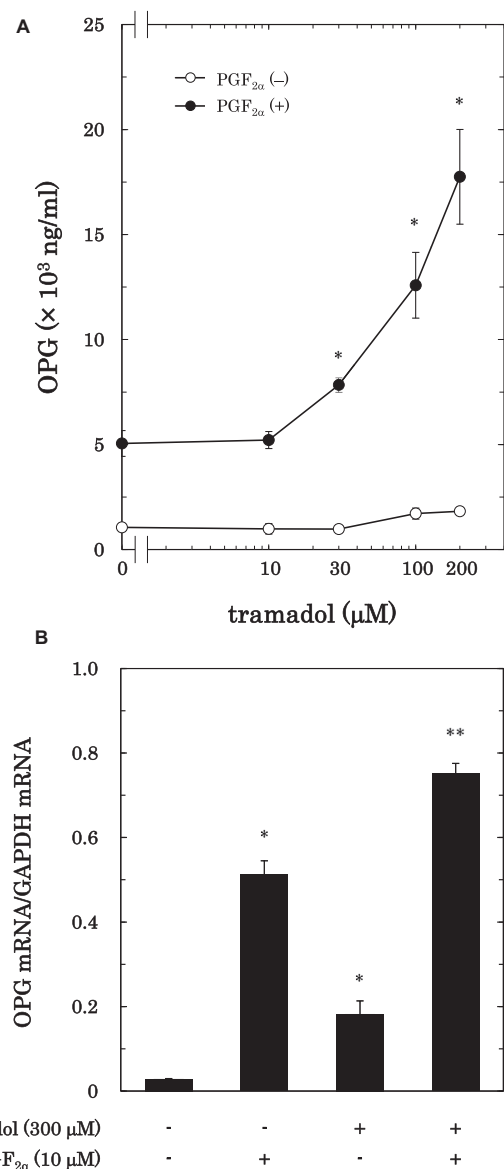
Figure 1. Effects of tramadol on the release and mRNA expression of OPG stimulated by PGF$_{2\alpha}$ in MC3T3-E1 cells. (A) The cells pretreated with 0, 10, 30, 100 and 200 μM of tramadol for 60 min were stimulated by 10 μM PGF$_{2\alpha}$ or vehicle for 48 h. The concentrations of OPG in the conditioned medium were determined. Each value represents the mean $\pm$ S.E.M. of three-fold determinations from three independently prepared cells. *$p < 0.05$, in comparison to the PGF$_{2\alpha}$ value alone. (B) The cells pretreated with 300 μM tramadol or vehicle for 60 min were stimulated by 10 μM PGF$_{2\alpha}$ or vehicle for 3 h. Thereafter, the total RNA was isolated in each and transcribed to cDNA. The expression of OPG mRNA and that of GAPDH mRNA were quantified by quantitative RT-PCR. We normalized the OPG mRNA levels to GAPDH mRNA levels. Each value represents the mean $\pm$ S.E.M. of three-fold determinations from three independently prepared cells. *$p < 0.05$ compared to the control value. **$p < 0.05$ compared to the PGF$_{2\alpha}$ value alone.

3.3. Effects of tramadol on the PGF$_{2\alpha}$-induced phosphorylation of p44/p42 MAPK, p38 MAPK, SAPK/JNK and MYPT in MC3T3-E1 cells

Regarding the signaling mechanism, we previously showed that PGF$_{2\alpha}$ stimulates OPG synthesis through p44/p42 MAPK, p38 MAPK and SAPK/JNK in osteoblast-like MC3T3-E1 cells (Kuroyanagi et al., 2014). We therefore examined the effects of tramadol on the PGF$_{2\alpha}$-induced phosphorylation of p44/p42 MAPK, p38 MAPK and SAPK/JNK in these cells. Tramadol did not affect the phosphorylation of p44/p42 MAPK
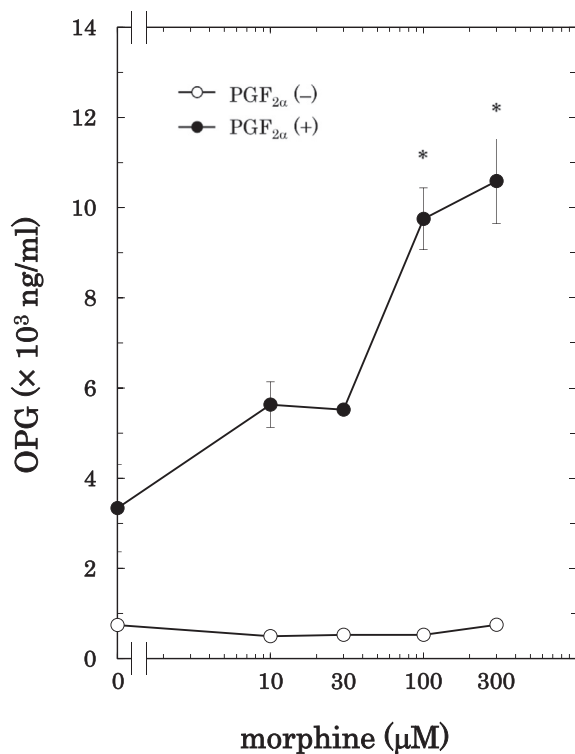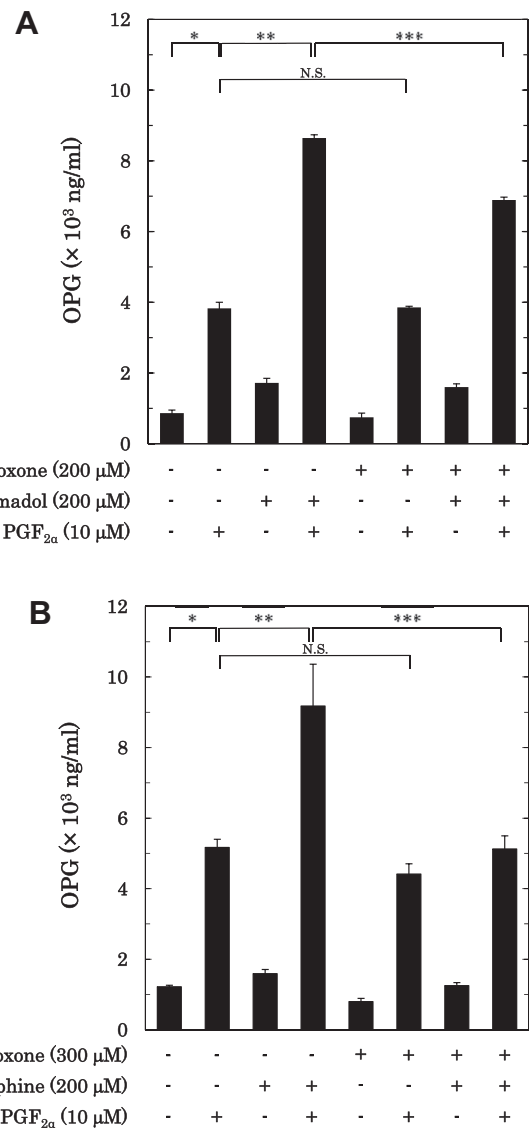
Figure 2. Effects of morphine on the PGF$_{2\alpha}$-stimulated OPG release in MC3T3-E1 cells. The cells pretreated with 0, 10, 30, 100 and 300 μM of morphine for 60 min were stimulated by 10 μM PGF$_{2\alpha}$ or vehicle for 48 h. The concentrations of OPG in the conditioned medium were determined. Each value represents the mean ± S.E.M. of three-fold determinations from three independently prepared cell preparations. *$p < 0.05$ compared to the PGF$_{2\alpha}$ value alone.

with or without PGF$_{2\alpha}$ stimulation (Figure 4A). However, while tramadol had little effect on the phosphorylation of p38 MAPK by itself, it significantly enhanced the PGF$_{2\alpha}$-stimulated phosphorylation of p38 MAPK dose-dependently in the range between 200 and 250 μM (Figure 4B). In addition, tramadol which alone hardly affected but markedly amplified the phosphorylation of SAPK/JNK stimulated by PGF$_{2\alpha}$ dose-dependently in the range between 200 and 250 μM (Figure 4C).

We previously reported that Rho-kinase regulates the PGF$_{2\alpha}$-stimulated IL-6 synthesis upstream of p38 MAPK in these cells (Minamitani et al., 2008) (Fujita et al., 2017). We therefore examined the effect of fasudil, a Rho-kinase inhibitor (Shimokawa and Rashid, 2007), on the OPG release induced by PGF$_{2\alpha}$ in osteoblast-like MC3T3-E1 cells and confirmed that fasudil significantly suppressed the PGF$_{2\alpha}$-induced OPG release in osteoblast-like MC3T3-E1 cells (Table 1). In addition, we also confirmed that fasudil suppressed the PGF$_{2\alpha}$-stimulated phosphorylation of SAPK/JNK in these cells (data not shown). Therefore, it is probable that Rho-kinase is involved in the PGF$_{2\alpha}$-stimulated OPG release at a point upstream of both p38 MAPK and SAPK/JNK in MC3T3-E1 cells. As MYPT, a myosin phosphatase component, is an established substrate of Rho-kinase (Fukata et al., 2001) (Ito et al., 2004), we next examined the effects of tramadol on the phosphorylation of MYPT induced by PGF$_{2\alpha}$ in osteoblast-like MC3T3-E1 cells. However, tramadol hardly affected the phosphorylation of MYPT with or without PGF$_{2\alpha}$ (Figure 4D).

3.4. Effects of SB203580 on the amplification by tramadol or morphine of the PGF$_{2\alpha}$-stimulated OPG release in MC3T3-E1 cells

We next examined the effect of SB203580, known as a p38 MAP kinase inhibitor (Cuenda et al., 1995), on the enhancement by tramadol or morphine of the PGF$_{2\alpha}$-stimulated OPG release in osteoblast-like


Figure 3. Effects of naloxone on the amplification by tramadol or morphine of the PGF$_{2\alpha}$-stimulated OPG release in MC3T3-E1 cells. The cells pre-incubated with (A) 200 μM naloxone or vehicle, (B) 300 μM naloxone or vehicle for 60 min were subsequently pretreated with (A) 200 μM tramadol or vehicle, (B) 200 μM morphine or vehicle for 60 min. The treated cells were then stimulated by 10 μM PGF$_{2\alpha}$ or vehicle for 48 h. The concentrations of OPG in the conditioned medium were determined. Each value represents the mean ± S.E.M. of three-fold determinations from three independently prepared cells. *$p < 0.05$ compared to the control value. **$p < 0.05$ compared to the PGF$_{2\alpha}$ value alone. ***$p < 0.05$ compared to the value of the combination of (A) tramadol and PGF$_{2\alpha}$, (B) morphine and PGF$_{2\alpha}$.

MC3T3-E1 cells. As previously reported (Kuroyanagi et al., 2014), SB203580 by itself had little effect on the OPG release but significantly suppressed the PGF$_{2\alpha}$-induced OPG release (Figure 5A, 5B). SB203580 markedly reduced the amplification by tramadol of the PGF$_{2\alpha}$-stimulated OPG release (Figure 5A) and also reduced the amplification by morphine of the PGF$_{2\alpha}$-stimulated OPG release, similar to tramadol (Figure 5B).

3.5. Effects of SP600125 on the amplification by tramadol or morphine of the PGF$_{2\alpha}$-stimulated OPG release in MC3T3-E1 cells

Furthermore, we examined the effect of SP600125, an inhibitor of SAPK/JNK (Bennett et al., 2001), on the amplification by tramadol or morphine of the OPG release stimulated by PGF$_{2\alpha}$ from osteoblast-like
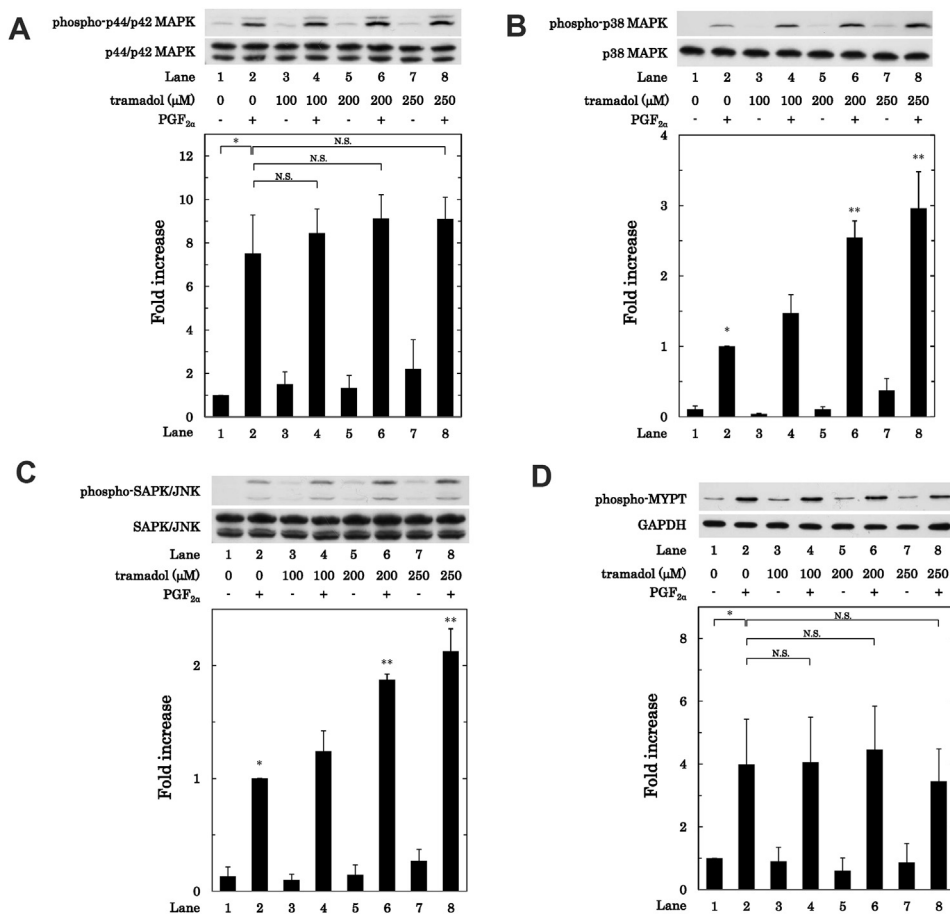
Figure 4. Effects of tramadol on the PGF$_{2\alpha}$-stimulated phosphorylation of p44/p42 MAPK, p38 MAPK, SAPK/JNK and MYPT in MC3T3-E1 cells. The cells pretreated with 0, 100, 200, and 250 μM of tramadol or vehicle for 60 min were stimulated by 10 μM PGF$_{2\alpha}$ or vehicle for (A) 20 min, (B) 10 min, (C) 20 min or (D) 2 min. The extracts of cells were subjected to SDS-PAGE with a subsequent Western blot analysis with antibodies against (A) phospho-specific p44/p42 MAPK or p44/p42 MAPK, (B) phospho-specific p38 MAPK or p38 MAPK, (C) phospho-specific SAPK/JNK or SAPK/JNK and (D) phospho-specific MYPT or GAPDH. The histogram shows the quantitative representation of the levels of PGF$_{2\alpha}$-induced phosphorylation with a laser densitometric analysis. The levels indicate the fold increase from the basal levels (lane 1). Each value represents the mean ± S.E.M. of threefold determinations from three independently prepared cells. *$p < 0.05$, in comparison to the control value (Lane 1). **$p < 0.05$, in comparison to the PGF$_{2\alpha}$ alone value (Lane 2). Full, non-adjusted images of Figure 4A, Figure 4B, Figure 4C and Figure 4D are provided as supplementary materials.

MC3T3-E1 cells. SP600125, which alone had little effect on OPG levels, significantly suppressed the PGF$_{2\alpha}$-induced OPG release as previously reported (Agas et al., 2013) (Figure 6A, 6B). SP600125 also markedly reduced the amplification of the PGF$_{2\alpha}$-stimulated OPG release caused by tramadol or morphine (Figure 6A) and also reduced the morphine-caused amplification of the OPG release stimulated by PGF$_{2\alpha}$ (Figure 6B).

4. Discussion

We demonstrated in the present study that tramadol clearly enhanced the OPG release induced by PGF$_{2\alpha}$ in osteoblast-like MC3T3-E1 cells. We also showed that the PGF$_{2\alpha}$-induced expression of OPG mRNA was markedly amplified by tramadol. The amplifying effect of tramadol on the PGF$_{2\alpha}$-stimulated OPG release likely appears at the transcriptional level. Tramadol is known to be a weak MOR agonist that exerts an analgesic effect on the central nervous system (Subedi et al., 2019). Using morphine (Bravo et al., 2017) and naloxone (Sadée et al., 2005), we
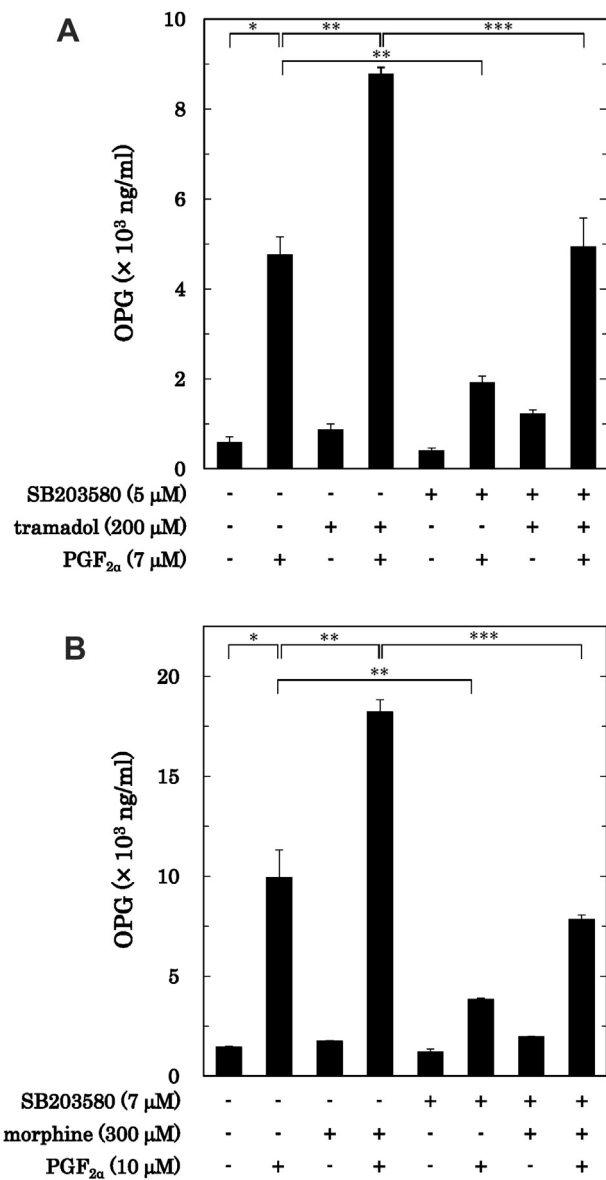
Table 1. Effect of fasudil on the PGF$_{2\alpha}$-stimulated OPG release from MC3T3-E1 cells.

Fasudil	PGF$_{2\alpha}$	OPG (pg/ml)
−	−	1089 ± 31
−	+	4368 ± 188*
+	−	1072 ± 75
+	+	2356 ± 64**

The cells pretreated with 10 μM fasudil or vehicle for 60 min were stimulated by 10 μM PGF$_{2\alpha}$ or vehicle for 48 h. The concentrations of OPG in the conditioned medium were determined. Each value represents the mean ± S.E.M. of three times determinations from three of independently prepared cells. *p < 0.05, compared to the control value. **p < 0.05, compared to the PGF$_{2\alpha}$ alone value.
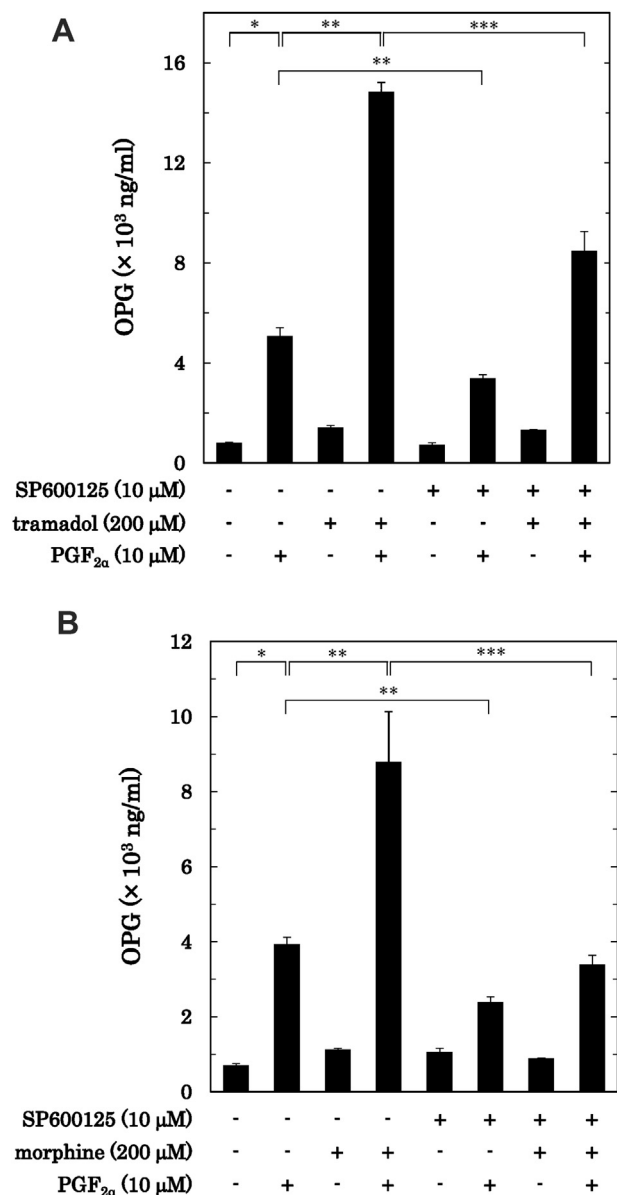
investigated whether or not the enhancing effect of tramadol on the PGF$_{2\alpha}$-stimulated OPG release was truly mediated through MOR in these cells. We found that morphine also enhanced the OPG release, whereas naloxone suppressed the amplifying effect by tramadol or morphine of the PGF$_{2\alpha}$-stimulated OPG release in these cells. Therefore, tramadol likely stimulates PGF$_{2\alpha}$-stimulated OPG synthesis through MOR, at least partially, in osteoblast-like MC3T3-E1 cells.

We next explored the mechanism of tramadol-amplifying effect on the synthesis of OPG stimulated by PGF$_{2\alpha}$ in MC3T3-E1 osteoblast-like cells. With regard to the intracellular signal transduction pathway of PGF$_{2\alpha}$, we previously reported that PGF$_{2\alpha}$ elicits the synthesis of OPG via the activation of p44/p42 MAPK, p38 MAPK and SAPK/JNK in these cells (Kuroyanagi et al., 2014). We found here that tramadol clearly amplified the PGF$_{2\alpha}$-upregulated phosphorylation of both p38 MAPK and SAPK/JNK, but had little effect on the p44/p42 MAPK phosphorylation in these cells. Thus, the enhancement of PGF$_{2\alpha}$-stimulated OPG synthesis caused by tramadol is probably mediated by the upregulation of the activities of p38 MAP kinase and SAPK/JNK, but not p44/p42 MAP kinase, in osteoblast-like MC3T3-E1 cells. Based on our previous findings that the IL-6 synthesis stimulated by PGF$_{2\alpha}$ is positively regulated by Rho-kinase upstream of p38 MAPK (Kuroyanagi et al., 2014) (Minamitani et al., 2008), we investigated the effects of fasudil (Shimokawa and Rashid, 2007) on the PGF$_{2\alpha}$-induced OPG release and SAPK/JNK phosphorylation in these cells, and found that tramadol truly suppressed both the release of OPG and the phosphorylation of SAPK/JNK stimulated by PGF$_{2\alpha}$. Rho-kinase probably positively regulates the synthesis of OPG stimulated by PGF$_{2\alpha}$ upstream of both p38 MAPK and SAPK/JNK in osteoblast-like MC3T3-E1 cells. We therefore examined the effect of tramadol on the PGF$_{2\alpha}$-induced phosphorylation of MYPT, a substrate of Rho-kinase (Shimokawa and Rashid, 2007) (Fukata et al., 2001), and found that the phosphorylation was not affected by tramadol in these

A

SB203580 (5 μM)	-	-	-	-	+	+	+	+
tramadol (200 μM)	-	-	+	+	-	-	+	+
PGF$_{2\alpha}$ (7 μM)	-	+	-	+	-	+	-	+

A

SP600125 (10 μM)	-	-	-	-	+	+	+	+
tramadol (200 μM)	-	-	+	+	-	-	+	+
PGF$_{2\alpha}$ (10 μM)	-	+	-	+	-	+	-	+

B

SB203580 (7 μM)	-	-	-	-	+	+	+	+
morphine (300 μM)	-	-	+	+	-	-	+	+
PGF$_{2\alpha}$ (10 μM)	-	+	-	+	-	+	-	+

B

SP600125 (10 μM)	-	-	-	-	+	+	+	+
morphine (200 μM)	-	-	+	+	-	-	+	+
PGF$_{2\alpha}$ (10 μM)	-	+	-	+	-	+	-	+

Figure 5. Effects of SB203580 on the amplification by tramadol or morphine of the PGF$_{2\alpha}$-stimulated OPG release in MC3T3-E1 cells. The cells pre-incubated with (A) 5 μM SB203580 or vehicle (B) 7 μM SB203580 or vehicle for 60 min were pretreated with (A) 200 μM tramadol or vehicle (B) 300 μM morphine or vehicle for a further 60 min. The treated cells were subsequently stimulated by 10 μM PGF$_{2\alpha}$ or vehicle for 48 h. The concentrations of OPG in the conditioned medium were determined. Each value represents the mean ± S.E.M. of three-fold determinations from three independently prepared cells. *$p < 0.05$ compared to the control value. **$p < 0.05$ compared to the PGF$_{2\alpha}$ value alone. ***$p < 0.05$ compared to the value of the combination of (A) tramadol and PGF$_{2\alpha}$ or (B) morphine and PGF$_{2\alpha}$.

Figure 6. Effects of SP600125 on the amplification by tramadol and morphine of the PGF$_{2\alpha}$-stimulated OPG release in MC3T3-E1 cells. The cells pre-incubated with 10 μM SP600125 or vehicle for 60 min were pretreated with (A) 200 μM tramadol or vehicle or (B) 200 μM morphine or vehicle for a further 60 min. The treated cells were subsequently stimulated by 10 μM PGF$_{2\alpha}$ or vehicle for 48 h. The OPG concentrations in the conditioned medium were determined. Each value represents the mean ± S.E.M. of three-fold determinations from three independently prepared cells. *$p < 0.05$ compared to the control value. **$p < 0.05$ compared to the PGF$_{2\alpha}$ alone value. ***$p < 0.05$ compared to the value of the combination of (A) tramadol and PGF$_{2\alpha}$ or (B) morphine and PGF$_{2\alpha}$.

cells. The PGF$_{2\alpha}$-activated Rho-kinase seems not to be modulated by tramadol in these cells. Therefore, the amplifying effect of tramadol on the PGF$_{2\alpha}$-induced OPG synthesis is likely to be exerted downstream of Rho-kinase in these cells. Tramadol appears to enhance the OPG synthesis induced by PGF$_{2\alpha}$, and the effect is probably exerted upstream of p38 MAPK and SAPK/JNK but downstream of Rho-kinase in osteoblast-like MC3T3-E1 cells. Furthermore, we showed in these cells that SB203580 (Cuenda et al., 1995) and SP600125 (Bennett et al., 2001) actually reduced the amplification of the PGF$_{2\alpha}$-stimulated OPG release caused by tramadol or morphine. Taking our present findings in osteoblast-like MC3T3-E1 cells into account as a whole, it is most likely that tramadol enhances the PGF$_{2\alpha}$-stimulated OPG synthesis through

MOR, an effect that is exerted downstream of Rho-kinase and upstream of p38 MAP kinase and SAPK/JNK. The supposed mechanism underlying the tramadol-amplifying effect on OPG synthesis in osteoblasts stimulated by PGF$_{2\alpha}$ is schematically summarized in Figure 7.

Low back pain is often observed in postmenopausal women suffering from osteoporosis without evident vertebral fracture, which is called osteoporotic pain (Deyo and Tsui-Wu, 1987) (Suzuki et al., 2013) (Ohtori et al., 2010). Although the mechanism and origin of such pain is still unknown, the proposed mechanism underlying such bone pain is related to the RANKL-OPG pathway and increased osteoclast activity (Vellucci et al., 2018). In fact, low serum levels of OPG are commonly observed in
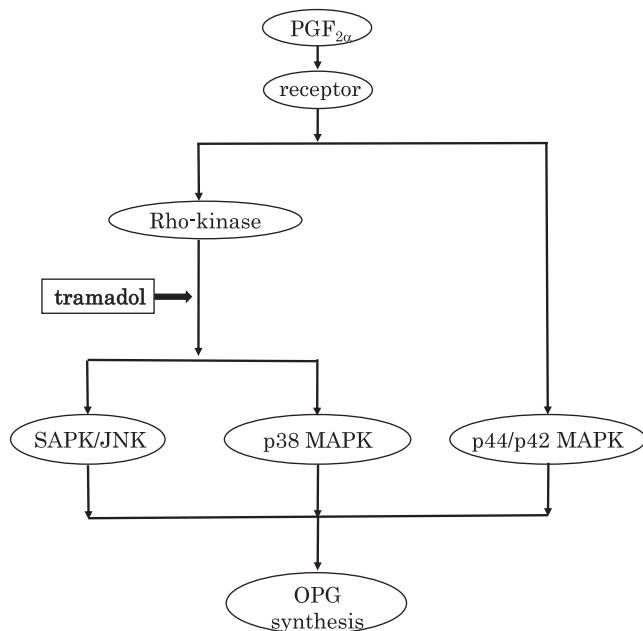
Figure 7. A schematic illustration of the regulatory mechanism of tramadol in PGF2α-stimulated in osteoblasts.

patients with multiple myeloma suffering from severe bone pain (Vellucci et al., 2018). In addition, a significant association was recently reported between the serum levels of OPG and the severity of radiographic space narrowing in subjects with low back pain (Goode et al., 2020). Thus, the upregulation of OPG synthesis might be deeply associated with the amelioration of low back pain.

Taking into account the enhancing effect of tramadol on the OPG synthesis by osteoblasts shown here, tramadol may be able to reduce bone pain through the upregulation of OPG levels in addition to its analgesic action in the central nervous system (Bravo et al., 2017). This is tramadol's hypothesized mechanism of action beyond the scope of the present results using osteoblasts, as peripheral and central changes related to pain may lead to different drug effects among healthy, acute and chronic pain subjects.

$PGF_{2\alpha}$, classically known to promote bone resorption, is currently recognized to act as a potent bone remodeling mediator and play pivotal roles in the maintenance of proper bone remodeling (Agas et al., 2013). OPG, as a decoy receptor of the RANKL, is known to block the interaction of RANK-RANKL required for the activation in addition to the differentiation of osteoclasts (Bucay et al., 1998). Thus, the enhancement of $PGF_{2\alpha}$-stimulated OPG synthesis by osteoblasts can reduce bone resorption and adjust bone remodeling to achieve a positive balance between the quality and quantity of bone. Based on our present findings, tramadol may be an ideal anti-analgesic agent for osteoporosis patients, exerting a favorable effect on adjusting disordered bone metabolism in osteoporosis. Further investigations will be necessary to clarify the detailed mechanism underlying the effects of tramadol on the functions of osteoblasts.

In conclusion, tramadol enhances the OPG synthesis stimulated by $PGF_{2\alpha}$ in osteoblasts, and this enhancing effect is mediated through MOR and the activation of p38 MAPK and SAPK/JNK. Tramadol may be a favorable anti-analgesic agent for use in treating osteoporosis patients, potentially reducing bone resorption by upregulating OPG synthesis by osteoblasts.

Declarations

Author contribution statement

H. Iida, H. Tokuda and O. Kozawa: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

W. Kim: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

K. Tanabe: Analyzed and interpreted the data; Wrote the paper.

R. Matsushima-Nishiwaki: Analyzed and interpreted the data.

K. Fujita, T. Kawabata, G. Sakai and T. Hioki: Performed the experiments; Analyzed and interpreted the data.

J. Tachi and D. Nakashima: Analyzed and interpreted the data.

S. Yamaguchi: Conceived and designed the experiments; Wrote the paper.

T. Otsuka: Conceived and designed the experiments.

Competing interest statement

The authors declare no conflict of interest.

Additional information

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References

Agas, D., Marchetti, L., Hurley, M.M., Sabbieti, M.G., 2013. Prostaglandin F2α: a bone remodeling mediator. J. Cell. Physiol.

Aghili, H., Yassaei, S., 2013. Effect of tramadol at different doses on orthodontic tooth movement and bone resorption in rats. Dent. Res. J. (Isfahan).

Bennett, B.L., Sasaki, D.T., Murray, B.W., O'Leary, E.C., Sakata, S.T., Xu, W., Leisten, J.C., Motiwala, A., Pierce, S., Satoh, Y., Bhagwat, S.S., Manning, A.M., Anderson, D.W., 2001. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc. Natl. Acad. Sci. U. S. A.

Boyce, B.F., Rosenberg, E., de Papp, A.E., Duong, L.T., 2012. The osteoclast, bone remodelling and treatment of metabolic bone disease. Eur. J. Clin. Invest.

Bravo, L., Mico, J.A., Berrocoso, E., 2017. Discovery and development of tramadol for the treatment of pain. Expet Opin. Drug Discov.

Bucay, N., Sarosi, I., Dunstan, C.R., Morony, S., Tarpley, J., Capparelli, C., Scully, S., Tan, H.L., Xu, W., Lacey, D.L., Boyle, W.J., Simonet, W.S., 1998. Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. Genes Dev.

Cuenda, A., Rouse, J., Doza, Y.N., Meier, R., Cohen, P., Gallagher, T.F., Young, P.R., Lee, J.C., 1995. SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. FEBS Lett.

Deyo, R.A., Tsui-Wu, Y.J., 1987. Descriptive epidemiology of low-back pain and its related medical care in the United States. Spine (Phila. Pa. 1976).

Fujita, K., Tokuda, H., Kuroyanagi, G., Yamamoto, N., Kainuma, S., Kawabata, T., Sakai, G., Matsushima-Nishiwaki, R., Kozawa, O., Otsuka, T., 2017. HSP90 inhibitors potentiate PGF2α-induced IL-6 synthesis via p38 MAP kinase in osteoblasts. PloS One.

Fukata, Y., Kaibuchi, K., Amano, M., Kaibuchi, K., 2001. Rho-Rho-kinase pathway in smooth muscle contraction and cytoskeletal reorganization of non-muscle cells. Trends Pharmacol. Sci.

Goode, A.P., Schwartz, T.A., Kraus, V.B., Huebner, J.L., George, S.Z., Cleveland, R.J., Gracely, R., Jimenez, M., DeFrate, L.E., Chen, J., Golightly, Y.M., Jordan, J.M., 2020. Inflammatory, structural, and pain biochemical biomarkers may reflect radiographic disc space narrowing: the johnston county osteoarthritis project. J. Orthop. Res.

Hikiji, H., Takato, T., Shimizu, T., Ishii, S., 2008. The roles of prostanoids, leukotrienes, and platelet-activating factor in bone metabolism and disease. Prog. Lipid Res.

Ito, M., Nakano, T., Erdodi, F., Hartshorne, D.J., 2004. Myosin phosphatase: structure, regulation and function. Mol. Cell. Biochem.

Karsenty, G., Wagner, E.F., 2002. Reaching a genetic and molecular understanding of skeletal development. Dev. Cell.

Kato, K., Ito, H., Hasegawa, K., Inaguma, Y., Kozawa, O., Asano, T., 2002. Modulation of the stress-induced synthesis of hsp27 and αb-crystallin by cyclic AMP in C6 rat glioma cells. J. Neurochem.

Kozawa, O., Suzuki, A., Tokuda, H., Uematsu, T., 1997. Prostaglandin F(2α) stimulates interleukin-6 synthesis via activation of PKC in osteoblast-like cells. Am. J. Physiol. Endocrinol. Metab.

Kuroyanagi, G., Tokuda, H., Matsushima-Nishiwaki, R., Kondo, A., Mizutani, J., Kozawa, O., Otsuka, T., 2014. Resveratrol suppresses prostaglandin F2α-induced osteoprotegerin synthesis in osteoblasts: inhibition of the MAP kinase signaling. Arch. Biochem. Biophys.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature.

Matziolis, G., Rau, H.M., Klever, P., Erli, H.J., Paar, O., 2002. Modification of human osteoblasts by various analgesics. Unfallchirurg.

Minamitani, C., Otsuka, T., Takai, S., Matsushima-Nishiwaki, R., Adachi, S., Hanai, Y., Mizutani, J., Tokuda, H., Kozawa, O., 2008. Involvement of Rho-kinase in prostaglandin F2α-stimulated interleukin-6 synthesis via p38 mitogen-activated protein kinase in osteoblasts. Mol. Cell. Endocrinol.

Ohtori, S., Akazawa, T., Murata, Y., Kinoshita, T., Yamashita, M., Nakagawa, K., Inoue, G., Nakamura, J., Orita, S., Ochiai, N., Kishida, S., Takaso, M., Eguchi, Y., Yamauchi, K., Suzuki, M., Aoki, Y., Takahashi, K., 2010. Risedronate decreases bone resorption and improves low back pain in postmenopausal osteoporosis patients without vertebral fractures. J. Clin. Neurosci.

Raisz, L.G., Alander, C.B., Fall, P.M., Simmons, H.A., 1990. Effects of prostaglandin f2α on bone formation and resorption in cultured neonatal mouse calvariae: role of prostaglandin e2 production. Endocrinology.

Sadée, W., Wang, D., Bilsky, E.J., 2005. Basal opioid receptor activity, neutral antagonists, and therapeutic opportunities. Life Sci.

Shimokawa, H., Rashid, M., 2007. Development of Rho-kinase inhibitors for cardiovascular medicine. Trends Pharmacol. Sci.

Simonet, W.S., Lacey, D.L., Dunstan, C.R., Kelley, M., Chang, M.S., Lüthy, R., Nguyen, H.Q., Wooden, S., Bennett, L., Boone, T., Shimamoto, G., DeRose, M., Elliott, R., Colombero, A., Tan, H.L., Trail, G., Sullivan, J., Davy, E., Bucay, N., Renshaw-Gegg, L., Hughes, T.M., Hill, D., Pattison, W., Campbell, P., Sander, S., Van, G., Tarpley, J., Derby, P., Lee, R., Boyle, W.J., 1997. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Cell.

Steeve, K.T., Marc, P., Sandrine, T., Dominique, H., Yannick, F., 2004. IL-6, RANKL, TNF-alpha/IL-1: interrelations in bone resorption pathophysiology. Cytokine Growth Factor Rev.

Subedi, M., Bajaj, S., Kumar, M.S., Yc, M., 2019. An overview of tramadol and its usage in pain management and future perspective. Biomed. Pharmacother.

Sudo, H., Kodama, H.A., Amagai, Y., Yamamoto, S., Kasai, S., 1983. In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J. Cell Biol.

Suzuki, M., Orita, S., Miyagi, M., Ishikawa, T., Kamoda, H., Eguchi, Y., Arai, G., Yamauchi, K., Sakuma, Y., Oikawa, Y., Kubota, G., Inage, K., Sainoh, T., Kawarai, Y., Yoshino, K., Ozawa, T., Aoki, Y., Toyone, T., Takahashi, K., Kawakami, M., Ohtori, S., Inoue, G., 2013. Vertebral compression exacerbates osteoporotic pain in an ovariectomy-induced osteoporosis rat model. Spine (Phila. Pa. 1976).

Vellucci, R., Terenzi, R., Kanis, J.A., Kress, H.G., Mediati, R.D., Reginster, J.Y., Rizzoli, R., Brandi, M.L., 2018. Understanding osteoporotic pain and its pharmacological treatment. Osteoporos. Int.