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 of 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). PGF2α 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 PGF2α 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 PGF2α-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 noradrenaline (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 (Matzolis et al., 2002) and reduction of the orthodontic tooth movement in rats (Aghili and Yassaee, 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 PGF2α in osteoblast-like MC3T3-E1 cells. We showed that tramadol enhances the PGF2α-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, naloxxone hydrochloride dihydrate, PGF2α 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 was obtained commercially. PGF2α 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 MgSO4, 1 mM CaCl2 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% CO2/95% air, were seeded into 35-mm diameter dishes (5 × 104 cells/dish) for an OPG assay and reverse transcription polymerase chain reaction (RT-PCR) or 90-mm diameter dishes (2 × 105 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 naloxxone; 0, 5 and 7 μM of SB203580; 0 and 10 μM of SP600125 for 60 min were stimulated with 10 μM of PGF2α 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 PGF2α or vehicle in α-MEM containing 0.3% FBS for 3 h. Using Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA) with Omniscript 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 PGF2α 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 ± standard error of the mean of triplicate determinations from three independent cell preparations.

3. Results

3.1. Effects of tramadol on the PGF2α-stimulated OPG synthesis in MC3T3-E1 cells

We previously showed that PGF2α stimulates OPG synthesis in osteoblast-like MC3T3-E1 cells (Kuroyanagi et al., 2014). Thus, we first examined the effect of tramadol on the PGF2α-stimulated OPG release. Tramadol, which by itself has little effect on OPG levels, significantly enhanced the PGF2α-stimulated OPG release in a dose-dependent manner between 30 and 200 \( \mu \text{M} \) (Figure 1A). To confirm whether or not the enhancing effects of tramadol on PGF2α-stimulated OPG release were mediated via transcriptional events, we next tested the effects of tramadol on the PGF2α-stimulated expression of OPG mRNA by real-time RT-PCR. We found that tramadol significantly enhanced the PGF2α-induced expression of OPG mRNA (Figure 1B).

3.2. Effects of morphine or naloxone on the PGF2α-stimulated OPG release in MC3T3-E1 cells

To explore whether or not the enhancing effects of tramadol on the PGF2α-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 PGF2α in osteoblast-like MC3T3-E1 cells. Similar to tramadol, morphine remarkably enhanced the OPG release in the stimulation with PGF2α (Figure 2). We next examined the effect of naloxone, an MOR antagonist (Sadde et al., 2005), on the amplification by tramadol or morphine of PGF2α-stimulated OPG release in these cells. Naloxone, which by itself did not influence the PGF2α-stimulated OPG release, significantly suppressed the tramadol-caused amplification of the PGF2α-stimulated OPG release (Figure 3A). Naloxone also markedly inhibited the amplification by morphine of the PGF2α-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 PGF2α-stimulated OPG release with or without naloxone to be similar in the different cell preparations. In addition, we used a dose of 200 \( \mu \text{M} \) of morphine, an intermediate dose between 100 and 300 \( \mu \text{M} \), which was adequate to clearly indicate the effect of naloxone on the amplification by morphine of the PGF2α-stimulated OPG release.

Figures 1 A and B show the results in osteoblast-like MC3T3-E1 cells. (A) The cells pretreated with 0, 10, 30, 100 and 200 \( \mu \text{M} \) of tramadol for 60 min were stimulated by 10 \( \mu \text{M} \) PGF2α 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-independent experiments. * \( p < 0.05 \), in comparison to the control value. (B) The cells pretreated with 300 \( \mu \text{M} \) tramadol or vehicle for 60 min were stimulated by 10 \( \mu \text{M} \) PGF2α 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 ± S.E.M. of three-independent determinations from three independently prepared cells. * \( p < 0.05 \), in comparison to the control value. ** \( p < 0.05 \) compared to the PGF2α value alone.

3.3. Effects of tramadol on the PGF2α-induced phosphorylation of p44/p42 MAPK, p38 MAPK, SAPK/JNK and MYPT in MC3T3-E1 cells

Regarding the signaling mechanism, we previously showed that PGF2α 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 PGF2α-induced phosphorylation of p44/p42 MAPK, p38 MAPK and SAPK/JNK in these cells. Tramadol did not affect the phosphorylation of p44/p42 MAPK.
with or without PGF2\textsubscript{a} stimulation (Figure 4A). However, while tramadol had little effect on the phosphorylation of p38 MAPK by itself, it significantly enhanced the PGF2\textsubscript{a}-stimulated phosphorylation of p38 MAPK dose-dependently in the range between 200 and 250 \(\mu\)M (Figure 4B). In addition, tramadol which alone hardly affected but markedly amplified the phosphorylation of SAPK/JNK stimulated by PGF2\textsubscript{a} dose-dependently in the range between 200 and 250 \(\mu\)M (Figure 4C).

We previously reported that Rho-kinase regulates the PGF2\textsubscript{a}-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 PGF2\textsubscript{a} in osteoblast-like MC3T3-E1 cells and confirmed that fasudil significantly suppressed the PGF2\textsubscript{a}-induced OPG release in osteoblast-like MC3T3-E1 cells (Table 1). In addition, we also confirmed that fasudil suppressed the PGF2\textsubscript{a}-stimulated phosphorylation of SAPK/JNK in these cells (data not shown). Therefore, it is probable that Rho-kinase is involved in the PGF2\textsubscript{a}-stimulated OPG release in osteoblast-like 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 PGF2\textsubscript{a} in osteoblast-like MC3T3-E1 cells. However, tramadol hardly affected the phosphorylation of MYPT with or without PGF2\textsubscript{a} (Figure 4D).

### 3.4. Effects of SB203580 on the amplification by tramadol or morphine of the PGF2\textsubscript{a}-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 PGF2\textsubscript{a}-stimulated OPG release in osteoblast-like MC3T3-E1 cells as previously reported (Kuroyanagi et al., 2014), SB203580 by itself had little effect on the OPG release but significantly suppressed the PGF2\textsubscript{a}-induced OPG release (Figure 5A, 5B). SB203580 markedly reduced the amplification by tramadol of the PGF2\textsubscript{a}-stimulated OPG release (Figure 5A) and also reduced the amplification by morphine of the PGF2\textsubscript{a}-stimulated OPG release, similar to tramadol (Figure 5B).

### 3.5. Effects of SP600125 on the amplification by tramadol or morphine of the PGF2\textsubscript{a}-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 PGF2\textsubscript{a} from osteoblast-like MC3T3-E1 cells. As previously reported (Kuroyanagi et al., 2014), SB203580 by itself had little effect on the OPG release but significantly suppressed the PGF2\textsubscript{a}-induced OPG release (Figure 5A, 5B). SB203580 markedly reduced the amplification by tramadol of the PGF2\textsubscript{a}-stimulated OPG release (Figure 5A) and also reduced the amplification by morphine of the PGF2\textsubscript{a}-stimulated OPG release, similar to tramadol (Figure 5B).
MC3T3-E1 cells. SP600125, which alone had little effect on OPG levels, significantly suppressed the PGF2α-induced OPG release as previously reported (Agas et al., 2013) (Figure 6A, 6B). SP600125 also markedly reduced the amplification of the PGF2α-stimulated OPG release caused by tramadol or morphine (Figure 6A) and also reduced the morphine-caused amplification of the OPG release stimulated by PGF2α (Figure 6B).

4. Discussion

We demonstrated in the present study that tramadol clearly enhanced the OPG release induced by PGF2α in osteoblast-like MC3T3-E1 cells. We also showed that the PGF2α-induced expression of OPG mRNA was markedly amplified by tramadol. The amplifying effect of tramadol on the PGF2α-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 (Sadé et al., 2005), we previously reported that PGF2α 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 amplifies the PGF2α-induced OPG release in osteoblast-like MC3T3-E1 cells. Therefore, tramadol likely stimulates PGF2α-induced OPG synthesis through MOR, at least partially, in osteoblast-like MC3T3-E1 cells.

With regard to the intracellular signal transduction pathway of PGF2α, we previously reported that PGF2α elicits the synthesis of OPG via the activation of p44/p42 MAPK, p38 MAPK and SAPK/JNK in osteoblast-like MC3T3-E1 cells. Kuroyanagi et al., 2014) We found here that tramadol clearly amplifies the PGF2α-upregulated phosphorylation of both p38 MAPK and SAPK/JNK, but had little effect on the p44/p42 MAPK phosphorylation in osteoblast-like MC3T3-E1 cells. Thus, the enhancement of PGF2α-stimulated OPG synthesis caused by tramadol is probably mediated by the upregulation of the activities of p38 MAPK and SAPK/JNK, but not p44/p42 MAPK kinase, in osteoblast-like MC3T3-E1 cells. Based on our previous findings that the IL-6 synthesis stimulated by PGF2α is positively regulated by Rho-kinase upstream of p38 MAPK (Kuroyanagi et al., 2014), we investigated whether or not the enhancing effect of tramadol on the PGF2α-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. We therefore examined the effect of tramadol on the PGF2α-induced OPG release in osteoblast-like MC3T3-E1 cells. W. Kim et al. Heliyon 6 (2020) e04779

| Table 1. Effect of fasudil on the PGF2α-stimulated OPG release from MC3T3-E1 cells. |
|---------------------------------|-----------------|-----------------|-----------------|
| Fasudil | PGF2α | OPG (pg/ml) | p44/p42 MAPK | p38 MAPK | SAPK/JNK | MYPT |
| - | - | 1089 ± 31 | - | - | - | - |
| + | + | 4368 ± 188** | + | + | + | + |
| **p < 0.05, compared to the control value. **p < 0.05, compared to the PGF2α alone value. |

The cells pretreated with 10 μM fasudil or vehicle for 60 min were stimulated by 10 μM PGF2α 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 independently prepared cells. Figure 4. Effects of tramadol on the PGF2α-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 PGF2α, 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 PGF2α-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 three fold determinations from three independently prepared cells. **p < 0.05, in comparison to the control value (Lane 1). *p < 0.05, in comparison to the PGF2α alone value (Lane 2). Full, non-adjusted images of Figure 4A, Figure 4B, Figure 4C and Figure 4D are provided as supplementary materials.
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 MAP kinase 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...
patients with multiple myeloma suffering from severe bone pain (Velucci 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 involving osteoblasts, as peripheral and central changes related to pain may lead to different drug effects among healthy, acute and chronic pain subjects.

PGF2α, 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 PGF2α-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 PGF2α 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.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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