Acetaminophen reduces osteoprotegerin synthesis stimulated by PGE$_2$ and PGF$_{2a}$ in osteoblasts: attenuation of SAPK/JNK but not p38 MAPK or p44/p42 MAPK

Woo Kim$^{1,2}$, Haruhiko Tokuda$^{2,3}$, Kumiko Tanabe$^1$, Shinobu Yamaguchi$^1$, Tomoyuki Hioki$^{1,4}$, Junko Tachi$^{1,2}$, Rie Matsushima-Nishiwaki$^2$, Osamu Kozawa$^2$, and Hiroki Iida$^1$

$^1$Department of Anesthesiology and Pain Medicine; $^2$Department of Pharmacology, Gifu University Graduate School of Medicine, Gifu 501-1194, Japan; $^3$Department of Clinical Laboratory/Medical Genome Center, National Center for Geriatrics and Gerontology, Obu 474-8511, Japan; and $^4$Department of Dermatology, Kizawa Memorial Hospital, Minokamo 505-0034, Japan

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ABSTRACT

Acetaminophen is one of the most widely used analgesic and antipyretic medicines, whose long-period use has reportedly been associated with an increased risk of bone fracture. However, the mechanism underlying this undesired effect remains to be investigated. The homeostatic control of bone tissue depends on the interaction between osteoblasts and osteoclasts. Osteoprotegerin produced by osteoblasts is known to play an essential role in suppressing osteoclast induction. We have previously reported that prostaglandin (PG) E$_2$ and PGF$_{2a}$ induce osteoprotegerin synthesis through p38 mitogen-activated protein kinase (MAPK), p44/p42 MAPK and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in osteoblast-like MC3T3-E1 cells. In the present study, we investigated the effects of acetaminophen on the osteoprotegerin synthesis induced by PGE$_2$ and PGF$_{2a}$ in MC3T3-E1 cells. Acetaminophen significantly suppressed the osteoprotegerin release stimulated by PGE$_2$ and PGF$_{2a}$. The PGE$_2$-induced expression of osteoprotegerin mRNA was also reduced by acetaminophen. Acetaminophen markedly downregulated the phosphorylation of SAPK/JNK stimulated by PGE$_2$ and PGF$_{2a}$ in MC3T3-E1 cells. Acetaminophen significantly suppressed the osteoprotegerin release stimulated by PGE$_2$ and PGF$_{2a}$. The PGE$_2$-induced expression of osteoprotegerin mRNA was also reduced by acetaminophen. Acetaminophen markedly downregulated the phosphorylation of SAPK/JNK stimulated by PGE$_2$ and PGF$_{2a}$, but not those of p38 MAPK or p44/p42 MAPK. SP600125, an inhibitor of SAPK/JNK, suppressed the levels of PGE$_2$- and PGF$_{2a}$-upregulated osteoprotegerin mRNA expression. Taken together, these results strongly suggest that acetaminophen reduces the PGE$_2$- and PGF$_{2a}$-stimulated synthesis of osteoprotegerin in osteoblasts, and that the suppressive effect is exerted via attenuation of SAPK/JNK. These findings provide a molecular basis for the possible effect of acetaminophen on bone tissue metabolism.

INTRODUCTION

Acetaminophen (N-acetyl-para-aminophenol, paracetamol) is currently the most utilized analgesic and antipyretic medicine which has been originally synthesized in 19$^{th}$ century (Ghanem et al. 2016). Although acetaminophen has been initially classified into a member of nonsteroidal anti-inflammatory drugs (NSAIDs), it was later proven to be ineffective as an anti-inflammatory agent (Brune et al. 2015; Ghanem et al. 2016). Acetaminophen easily crosses the blood brain barrier and is distributed homogenously into the central nervous system (Ghanem et al. 2016). It is currently recognized that the antipyretic effect of acetaminophen is exerted through the selective inhibition of the cyclooxygenase (COX) pathway in the central nervous system (Ghanem et al. 2016). Regarding the analgesic effect of acetaminophen, the direct action of a metabolite N-arachidonoyl-phenolamine to vanilloid system
and the indirect action to modulate the endogenous cannabinoid system are recently proposed as candidates of signaling mechanism (Ghanem et al. 2016). As for undesired effects of acetaminophen, it has been reported that acetaminophen use could be a high risk factor for bone fracture as well as the decline of bone mineral density (Williams et al. 2011). However, it remains to be investigated how acetaminophen affects bone tissue. Metabolic bone diseases like fragile bone as osteoporosis and fracture healing distress are caused by the imbalance of bone remodeling (Karsenty and Wagner 2002). The bone remodeling is strictly performed by two types of bone cells known as osteoclasts derived from myeloid stem cells and osteoblasts derived from mesenchymal stem cells, which are responsible for bone resorption and bone formation, respectively (Karsenty and Wagner 2002). Indeed, osteoblasts play a pivotal role not only in bone formation but also in the control of bone resorption through receptor activator of nuclear factor-κB (RANK) ligand (RANKL) expression in response to bone resorptive stimuli (Boyce et al. 2012). The binding of RANKL to RANK expressed on the surface of osteoclastic precursors and mature osteoclasts, evokes them to osteoclastogenesis, upregulating bone resorption (Boyce et al. 2012). On the other hand, osteoprotegerin which belongs to the tumor necrosis factor receptor family, is synthesized and secreted by osteoblasts, and binds RANKL as a decoy receptor to inhibit the binding of RANK-RANKL, leading to suppression of bone resorption (Simonet et al. 1997). In fact, osteoprotegerin knockout mice show severe osteoporosis (Bucay et al. 1998). Thus, it is currently recognized that osteoprotegerin produced by osteoblasts functions as an essential regulator in bone metabolism (Steeve et al. 2004).

Prostaglandins (PGs), lipid mediators catalyzed from membrane glycerophospholipid-derived arachidonic acid, are firmly established to act as autacoids and play crucial roles not only in bone resorption but also in bone formation associated with fracture healing (Hijiki et al. 2008). Among PGs, PGE$_2$ is known as a potent stimulator of osteoclastic bone resorption via modulating the functions of osteoblasts including RANKL up-regulation on which the specific receptors for PGE$_2$ named EP are expressed (Blackwell et al. 2010). We have previously reported that PGE$_2$ stimulates the synthesis of osteoprotegerin through the activation 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 (Yamamoto et al. 2014). On the other hand, the receptor for PGF$_{2\alpha}$ named FP is also expressed on osteoblasts (Hijiki et al. 2008), and PGF$_{2\alpha}$ acts as a potent bone remodeling mediator in bone metabolism through regulating a variety of signaling pathways (Agas et al. 2013). We have additionally demonstrated that PGF$_{2\alpha}$ induces the synthesis of osteoprotegerin in MC3T3-E1 cells, and three MAP kinases as well as PGE$_2$ positively regulate the synthesis (Kuroyanagi et al. 2014).

In the present study, we investigated the effect of acetaminophen on the synthesis of osteoprotegerin stimulated by PGE$_2$ and PGF$_{2\alpha}$ in osteoblast-like MC3T3-E1 cells. We showed here that acetaminophen reduced the PGE$_2$ and PGF$_{2\alpha}$-stimulated osteoprotegerin synthesis in MC3T3-E1 cells, and that the suppression by acetaminophen was due to the inhibition of SAPK/JNK.

MATERIALS AND METHODS

Materials. Acetaminophen, PGE$_2$ and PGF$_{2\alpha}$ were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Mouse osteoprotegerin ELISA kits were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). The antibodies against phospho-specific p38 MAPK, p38 MAPK, phospho-specific p44/p42 MAPK, p44/p42 MAPK, phospho-specific SAPK/JNK and SAPK/JNK were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). The antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 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). SP600125 was obtained from Calbiochem-Novabiochem Co. (La Jolla, CA, USA). PGE$_2$ and PGF$_{2\alpha}$ were dissolved in ethanol. The maximum concentration of ethanol was 0.1%, which did not affect the assay for osteoprotegerin or the detection of the protein level using Western blot analysis (Yamamoto et al. 2014).

Cell culture. Osteoblast-like MC3T3-E1 cells, a clonal cell line established from newborn mouse calvaria (Sudo et al. 1983), were maintained as previously described (Kozawa et al. 1997). Briefly, the cells cultured in α-minimum essential medium (α-MEM) containing 10% fetal bovine serum (FBS) at 37°C under humidified conditions of 5% CO$_2$/95% air were seeded into 35-mm diameter dishes ($5 \times 10^4$ cells/dish) for an osteoprotegerin assay and reverse
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transcription polymerase chain reaction (RT-PCR) or 90-mm diameter dishes (2 × 10^5 cells/dish) for Western blotting. The medium was switched to 0.3% FBS-containing α-MEM after 5 days, and the cells were then used for experiments 48 h later (Kozawa et al. 1997).

Assay for osteoprotegerin. The cells pretreated with acetaminophen in a variety of doses for 60 min were stimulated with 10 μM of PGE\(_2\), 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 osteoprotegerin in the medium were then determined with a mouse osteoprotegerin ELISA kit according to the manufacturer’s protocol (Kuroyanagi et al. 2014).

Real-time RT-PCR. The cultured cells were pretreated with 1,000 μM of acetaminophen, 10 μM of SP600125 or vehicle for 60 min, and then stimulated with 10 μM of PGE\(_2\), 10 μM of PGF\(_{2\alpha}\) or vehicle in α-MEM containing 0.3% FBS for 3 h. By the use of Omniscript Reverse Transcriptase kit (QIAGEN Inc., Valencia, CA, USA) with Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA), the isolated total RNA was transcribed into cDNA. Real-time RT-PCR using a Light Cycler system was performed in capillaries and Fast Start DNA Master SYBR Green I provided with the kit (Roche Diagnostics, Basel, Switzerland) (Kuroyanagi et al. 2014). Sense and antisense primers for mouse osteoprotegerin mRNA or GAPDH mRNA were purchased from Takara Bio Inc. (Tokyo, Japan). The amplified products were determined using a melting curve analysis. The osteoprotegerin mRNA levels were adjusted to those of GAPDH mRNA (Kuroyanagi et al. 2014).

Western blot analysis. The cells pretreated with various doses of acetaminophen for 60 min were stimulated by 10 μM of PGE\(_2\), 10 μM of PGF\(_{2\alpha}\) or vehicle in α-MEM containing 0.3% FBS for the indicated periods. The cells were subsequently washed twice with phosphate-buffered saline, and then lysed, homogenized and sonicated in a lysis buffer containing 62.5 mM Tris/HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol. SDS-polyacrylamide gel electrophoresis (PAGE) in 10% polyacrylamide gel was performed (Kuroyanagi et al. 2014) according to the Laemmli’s method (Laemmli 1970). The protein was fractionated and transferred onto an Immun-Blot polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Before incubation with primary antibodies, the membranes were blocked for 1 h with 5% fat-free dry milk in Tris-buffered saline-Tween (TBS-T; 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) for 1 h. Using the rabbit antibodies against phospho-specific p38 MAPK, p38 MAPK, phospho-specific p44/p42 MAPK, p44/p42 MAPK, phospho-specific SAPK/JNK, SAPK/JNK and GAPDH as primary antibodies, and peroxidase-labeled antibodies raised in goat against rabbit IgG as secondary antibodies, Western blot analysis was performed (Kato et al. 1996). The activity of peroxidase on the membrane was visualized on X-ray film with the ECL Western blotting detection system as previously described (Kuroyanagi et al. 2014).

Densitometric analysis. Densitometric analysis of the Western blots was performed using a scanner and image analysis software program (image J version 1.51; NIH, Bethesda, MD, USA) (Kuroyanagi et al. 2014). The phosphorylated protein levels were calculated as follows: the background-subtracted signal intensity of each phosphorylation signal of p38 MAPK, p44/p42 MAPK and SAPK/JNK was respectively normalized to the corresponding total protein signal. The levels were plotted as the fold increase in comparison to those of the control cells without stimulation.

Statistical analysis. The analysis of variance method for multiple comparisons between pairs was adopted, and \( P < 0.05 \) was considered to be statistically significant. All data are presented as the mean ± SEM of triplicate determinations from three independent cell preparations.

RESULTS

Effects of acetaminophen on the osteoprotegerin release stimulated by PGE\(_2\) or PGF\(_{2\alpha}\) in MC3T3-E1 cells

Based on our previous studies (Kuroyanagi et al. 2014; Yamamoto et al. 2014), we first examined the effect of acetaminophen on the PGE\(_2\)-stimulated osteoprotegerin release in osteoblast-like MC3T3-E1 cells. Acetaminophen, which alone had little effect on the osteoprotegerin levels, significantly suppressed the PGE\(_2\)-stimulated release of osteoprotegerin in a dose-dependent manner between 300 and 1,000 μM (Fig. 1A). We next examined whether or not acetaminophen affects the PGF\(_{2\alpha}\)-stimulated osteoprotegerin release in these cells. One thousand μM of acetaminophen, which by itself did not affect the basal levels of osteoprotegerin, markedly reduced the PGF\(_{2\alpha}\)-stimulated osteoprotegerin release (Fig. 1B).
Effects of acetaminophen on the expression levels of osteoprotegerin mRNA stimulated by PGE$_2$ in MC3T3-E1 cells
To elucidate whether or not the suppressive effect of acetaminophen on the PGE$_2$-stimulated release of osteoprotegerin is mediated through the transcriptional events, we examined the effects of acetaminophen on the PGE$_2$-induced expression of osteoprotegerin mRNA by real-time RT-PCR. Acetaminophen (1,000 μM), which alone failed to affect the levels of osteoprotegerin mRNA, significantly decreased the PGE$_2$-induced expression levels of osteoprotegerin mRNA (Fig. 2).

Effects of acetaminophen on the PGE$_2$-induced phosphorylation of p38 MAPK, p44/p42 MAPK and SAPK/JNK in MC3T3-E1 cells
With regard to the intracellular signaling in osteoblasts, we previously reported that PGE$_2$ stimulates osteoprotegerin synthesis through the activation of p38 MAPK, p44/p42 MAPK and SAPK/JNK in osteoblast-like MC3T3-E1 cells (Yamamoto et al. 2014). Thus, we examined the effects of acetaminophen on the PGE$_2$-induced phosphorylation of p38 MAPK, p44/p42 MAPK and SAPK/JNK in these cells. Acetaminophen hardly affected the PGE$_2$-induced phosphorylation of p38 MAPK (Fig. 3A) or p44/p42 MAPK (Fig. 3B). On the other hand, acetaminophen significantly suppressed the PGE$_2$-induced phosphorylation of SAPK/JNK (Fig. 3C).

Effects of acetaminophen on the PGF$_{2α}$-induced phosphorylation of p38 MAPK, p44/p42 MAPK and SAPK/JNK in MC3T3-E1 cells
We have shown that PGF$_{2α}$ as well as PGE$_2$ stimulates osteoprotegerin synthesis through p38 MAPK, p44/p42 MAPK and SAPK/JNK in osteoblast-like MC3T3-E1 cells (Kuroyanagi et al. 2014). Therefore, we examined the effects of acetaminophen on the PGF$_{2α}$-induced phosphorylation of p38 MAPK, p44/p42 MAPK and SAPK/JNK in MC3T3-E1 cells. Acetaminophen significantly attenuated the PGF$_{2α}$-stimulated phosphorylation of SAPK/JNK (Fig. 4C) without affecting the PGF$_{2α}$-induced phosphorylation of p38 MAPK (Fig. 4A) or p44/p42 MAPK (Fig. 4B).
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**Fig. 3** Effects of acetaminophen on the PGE$_2$-induced phosphorylation of p38 MAPK (A), p44/p42 MAPK (B) and SAPK/JNK (C) in MC3T3-E1 cells. The cells pretreated with 1,000 μM of acetaminophen or vehicle for 60 min were stimulated by 10 μM PGE$_2$ or vehicle for 3 min (A), 10 min (B) or 20 min (C). Western blot analysis was performed using antibodies against phospho-specific p38 MAPK or p38 MAPK (A), phospho-specific p44/p42 MAPK or p44/p42 MAPK (B), and phospho-specific SAPK/JNK or SAPK/JNK (C). The histogram shows the quantitative representation of the levels of PGE$_2$-induced phosphorylation obtained from a laser densitometric analysis. The density levels were expressed as the fold increase to the basal levels presented as lane 1. Each value represents the mean ± SEM of triplicate determinations from three independent cell preparations. *P < 0.05 compared to the value of the control cells without PGE$_2$-stimulation. **P < 0.05 compared to the value of PGE$_2$ alone. N.S. designates no significant difference between the indicated pairs. APAP, acetaminophen.

**Fig. 4** Effects of acetaminophen on the PGF$_{2\alpha}$-induced phosphorylation of p38 MAPK (A), p44/p42 MAPK (B) and SAPK/JNK (C) in MC3T3-E1 cells. The cells pretreated with 1,000 μM of acetaminophen or vehicle for 60 min were stimulated by 10 μM PGF$_{2\alpha}$ or vehicle for 3 min (A), 10 min (B) or 20 min (C). Western blot analysis was performed using antibodies against phospho-specific p38 MAPK or p38 MAPK (A), phospho-specific p44/p42 MAPK or p44/p42 MAPK (B), and phospho-specific SAPK/JNK or SAPK/JNK (C). The histogram shows the quantitative representation of the levels of PGF$_{2\alpha}$-induced phosphorylation obtained from a laser densitometric analysis. The density levels were expressed as the fold increase to the basal levels presented as lane 1. Each value represents the mean ± SEM of triplicate determinations from three independent cell preparations. *P < 0.05 compared to the value of the control cells without PGF$_{2\alpha}$-stimulation. **P < 0.05 compared to the value of PGF$_{2\alpha}$ alone. N.S. designates no significant difference between the indicated pairs. APAP, acetaminophen.

**Effects of SP600125 on the expression levels of osteoprotegerin mRNA stimulated by PGE$_2$ or PGF$_{2\alpha}$ in MC3T3-E1 cells**

To clarify the causal relation between SAPK/JNK inhibition and suppression of osteoprotegerin expression, we examined the effect of SP600125, an inhibitor of SAPK/JNK (Bennet et al. 2001), on the levels of osteoprotegerin mRNA expression stimulated by PGE$_2$ or PGF$_{2\alpha}$ in osteoblast-like MC3T3-E1 cells. SP600125 significantly suppressed the PGE$_2$- and PGF$_{2\alpha}$-upregulated levels of osteoprotegerin mRNA expression in these cells (Fig. 5).

**DISCUSSION**

In the present study, we first demonstrated that acetaminophen suppressed the osteoprotegerin release and the expression of osteoprotegerin mRNA stimulated by PGE$_2$ in osteoblast-like MC3T3-E1 cells. These findings suggest that the suppression by acet-
aminophen of PGE$_{2α}$-stimulated osteoprotegerin release is mediated through the gene transcriptional event. In addition, we presented here that acetaminophen inhibited the PGF$_{2α}$-stimulated osteoprotegerin release in MC3T3-E1 cells. Regarding as the analogy of PGE$_2$, the inhibition by acetaminophen of PGF$_{2α}$-stimulated osteoprotegerin release is probably mediated via a transcriptional event in these cells. It is well recognized that the specific receptors for both PGE$_2$ and PGF$_{2α}$, EP and FP, respectively, are expressed on cultured osteoblasts including MC3T3-E1 cells, and transduce their signaling separately to the intracellular effectors (Hijiki et al. 2008). Therefore, it is most likely that the suppression by acetaminophen in osteoprotegerin synthesis is exerted at a point downstream of EP and FP in osteoblast-like MC3T3-E1 cells.

We have previously reported that both PGE$_2$ and PGF$_{2α}$ induce the osteoprotegerin synthesis commonly through the activation of p38 MAPK, SAPK/JNK and p44/p42 MAPK activation in osteoblast-like MC3T3-E1 cells (Kuroyanagi et al. 2014; Yamamoto et al. 2014). We found here that acetaminophen markedly suppressed the PGE$_2$-induced phosphorylation of SAPK/JNK without affecting the phosphorylation of either p38 MAPK or p44/p42 MAPK. Therefore, it is probable that the suppressive effect of acetaminophen on the PGE$_2$-stimulated osteoprotegerin synthesis could be resulting from the decrease of SAPK/JNK activation in osteoblast-like MC3T3-E1 cells. We also showed that the PGF$_{2α}$-induced phosphorylation of SAPK/JNK but not p38 MAPK nor p44/p42 MAPK was inhibited by acetaminophen in these cells. It seems that the inhibition by acetaminophen of the PGF$_{2α}$-stimulated osteoprotegerin synthesis could be exerted via the diminishment of SAPK/JNK activity in MC3T3-E1 cells, in a fashion similar to PGE$_2$-stimulation. We confirmed that the inhibitor of SAPK/JNK SP600125 (Bennewt et al. 2001) truly suppressed the levels of osteoprotegerin mRNA upregulated by PGE$_2$ and PGF$_{2α}$, suggesting that the inhibition of SAPK/JNK is closely related to the suppression of osteoprotegerin expression in these cells. Taking our present findings of all into account, it is most likely that acetaminophen suppresses the synthesis of osteoprotegerin stimulated by PGE$_2$ and PGF$_{2α}$ in osteoblast-like MC3T3-E1 cells, and the effect of acetaminophen is commonly exerted through the inhibition of not p38 MAPK or p44/p42 MAPK but SAPK/JNK activity. The supposed mechanism of the acetaminophen-suppressing effect on the osteoprotegerin synthesis stimulated by PGE$_2$ and PGF$_{2α}$ in osteoblasts is schematically summarized in Fig. 6.

As for the acetaminophen effect on SAPK/JNK signaling, hepatotoxicity of this agent is considered to be due to the upregulation of SAPK/JNK phosphorylation caused by the upregulation of apoptosis signal-regulating kinase 1 (ASK1), which is interacted with receptor interacting protein kinase-1 and the cytoprotective protein A20 in mouse hepatocyte (Iorga et al. 2021). Although the effect of acetaminophen exerted on SAPK/JNK is opposite, it is possible that the suppressive effect of acetaminophen on the PGE$_2$ or PGF$_{2α}$-stimulated SAPK/JNK in osteoblasts shown here is caused by the downregulation of ASK1. In addition, our findings that the reduction of SAPK/JNK without p38 MAPK or p44/p42 MAPK elicited the remarkable suppression of osteoprotegerin synthesis might indicate the importance of SAPK/JNK axis in the osteoblast function. Regarding the effects of analgesics on osteoblasts with interest, we have recently reported that tramadol, a weak μ-opioid receptor agonist with an inhibitory effect on the reuptake of serotonin and norepinephrine (Bravo et al. 2017; Subedi et al. 2019), enhances PGF$_{2α}$-stimulated osteoprotegerin synthesis due to the amplification of SAPK/JNK and p38 MAPK ac-

![Fig. 5](image-url) Fig. 5 Effects of SP600125 on the expression levels of osteoprotegerin mRNA stimulated by PGE$_2$ or PGF$_{2α}$ in MC3T3-E1 cells. The cells pretreated with 10 μM SP600125 or vehicle for 60 min were stimulated by 10 μM PGE$_2$, 10 μM PGF$_{2α}$ or vehicle for 3 h. The total RNA was then isolated in each, and then transcribed into cDNA. The expression of osteoprotegerin mRNA and that of GAPDH mRNA were quantified by real time RT-PCR. The osteoprotegerin mRNA levels were normalized to GAPDH mRNA levels. Each value represents the mean ± SEM of triplicate determinations. *P < 0.05 compared to the control without stimulation. **P < 0.05 compared to the value of the stimulator alone. OPG, osteoprotegerin.
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![Diagram](attachment:Fig_6.png)

**Fig. 6** A schematic illustration of the suppression by acetaminophen of PGE$_2$ and PGF$_{2\alpha}$-stimulated osteoprotegerin synthesis in osteoblasts. PGE$_{2\alpha}$, prostaglandin E$_2$; PGF$_{2\alpha}$, prostaglandin F$_{2\alpha}$; EP receptor, receptor for prostaglandin E$_2$; FP receptor, receptor for prostaglandin F$_{2\alpha}$; SAPK/JNK, stress-activating protein kinase/c-Jun N-terminal kinase; p38 MAPK, p38 mitogen-activating protein kinase; p44/p42 MAPK, p44/p42 mitogen-activating protein kinase; OPG, osteoprotegerin.

Activities in osteoblast-like MC3T3-E1 cells (Kim et al. 2020). Thus, the effects of analgesic agents on the osteoprotegerin synthesis in osteoblasts might be quite different in each other. Further investigations are necessary to clarify the exact mechanism of analgesics in osteoblasts.

Regarding the mechanism of acetaminophen as an analgesic agent, it is generally recognized that acetaminophen possesses a weak inhibitory effect on COXs (Ghanem et al. 2016). In addition, it has been reported that an indirect action of endogenous cannabinoid system via cannabinoid receptor CB1 and CB2 and a direct action of the metabolite to vanilloid system via transient vanilloid 1 (TRPV1) and TRP ankyrin 1 (TRPA1) are involved in the effects of acetaminophen (Ghanem et al. 2016). It is currently recognized that CB1 and TRPV1 stimulation is osteoclastogenic in bone metabolism, whereas CB2 stimulation is anti-osteoclastogenic (Rossi et al. 2019). As for the effects of acetaminophen on osteoblasts, it has been shown that acetaminophen inhibits the differentiation in the relationship with the COX inhibition but promotes the migration of osteoblast-like MC3T3-E1 cells independently of COX, TRPV1, TRPA1 or CB1 (Nakatsu et al. 2018). TRPV1 stimulation reportedly reduces osteoprotegerin and inhibits calcium deposition by osteoblasts (Rossi et al. 2019). On the other hand, it has been shown that CB2 stimulation ameliorates the suppression of osteoprotegerin/RANKL ratio induced by titanium particles in MC3T3-E1 cells (Qui et al. 2015). In this study, we demonstrated that acetaminophen inhibited the osteoprotegerin synthesis by PGE$_2$ or PGF$_{2\alpha}$ through the suppression of SAPK/JNK in MC3T3-E1 cells. Although the rapid effect of acetaminophen on the phosphorylation of SAPK/JNK is considered to be exerted directly by itself but not by the metabolite, it is possible that the inhibition by acetaminophen of the osteoprotegerin synthesis stimulated by PGE$_2$ or PGF$_{2\alpha}$ might be caused by the metabolite-mediated activation of vanilloid system.

It is firmly established that osteoprotegerin, as a decoy receptor of the RANKL, blocks the binding of RANK and RANKL, which is the crucial step for osteoclastogenesis and osteoclastic bone resorption, leading to the suppression of bone resorption (Simonet et al. 1997). In addition, PGE$_2$ and PGF$_{2\alpha}$ act as the modulators of bone remodeling in the process of up-regulating bone turnover such as fracture healing (Hijiki et al. 2008). Based on our present findings in osteoblast-like MC3T3-E1 cells that acetaminophen inhibited the osteoprotegerin synthesis stimulated by PGE$_2$ and PGF$_{2\alpha}$, it seems likely that acetaminophen, an analgesic agent, might accelerate bone resorption and impaired bone remodeling. Therefore, it is possible that the novel mechanism of acetaminophen shown here is implicated in the high risk of the development of bone fracture and fragile bone observed in the population prescribed with the widely used analgesics (Warner et al. 1999; Williams et al. 2011; Wheatley et al. 2019). The clinically critical acetaminophen concentration of serum was considered to be ranged between 50 to 250 μg/mL (approximately 0.3 to 1.6 μM (Abuknesha et al. 2011). It has also been reported that high-dose rectal and oral acetaminophen administration (4,000 mg in total) caused 141.2 ± 52.1 μM in its maximum serum concentration (Hahn et al. 2000). Although the concentration of acetaminophen (1,000 μM in maximum) used in our experiments seems to be extremely high, it could be possible that the failure of local circulation in the skeletal tissue damaged by micro-injury might provoke the accumulation to the levels much higher than the serum levels in the pathophysiological condition by osteoporosis. Further investigation would be required to clarify the details behind acetaminophen-effects on bone metabolism.

Taken together, our findings strongly suggest that acetaminophen suppresses the osteoprotegerin synthesis stimulated by PGE$_2$ and PGF$_{2\alpha}$ in osteoblasts, and that the suppressive effect is exerted via inhibition of SAPK/JNK but not p38 MAPK or p44/p42 MAPK.
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CONFLICT OF INTEREST
The authors declare that they have no conflicts of interest.

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