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Original article

Effect of acetaminophen on osteoblastic differentiation and migration of MC3T3-E1 cells

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ABSTRACT

Background: *N*-acetyl-p-aminophenol (APAP, acetaminophen, paracetamol) is a widely used analgesic/antipyretic with weak inhibitory effects on cyclooxygenase (COX) compared to non-steroidal anti-inflammatory drugs (NSAIDs). The mechanism of action of APAP is mediated by its metabolite that activates transient receptor potential channels, including transient receptor potential vanilloid 1 (TRPV1) and TRP ankyrin 1 (TRPA1) or the cannabinoid receptor type 1 (CB1). However, the exact molecular mechanism and target underlying the cellular actions of APAP remain unclear. Therefore, we investigated the effect of APAP on osteoblastic differentiation and cell migration, with a particular focus on TRP channels and CB1.
Methods: Effects of APAP on osteoblastic differentiation and cell migration of MC3T3-E1, a mouse pre-osteoblast cell line, were assessed by the increase in alkaline phosphatase (ALP) activity, and both wound-healing and transwell-migration assays, respectively.
Results: APAP dose-dependently inhibited osteoblastic differentiation, which was well correlated with the effects on COX activity compared with other NSAIDs. In contrast, cell migration was promoted by APAP, and this effect was not correlated with COX inhibition. None of the agonists or antagonists of TRP channels and the CB receptor affected the APAP-induced cell migration, while the effect of APAP on cell migration was abolished by down-regulating TRPV4 gene expression.
Conclusion: APAP inhibited osteoblastic differentiation *via* COX inactivation while it promoted cell migration independently of previously known targets such as COX, TRPV1, TRPA1 channels, and CB receptors, but through the mechanism involving TRPV4. APAP may have still unidentified molecular targets that modify cellular functions.
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Introduction

Acetaminophen (*N*-acetyl-p-aminophenol [APAP], paracetamol) is one of the most common drugs used to treat pain and fever. APAP shows different characteristics from those of other analgesic non-steroidal anti-inflammatory drugs (NSAIDs), and owing to its poor inhibitory effect on cyclooxygenase (COX), it lacks anti-inflammatory effects and gastrointestinal side effects [1]. An impressive study by Högestätt et al. [2] showed the two-step metabolic transformation of APAP to N-acylphenolamine AM404, which required fatty acid amide hydrolase (FAAH) activity. This finding appeared credible because AM404 is already known to activate the transient receptor potential vanilloid 1 (TRPV1) channel and the cannabinoid receptor CB1, which are both involved in the regulation of pain and body temperature [3,4]. This observation suggests that TRPV1 and CB1 are candidate targets of APAP. The requirement of TRPV1 and CB1 for the analgesic/antipyretic effect of APAP was demonstrated in follow-up and independent studies, respectively [5,6]. Gentry et al. [7] recently updated the mechanism of action of APAP, which they attributed to TRP ankyrin 1 (TRPA1) but not TRPV1. However, the exact mechanism of the analgesic/antipyretic effect of APAP has not been elucidated and requires further investigation.

In addition to its effects on pain and fever, APAP is considered to have other actions. Several studies have been conducted to verify whether APAP affects bone remodeling analogously to NSAIDs,

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which are suspected to have adverse effects on bone healing [8,9]. This is based on the widely accepted evidence that COX activity and its product, prostaglandin (PG) E_2, are important modulators of bone remodeling [10]. Indeed, it has been suggested that NSAIDs interfere with the healing of bone fractures in *in vitro* and animal studies, although the effects would be minimal and limited to their long-term use [10]. In contrast, the effect of APAP on bone healing remains controversial because of its low inhibitory effect on COX enzymatic activity [8,9]. Díaz-Rodríguez et al. [11] examined the effect of APAP on osteoblasts using the human MG63 osteosarcoma cell line and showed that it suppressed both cell proliferation rate and synthesis of osteocalcin, a marker of osteoblastic differentiation. However, the mechanism of this action was not investigated.

In this study, we examined the effect of APAP on osteoblast differentiation and cell migration to clarify if the proposed candidate targets of APAP, particularly TRPV1, TRPA1, and CB1, mediate its cellular functions other than as an analgesic and antipyretic.

Materials and methods

This study was approved by and performed in accordance with guidelines of the institutional animal research ethics committee of the institution (approval number 16-013).

Chemicals

AM404, AMG9810, BCTC, AP18, capsaicin, GSK1016790A, ruthenium red, cinnamaldehyde, indomethacin were obtained from Wako Pure Chemical (Osaka, Japan). SR141716A and diclofenac were from Tocris Bioscience (Bristol, UK). Celecoxib was from Cayman Chemical (Ann Arbor, MI, USA). Fetal bovine serum (FBS) was from Life Technologies/Gibco (Carlsbad, CA, USA). L-ascorbic acid and β-glycerophosphate disodium salt hydrate were from Sigma-Aldrich (St.Louis, MO, USA). Acetaminophen (APAP) was from SHOWA YAKUHIN KAKO (Tokyo, Japan).

Cell culture

Mouse calvaria-derived cell line MC3T3-E1 was provided by the RIKEN BRC, Japan. Cells were cultured under humidified atmosphere of 5% CO_2 and 95% air at 37 °C in Minimum Essential Medium Alpha Modification (MEMα, Wako) supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (0.1 mg/mL).

Osteoblastic differentiation

The effect of APAP on the osteoblastic differentiation of MC3T3-E1 cells was evaluated by determining the increase in the enzymatic activity of alkaline phosphatase (ALP) associated with the cell.

The cells were seeded into a 24-well plate, and osteoblastic differentiation was induced by replacing the growth medium with differentiation medium that is the growth medium supplemented with 10 mM β-glycerophosphate and 50 μg/mL L-ascorbic acid. The differentiation medium was replaced every 2 days. The cells were cultured in the differentiation medium for 7 days in the presence or absence of APAP. Then, the cells were fixed with 4% paraformaldehyde, dehydrated with 1:1 ethanol: acetone, washed with phosphate-buffered saline, and then stained with the ALP substrate (Wako). Alternatively, the cells induced differentiation for 7 days were lysed in 0.1% Triton X-100 and subjected to measuring ALP activity using LabAssay kit (Wako). Protein concentration of the lysate was also measured using BCA Protein Assay (Wako) and used for normalizing the ALP activity.

Cell proliferation

Cell proliferation was assessed by a colorimetric tetrazolium salt-based assay. MC3T3-E1 cells were seeded at 5000 cells/well into a 96-well plate, then cultured for 3 days in the absence or presence of different concentrations of APAP (33–330 μM). Then 10 μL of Cell Counting Kit-8 (Dojindo) was added to each well and the plate was incubated at 37 °C in a CO_2 incubator for 3 h, followed by measuring the absorbance at 450 nm using a microplate reader (BioRad, Hercules, CA, USA).

Measurement of prostaglandin (PG) E_2 release

MC3T3-E1 cells cultured in a 24-well plate were washed twice with serum-free MEMα, followed by the incubation in the growth medium with or without various chemicals for 3 h. Then the conditioned media were collected and subjected to measurement of PGE_2 contents using enzyme-linked immunosorbent assay kit for PGE_2 (Cayman Chemical, Ann Arbor, MI, USA).

Wound-healing assay

The cells were seeded into a six-well plate the day before the assay, then the cells were pre-treated with APAP for 8 h before the scratches were created. The "wound" was created by scratching the cell monolayer using a pipette tip, and the images were captured at the beginning and 12 h later. The cell migration required to close the wound was analyzed using the National Institutes of Health (NIH) ImageJ software.

Transwell cell migration assay

The Falcon cell culture inserts with 8-μm pore membrane (Corning, Corning, NY, USA) were placed in the lower chamber of the 24-well plate containing 700 μL of MEMα supplemented with 10% FBS. The cells (1000 cells) were resuspended in 300 μL of serum-free MEMα and placed in the top chamber to allow them to migrate towards the underside of the membrane. After 3-h incubation at 37 °C, the cells were fixed with 4% paraformaldehyde, the non-migrated cells on the upper side of the chamber were removed using cotton swabs. Then the migrated cells that were attached to the bottom surface of the membrane were stained with 0.1% crystal violet, 0.1 M borate (pH 9.0), and 2% ethanol for 5 min at 25 °C. The number of migrated cells was counted in 5 randomly selected fields of the membrane by capturing the images using Olympus IX71 inverted microscope using the 20 × objective lens equipped with DP71 digital camera (Olympus, Tokyo, Japan). To down-regulate TRPV4 expression, cells were transfected with small interfering RNAs (siRNAs) for TRPV4 or the universal negative control siRNA (Mission Predesigned siRNA, Sigma-Aldrich) at 20 nM using ScreenFect siRNA (WAKO) and incubated for 24 h, then were subjected to the transwell cell migration assay.

Reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR analysis

Total RNA was extracted from monolayer of the cells or the mouse tissues with the use of a NucleoSpin RNA (Takara Bio, Shiga, Japan). The RNA concentration of each sample was determined by the absorbance at 260 nm. Isolated total RNA (100 ng) was subjected to reverse transcription with or without PrimeScript Reverse Transcriptase (Takara Bio), then PCR was performed using a GeneAtlas G Thermal Cycler (ASTEC, Fukuoka, Japan). Real-time PCR was also performed using THUNDERBIRD Probe qPCR Mix (TOYOBO, Osaka, Japan) and the 7300 Real-time PCR system (Applied Biosystems, Foster City, CA, USA) according to the manufacture's instructions,

with β-actin expression as an internal control. The primer sets used were as follows, alkaline phosphatase, 5′-GGGCGTCTCCACAG-TAAGCG-3′ and 5′-ACTCCCACTGTGCCCTCGTT-3′; osteocalcin, 5′-AAGCAGGAGGGCAATAAGGT-3′ and 5′-TTTGTAGGCGGTCTTCAAGC-3′, β-actin, 5′-GCCAACCGTGAAAAGATGACC-3′ and 5′-TGCCAATAGT-GATGACCTGGCC-3′; TRPV1, 5′-GGCTGTCTTCATCATCCTGTTA-3′ and 5′-GTTCTTGCTCTCTTGTGCAATC-3′; TRPV4, 5′-TTCTTCTCTCTAC-GACCTCTCC-3′ and 5′-ACAGTTCGTTAATGGGCTCTAC-3′; TRPA1, 5′-GGTCCAACATAACCGCATAGA-3′ and 5′-TGGGTATGAGACCAAGA-CAATAAG-3′; CB1, 5′-GTACTCTGGCATCAGGGTTATC-3′ and 5′-GTCTAGGACCCAGTCTTTGTTT-3′; CB2, 5′-GGATGCCGGGAGACA-GAAGTGA-3′ and 5′-CCCATGAGCGGCAGGTAAGAAAT-3′, COX1, 5′-CTTCTATGCTGGTGGACTATGG-3′ and 5′- GCTCCTGGAAAGAGGTG-TAAG-3′; COX2, 5′- CATTGACCAGAGCAGAGAGATG-3′ and 5′-GGCTTCCAGTATTGAGGAGAAC-3′, FAAH, 5′- TGAACGAGGGTGTGA-CATCG-3′ and 5′- TTCCACGGGTTCATGGTCTG-3′.

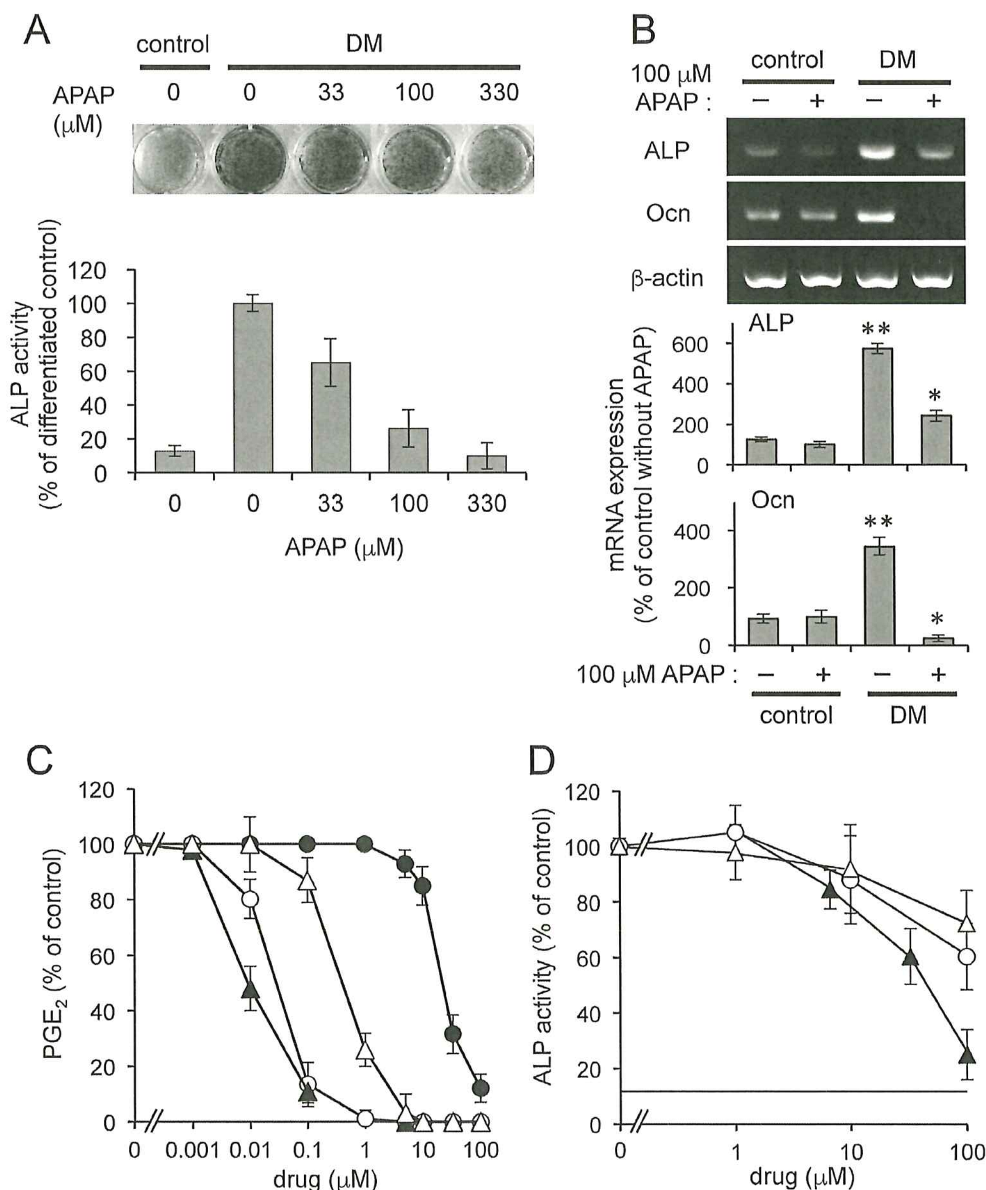

Fig. 1. Effect of *N*-acetyl-p-aminophenol (APAP) on osteoblastic differentiation of MC3T3-E1.
(A) MC3T3-E1 cells were continuously cultured in control or differentiation medium (DM) with or without indicated concentrations of APAP for 7 days. Typical image of alkaline phosphatase (ALP) staining of cells is presented in upper panel. ALP activities of cell lysates were normalized to protein concentrations and calculated as percentage of differentiated control, and then presented as mean ± standard deviation (SD) of four separate experiments (bottom graph). (B) Equal amounts of total RNA from MC3T3-E1 cells cultured in control or differentiation medium with or without 100 μM of APAP for 7 days were subjected to reverse transcription, followed by general PCR and real-time PCR analyses. Upper panel shows typical result of PCR for ALP, osteocalcin (Ocn) and β-actin, and similar results were obtained in three independent experiments. Bottom panels show the mRNA levels of ALP and Ocn analyzed using real-time PCR. The results are presented as the mean (%) ratio against the control without APAP ± SD of three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ *versus* vehicle. (C) MC3T3-E1 cells were incubated with fresh medium for 1 h in the presence or absence of indicated concentrations of APAP (closed circle), AM404 (open triangle), indomethacin (open circle), or diclofenac (closed circle), and then subjected to enzyme-linked immunosorbent assay to measure prostaglandin (PG) E_2 concentrations. Results are mean ± SD of three independent experiments expressed as percentage (%) ratio of control without any drug. (D) Osteoblastic differentiation of MC3T3-E1 was induced as in (A) in the presence or absence of the indicated concentrations of AM404 (open triangle), indomethacin (open circle), or diclofenac (closed circle) for 7 days. ALP activities associated with the cell lysates were normalized with protein concentrations. Results are mean ± SD of three independent experiments expressed as percentage (%) ratio of differentiated cells without any drug treatments. Horizontal line in the graph indicates basal ALP activity associated with undifferentiated MC3T3-E1 cells.

Statistical analysis

Unless indicated otherwise, data were analyzed with the unpaired two-tailed Student's t test and are presented as mean values ± standard deviation (SD).

Results

Effect of APAP on osteoblastic differentiation and COX activity of MC3T3-E1

We first examined the effect of APAP on the osteoblastic differentiation of MC3T3-E1 cells. Osteoblastic differentiation was induced by culturing the cells in the presence of β-glycerophosphate and ascorbic acid (differentiation medium) with or without APAP. After a 7-day incubation, the cells cultured in the differentiation medium were strongly stained positive for ALP activity while the control cells cultured in the growth medium were barely stained (Fig. 1A, left two wells). This observation was indicative of osteoblastic cell differentiation. The ALP-staining of the cells was dose-dependently inhibited by APAP (Fig. 1A, top panel), which was in agreement with the ALP activity observed in the cell lysates (Fig. 1A, bottom graph).

The inhibitory effect of APAP on osteoblastic differentiation was also confirmed by the expression levels of osteoblast marker genes. The expression of both alkaline phosphatase and osteocalcin increased in the differentiation-induced cells in the absence but not in the presence of APAP (Fig. 1B). These results indicate that

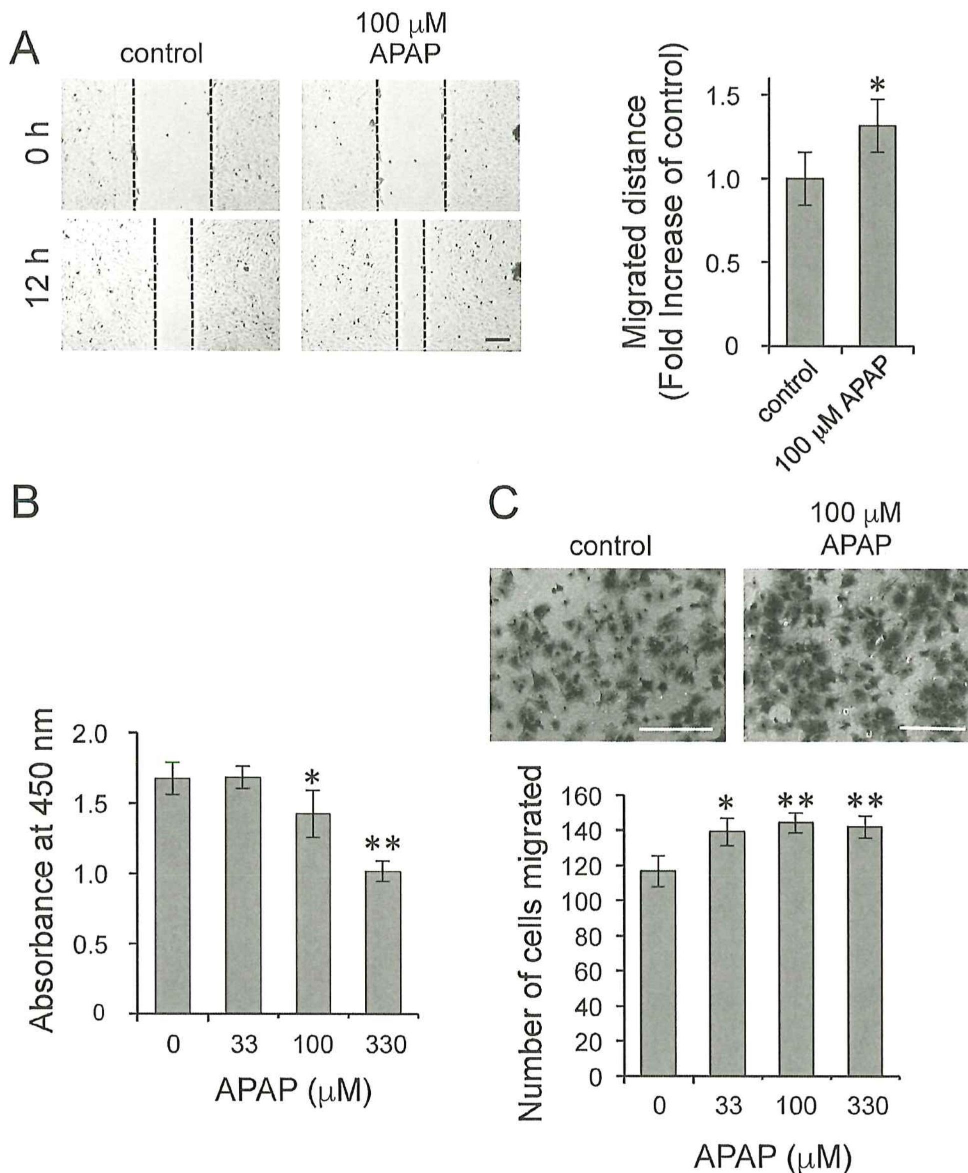

Fig. 2. Effect of N-acetyl-p-aminophenol (APAP) on migration of MC3T3-E1 cells.
(A) Typical images of cell monolayer from wound-healing assay with or without 100 μM of APAP at time 0 and 12 h are presented. Dashed lines indicate front edges of scratched line. Bar, 200 μm. Distances cells migrated are presented as fold increase compared to control and are mean ± standard deviation (SD, bar graph); $^{*}p < 0.05$ *versus* control (Student's *t*-test). (B) Number of live cells after culturing for 72 h with or without APAP was assessed using a colorimetric cell counting kit. Data are mean ± SD of triplicate results of three independent experiments; $^{*}p < 0.05$ and $^{**}p < 0.01$ *versus* control without APAP (Student's *t*-test). (C) Typical images of migrated cells on bottom surface of filter are presented. Bars, 200 μm Where indicated, APAP was added to both upper and lower compartment of well. Cell numbers per five randomly selected fields from every filter were counted and presented as mean ± SD of at least three independent experiments; $^{*}p < 0.05$ and $^{**}p < 0.01$ *versus* control without APAP (Student's *t*-test).

APAP had an inhibitory effect on the osteoblastic differentiation of MC3T3-E1. Although APAP is believed to have little inhibitory activity against COX [12], we assessed the COX-inhibiting activity of the APAP used in this study by measuring PGE_2 production in MC3T3-E1. The amount of PGE_2 released into the medium by the cells during the 3-h incubation in the presence or absence of APAP or NSAIDs was determined. APAP dose-dependently inhibited PGE_2 release from the cells at the concentration used in this study, although its half-maximal inhibitory concentration (IC_{50}) was almost 1000 times higher than those of the other NSAIDs, indomethacin, and diclofenac (IC_{50}, 20, 0.02, and 0.015 μM for APAP, indomethacin, and diclofenac, respectively).

AM404, a metabolite of APAP considered to activate TRP channels to exert antipyretic/analgesic effects, also inhibited PGE_2 release with a higher potency than that of APAP (Fig. 1C) as reported previously [2]. Because AM404 suppressed COX activity, inhibition of osteoblastic differentiation by APAP could be mediated through inhibiting COX. Thus, we next examined if other NSAIDs also suppress osteoblastic differentiation similar to APAP. Both indomethacin and diclofenac dose-dependently suppressed ALP activity in the differentiation-induced cells (Fig. 1D). Moreover, the order of the inhibition magnitude of NSAIDs and AM404/APAP on ALP activity was well correlated with that on PGE_2-release (Fig. 1C), suggesting that APAP inhibits osteoblastic differentiation through COX after being metabolized to AM404.

Effect of APAP on migration of MC3T3-E1 cells

Next, we investigated the effect of APAP on the migration of MC3T3-E1 using two different methods, namely, wound-healing and transwell migration assays. In the first assay, after the scratches were created on the confluent cell monolayers, cells were allowed to migrate for 12 h in the presence or absence of 100 μM APAP. The distance of migration was significantly increased by 1.3-fold in the presence of APAP (Fig. 2A). To verify that this effect of APAP on cell migration was not due to accelerated cell proliferation, we examined the effect of APAP on cell proliferation using a tetrazolium salt-based colorimetric assay. As shown in Fig. 2B, APAP did not increase, but rather, decreased the cell proliferation, confirming that the data shown in Fig. 2A was indeed the result of increased cell migration.

The effect of APAP on cell migration was further confirmed using the transwell migration assay. The cells suspended in serum-free medium were placed on the upper surface of the transwell filter, and medium containing 2% serum was added to the lower compartment, followed by a 3-h incubation to enable the cells to migrate to the bottom surface of the filter. The addition of APAP to both the upper and lower compartments of the transwell filter resulted in a 1.2-fold increase in the number of cells that migrated to the bottom surface of the membrane (Fig. 2C). The cell migratory period in this assay was only 3 h, which was short enough to exclude the effect of cell proliferation. These results clearly indicate that APAP promoted the migration of MC3T3-E1 cells.

Comparison of effects of APAP and NSAIDs on migration of MC3T3-E1 cells

The release of PGE_2 from the MC3T3-E1 was suppressed by APAP at the same concentrations used in the migration assay (Figs. 1C and 2). Therefore, we determined if cell migration was also promoted by inhibiting PGE_2 production using other NSAIDs. The results shown in Fig. 3 reveal that indomethacin promoted cell migration at a comparable level to APAP while diclofenac and celecoxib did not. Although the concentrations of the NSAIDs used were adequate to completely inhibit COX activity, their effects on cell migration varied. The result suggests that the effect of APAP on cell migration was not mediated by inhibiting COX activity, but likely through another molecular target.

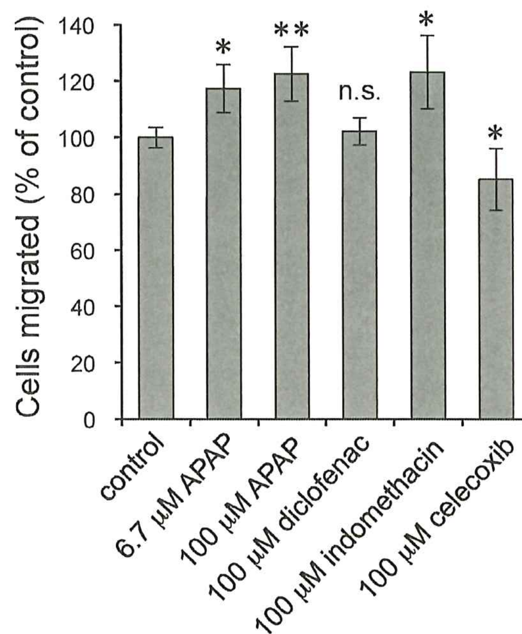

Fig. 3. Effect of *N*-acetyl-p-aminophenol (APAP), AM404, and non-steroidal anti-inflammatory drugs (NSAIDs) on migration of MC3T3-E1 cells.
Transwell migration assay was performed as in Fig. 2C in the presence or absence of indicated drugs. Cell numbers per five randomly selected fields from every filter were counted. Results are presented as mean ratio (%) against control ± standard deviation (SD) of at least three independent experiments; $^{*}p < 0.05$ and $^{**}p < 0.01$ *versus* control without drug (Student's *t*-test).

Effects of agonists and antagonists of previously known APAP targets on migration of MC3T3-E1 cells

There are several putative targets for APAP including TRPV1, TRPA1, and CB1 [2,5–7]. Therefore, we next examined the mRNA expressions of these target and other related molecules in undifferentiated MC3T3-E1. The mRNA expression of FAAH, the enzyme responsible for converting APAP to AM404, was detected at a similar level to that in the mouse brain (Fig. 4A, left bottom panels). The general enzyme targets of NSAIDs, COX-1 and −2, were both expressed in the cells (Fig. 4, right panels). However, the expression of TRPV1, TRPA1, and CB1 was not detectable in MC3T3-E1 cell. However, the expression of TRPV4, which has not been reported to be involved in the actions APAP, was strongly detected.

Although we did not detect the mRNA expression of the previously known targets of APAP in MC3T3-E1 cells, we pharmacologically determined if the effect of APAP on cell migration was mediated by these molecules. First, the direct activation of TRPV1 and TRPA1 by their respective agonist was tested to determine if these channels are involved in the regulation of MC3T3-E1 cell migration. As shown in Fig. 4B, neither capsaicin for TRPV1 nor cinnamaldehyde for TRPA1 affected the cell migration. Furthermore, AM404, an APAP metabolite that modulates TRPV1 and CB1, failed to promote cell migration while APAP accelerated cell migration. In contrast, a specific agonist for TRPV4, GSK1016790A, suppressed the basal migration of MC3T3-E1 cells without affecting the APAP-induced cell migration.

Next, we examined whether inhibitors of TRPV1, TRPA1, or CB1 impaired the APAP-induced cell migration. The TRPV1 antagonists,

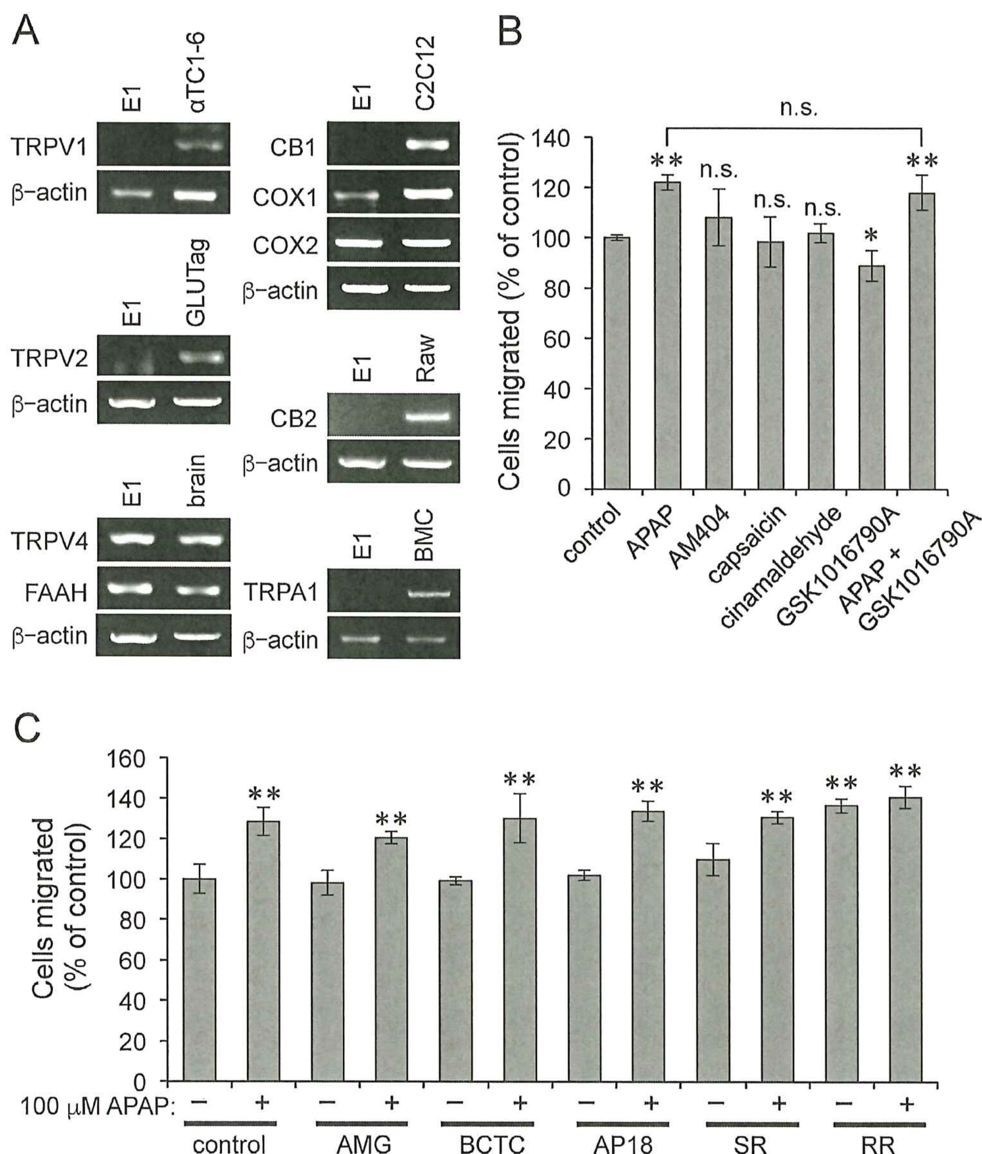

Fig. 4. Expression of putative *N*-acetyl-p-aminophenol (APAP) targets and related genes and effect of APAP and agonists/antagonists of transient receptor potential (TRP) channel or cannabinoid receptor type 1 (CB1) on migration of MC3T3-E1 cells.
(A) Typical images of reverse transcription-polymerase chain reaction (RT-PCR) products separated on agarose gels. Expression of mRNA of indicated molecules in MC3T3-E1 cells is compared with those of other cell lines or tissue used as positive controls. E1, MC3T3-E1; brain, mouse brain; Raw, Raw 264.7; BMM, mouse bone marrow cells. (B) Transwell migration assay was performed in the presence or absence of 100 μM of APAP, 100 μM AM404, 1 μM capsaicin, 30 μM cinnamaldehyde, and 100 nM GSK1016790A with or without 100 μM of APAP. Cell numbers per five randomly selected fields from every filter were counted. Results are presented as mean (%) ratio against control ± standard deviation (SD) of at least three independent experiments; $^{**}p < 0.01$ and n.s. (not significant) *versus* vehicle (Student's *t*-test). (C) Transwell migration assay was performed in the presence or absence of 100 μM APAP and indicated chemicals. Cell numbers per five randomly selected fields from every filter were counted. AMG, 20 μM AMG9810; BCTC, 0.5 μM BCTC; AP18, 10 μM AP18; SR, 10 μM SR141716A; RR, 10 μM ruthenium red. Results are mean (%) ratio against control ± SD of at least three independent experiments; $^{*}p < 0.05$ *versus* vehicle control. No significant differences occurred between cells treated with APAP alone and those treated with both APAP and indicated chemicals (Student's *t*-test). No significant differences occurred between untreated control cells and those treated with each chemical alone, except for ruthenium red alone.

AMG9810 and BCTC, as well as the TRPA1 and CB1 antagonists, AP18 and SR141716A, respectively, were applied either alone or in combination with APAP in the transwell migration assay. None of these antagonists inhibited the APAP-induced cell migration or affected the basal cell migration in the absence of APAP (Fig. 4C). The exception was ruthenium red that non-selectively blocks Ca^{2+} channels, including TRPV4, and robustly increased basal cell migration in this study without affecting the APAP-induced cell migration (Fig. 4C, right two bars).

Effects of down-regulation of TRPV4 on APAP-induced migration of MC3T3-E1 cells

The pharmacological approach failed to show the involvement of TRP channels and CB1 in the effect of APAP on the cell migration. However, as both agonist and antagonist of TRPV4 affected cell migration of MC3T3-E1 (Fig. 4), we further examined if TRPV4 was involved in APAP-induced cell migration by down-regulation of TRPV4 expression. Both of two different siRNAs for TRPV4

examined down-regulated mRNA expression of TRPV4 about 80% in MC3T3-E1 cells (Fig. 5A), thus transwell-migration assay was performed using the cells transfected with these siRNAs. Effect of APAP on cell migration was abolished in the cells treated with either of these siRNAs for TRPV4, but not with the control siRNA (Fig. 5B).

Discussion

In this study, we investigated the non-analgesic/antipyretic effect of APAP using a mouse pre-osteoblastic MC3T3-E1 cell line and found that APAP suppressed osteoblastic differentiation and, surprisingly, accelerated cell migration. The osteoblast differentiating effect of APAP was assumed based on numerous reports of the effect of long-term NSAID use on bone fracture healing [8,9], which were related to the pivotal role of COX activity in maintaining bone metabolism [13,14]. However, because APAP does not inhibit COX as strongly as other NSAIDs do [1], its effect on bone are still unclear [8,9,11,15]. In this study, we showed that AM404, a metabolite of APAP, suppressed COX activity and inhibited osteoblastic differentiation. The magnitude of the inhibitory effects of AM404, indomethacin, and diclofenac on osteoblastic differentiation were in the same order as their COX-inhibiting activities. Therefore, APAP likely inhibited osteoblastic differentiation by suppressing COX activity following its conversion to AM404. This notion was supported by our observation that the MC3T3-E1 cells expressed FAAH, which is required for metabolizing APAP to AM404 [2].

This is the first study to report that APAP promotes cell migration. This effect was confirmed using two different methods, which were the scratch wound healing and transwell cell migration assays. Although the cell proliferation rate could affect the result obtained from these methods, we excluded that the possibility and confirmed that cell migration and not proliferation was increased by APAP. First, in the transwell migration assay, the cells were allowed to migrate for only 3 h, which was short enough to preclude the effect of cell proliferation. Second, we observed that APAP did not accelerate, but rather, suppressed cell proliferation. This inhibitory effect of APAP on osteoblast proliferation was in agreement with the previous reports [11,15]. Therefore, the data obtained in this study showing the enhanced cell migration of MC3T3-E1 by APAP is reliable. On the other hand, given the therapeutic application of APAP-induced cell migration, inhibitory effect of APAP on cell proliferation would not be favorable. Thus, it should be clarified if APAP also inhibit proliferation of other type of cells including fibroblasts.

While APAP inhibited osteoblastic differentiation, it is unlikely that it promoted cell migration *via* COX activity for the following reasons. First, the effects of APAP and NSAIDs on cell migration were different, although the concentrations of all inhibitors used in the assay were high enough to completely block COX activity. Second, PGE_2 was reported to promote, but not inhibit, the migration of various cell types [16]. Collectively, these results indicate that COX is not a favorable target of APAP in promoting cell migration.

Therefore, we tried to clarify the molecular target mediating the APAP-induced migration of MC3T3-E1 cells, with a particular focus

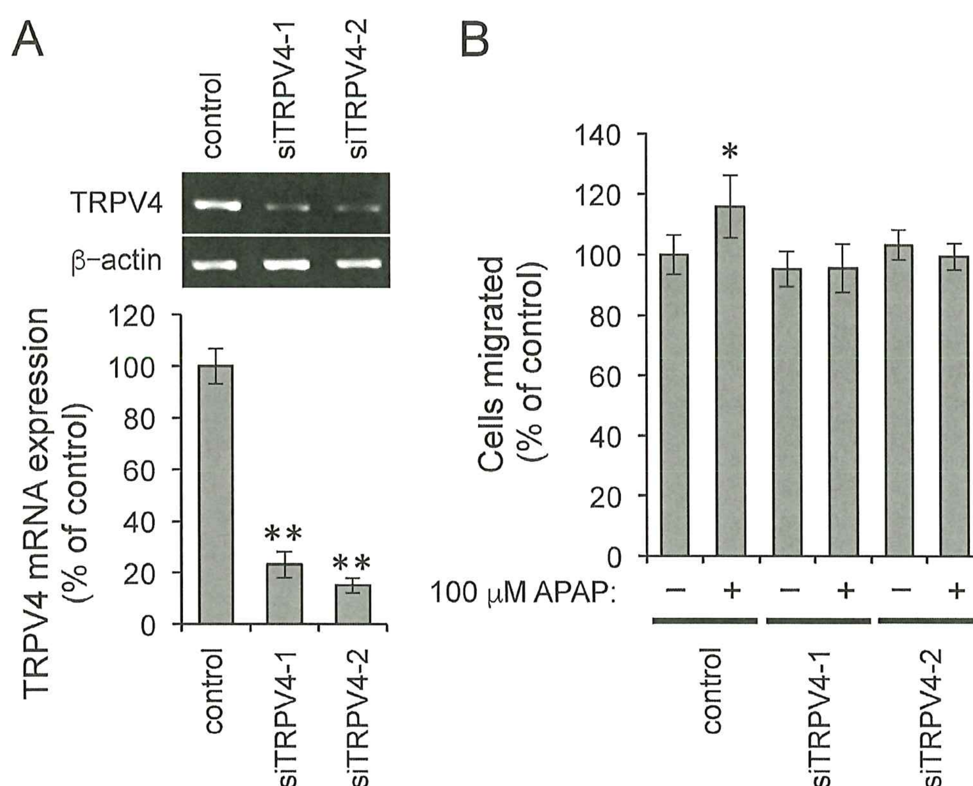

Fig. 5. Effects of down-regulation of TRPV4 on APAP-induced migration of MC3T3-E1 cells.
(A) MC3T3-E1 cells were transfected with control siRNA or two different siRNAs for TRPV4 (siTRPV4-1 and siTRPV4-2). Total RNA was isolated from the cells and subjected to reverse transcription followed by general PCR and real-time PCR analyses for TRPV4. Typical result of PCR is shown in the upper panel and mRNA levels of TRPV4 assessed by real-time PCR are normalized with β-actin gene and shown as bar graph. The results are presented as the mean (%) ratio against the control ± SD of three independent experiments; $^{**}p < 0.01$ *versus* control. (B) Transwell migration assay was performed and the results are presented as in Fig. 4B using the cells transfected with the negative control siRNA or siRNAs for TRPV4; $^{*}p < 0.05$ *versus* control without APAP (Student's *t*-test).

on the possible involvement of TRPV1, TRPA1, and CB1, which are all involved in the antipyretic and analgesic effects of APAP [7,12]. As an activator of these receptors, AM404, was detected in the rat brain 20 min after an intraperitoneal injection of labeled APAP [2], suggesting that the conversion occurred rapidly enough to affect cell migration when it was added to the culture medium. The involvement of TRPV1, TRPA1, and CB1 in cell migration has been reported in various cell types, although it is still controversial whether they upregulate or downregulate cell migration [17,18]. Thus, we pharmacologically tested the potential mediation of APAP-induced cell migration by these channels, although their mRNA expression levels were barely detected using RT-PCR analysis. However, all the antagonists for TRPV1, TRPA1, and CB1 failed to block APAP-induced cell migration. Furthermore, treatment of the cells with capsaicin or cinnamaldehyde to activate TRPV1 or TRPA1, respectively, in addition to AM404, did not show any effect on the cell migration, which agreed with the low expression levels of these channels.

Contrary to the previously known targets of APAP, TRPV4, which has a critical role in skeletal development and homeostasis, was strongly expressed in MC3T3-E1 cells [19–21]. The involvement of TRPV4 in cell migration has been previously reported in various studies such as that of Martin et al. [22], which showed that TRPV4 induced migratory responses in pulmonary arterial smooth muscle cells. In contrast, Zaninetti et al. [23] showed that TRPV4 negatively regulated migration of the immortalized neuroendocrine GN11 cell line. In this study, although the activation and inhibition of TRPV4 by the agonist GSK1016790A and the non-specific calcium channel blocker ruthenium red, respectively showed that TRPV4 is involved in the negative regulation of MC3T3-E1 cell migration, neither GSK1016790 nor ruthenium red did not affect the APAP-induced cell migration. However, down-regulation of TRPV4 using siRNA abolished the effect of APAP on cell migration, suggesting that TRPV4 is involved in the mechanism for APAP-induced cell migration. As APAP did not show any effect on TRPV4-mediated intracellular Ca^{2+} mobilization in MC3T3-E1 cells at the concentration used in this study (data not shown), it is currently unclear whether APAP directly or indirectly affects TRPV4.

Collectively, these results suggest that the effect of APAP on MC3T3-E1 cell migration is not mediated by previously reported targets of APAP such as COX, TRPV1, TRPA1, CB1, but is likely mediated *via* another unidentified target molecule including TRPV4. The concentration of APAP used in this study was in the therapeutic range [12] and, therefore, the observed effects in this study should be carefully considered during the clinical use of APAP. Furthermore, the newly identified effect of APAP on cell migration could be useful not only for the treatment of pain and fever but also for novel therapeutic applications such as acceleration of wound healing by accumulating to the bone fracture site. Therefore, further studies to clarify the effect of APAP on TRPV4 and other target molecules mediating the APAP-induced cell migration are required.

Conflicts of interest

The authors have no potential conflict of interest to declare.

Author contributions

Study conception and design: Y.N., S.W. and H.T.
Acquisition of data: Y.N., F.N., S.H. T.O.
Analysis and interpretation: Y.N., S.S., S.W. and H.T.
Drafting of manuscript: Y.N., S.W. and H.T.
All authors provided final approval of the final version of the manuscript.

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