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Activation of osteoblastic functions by a mediator of pain, bradykinin

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Abstract

We investigated the effects of bradykinin (BK) on the production of interleukin (IL)-6 and prostaglandin PGE_2, whose molecules are capable of stimulating the development of osteoclasts from their hematopoietic precursors as well as the signal transduction systems involved, in human osteoblasts (SaM-1 cells). BK receptors B1 (B1R) and B2 (B2R) were expressed in SaM-1 and osteosarcoma (SaOS-2, HOS, and MG-63) cells. Treatment of SaM-1 cells with BK increased the synthesis of both IL-6 and PGE_2 and the increase in both was blocked by HOE140 (B2R antagonist), but not by Des-Arg^9-[Leu^8]-BK (B1R antagonist). U-73122, a phospholipase C (PLC) inhibitor, suppressed BK-induced IL-6 and PGE_2 synthesis in SaM-1 cells. In addition, BK caused an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$), which was inhibited by pretreatment with HOE140 or 2-aminoethoxydiphenyl borate (2-APB), an inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R) blocker. Furthermore, both SB203580 (an inhibitor of p38 mitogen-activated protein kinase [MAPK]) and PD98059 (an inhibitor of MEK, upstream of ERK) attenuated the BK-induced IL-6 and PGE_2 synthesis. BK treatment resulted in the phosphorylation of p38 MAPK and extracellular signal-regulated kinase (ERK)1/2, and 2-APB could suppress BK-induced phosphorylation of ERK1/2. These findings suggest that BK increased both IL-6 and PGE_2 synthesis in osteoblastic cells via B2R and that PLC, IP_3-induced $[Ca^{2+}]i$, MEK, and MAPKs were involved in the signal transduction in these cells.

Keywords: Bradykinin; Osteoblast; Interleukin-6; Prostaglandin E_2; MAPK; Calcium; Inositol 1,4,5-trisphosphate

1. Introduction

Orthodontic treatment is based on the biologic principle that prolonged pressure on teeth results in the remodeling of periodontal structures to allow for tooth movement. Unfortunately, pain is associated with such treatment. The pain caused by orthodontic tooth movement can be major negative component of the entire therapy [1,2]. In addition, tooth movement may be mediated through the local production and action of prostaglandins (PGs) [3–5]. Prostaglandin E_2 (PGE_2), either acting alone or in concert with other inflammatory agents, plays a major role in the inflammatory response [6]. However, it also promotes osteoclastogenesis and bone resorption in bone marrow or organ cultures [7,8]. Local injection of PGE_2 results in an increased number of osteoclasts at the site of tooth movement [9], and a decrease in local PGE_2 production is associated with a decreased rate of orthodontic tooth movement [10]. Although local injection of PGs is well known to elicit significant pain [11], the details of the mechanism of PG-induced tooth movement have not yet been determined.

Bradykinin (BK) is vasoactive peptide formed by the cleavage of kininogen by plasma kallikrein, and it is a very potent mediator of pain and smooth muscle contraction in a variety of tissues [12]. This peptide is known to increase the level of intracellular calcium concentration ($[Ca^{2+}]i$) and to induce inositol 1,4,5-trisphosphate (IP_3) production [13–16] in several cell lines. Furthermore, both BK and Lsy-BK stimulate bone resorption and the release of lysosomal enzymes in mouse calvaria in vitro [17–19]. BK also stimulates PGE_2 synthesis in murine osteoblast-like cell line MC3T3-E1 cells [20] and elevates interleukin (IL)-6 and PGE_2 synthesis in human osteoblast-like cells [21]. However, the signal transduction pathways involved in BK-induced IL-6 and PGE_2 synthesis in osteoblasts

Abbreviations: BK, bradykinin; IL, interleukin; COX, cyclooxygenase; PGE_2, prostaglandin E_2; PLC, phospholipase C; IP_3, inositol 1,4,5-trisphosphate; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase.

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have not yet been clarified. IL-6 is also a potent regulator for osteoclast differentiation, like PGE_2, and is synthesized by osteoblasts stimulated with IL-1β, parathyroid hormone, lipopolysaccharide (LPS), epinephrine, or PGE_2 [22–25].

Recently, BK was reported to increase IL-6 and IL-8 synthesis via phosphorylation of p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK)1/2 in human lung fibroblasts [26]. However, the nature of phosphorylation systems leading to BK-induced PGE_2 and IL-6 synthesis in human osteoblasts, especially the interaction between intracellular calcium and MAPK systems, has not yet been defined.

In this study, to investigate whether BK, a potent pain mediator, acts to increase osteoclast bone resorption during orthodontic tooth movement, we sought to determine the effects of BK on the synthesis of mediators of bone resorption in human osteoblastic SaM-1 cells and to examine the mechanism of signal transduction leading to such synthesis.

2. Material and methods

2.1. Materials

SaM-1 cells were used in this study. They were provided by Dr. Koshihara, who prepared them from an explant of ulnar periosteum obtained with informed consent from a 20-year-old male patient undergoing curative surgery [27]. These cells have a mitotic life span of 34 population-doubling levels (PDLs), and we used them at 22–23 PDL for our experiments. SaOS-2 cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan). HOS and MG-63 cell lines were from the American Type Culture Collection (Rockville, MD, USA). Alpha-modified minimum essential medium (α-MEM) was obtained from Gibco BRL (Grand Island, NY, USA) and fetal calf serum (FCS) from Cell Culture Laboratories (Cleveland, OH, USA) and Irnine Scientific (Santa Ana, CA, USA). A human IL-6 ELISA kit was obtained from R&D Systems (Minneapolis, MN, USA) and a PGE_2 ELISA kit from Cayman (Ann Arbor, MI, USA). p38 MAPK [pTpY180/182] and ERK1/2 [pTpY185/187] ELISA kits were from Biosource International (Camarillo, CA, USA). PD98059 (2′-amino-3′-methoxyflavone) and SB203580 ((4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole) were purchased from Biomol (Plymouth Meeting, PA, USA). Actinomycin D (AD) was from Sigma (St. Louis, MO, USA). BK, Lsy-BK, [des-Arg^{10}]kallidin, Des-Arg^9-[Leu^8]-BK, and HOE140 were obtained from Peptide Institute, Inc. (Osaka, Japan). A selective inhibitor of cyclooxygenase (COX)-2, NS398, was supplied by Taisho Pharmaceutical Co. U-73122 and 2-APB were from CALBIOCHEM (San Diego, CA, USA). Fura-2AM (1-[6-amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]-2-(2′-amino-5′-methylphenoxy)ethane-*N*,*N*,*N*′,*N*′,-tetraacetic acid pentaacetoxymethyl ester) was from DOJINDO (Kumamoto, Japan). BK, Lsy-BK, [des-Arg^{10}]kallidin, Des-Arg^9-[Leu^8]-BK, and HOE140 were dissolved in distilled water, and all other chemicals in dimethyl sulphoxide (DMSO; Sigma, St. Louis, MO, USA). All other chemicals used were of reagent grade.

2.2. SaM-1, SaOS-2, HOS, and MG-63 cell cultures

SaM-1 cells were maintained in α-MEM containing 10% FCS and 60 μg/mL kanamycin at 37 °C in a humidified atmosphere containing 5% CO_2 in air. Osteosarcoma cells were cultured in α-MEM containing 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin. For experiments to examine the expression of certain genes, cells were grown to confluence for extraction of total RNAs. For IL-6 or PGE_2 ELISA analysis, SaM-1 cells were cultured in 24-well plates until they were almost confluent. Before the stimulation, the maintenance medium was replaced with α-MEM containing 0.1% FCS and kanamycin. For p38 MAPK or ERK1/2 ELISA analysis, SaM-1 cells were cultured in the maintenance medium in 10-cm dishes until confluent, and then they were incubated in α-MEM containing 0.1% FCS and kanamycin for 18 h.

2.3. Analysis of mRNA levels by RT-PCR

Total RNAs were extracted from SaM-1 and osteosarcoma cells in 6-cm dishes (Falcon Plastics, Los Angeles, CA, USA) by the guanidinium–thiocyanate method [28]. Total RNAs were solubilized in guanidinium thiocyanate buffer and then phenol extracted and DNase I (Boehringer Mannheim, GmbH, Germany) treated. cDNA was synthesized by using random primers and Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Grand Island, NY, USA), whose synthesis was followed by PCR amplification using synthetic gene primers specific for human IL-6, COX-2, B1R, B2R, and human glyceraldehyde 3-phosphate dehydrogenase (GAPDH). These primers were designed with reference to the respective reported cDNA sequences [29–32]. The oligonucleotide primers were synthesized on a DNA synthesizer (Expedite model 8909; PerSeptiv Biosystem, Cambridge, MA, USA) and purified on a polypropylene filter (Oligo Prep kit; Pharmacia Biotech, Uppsala, Sweden). The genes examined, respective forward and reverse primers, and size of amplified product were the following. GAPDH, 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′, 452-bp cDNA fragment; IL-6, 5′-CATCCTCGACGGCATCTCAGC-3′ and 5′-TTGGGTCAGGGGTGGTTATTG-3′, 332-bp cDNA fragment; COX-2, 5′-TGTATCCTGCCCTTCTGGTAG-3′ and 5′-GCATTGATGGTGACTGTTTTA-3′, 277-bp cDNA fragment; B1R, 5′-TGGACCCAGTTTAACTGGCC-3′ and 5′-ATGAAGTCCCAAAAGCA-3′, 578-bp cDNA fragment; and

B2R, 5′-TGGGGACGGTTCTGACGGTG-3′, and 5′-GCCAGGATCAGGTCTGTCG-3′, 447-bp DNA fragment. PCR amplification was performed with a GeneAmp PCR System (Perkin Elmer/Cetus, Norwalk, CT, USA) according to the following setting: denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 30 s for the appropriate cycles. PCR products were electrophoresed on a 2% Nusive GTG agarose gel (FMC BioProducts, Rockland, ME, USA), stained with ethidium bromide, and detected with a fluoroimage analyzer (FluorImager 575; Molecular Dynamics, Sunnyvale, CA, USA). Relative expression of these increases. The mRNA level of IL-6 was calculated by dividing the intensity of the IL-6 band by that of the GAPDH band as determined by fluorescent image analyzer.

2.4. Measurement of IL-6 and PGE_2 levels and analysis of phosphorylated p38 MAPK and ERK1/2

Levels of IL-6 and PGE_2 in conditioned media were quantified by using ELISA kits. For assessing the quantity of phosphorylated p38 MAPK and ERK1/2, SaM-1 cells were cultured until confluent. The cells were preincubated in 0.1% FCS for 18 h and then treated with BK for 15 min. The desired inhibitor was added 30 min before the addition of BK. After the stimulation by BK, the cells were collected in PBS and dissolved in cell extraction buffer. These samples were used for ELISA analysis. Data were presented as the mean ± S.E.M. of three cultures. Differences between control and experiment were determined by using Student's *t*-test.

2.5. Optical measurements of $[Ca^{2+}]i$

SaM-1 cells were cultured in glass-bottomed microwell dishes (MatTek Co., MA, USA) until semiconfluent and then washed with BSS and loaded with Fura-2AM for 30 min. After that, they were washed with BSS and incubated with BSS for 1 h prior to observation. Fura-2 fluorescence was monitored with ARUGAS 50/CA (Hamamatsu Fotonix, Japan) spectrofluorophotometer by recording excitation signal at 340 and 380 nm at 2-s intervals. SaM-1 cells were pretreated with B1R antagonist, HOE140 or 2-APB, for 10 min before the stimulation by BK. Data were expressed as the 340–380-nm fluorescence ratio (F_{340}/F_{380}) relative to the value of F_{340}/F_{380} in the resting state.

3. Results

3.1. Expression of B1R and B2R mRNAs in SaM-1 and osteosarcoma cells

Human osteoblasts SaM-1 cells and osteosarcoma (SaOS-2, HOS, and MG-63) cells expressed BK receptor,

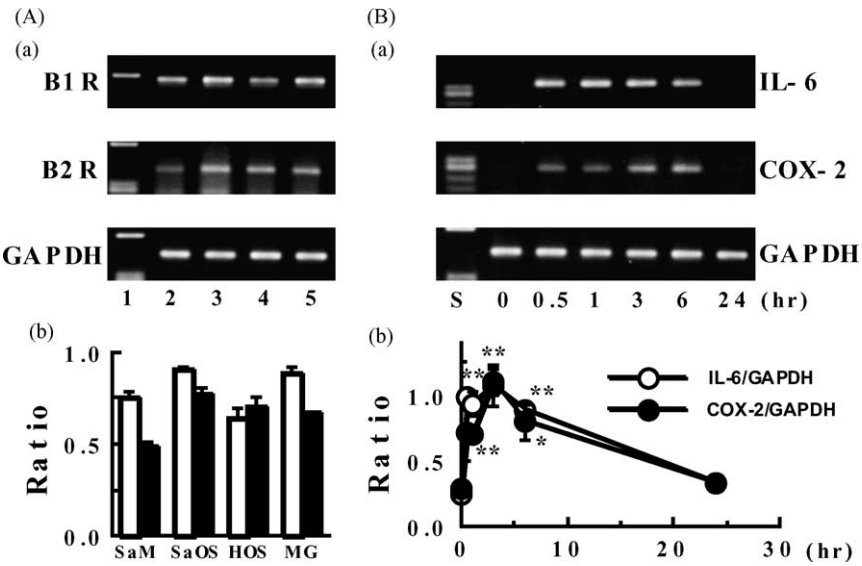

Fig. 1. Expression of B1R and B2R mRNAs and of BK-induced IL-6 and COX-2 mRNAs in human osteoblastic cells. A: (a) Expression of B1R and B2R mRNAs in SaM-1 (lane 2), SaOS-2 (lane 3), HOS (lane 4), and MG-63 (lane 5) cells. Cells were cultured until almost confluent, and then total RNA was extracted and subjected to RT-PCR analysis. DNA size markers (ϕX 174/*Hae* III digest) are shown in lane 1. (b) Relative expression of these genes. The mRNA levels of B1R (white column) or B2R (black column) were calculated by dividing the intensity of B1R or B2R band by that of the GAPDH band as determined by fluorescent image analyzer. Values are means ± S.E.M. ($n = 3$). SaM-1, SaM; SaOS-2, SaOS; and MG-63, MG. B: (a) Effects of BK on the expression of IL-6 and COX-2 mRNAs in SaM-1 cells. Cells were incubated for 0.5, 1, 3, 6, or 24 h with 1 μM BK, and then total RNA was extracted and subjected to RT-PCR analysis. DNA size markers (ϕX 174/*Hae* III digest) are shown in the left lanes (S). Data shown are representative of three similar experiments. (b) Relative expression of these increases. The mRNA levels of IL-6 or COX-2 were calculated by dividing the intensity of IL-6 or COX-2 band by that of the GAPDH band as determined by fluorescent image analyzer. Values are means ± S.E.M. ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$.

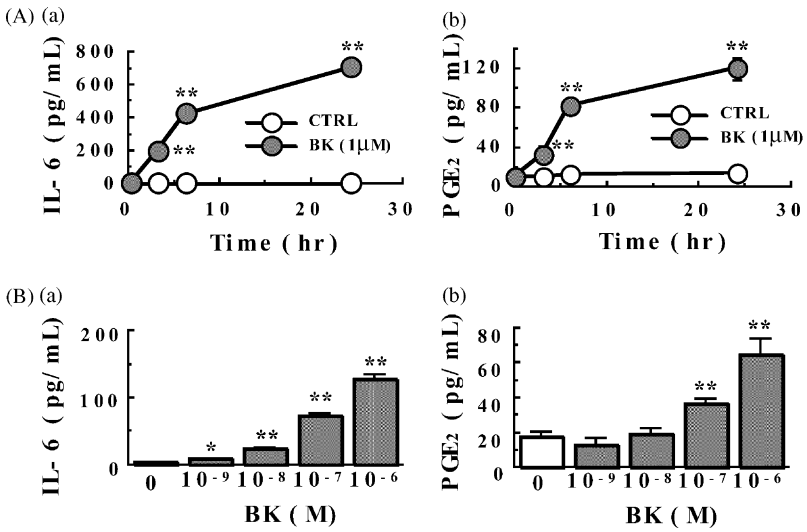

Fig. 2. Effects of BK on IL-6 and PGE_2 production/secretion in SaM-1 cells. (A) Cells were incubated for 3, 6, or 24 h with 1 μM BK in the presence of 0.1% FCS. Conditioned medium was used for analysis of IL-6 (a) and PGE_2 (b) production/secretion with specific ELISA systems. $^{**}P < 0.01$ vs. control (CTRL). (B) Cells were incubated for 24 h with the indicated doses of BK in the presence of 0.1% FCS. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. non-treated cells (0).

B1R, and B2R mRNAs (Fig. 1A(a)). Although the magnitude of B1 and B2 mRNA expressions was almost same level in all cell lines (Fig. 1A(b)), the expression of B2R gene in osteosarcoma cells was a little stronger than that in SaM-1 cells.

3.2. Effects of BK on expression of IL-6 and COX-2 mRNAs in osteoblasts

Fig. 1B(a) and (b) shows the effects of BK on the expression of the IL-6 and COX-2 genes in SaM-1 cells. When cells were treated with BK (1 μM) for the indicated periods, BK increased the steady-state level of both IL-6 and COX-2 mRNAs in a time-dependent manner. The expression of each gene was first observed at 0.5 h after the start of treatment with BK, and the expression remained elevated until 6 h (Fig. 1B).

3.3. Effects of BK on IL-6 and PGE_2 synthesis in SaM-1 cells

SaM-1 cells were treated with BK at a fixed concentration for various periods or at various concentrations for 24 h, and the conditioned medium was then analyzed for IL-6 and PGE_2 contents by using specific ELISA systems. BK (1 μM) significantly increased both IL-6 and PGE_2 production/secretion in a time-dependent manner (Fig. 2A(a) and (b)). The induction of both IL-6 and PGE_2 was relatively rapid starting (as early as 3 h after stimulation), and maximum levels were reached by 24 h. The effect of BK on IL-6 and PGE_2 synthesis by the cells was dose dependent in the range between 10^{-9} and 10^{-6} M (Fig. 2B(a) and (b)). When we examined the effect of BK on cell proliferation using BrdU-uptake analysis, BK did not show any influence on cell proliferation (treated BK; 97.0 ± 5.5%, as 100% of control). Similarly, the natural human B2R agonist Lsy-BK (kallidin) as well as BK significantly increased IL-6 and PGE_2 synthesis in a dose-dependent manner (10^{-9}–10^{-6} M), and maximum levels were reached at the dose of 10^{-6} M (IL-6: control, 0.41 ± 0.2 pg/mL, Lsy-BK, 158.9 ± 3.4 pg/mL, $P < 0.01$; PGE_2: control, 11.0 ± 1.7 pg/mL, Lsy-BK; 55.3 ± 3.9 pg/mL, $P < 0.01$).

3.4. Effects of B1R and B2R antagonists on BK-induced IL-6 and PGE_2 synthesis

SaM-1 cells were treated with B1R or B2R antagonist (10^{-5}–10^{-7} M) for 1 h before treatment with BK. Although the B1R antagonist, Des-Arg^9-[Leu^8]-BK (10^{-5}M), did not inhibit BK-induced IL-6 and PGE_2 synthesis (data not shown), the B2R one, HOE140, inhibited them in a dose-dependent manner (Fig. 3A and B). HOE140 at 10^{-5} M decreased BK-induced IL-6 and PGE_2 synthesis to the steady-state level. These data indicate that BK bound to B2R to induce IL-6 and PGE_2 synthesis in SaM-1 cells. In addition, since 10^{-6} M B1R agonist, [des-Arg^{10}]kallidin, could not increase IL-6 and PGE_2 synthesis (IL-6: control, 0.4 ± 0.2 pg/mL, B1R agonist, 1.0 ± 0.53 pg/mL, $P < 0.33$; PGE_2: control, 11.0 ± 1.7 pg/mL, B1R agonist, 18.8 ± 6.2 pg/mL, $P < 0.30$), these data suggest that BK increased IL-6 and PGE_2 synthesis via B2R but not B1R.

3.5. Effects of phospholipase C (PLC) inhibitor on BK-induced IL-6 and PGE_2 synthesis

Fig. 4A and B shows the effects of the PLC inhibitor U-73122 on BK-induced IL-6 and PGE_2 synthesis in SaM-1 cells. U-73122 (1 and 2 μM) significantly decreased

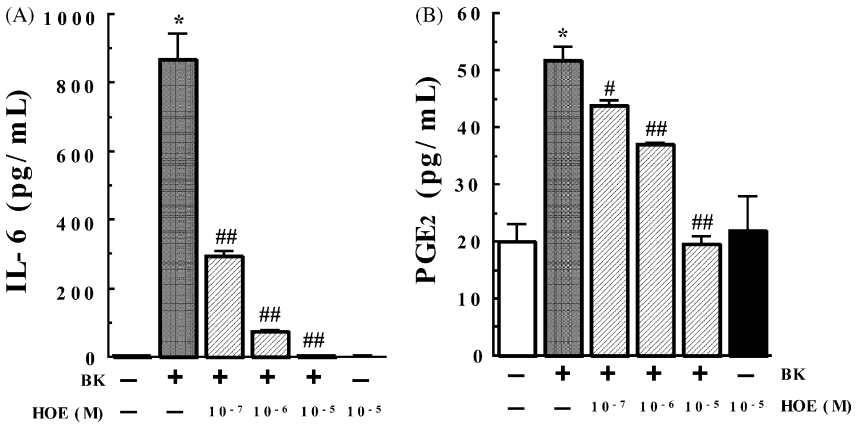

Fig. 3. Effects of HOE140 on BK-induced IL-6 (A) and PGE_2 (B) synthesis in SaM-1 cells. Cells were pretreated with the indicated doses of HOE140 (HOE) in the presence of 0.1% FCS for 1 h before the addition of 1 μM BK. After the treatment with BK for 24 h, the conditioned medium was collected and used for determination of IL-6 and PGE_2 concentrations. $^{*}P < 0.01$ vs. non-treated cells, $^{\#}P < 0.05$, and $^{\#\#}P < 0.01$ vs. treatment with BK only.

BK-induced IL-6 and PGE_2 synthesis, suggesting that BK caused the increase via activation of PLC in the cells.

3.6. Effect of BK on $[Ca^{2+}]i$ in SaM-1 cells

Next, we examined the alteration of the intracellular calcium level induced by BK alone and that by pretreatment with HOE140 or 2-APB prior to BK in SaM-1 cells. As shown in Fig. 5A and B, BK caused a rapid rise in $[Ca^{2+}]i$. Although the pretreatment with B2R antagonist HOE140 (10 μM) decreased the BK-increased $[Ca^{2+}]i$, the B1R antagonist could not attenuate the BK-induced increase in $[Ca^{2+}]i$ (data not shown). Fig. 5B shows that the pretreatment with 2-APB (IP_3R blocker: 100 μM) also inhibited the BK-induced increase in $[Ca^{2+}]i$, suggesting that BK increased the intracellular calcium level via B2R and IP_3 in SaM-1 cells. Although extracellular calcium was removed by 2.5 mM EGTA, BK could increase $[Ca^{2+}]i$ in the cells (Fig. 5C), suggesting that BK-increased $[Ca^{2+}]i$ was not derived from extracellular calcium.

3.7. Effects of IP_3R blocker and MAPK inhibitors on BK-induced IL-6 and PGE_2 synthesis

The BK-induced synthesis of both IL-6 and PGE_2 was significantly inhibited by 2-APB in a dose-dependent manner in SaM-1 cells (Fig. 6A and B). 2-APB (100 μM) caused a 90% decrease in BK-induced IL-6 synthesis and a 40% decrease in BK-induced PGE_2 synthesis, suggesting that the induction in both cases was associated with an IP_3-dependent increase in intracellular calcium. Although MEK inhibitor PD98059 (10 μM) completely decreased BK-induced PGE_2 synthesis (Fig. 7B), p38 MAPK inhibitor SB203580 only partially decreased its synthesis. In contrast, neither SB203580 nor PD98059 decreased BK-induced IL-6 synthesis below 50%. Also, the inhibition by PD98059 was stronger than that by SB203580 toward either IL-6 or PGE_2 synthesis induced by BK in SaM-1 cells. In addition, when cells were treated with simultaneous addition of SB203580 and PD98059, BK-increased IL-6 synthesis was not completely inhibited by simultaneous use, suggesting that there is another

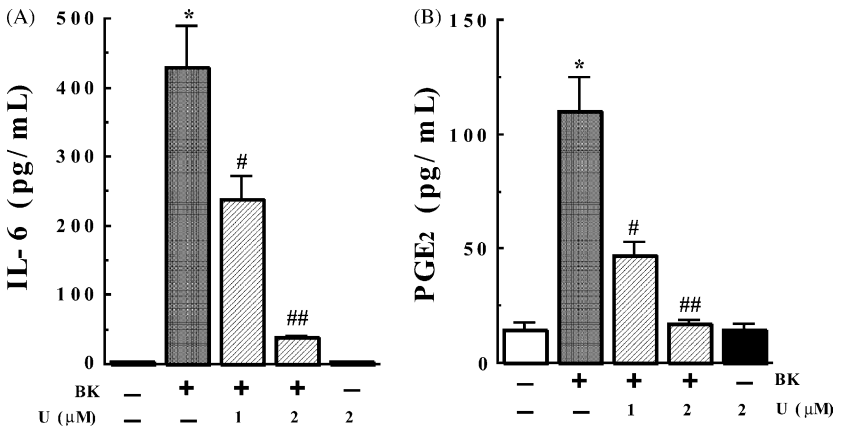

Fig. 4. Effects of U-73122 on BK-induced IL-6 (A) and PGE_2 (B) synthesis in SaM-1 cells. Cells were pretreated with the indicated doses of U-73122 (U) in the presence of 0.1% FCS for 1 h before addition of 1 μM BK. Incubations were conducted for 24 h. $^{*}P < 0.01$ vs. non-treated cells, $^{\#}P < 0.05$, and $^{\#\#}P < 0.01$ vs. treatment with BK only.

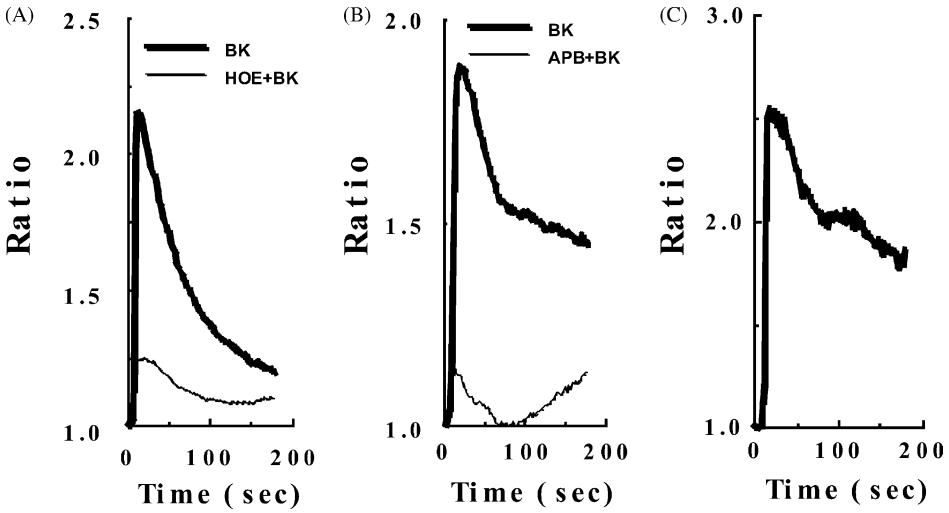

Fig. 5. Effects of HOE140 or 2-APB on BK-increased $[Ca^{2+}]i$ in SaM-1 cells. BK was added to SaM-1 cell cultures at 10 s after the start of recording the fluorescence. (A) Cells were pretreated with HOE140 (10 μM) for 10 min before the addition of 1 μM BK. (B) Cells were incubated with 100 μM 2-APB (APB) for 10 min before the addition of 1 μM BK. The experiments were performed in the presence of extracellular calcium. (C) The effect of extracellular calcium removal on BK-increased $[Ca^{2+}]i$ increases. Data are representative of three independent experiments.

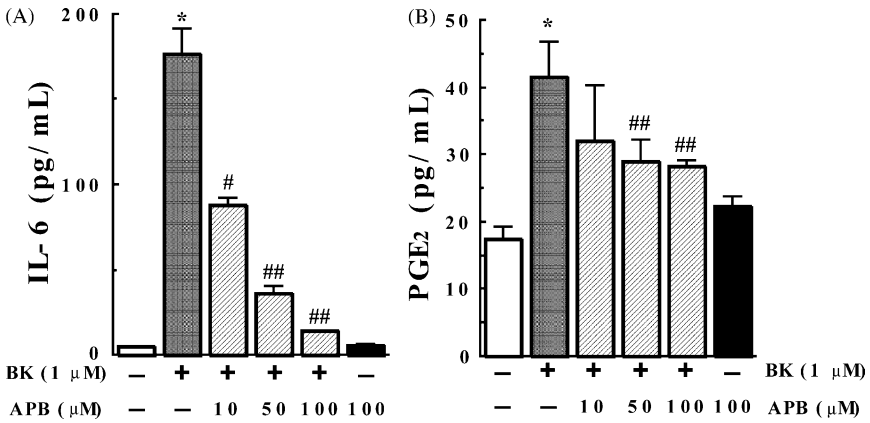

Fig. 6. Effects of 2-APB on BK-induced IL-6 (A) and PGE_2 (B) synthesis in SaM-1 cells. Cells were pretreated with the indicated doses of 2-APB (APB) in the presence of 0.1% FCS for 1 h before the addition of 1 μM BK. Incubations were for 24 h. $^{*}P < 0.01$ vs. non-treated cells, $^{\#}P < 0.05$, and $^{\#\#}P < 0.01$ vs. treatment with BK only.

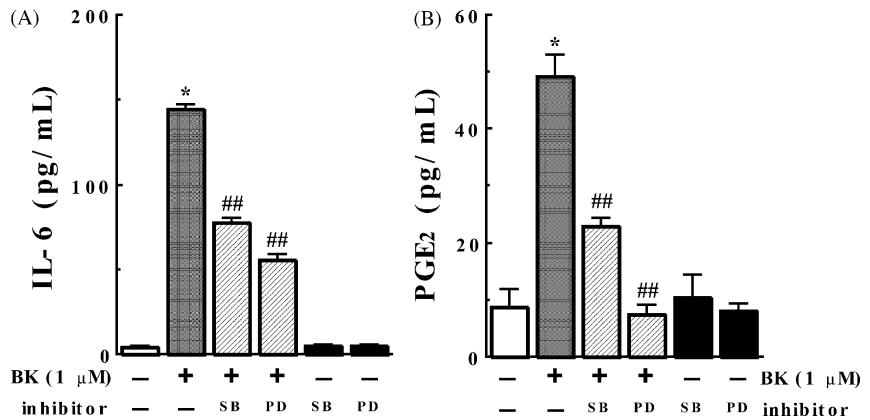

Fig. 7. Effects of SB203580 and PD98059 on BK-induced IL-6 (A) and PGE_2 (B) synthesis in SaM-1 cells. Cells were pretreated with SB203580 (SB, 10 μM) or PD98059 (PD, 10 μM) in the presence of 0.1% FCS for 1 h before the addition of 1 μM BK. Incubations were for 24 h. $^{*}P < 0.01$ vs. non-treated cells, and $^{\#\#}P < 0.01$ vs. treatment with BK only.

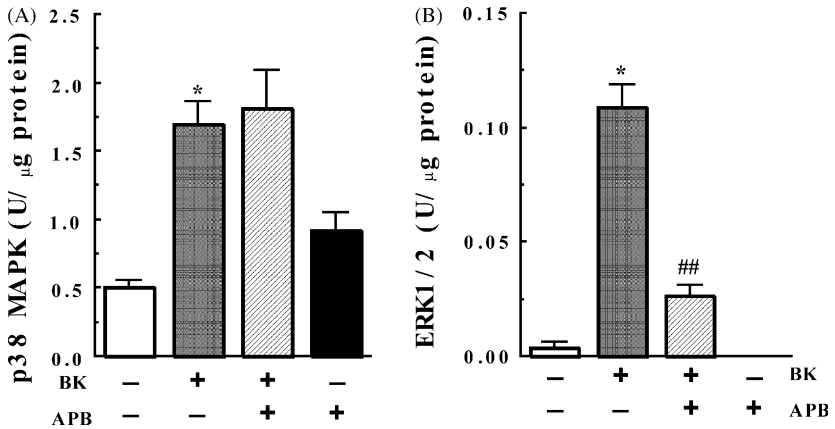

Fig. 8. Effects of BK on p38 MAPK (A) and ERK1/2 (B) phosphorylation and effects of 2-APB on BK-activated MAPKs in SaM-1 cells. Cells were incubated with 100 μM 2-APB (APB) in the presence of 0.1% FCS for 30 min before the addition of 1 μM BK. After the treatment with BK for 15 min, cytoplasm was extracted and used for determination of phosphorylated p38 MAPK and ERK1/2 levels by using the respective ELISA systems. $^{*}P < 0.01$ vs. non-treated cells, and $^{\#\#}P < 0.01$ vs. treatment with BK only.

signaling pathway in addition to p38 MAPK and MEK in this response. Taken together, the data indicate that a pathway involving IP_3-induced $[Ca^{2+}]i$, MEK, and p38 MAPK is at least in part involved in BK-induced IL-6 and PGE_2 synthesis in SaM-1 cells.

3.8. Effects of BK on p38 MAPK and ERK1/2 phosphorylation

Finally, we demonstrated that BK increased both p38 MAPK and ERK1/2 phosphorylation in SaM-1 cells (Fig. 8). Although 2-APB (100 μM) inhibited BK-induced ERK1/2 phosphorylation, it had no effect on BK-induced p38 MAPK phosphorylation. In addition, PLC inhibitor U-73122 blocked BK-induced ERK1/2 phosphorylation (BK: 0.76 ± 0.15 U/μg protein versus BK and U-73122: 0.03 ± 0.002 U/μg protein, $P < 0.01$), but did not inhibit BK-induced p38 MAPK phosphorylation (data not shown), suggesting that BK induced an IP_3-dependent intracellular calcium increase, leading to ERK1/2 phosphorylation in SaM-1 cells.

4. Discussion

In the present study, we demonstrated the effects of BK, a very potent mediator of pain, on IL-6 and PGE_2 synthesis in osteoblasts. The pain is caused by orthodontic tooth movement, and this tooth movement may be mediated by the activation of PGE_2 production [3–5]. The injection of PGs results in significant pain and tooth movement [9,11], and non-steroidal anti-inflammatory drugs reduce the rate of orthodontic tooth movement in cats [33]. In addition, PGs are known as biochemical mediators of bone resorption [7,8], and PGE_2 is synthesized in osteoblasts stimulated by LPS, proinflammatory cytokines (IL-1α, -β, and tumor necrosis factor-α) [34,35], or transforming growth factor-β [36]. However, the relationship between pain-inducing PGs and BK, which is also a pain mediator, in the enhancement of bone resorption resulting in tooth movement has not yet been made clear.

IL-6 is known to be present in the synovial fluid from patients with rheumatoid arthritis and osteoarthritis [37], and the concentrations of IL-6 and other inflammatory cytokines are significantly elevated in gingival crevicular fluid during orthodontic tooth movement [38]. These findings suggest that orthodontic tooth movement is mediated by increased production of IL-6 or other local factors. Since it is possible that BK increases tooth movement via mediators of bone resorption such as IL-6 or PGE_2, we investigated whether BK could induce mediators of bone resorption in the human osteoblastic SaM-1 cell and examined their signal transduction systems by using pharmacological inhibitors.

We demonstrated that SaM-1 cells expressed B1R and B2R mRNAs and that BK significantly elevated the synthesis of both IL-6 and PGE_2 in these cells. However, since BK did not increase the expression of genes of other osteoclastogenic factors such as IL-11, insulin growth factor, receptor activator of NF-κB ligand (RANKL), and osteoprotegerin (OPG), a decoy receptor for RANKL, in SaM-1 cells (data not shown), we focused on the signaling pathway involved in BK-increased IL-6 and PGE_2 synthesis. Two types of BK receptor subtypes, B1Rs and B2Rs, have been cloned and pharmacologically defined [39]. Although B1Rs are generally absent under non-pathological conditions, inflammatory cytokines can induce B1Rs [40,41]. In contrast, B2Rs are expressed constitutively in several tissues [42]. Although the B2R antagonist inhibited BK-induced IL-6 and PGE_2 synthesis in SaM-1 cells (Fig. 3), the B1R one was ineffective, indicating that a common signal transduction system via B2R is involved in the BK-induced synthesis of both IL-6 and PGE_2. In accordance with our data, BK-induced IL-6

production in human airway smooth muscle cells was earlier reported to be inhibited by HOE140 [43].

Furthermore, we demonstrated that BK increased $[Ca^{2+}]i$ and that HOE140 or 2-APB reduced the BK-increased $[Ca^{2+}]i$, IL-6, and PGE_2 production (Figs. 5 and 6). Since 2-APB is generally used as an IP_3R blocker at the dose of 5–100 μM [44–46], these data suggest that BK induced IL-6 and PGE_2 synthesis via an IP_3-induced increase in intracellular calcium in SaM-1 cells.

PGE_2 is well known to induce IL-6 production in mouse osteoblastic cells [25]. To examine whether BK-induced PGE_2 elevated IL-6 synthesis in SaM-1 cells, we investigated the BK-induced IL-6 synthesis in the absence of PGE_2. Although the pretreatment with NS398 (COX-2 inhibitor: 1 μM) inhibited completely BK-induced PGE_2 synthesis, NS398 could not suppress BK-induced IL-6 synthesis (BK: 144.21 ± 2.98 pg/mL versus NS398 and BK: 129.94 ± 6.31 pg/mL), indicating that PGE_2 was not involved in the action of BK on IL-6 synthesis in SaM-1 cells. In addition, transcription inhibitor actinomycin D (0.5 μg/mL) inhibited BK-increased IL-6 and PGE_2 synthesis (IL-6: BK, 195.9 ± 3.3 pg/mL, BK + AD, 4.4 ± 0.4 pg/mL, $P < 0.01$; PGE_2: BK, 56.4 ± 2.2 pg/mL, BK + AD, 21.0 ± 1.0 pg/mL, $P < 0.01$), suggesting that BK-increased IL-6 and PGE_2 synthesis is transcription-dependent in the cells.

As was shown in Fig. 8, BK increased ERK1/2 phosphorylation, and this phosphorylation inhibited 2-APB, suggesting that BK-increased $[Ca^{2+}]i$ led to phosphorylation of ERK1/2, but not to that of p38 MAPK. It was previously reported that BK increased IP_3 production and $[Ca^{2+}]i$ and that BK-increased intracellular calcium activated MAPK in vascular cells [47], findings that are in accord with our data.

In conclusion, we found that BK increased IL-6 and PGE_2 synthesis and that there were two signaling pathways involved in BK-induced synthesis in SaM-1 cells. Principally, BK bound to B2R results in PLC, IP_3, IP_3-induced intracellular calcium release, and ERK1/2 phosphorylation by MEK, leading to IL-6 and PGE_2 synthesis. In contrast, there is another pathway by which BK bound to B2R leads to IL-6 and PGE_2 synthesis through $[Ca^{2+}]i$-independent p38 MAPK phosphorylation. Understanding the signaling pathways by which BK regulates mediators of bone resorption may lead to painless therapy and new types of orthodontic treatment.

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