Molecules and Cells

The Inactivation of ERK1/2, p38 and NF-kB Is Involved in the Down-Regulation of Osteoclastogenesis and Function by A2B Adenosine Receptor Stimulation

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A2B adenosine receptor (A2BAR) is known to be the regulator of bone homeostasis, but its regulatory mechanisms in osteoclast formation are less well-defined. Here, we demonstrate the effect of A2BAR stimulation on osteoclast differentiation and activity by RANKL. A2BAR was expressed in bone marrow-derived monocyte/macrophage (BMM) and RANKL increased A2BAR expression during osteoclastogenesis. A2BAR stimulation with its specific agonist BAY 60-6583 was sufficient to inhibit the activation of ERK1/2, p38 MAP kinases and NF-κB by RANKL as well as it abrogated cell-cell fusion in the late stage of osteoclast differentiation. Stimulation of A2BAR suppressed the expression of osteoclast marker genes, such as *c-Fos*, *TRAP*, *Cathepsin-K* and *NFATc1*, induced by RANKL, and transcriptional activity of NFATc1 was also inhibited by stimulation of A2BAR. A2BAR stimulation caused a notable reduction in the expression of Atp6v0d2 and DC-STAMP related to cell-cell fusion of osteoclasts. Especially, a decrease in bone resorption activity through suppression of actin ring formation by A2BAR stimulation was observed. Taken together, these results suggest that A2BAR stimulation inhibits the activation of ERK1/2, p38 and NF-κB by RANKL, which suppresses the induction of osteoclast marker genes, thus contributing to the decrease in osteoclast cell-cell fusion and bone resorption activity.

Keywords: A2B adenosine receptor, osteoclastogenesis, RANKL

INTRODUCTION

Bone homeostasis is maintained by the balance between bone formation by osteoblasts and destruction of mineralized bone matrix by osteoclasts (Boyle et al., 2003; Suda et al., 1999; Takayanagi, 2007). Thus, bone remodeling is a costly process and it requires a considerable amount of energy. Indeed, there is a constant supply of energy to osteoblasts and osteoclasts, and extracellular nucleotide molecules such as ATP have been shown to be increased during bone fracture and bone repair (Bowler et al., 2001; Orriss et al., 2010).

The biological actions of adenosine, which is formed from its precursor ATP are mediated via A1, A2A, A2B and A3 adenosine receptors (A1R, A2AR, A2BAR and A3R, respectively) that are members of the G protein-coupled receptor family (Evans et al., 2006; Fredholm et al., 2001; Ham and Evans, 2012). It was shown that all four adenosine receptors are expressed in both osteoblasts and osteoclasts, in which stimulation of A2AR, A2BAR and A3R seems to inhibit osteoclast differentiation whereas A1R shows opposite effects (Ham and Evans, 2012; He et al., 2013b; Kara et al., 2010a; 2016b; Mediero and Cronstein, 2013). Especially, differentiation of osteoblasts was impaired in *A2BAR* KO mice, which showed decreased bone mineral density (Carroll et al., 2012; Corciulo et al., 2016) and A2BAR stimulation with its specific

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agonist BAY 60-6583 promoted osteoblast differentiation through modulation of levels of Runx2 and Osterix, and partially via cAMP signaling and decreased osteoclast differentiation (Carroll et al., 2012; Corciulo et al., 2016; Trincavelli et al., 2014). However, regulatory mechanisms of A2BAR stimulation on the osteoclast differentiation and functional activity are still less well-defined.

Osteoclasts are derived from hematopoietic progenitors of the bone marrow-derived monocyte/macrophage (BMM) lineage. Mononuclear osteoclast precursors become multinucleated mature cells that degrade bone matrix via two essential cytokines, the receptor activator of nuclear factor-kB (NF-kB) ligand (RANKL) and macrophage-colony stimuloating factor (M-CSF). RANKL results in downstream activation of distinct signaling cascades such as extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase 1/2 (JNK1/2) and p38 MAP kinases (Boyle et al., 2003). The activation of these MAP kinases finally induces the expression of various osteoclast marker genes including nuclear factor of activated T-cells cytoplasmic 1 (NFATc1), tartrate-resistant acid phosphatase (TRAP), c-Fos, cathepsin K and microphthalmia transcription factor (MITF) (Boyle et al., 2003; Teitelbaum, 2000; Teitelbaum and Ross, 2003). In particular, NFATc1 is known to be a master transcription factor for osteoclastogenesis (Takayanagi et al., 2002). NFATc1 undergoes nuclear translocation and regulates the expression of many osteoclast-specific genes.

To become mature osteoclasts, mononuclear osteoclast precursor cells fuse together. The d2 isoform of vacuolar (H+) ATPase (v-ATPase) Vo domain (Atp6v0d2) and dendritic cell-specific transmembrane protein (DC-STAMP) are crucial for osteoclast cell-cell fusion since deficiency of these genes causes osteopetrotic phenotypes in mice because of defects in the cell-cell fusion process (Lee et al., 2006; Yagi et al., 2005). To solubilize the mineral component of bone, osteoclasts form a filamentous actin ring for tight attachment to the substrate and construct a resorption space called the sealing zone (Burgess et al., 1999; Vaananen et al., 2000).

Here, our data suggest that the inactivation of ERK1/2, p38 MAP kinase and NF-kB is involved in the down-regulation of osteoclastogenesis and function by A2BAR stimulation, which implies the potential of A2BAR as a therapeutic target for bone-related diseases.

MATERIALS AND METHODS

Isolation of bone marrow precursors and *in vitro* osteoclastogenesis

Isolation of bone marrow precursors and the in vitro osteoclastogenesis experiment were performed as described previously (Choi et al., 2013). In brief, bone marrow cells isolated from femurs of 4-6 week-old C57BL/6 male mice were cultured in the presence of M-CSF (20 ng/ml, R&D Systems) for 3 days. After washing out the non-adherent cells, the adherent cells were used as BMMs. For osteoclast formation, the isolated preosteoclasts were further cultured in the presence of 200 ng/ml of RANKL (Huh et al., 2016), 30 ng/ml of M-CSF and/or BAY 60-6583 (Tocris). After 3 days, the cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) using a TRAP staining kit (Sigma). The cells were observed using a Zeiss Axiovert 200 microscope and images were obtained with an AxioCam HR (Carl Zeiss) equipped with Axio Vision 3.1 software (Carl Zeiss). TRAP-positive multinucleated cells ($TRAP^+$ MNCs) larger than 100 μm in diameter containing more than 20 nuclei and $TRAP^+$ mononuclear cells were counted, and the number was presented as relative percentage (%).

RNA isolation and real-time PCR

The total RNA was isolated from BMMs treated with RANKL (200 ng/ml) and reverse transcribed using SuperScriptIII reverse transcriptase (RT) (Invitrogen) in accordance with the manufacturer's protocol. Real-time PCR was performed with the Brilliant UltraFast SYBR Green QPCR Master Mix (Agilent Technologies) in triplicate via an Mx3000P instrument (Agilent Technologies). Specific primers for the indicated genes and *hprt* (for endogenous control) were purchased from QIAGEN. The thermal cycling conditions were as follows: 3 min at 95℃, followed by 40 cycles of 95℃ for 10 s, 60℃ for 20 s, and 1 cycle of 95℃ for 1 min, 55℃ for 30 s, and 95℃ for 30 s. All quantitation were normalized to the *hprt* expression (Choi et al., 2013).

Western blot analysis

BMMs stimulated with RANKL (200 ng/ml) and/or BAY 60-6583 as indicated were lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonylfluoride) and whole cell extracts were prepared by centrifugation. The supernatants were electrophoresed on a 10% SDS-polyacrylamide gel and blotted onto a polyvinylidene difluoride membrane. Immunoblot detection was performed with polyclonal antibodies (Abs) specific to A2BAR, NFATc1, c-Fms, β-actin (as a loading control), α-tubulin, TBP (Santa Cruz Biotechnology Inc), Atp6v0d2 (Sigma-Aldrich), ERK1/2, phospho-ERK1/2, JNK1/2, phospho-JNK1/2, p38, phospho-p38 and phospho-IκBα (Cell Signaling Technology, Beverly, MA), followed by HRP-conjugated secondary antibodies and enhanced using an ECL detection kit (Amersham Biosciences) (Choi et al., 2013).

Cytosolic and nuclear fractionation

Cells were lysed with cytosolic extraction buffer (10 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 M dithiothreitol, 0.05% NP-40) containing protease inhibitors and phosphatase inhibitors. After centrifugation at 8,000 r.p.m. for 5 min at 4℃, supernatants were collected for the cytosolic fraction. Pellets were washed with cytosol extraction buffer and then lysed with nuclear extraction buffer (5 mM HEPES, pH 7.4, 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol) containing protease inhibitors and phosphatase inhibitors. After incubation on ice for 30 min, the nuclear fraction was obtained by centrifugation at 14,000 r.p.m. for 30 min at 4℃ (Ko et al., 2015).

Transfection and luciferase reporter assay

RAW264.7 cells were plated into 12-well plates at 1×10^5

cells/well 24 h before transfection. NFATc1-luciferase reporter plasmid (Jang et al., 2011) or NF-κB-luciferase reporter plasmid (Lee et al., 2006) and β-galactosidase plasmid as an internal control were mixed with FuGENE 6 (Roche) and transfected in accordance with the manufacturer's instructions. After 36 h of transfection, the cells were treated with 200 ng/ml of RANKL and/or BAY 60-6583 as indicated for 12 h, lysed in reporter lysis buffer (Promega) and luciferase reporter assay was performed using a dual-luciferase reporter assay system (Promega) according to the manufacturer's protocol (Choi et al., 2013).

Actin ring staining

After differentiation of osteoclasts, the cells were fixed with 3.7% formaldehyde solution in PBS, permeabilized with 0.1% Triton X-100, and incubated with Alexa Fluor 488-phalloidin (Invitrogen) for 20 min. After washing with PBS, the cells were photographed under a fluorescence microscope (Suda et al., 1997).

Bone resorption assay

Mature osteoclasts on dentine discs (Immunodiagnostic Systems) were treated without or with BAY 60-6583 (1 μM) in the presence of 200 ng/ml of RANKL for 48 h. The cells were completely removed from the dentine discs via abrasion with a cotton tip, and the dentine discs were stained with hematoxylin. The resorption pit areas were measured using Image-Pro Plus 4.5 software (Media Cybernetics) and presented as relative pit area (%) (Huh et al., 2016).

Statistical analysis

Results are presented as means ± standard deviations (SD) obtained from at least 3 independent experiments, and statistical analyses were performed using the Student's *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

A2BAR expression is induced by RANKL treatment during osteoclastogenesis

Prior to determining the regulatory mechanism of A2BAR on the osteoclast differentiation, we first examined the expression of A2BAR during osteoclastogenesis. A2BAR was expressed in BMMs and stimulation with RANKL notably increased the expression of A2BAR (Figs. 1A and 1C). M-CSF failed to regulate A2BAR expression whereas *c-Fms*, a receptor of M-CSF, was induced by M-CSF treatment as expected in BMMs (Fig. 1B).

A2BAR stimulation inhibits osteoclastic cell-cell fusion

To confirm whether A2BAR regulates osteoclast differentiation, BAY 60-6583, a selective A2BAR agonist, was used. Simultaneous treatment with BAY 60-6583 and RANKL significantly suppressed osteoclast differentiation by RANKL in a dose-dependent manner, whereas BAY 60-6583 alone did not show any effect on osteoclast differentiation (Fig. 2A). Especially, 1 and 10 μM of BAY 60-6583 induced a profound reduction of the number of fused, multinucleated cells (MNCs) larger than 100 μm in diameter containing more than 20 nuclei about 90% and 99%, respectively, whereas the number of TRAP-positive mononuclear cells was increased because of decrease of larger MNCs per well (Figs. 2B and 2C).

To determine whether A2BAR stimulation regulates osteoclastic cell-cell fusion, BAY 60-6583 was added along with RANKL at the beginning of culture or after RANKL treatment for 2 days (Fig. 2D). Both treatments resulted in a similar decrease in the number of larger MNCs and a similar increase in the number of TRAP-positive mononuclear cells (Figs. 2E-2G). These results demonstrate that A2BAR stimulation down-regulates osteoclastic cell-cell fusion.

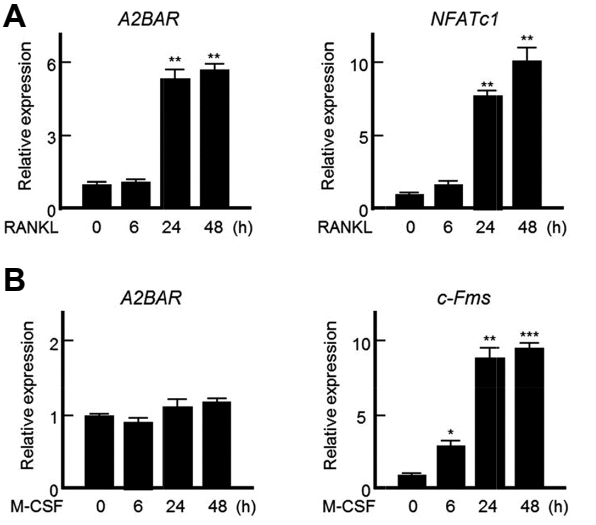

Fig. 1. RANKL induces A2BAR expression in BMMs. (A, B) BMMs were incubated with RANKL (200 ng/ml, A) or M-CSF (20 ng/ml, B) for the indicated time and then harvested, and the RNA isolates were analyzed by real-time PCR using *A2BAR*-, *NFATc1*- and *c-Fms*-specific primers. All quantitations were normalized to *hprt* expression. Results are representative of at least three independent experiments. $*p < 0.05$, $**p < 0.005$ and $***p < 0.001$ vs. non-treated cells. (C) BMMs were incubated with RANKL (200 ng/ml) for indicated times, and then total proteins from the cells were isolated and subjected to Western blot analyses. NFATc1 was used as a positive control for RANKL stimulation. The β-actin is shown as a loading control.

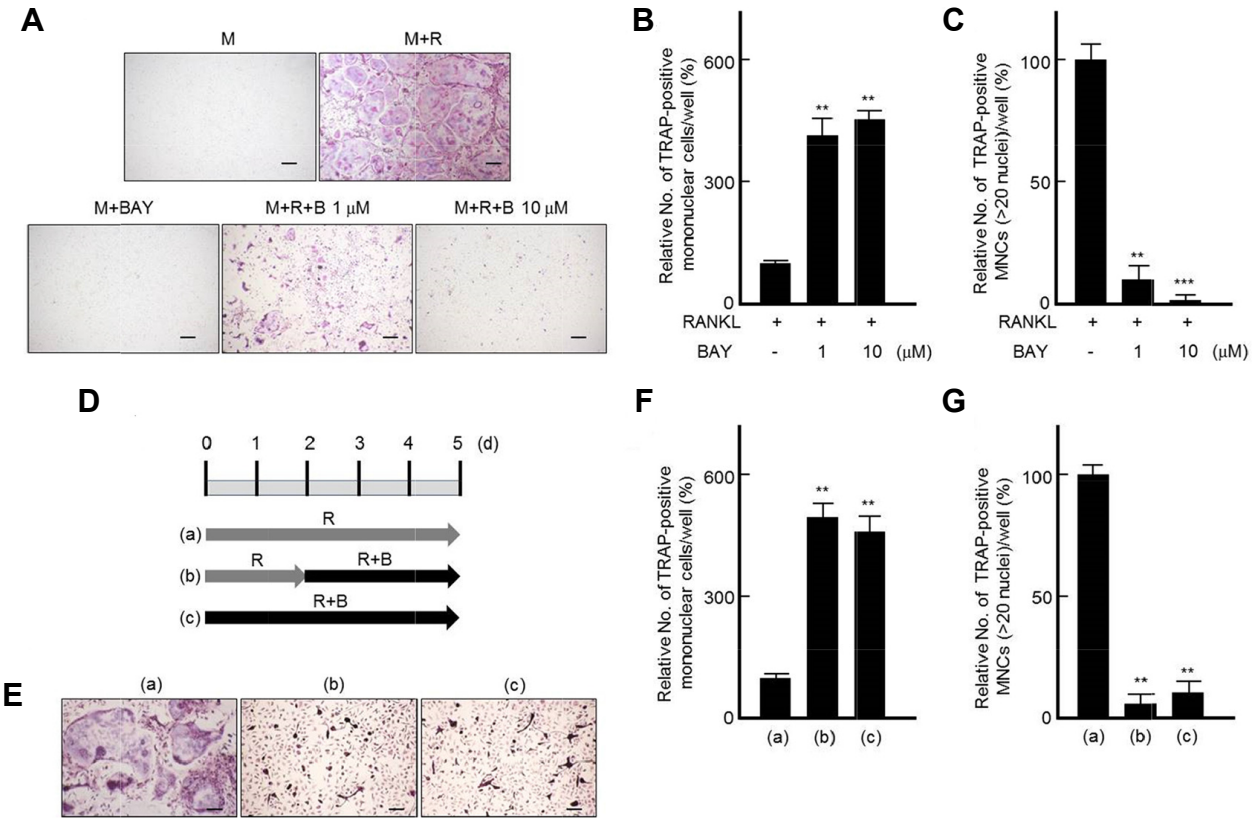

Fig. 2. A2BAR stimulation inhibits osteoclastic cell-cell fusion. (A-C) BMMs were incubated with or without indicated doses of BAY 60-6583 in the presence or absence of RANKL (200 ng/ml) for 5 days. TRAP staining was performed (original magnification, X 100) (A) and TRAP$^+$ mononuclear cells (B) or TRAP$^+$ MNCs larger than 100 μm in diameter containing more than 20 nuclei (C) were counted and presented as relative percentage (%). Scale bars, 100 μm. $^{**}p < 0.005$ and $^{***}p < 0.001$ vs. RANKL-treated cells. (D-G) Experimental scheme of mature osteoclast formation from BMMs (D). 5 μM of BAY 60-6583 was added on d 0, 2 after RANKL (200 ng/ml) treatment to culture media. The cells were incubated for 5 days, and then TRAP staining was performed (original magnification, X 100) (E). TRAP$^+$ mononuclear cells (F) or TRAP$^+$ MNCs larger than 100 μm in diameter containing more than 20 nuclei (G) were counted and presented as relative percentage (%). Scale bars, 100 μm. $^{**}p < 0.005$ vs. RANKL-treated cells.

A2BAR stimulation represses the expression of osteoclast marker genes

The results suggesting that stimulation of A2BAR regulated osteoclast differentiation led us to examine whether it regulates the expression of osteoclast marker genes including *NFATc1*, a master regulator of osteoclast differentiation, and *Atp6v0d2* and *DC-STAMP* which are both known to control the fusion of osteoclasts. Co-treatment with BAY 60-6583 and RANKL significantly suppressed the expression of osteoclast marker genes, *NFATc1, TRAP, c-Fos, CtsK and Mitf,* induced by RANKL (Fig. 3A). However, the expression of *Fosl1* (encoding Fra-1) was not changed by the co-treatment (Fig. 3A). Especially, A2BAR stimulation caused a notable reduction in the expression of *Atp6v0d2* and *DC-STAMP,* which are related to cell-cell fusion of osteoclasts (Fig. 3B). Western blot analysis supported the finding that BAY 60-6583 blocks the expression of NFATc1 and Atp6v0d2 induced by RANKL (Fig. 3C).

NFATc1 undergoes nuclear translocation and regulates the expression of various transcription factors. To investigate whether A2BAR stimulation regulates NFATc1 translocation, cells were treated with BAY 60-6583 in the presence of RANKL. Although RANKL increased the translocation of NFATc1, BAY 60-6583 blocked the nuclear translocation of NFATc1 induced by RANKL (Fig. 3D). Moreover, luciferase assay using NFATc1-luciferase plasmids supported the finding that stimulation of A2BAR inhibits the activity of NFATc1 induced by RANKL (Fig. 3E). Taken together, these results suggest that stimulation of A2BAR suppresses osteoclastogenesis by inhibiting the expression of osteoclast marker genes.

A2BAR stimulation inhibits the activation of ERK1/2, p38 MAP kinase and NF-kB by RANKL

The activation of MAP kinases is critical for RANKL-induced osteoclast differentiation. To investigate the underlying mechanism by which A2BAR regulates osteoclast differentiation, the change in MAP kinase activation by A2BAR stimulation was examined. Interestingly, the activation of ERK1/2 and p38 MAP kinases by RANKL was dramatically decreased

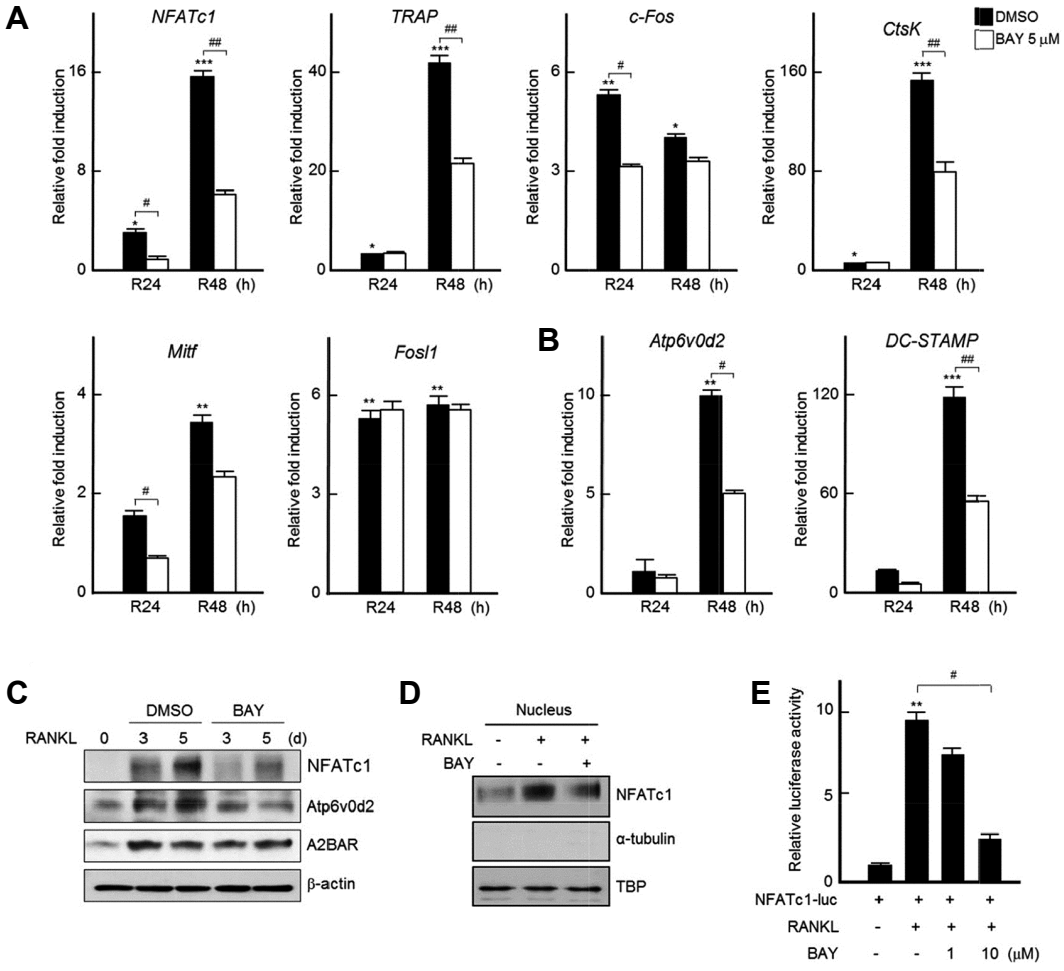

Fig. 3. BAY 60-6583 suppresses the expression of osteoclast marker genes. (A, B) BMMs were stimulated with or without 5 μM of BAY 60-6583 in the presence of RANKL for 24 h and 48 h. Isolated total RNAs from the cells were subjected to real-time PCR using the indicated gene-specific primers. All quantitations were normalized to *hprt* expression. Results are representative of at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.005$ and $^{***}p < 0.001$ vs. non-treated cells. $^{\#}p < 0.05$ and $^{\#\#}p < 0.005$ vs. RANKL-treated cells. (C) BMMs were treated with RANKL in the presence or absence of BAY 60-6583 for 3 days or 5 days. Total cell lysates were analyzed by Western blot analysis using specific antibodies against NFATc1, Atp6v0d2 and A2BAR. β-actin was shown as a loading control. (D) BMMs were treated with RANKL in the presence or absence of BAY 60-6583 (BAY, 5 μM) for 20 min. Isolated nuclear fractions were analyzed by Western blot analysis using specific antibodies against NFATc1. α-tubulin and TBP were shown as loading controls of cytosol and nuclear faction, respectively. (E) RAW264.7 cells were transfected with NFATc1-luciferase plasmid. After 36 h of transfection, cells were treated with RANKL and/or BAY 60-6583 for 12 h and then they were followed by luciferase assay. Results are representative of at least three independent experiments. $^{**}p < 0.005$ vs. non-treated cells, $^{\#}p < 0.05$ vs. RANKL-treated cells.

at 5 min and 15 min after BAY 60-6583 treatment, whereas JNK1/2 MAP kinase activity was not affected by the treatment (Fig. 4A). Moreover, BAY 60-6583 notably inhibited IκBα phosphorylation (Fig. 4A) and the activation of NF-kB, transcription factor critical for osteoclast formation, in a dose-dependent manner (Fig. 4B). These results imply that A2BAR stimulation represses osteoclastogenesis by inhibiting the activation of ERK1/2, p38 MAP kinases and NF-kB by RANKL.

JNK 1/2 MAP kinase signaling is involved in the expression of A2BAR by RANKL

To gain an insight into how A2BAR expression is regulated during osteoclastogenesis, the cells were pretreated with the inhibitors of MAP kinases and then incubated with RANKL for 5 days. As shown in Fig. 5, A2BAR expression was significantly abrogated by the pretreatment of SP600125, a JNK1/2 inhibitor. The pretreatment of SB203580, a p38 inhibitor, or PD98059, a MEK inhibitor, had little effect on the expression of A2BAR during RANKL-induced osteoclast differentiation. Accordingly, this result demonstrates that A2BAR expression is regulated by the JNK1/2 MAP kinase signaling pathway during RANKL-induced osteoclast differentiation.

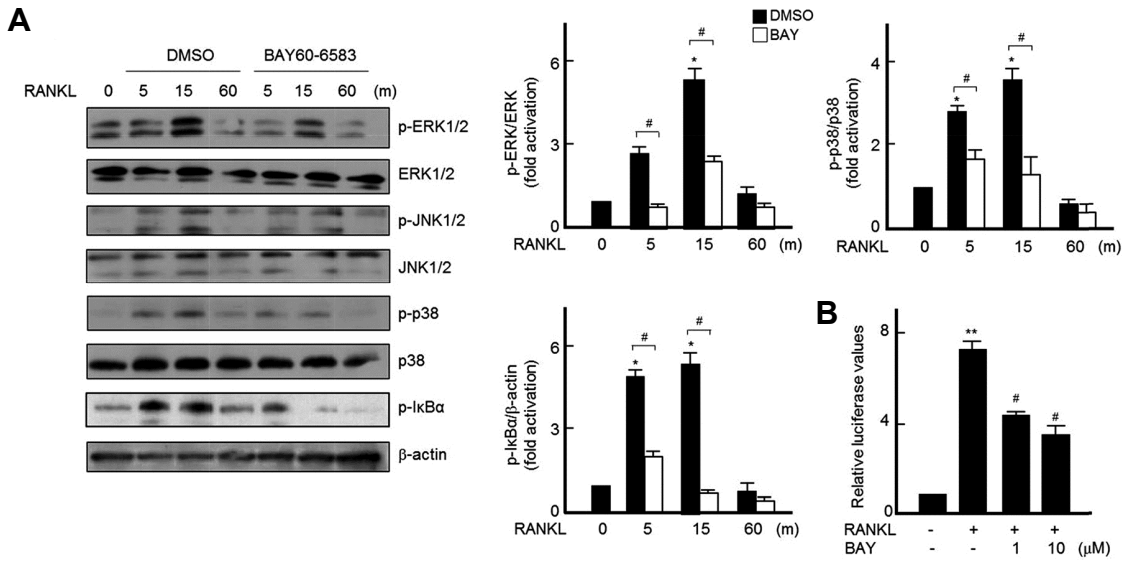

Fig. 4. BAY 60-6583 inhibits the activation of ERK1/2, p38 MAP kinase and NF-κB by RANKL. (A) Isolated BMMs were serum-starved with culture media containing 1% FBS for 6 h. RANKL (200 ng/ml) or BAY 60-6583 (5 μM) with RANKL was added for indicated times and total cell lysates were analyzed by Western blot analysis. Phosphorylated forms of ERK1/2, JNK1/2, p38 MAP kinases and IκBα were detected with phosphopeptide-specific antibodies. β-actin was shown as a loading control (Left). Protein bands were quantified by densitometry and presented (Right). $^{*}p < 0.05$ vs. non-treated cells, $^{\#}p < 0.05$ vs. RANKL-treated cells. (B) RAW264.7 cells were transfected with NF-κB-luciferase plasmid. After 36 h of transfection, cells were treated with BAY 60-6583 in the presence of RANKL for 12 h and then they were followed by luciferase assay. Results are representative of at least three independent experiments. $^{**}p < 0.005$ vs. non-treated cells, $^{\#}p < 0.05$ vs. RANKL-treated cells.

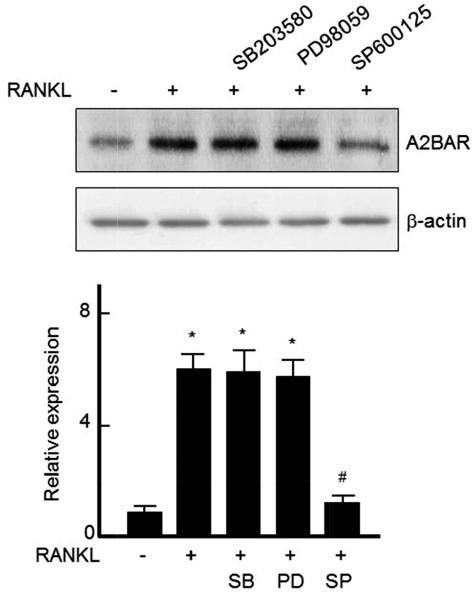

Fig. 5. RANKL increases A2BAR expression through JNK1/2 MAP kinase in BMMs. BMMs were pretreated with SB203580 (p38 inhibitor, 20 μM), PD98059 (ERK 1/2 inhibitor, 20 μM) or SP600125 (JNK 1/2 inhibitor, 20 μM) for 1 h and then incubated with RANKL for 5 days. Total cell lysates were analyzed by Western blot analysis. β-actin was shown as a loading control (upper panel). Protein bands were quantified by densitometry, and the level of A2BAR was normalized to that of β-actin (lower panel). $^{*}p < 0.05$ vs. non-treated cells, $^{\#}p < 0.05$ vs. RANKL-treated cells.

Abrogation of actin ring formation and bone resorption by A2BAR stimulation

Mature osteoclasts show bone resorption activity by constructing a sealing zone with a filamentous actin ring (Wilson et al., 2009). Therefore, we decided to determine the effects of A2BAR stimulation on actin ring formation and bone resorption. The addition of BAY 60-6583 abrogated the formation of actin ring with clear and dense margins induced by RANKL (Fig. 6A). Moreover, it caused a decrease in the RANKL-induced resorption ability of osteoclasts (Fig. 6B). These results suggest that A2BAR stimulation in osteoclasts inhibits actin ring formation and bone resorption induced by RANKL.

DISCUSSION

Bone remodeling is a process that requires a constant supply of energy and, when bones are damaged or repaired, increased extracellular nucleotide molecules such as ATP produce adenosine, a purine nucleoside (Bowler et al., 2001; Ham and Evans, 2012; Mediero and Cronstein, 2013; Orriss et al., 2010). Despite the importance of A2BAR on bone remodeling (Carroll et al., 2012; Corciulo et al., 2016; Trincavelli et al., 2014), regulatory mechanisms of A2BAR stimulation on the osteoclastogenesis remain unclear. The present study aimed to demonstrate the underlying mechanism of A2BAR stimulation on the osteoclastogenesis and finally assess the potential of A2BAR agonist as a therapeutics for bone-related diseases.

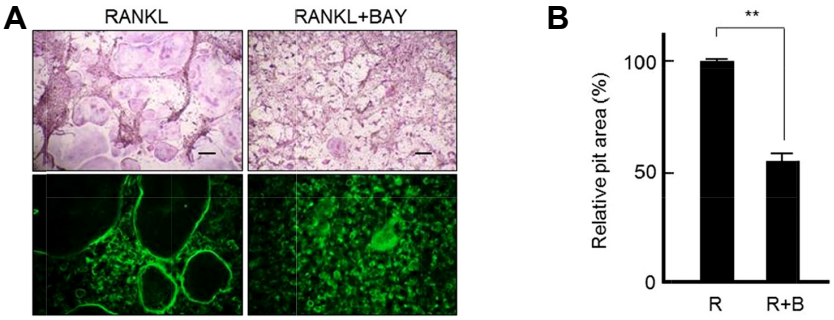

Fig. 6. The ablation of RANKL-induced actin ring formation and bone resorption in BAY 60-6583-treated cells. (A) After incubation of BMMs with RANKL or RANKL and BAY 60-6583 (BAY, 1 μM) for 5 days, the cells were subjected for TRAP staining (upper, original magnification, X 40) or incubated with Alexa Fluor 488-phalloidin for 20 min. After washing with PBS, the cells were photographed under a fluorescence microscope (lower, original magnification, X 200). Scale bars, 50 μm. (B) Mature osteoclasts on dentine discs were treated without or with BAY 60-6583 (R+B, 1 μM) in the presence of RANKL (R) for 24 h. The cells were removed from the dentine discs, and the resorption pits were stained with hematoxylin and the relative pit area was presented. $^{**}p < 0.005$ vs. RANKL-treated cells.

We found that A2BAR expression was notably increased by RANKL stimulation during osteoclast differentiation whereas M-CSF alone failed to affect A2BAR expression. Moreover, the expression of A2BAR was regulated through JNK1/2 MAP kinase signaling pathway since SP600125, an inhibitor of JNK1/2 MAP kinase, completely decreased the expression of A2BAR induced by RANKL. These results suggest that A2BAR expression is regulated through JNK1/2 MAP kinase signaling pathway during osteoclastogenesis by RANKL.

Binding of RANKL to RANK leads to recruitment of TNF receptor-associated factor (TRAF) adaptor proteins including TRAF6 and transmits signals to downstream targets such as JNK1/2, ERK1/2, p38 and NF-κB to induce osteoclastogenesis (Boyle et al., 2003; Burgess et al., 1999; Ko et al., 2015). A2BAR agonist seems to regulate osteoclastogenesis through ERK1/2, p38 and NF-κB since A2BAR stimulation with BAY 60-6583 effectively suppressed osteoclast formation in a dose-dependent manner and decreased the activation of ERK1/2 and p38 MAP kinases. However, JNK1/2 activation was not affected by it. In addition, BAY 60-6583 treatment effectively prevented IκBα phosphorylation, and ultimately resulted in the inhibition of NF-κB activation by RANKL.

A2BAR stimulation inhibited the expression of osteoclast marker genes, TRAP, c-Fos, cathepsin K and MITF in BMMs. Moreover, not only the expression of NFATc1, a master transcription factor for the osteoclast differentiation, but also nuclear translocation and activity of NFATc1 were also prevented by it, which supports the possibility that A2BAR may serve as an endogenous therapeutic target in various bone pathological states.

BAY 60-6583 dramatically reduced the cell-cell fusion by RANKL stimulation via blocking the expression of DC-STAMP and Atp6v0d2 since A2BAR stimulation significantly suppressed the expression of DC-STAMP and Atp6v0d2 induced by RANKL, and the formation of giant MNCs was also decreased by A2BAR stimulation. However, TRAP-positive mononuclear cells were relatively increased compared to when RANKL alone was treated because RANKL markedly increased the number of giant MNCs.

A2BAR stimulation effectively prevented the actin ring formation and bone-resorbing activity of osteoclasts. Moreover, the result that A2BAR stimulation inhibited the expression of TRAP and cathepsin K affecting the functional activity of osteoclast by regulating bone matrix resorption (Roberts et al., 2007) strongly supports that A2BAR stimulation regulates not only osteoclast formation but also osteoclast function. It may be possible that BAY60-6583 suppresses p-ERK and p-p38 through independent pathway of A2BR.

Different from BAY 60-6583, a selective antagonist of A2BAR, MRS 1754, had little influence on osteoclast differentiation (Supplementary Fig. S1). Instead, MRS 1754 pretreatment reversed the effect induced by BAY 60-6583 during RANKL-induced osteoclast differentiation (Supplementary Fig. S1). Indeed, adenosine receptor antagonists seem to work as a reverse agonist (Chen et al., 2013; He et al., 2013a; 2013b). For example, rolofylline, an adenosine A1 receptor antagonist, diminishes osteoclast differentiation as an inverse agonist (Chen et al., 2013; He et al., 2013a; 2013b).

Several reports have shown that caffeine has profound biological effects as an antagonist of adenosine receptors and many of its actions are due to reduction in the number of adenosine receptors to half of the normal levels (Chen et al., 2013; Yang et al., 2009). In the previous study, we demonstrated that caffeine enhanced osteoclast differentiation and maturation by RANKL through p38 MAP kinase and by inducing Mitf, DC-STAMP, CtsK and TRAP genes (Choi et al., 2013), which occurs via a mirror regulatory mechanism compared to the mechanism of action of BAY 60-6583. The regulatory association between caffeine and A2BAR during osteoclastogenesis needs to be studied further.

Collectively, this study contributes to better understanding on the regulatory mechanisms of osteoclastogenesis by A2BAR stimulation. Ultimately, our results suggest the possibility that A2BAR could be a good therapeutic target for bone loss, and the therapeutic potential of A2BAR agonists in numerous bone and metabolic diseases.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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