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The effect of P2X7R-mediated Ca^{2+} signaling in OPG-induced osteoclasts adhesive structure damage

Yonggang Ma[a,b,c,1], Hongyan Zhao[a,b,c,1], Chung Chile[a,b,c], Chao Wang[a,b,c], Jiaming Zheng[a,b,c], Ruilong Song[a,b,c], Hui Zou[a,b,c], Jianhong Gu[a,b,c], YanYuan[a,b,c], Jianchun Bian[a,b,c], Zongping Liu[a,b,c,*]

[a] College of Veterinary Medicine, Yangzhou University, Yangzhou, Jiangsu, 225009, PR China
[b] Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou, Jiangsu, 225009, PR China
[c] Joint International Research Laboratory of Agriculture and Agri-Product Safety of the Ministry of Education of China, Yangzhou University, Yangzhou, Jiangsu, 225009, PR China

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ABSTRACT

Osteoclast adhesion is important for bone resorption. Osteoprotegerin inhibits osteoclast differentiation and bone resorption via Ca^{2+} signaling. Purinergic receptor P2X7 (P2X7R) affects osteoclastogenesis by activating transcription factor nuclear factor of activated T cells 1 (NFATc1). However, the detailed mechanism of osteoprotegerin-mediated P2X7R modulation of osteoclast adhesion is unclear. This study aimed to determine the effect of P2X7R on osteoprotegerin-induced damage to osteoclast adhesion. Osteoprotegerin reduced the expression of P2X7R, and protein tyrosine kinase 2 (PYK2) and SRC phosphorylation, and reduced calcium concentration, significantly decreasing Ca^{2+}-NFATc1 signaling. 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N'-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA-AM)/*N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7) partly or absolutely recovered osteoprotegerin-induced osteoclasts adhesion structure damage, including increased the PYK2 and SRC phosphorylation, changed the distribution of PYK2/SRC and integrinαvβ3, and inhibited retraction of lamellipodia and filopodia and recovered osteoclast bone resorption activity. In addition, BAPTA-AM/W-7 also increased osteoprotegerin-induced activation of Ca^{2+}-NFATc1 signaling, and restored normal P2X7R levels. P2X7R knockdown significantly inhibited osteoclast differentiation, and the formation of lamellipodia and filopodia, reduced the PYK2 and SRC phosphorylation, and inhibited Ca^{2+}-related protein activation. However, P2X7R knockdown aggravated osteoprotegerin-induced osteoclast adhesion damage via Ca^{2+} signaling. In conclusion, the P2X7R–Ca^{2+} NFATc1 signaling pathway has a key functional role in osteoprotegerin-induced osteoclast adhesion structure damage.

1. Introduction

Bones are dynamic organs that are constantly renewed throughout life. This constant and balanced bone metabolism relies on bone remodeling mediated by osteoblasts (OB) that form bone and osteoclasts (OC) that reabsorb bone [1]. An imbalance between OBs and OCs occurs in many mammalian bone diseases, such as osteoporosis, arthritis, and bone tumors [2]. Osteoclasts are multinucleated macrophages that originate from hematopoietic stem cells [3]. A soluble tumor necrosis factor (TNF)-receptor family member, termed osteoprotegerin (OPG), is a decoy receptor for the pro-osteoclastic cytokine receptor activator of nuclear factor kappa-B ligand (RANKL) with a similarly high affinity to RANKL for its receptor, RANK, which can inhibit OC formation and bone resorption [4]. A previous study also showed that OPG inhibits OC differentiation and formation via calcium signaling [5]. Our team found that OPG induces podosome disassembly in OCs through calcium signaling pathways [6]. Osteoclasts resorb bone through the formation of a unique adhesion structure called the sealing zone. The sealing zone facilitates tight adhesion to bone to form an acidic, protease-rich microenvironment at the bone surface that promotes its resorption. Thus, the OC sealing zone is essential for OC function [7]. Adherence of OCs to the bone matrix activates integrin molecules, resulting in increased phosphorylation of podosomal-associated proteins, such as protein tyrosine kinase 2 (PYK2) and SRC [8]. Our previous research showed

* Corresponding author. 88 South University Ave., Yangzhou, Jiangsu, 225009, PR China.
E-mail address: liuzongping@yzu.edu.cn (Z. Liu).
[1] These authors contributed equally to the this work.

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that SRC functions as an adaptor protein that competes for PYK2 and relocates it from the peripheral adhesive zone to the central region of OCs in response to OPG treatment. OPG induces podosome reassembly and peripheral adhesive structure detachment by modulating the phosphorylation of PYK2 and SRC and their intracellular distribution in osteoclasts [9]. Although OPG clearly inhibits OC bone resorption, the molecular mechanisms underlying its effects remain largely unknown.

Several studies have indicated that ATP, as a stimulator, triggers a transient rise in intracellular Ca^{2+} concentrations in OCs. P2 purinergic receptors are involved in this rise in the Ca^{2+} concentration [10]. Herefore, P2 purinergic receptors might have a critical role in OC development. Purinergic receptor P2X 7 (P2X7R) is one of the seven subtypes of P2X preceptors. P2X7R is an ATP sensitive, non-selective cationic gate channel receptor that is widely expressed in various tissues, including in mature osteoclasts [11]. P2X7 activation is thought to be important for the differentiation, survival, adhesion, and fusion of OCs [12]. ATP signaling induces membrane depolarization in OCs, leading to increase in intracellular Ca^{2+}. This increase results in the activation of key signaling molecules, such as protein kinase C (PKC), nuclear factor kappa B (NF-κB), and nuclear factor of activated T cells 1 (NFATc1), which rely on elevated cytosolic Ca^{2+} to regulate OC resorption [13]. Thus, we speculated that P2X7R-mediated Ca^{2+} signaling might affect the function of the OC adhesion structure.

The present study aimed to investigate the damaging effects of OPG on P2X7R-mediated Ca^{2+} signaling and to determine the molecular mechanism. The results may identify a new target for the clinical treatment of bone metabolism diseases.

2. Materials and methods

2.1. Experimental animals and reagents

BALB/c mice were purchased from the Comparative Medicine Centre of Yangzhou University (Yangzhou China). All cell culture media and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). Recombinant mouse OPG, recombinant mouse RANKL, and recombinant mouse macrophage colony-stimulating factor (M-CSF) were obtained from R&D systems (Minneapolis, MN, USA). The calcium chelator 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N'-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA-AM) was supplied by AMQUAR (Denver, CO, USA). The calmodulin antagonist *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7) was purchased from R&D systems. The mouse ATP ELISA kit and mouse Ca^{2+} ATPase ELISA kit were purchased from Mibio (Shanghai, China). The tartrate-resistant acid phosphatase (TRAP) kit 387-A; rhodamine phalloidin; 4,6-diamidino-2-phenylindole (DAPI); L-glutamine; penicillin; and streptomycin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The anti-calcium/calmodulin dependent protein kinase IV (CAMKIV) (phosphoT196 + T200), *anti*-CAMKIV, anti-calcium/calmodulin dependent protein kinase II (CAMKII), anti-Calmodulin, *anti*-NFATc1, *anti*-PYK2 (PhosphoY402), anti-peroxisome proliferator-activated receptor-gamma (PPAR-gamma) coactivator-1 beta (PGC-1 beta), anti-plasma membrane calcium-transporting ATPase (PMCA), and *anti*-PYK2 antibodies were obtained from Abcam (Cambridge, UK). The anti-rabbit c-Fos, anti-rabbit phospholipase C gamma 2 (PLC γ2), anti-rabbit SRC, anti-rabbit phospho-SRC Family (Tyr416)., *anti*-β-actin, anti-rabbit phospho- Ca^{2+}-CAM-dependent kinase (CAMK)-cAMP response element-binding protein (CREB) (Ser133), *anti*-phospho-CAMKII (Thr286), anti-rabbit phospho-SRC Family (Tyr527) rabbit, *anti*-CREB, and anti-rabbit phospho-PLC γ antibodies were purchased from Cell Signaling Technology (Boston, MA, USA). The P2X7R agonist 2′ (3′)-O-(4-Benzoylbenzoyl) ATP (BzATP) was obtained from Abcam. The AD-P2X7R-short hairpin RNA (shRNA) was obtained from Hanhen (Shanghai, China). Other chemicals and reagents were purchased locally at analytical grade.

2.2. Cell culture

Bone marrow cells were obtained from the femurs and tibias of male Balb/c mice at 4–6 weeks old, and bone marrow macrophages (BMMs) were cultured in α-minimal essential medium (MEM) containing 10% FBS in the presence of 30 ng/ml M-CSF and 60 ng/ml RANKL for 5–6 days. The medium was replaced every 2 days. After 5 days of incubation, the cells were incubated with OPG at the indicated concentrations for another 30 min.

2.3. TRAP staining

Multinucleated cells were stained for TRAP, an enzyme released by mature OCs during bone resorption. Multinucleated TRAP-positive cells containing at least three nuclei were identified as OC-like cells using an inverted phase contrast microscope (LEICADMI 3000B, Wetzlar, Germany).

2.4. Intracellular Ca^{2+} measurement

Isolated BMMs were plated on specialized cell culture dishes for laser confocal microscopy in the presence of M-CSF (30 ng/ml) and RANKL (60 ng/ml) for 5 days. Cells were then incubated in the presence of 5 μM fluo-3 acetomethyl (AM), a fluorescent indicator of intracellular calcium, for 30 min in α-MEM with 10% FBS. After washing three times with α-MEM, the cells were observed in the presence of OPG (80 ng/ml) under a laser scanning confocal microscope for 30 min, at an excitation wavelength of 488 nm, and emission at 525–530 nm for fluo-3. The images were digitized and analyzed by Nis-Elements (Nikon, Japan).

2.5. Immunofluorescence staining

Osteoclasts were generated on bone slices from bone marrow in the presence of M-CSF (30 ng/ml) and RANKL (60 ng/ml). Cells were fixed in 4% paraformaldehyde for 30 min and permeabilized in 0.1% Triton X-100 for 10 min. Cells were then blocked in 5% normal goat serum for 20 min, and incubated with rabbit monoclonal antibodies diluted in 5% bovine serum albumin-phosphate buffered saline (BSA-PBS) overnight at 4 °C. Thereafter, cells were washed in PBS, incubated with fluorescein isothiocyanate (FITC)- labeled Goat Anti-Rabbit IgG antibodies for 1 h and washed. F-actin was stained with phalloidin at a 1:40 dilution and nuclei were visualized using 1 mg/ml DAPI. Cells were examined using a scanning laser confocal imaging system.

2.6. Western blotting and immunoprecipitation

BMMs were cultured with M-CSF (30 ng/ml) and RANKL (60 ng/ml) for 5 days. After OPG treatment, the cells were washed with cold PBS and lysed in Radioimmunoprecipitation assay (RIPA) buffer on ice. The cells were collected by centrifugation at 12,000 × *g* for 10 min at 4 °C. The protein concentration was determined using a BCA protein assay kit (Shang hai, China). Immunoprecipitation was achieved using SureBeads ProteinG (BIO-RAD, USA). Total lysates were diluted with 6 × SDS sample buffer and boiled for 10 min. Equivalent amounts of protein were separated on 10% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes. Each membrane was blocked for 2 h using 5% Skim milk and then incubated with a 1:1000 dilution of the primary antibody and then with peroxidase-coupled secondary antibodies (1:5000). Immunoreactive proteins were detected using chemiluminescence. The detected bands were quantified using the ImageJ Software (NIH, Bethesda, MD, USA).

2.7. Scanning electron microscopy (SEM)

BMMs were plated on coverslips for 5 days. After OPG treatment, the cells were fixed in 2.5% glutaraldehyde solution overnight at 4 °C,

and then dehydrated through an alcohol gradient and dried. To observe their morphology, the specimens were coated with gold using an SCD 500 sputter-coater (Leica, Wetzlar, Germany) and examined using a Hitachi S-4800 field-emission environmental scanning electron microscope (Hitachi, Tokyo, Japan).

2.8. Osteoclast absorption activity assay

BMMs were seeded to 48-well plates with bovine cortical bone slices in the presence of M-CSF and RANKL. After different reagent treatment, the cells were fixed in 2.5% glutaraldehyde solution overnight at 4 °C, and then dehydrated through an alcohol gradient and dried. To observe their morphology, the specimens were coated with gold using an SCD 500 sputter-coater (Leica, Wetzlar, Germany) and examined using a GeminiSEM 300 field-emission environmental scanning electron microscope (Carl Zeiss, Germany).

2.9. Measurement of ATP and ATPase

BMMs were seeded at a density of 1.2×10^5 cells/well in 6-well plates for 5 days. After OPG treatment for 30 min, the samples were collected and measured using mouse ATP ELISA and ATPase ELISA kits. The data were read using a multi-mode plate reader (Vermont, USA).

2.10. Statistical analysis

Statistical analyses were performed using ANOVA or Student's *t*-test in Prism4.03 (GraphPad Software, La Jolla,CA,USA). Values of $P < 0.05$ were considered significant. Each experiment consisted of at least 3 biologic replicates. Results are presented as the mean ± SD.

3. Results

3.1. OPG impaired osteoclast adhesion structure and inhibits the phosphorylation of Ca^{2+} signaling related proteins in osteoclasts

PYK2 and SRC are both required for osteoclast cytoskeletal organization [14], which regulate osteoclast adhesion function, As show in Fig. 1A and B, OPG significantly inhibited the phosphorylation of PYK2 and SRC (Fig. 1A). Meanwhile, SEM analysis indicated the OPG impaired the formation of osteoclast lamellipodia and filopodia. However, Calcium signaling plays a critical role in the differentiation of OCs [15], which cytoplasmic Ca^{2+} oscillations occur during RANKL-mediated osteoclastogenesis; therefore, we examined the effects of OPG on calcium concentration in OCs. As show in Fig. 2A, the result showed that OPG significantly reduced calcium concentration. To further investigate the effect of Ca^{2+} signaling in OPG-treated OCs, RANKL-induced OCs were treated with of OPG for 30 min. We first examined the levels of calmodulin/calmodulin kinase in mature OCs. As show in Fig. 2B, the results showed that OPG significantly inhibited the phosphorylation of calmodulin/calmodulin kinase in a dose-dependent manner.

We then further investigate the effects of calcium downstream signaling in mature OCs. CREB is essential in the regulation of RANKL-induced NFATc1 activity [16]. As show in Fig. 2B, OPG significantly suppressed CREB phosphorylation and obviously inhibited the activation of PLC. Peroxisome proliferator-activated receptor gamma coactivators-1β (PGC1β) is a master regulator of mitochondrial biogenesis and respiration. Our results showed that the expression level of PCG1β was significantly reduced by OPG in a dose-dependent manner. Therefore, these results indicated that OPG inhibits the Ca^{2+}/CREB/PLC/PCG1β signaling pathways. c-Fos and NFATc1 is the most important OC-specific transcription factors. The levels of c-Fos and NFATc1 were significantly reduced by OPG treatment. The above results indicated that OPG impaired osteoclast adhesion structure and blocks the Ca^{2+}/CREB/PLC/PCG1β-NFATc1 signaling pathway.

3.2. BAPTA-AM/W-7 relieved OPG-induced disruption of OC adhesion structures and recovered OPG-induced location of adhesion key protein and bone resorption activity

To study the effect of BAPTA-AM (a Ca^{2+} specific chelator) on the OPG-induced damage to OC adhesion structures, OC morphology was observed using SEM. As show in Fig. 3A, the control OCs had a characteristic morphology, showing an adhesion structure with a large circular zone containing multiple lamellipodia and filopodia. Treatment with BAPTA-AM(5uM) no affected cell viability Fig. s1, caused no apparent changes to the OC adhesion structure. However, treatment of OCs with 80 ng/ml OPG caused damage to the cell adhesion structure, including retraction of lamellipodia and filopodia. BAPTA-AM clearly attenuated the effects of OPG on the retraction of OC adhesion structures. However, W-7 (a calmodulin antagonist, 10uM) no affected cell viability Fig. s1, which also had the same effect. However, the intact adhesion structure is the premise of osteoclast bone resorption. As show in Fig. 3B, BAPTA-AM and W-7 obviously recovered OPG-induced osteoclast bone resorption function. In previous studies, SRC/PYK2 and integrins was shown to play a critical role in forming the actin ring structure in the peripheral belt of podosomes. To gain further insights into the mechanism underlying the dysfunction of OCs after treatment with OPG, confocal microscopy analysis of immunofluorescent labeling of actin, PYK2/SRC and integrin αvβ3 in OCs was performed. As show in Fig. 3C, the results suggested that PYK2/SRC and integrin αvβ3 were localized to the peripheral adhesive region in the control and BAPTA-AM groups. OPG treatment disrupted the peripheral distribution of ring-like, actin-containing podosome structures, and the location of PYK2/SRC and integrin αvβ3. BAPTA-AM treatment mostly recovered the location of PYK2/SRC and integrin αvβ3. However, W-7 (a calmodulin antagonist) also had the same effect. These data suggested that OPG disturbed actin ring formation and inhibited the function of OCs by changing the location of PYK2/SRC and integrin αvβ3 and bone resorption.

3.3. BAPTA-AM/W-7 inhibited OPG-induced reduction in phosphorylation of SRC/PYK2 and key Ca^{2+} signaling proteins

BAPTA-AM (a Ca^{2+} specific chelator) was used to further investigate whether OPG impaired osteoclast adhesion structure via disrupting Ca^{2+} signaling in OCs. PYK2 and SRC are both required for OC cytoskeletal organization [17]. Osteoclasts were pretreated with BAPTA-AM (5 μm) for 30 min and then treated with 80 ng/ml OPG for another 30 min. As show in Fig. 3D, compared with the control, a significant decrease in phosphorylation of PYK2 at Tyr402 and SRC at Tyr416 and Tyr527 were observed in OPG-treated OCs. However, the addition of BAPTA-AM and W-7 increased phosphorylation of PYK2 at Tyr402 and SRC at Tyr416 and Tyr527 in OPG-treated OCs. Our results showed BAPTA-AM and W-7 effectively recovered the phosphorylation level of SRC and PYK2 after OPG treatment, Next, we investigated whether OPG regulates the PYK2-SRC complex, as assessed using an immunoprecipitation assay. As show in Fig. 3D, the results showed that OPG significantly reduced the protein interaction between PYK2 and SRC, whereas BAPTA-AM significantly enhanced PYK2 and SRC protein association. Interestingly, As shown in Fig. 3E, Co-treatment with OPG and BAPTA-AM/W-7 also significantly restored the phosphorylation level of Ca^{2+} signaling proteins (CAM, CAMKII, CAMKIV, CREB, PLC) and expression of PCG1β, NFAT1, and c-fos. Therefore, Ca^{2+} signaling plays an essential role in regulating osteoclast function.

3.4. OPG increases ATP release from osteoclasts and the expression of PMCA, but reduces Ca^{2+}-ATPase activity and expression of P2X7R in mature osteoclasts

Previous studies indicated that ATP could be released from the mechanically stimulated osteoblasts or osteocytes, and which further

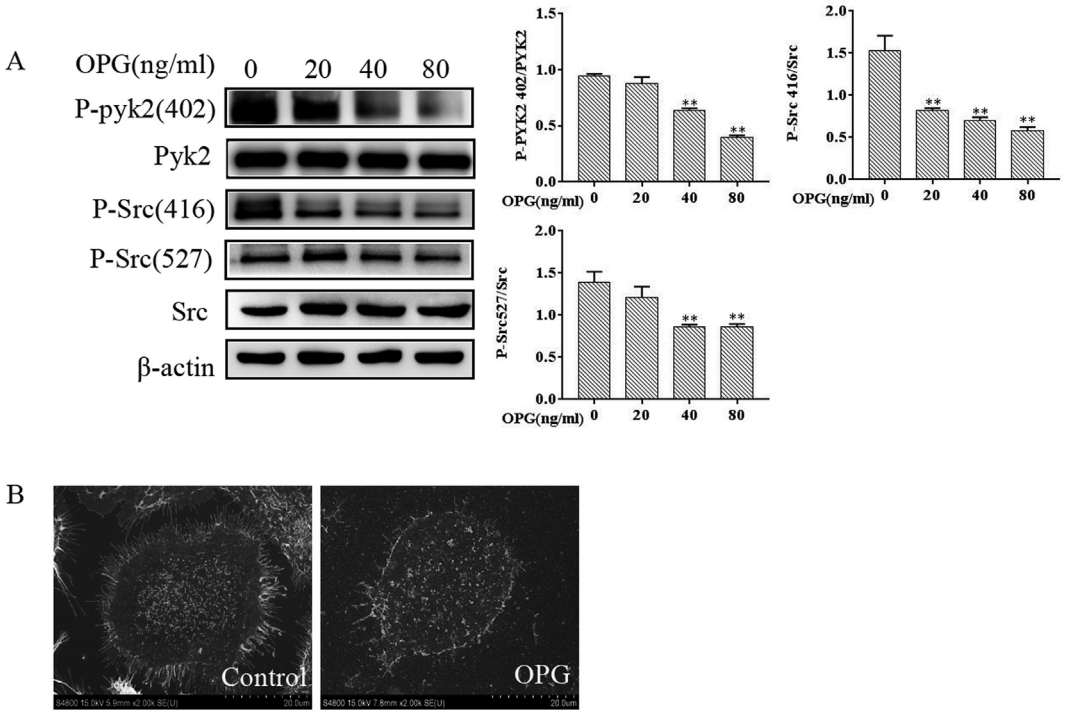

Fig. 1. OPG impaired osteoclast adhesion. BMMs were cultured in the presence of M-CSF (30 ng/ml) and RANKL (60 ng/ml) for 5 days. (A) Osteoclasts were treated with OPG (0, 20, 40, and 80 ng/ml) for 30 min and lysed; the phosphorylation level of PYK2 and SRC were determined using western blotting, $^{**}p < 0.05$ *vs.* the control. (B) Osteoclasts were treated with 80 ng/ml OPG for 30 min, and then the osteoclast adhesion structure were observed using SEM. All experiments were repeated at least three times.

binds the purinergic P2 receptor on the neighboring cells to cause calcium release from the ER [18]. To determine whether OPG affects ATP release and Ca^{2+}-ATPase activity in mature osteoclast. As shown in Fig. 4A, the results suggested that OPG increased ATP release from intracellular stores, significantly increasing the extracellular ATP levels. Meanwhile, OPG remarkably inhibited Ca^{2+}-ATPase activity in a dose-dependent manner. However, the protein level of PMCA significantly increased. These results demonstrated that OPG strongly affects OC homeostasis via disrupting ATP levels and Ca^{2+}-ATPase. Then, we investigated the effect of OPG on P2X7R expression in RANKL-induced OCs. As shown in Fig. 4B, the results showed that OPG significantly reduced the expression of P2X7R in time and dose-dependent manners.

3.5. P2X7R knockdown inhibits osteoclast differentiation and reduced intracellular ATP level and Ca^{2+}-ATPase activity

To further demonstrate whether functional P2X7R affects ATP release and Ca^{2+}-ATPase activity. As shown in Fig. 5A, the result showed that P2X7R knockdown obviously reduced intracellular ATP level compared with NC-shRNA, and significantly inhibited the Ca^{2+}-ATPase activity. However, the expression of PMCA increased remarkably. P2X7R knockdown intensified OPG-induced ATP release from the cells. Meanwhile, P2X7R knockdown further inhibited OPG-induced Ca^{2+}-ATPase activity, but significantly increased the OPG-induced expression of PMCA. These findings indicated that P2X7R modulated the OPG-induced ATP level and Ca^{2+}-ATPase activity. However, to further examine the biological function of P2X7R during OC differentiation. As shown in Fig. 5B, TRAP staining revealed that P2X7R knockdown reduced the number of TRAP-positive multinucleated cell. Therefore, our results indicated that P2X7R knockdown affected OC differentiation.

3.6. P2X7R knockdown aggravated OPG-induced OC adhesion structure damage and promoted OPG-induced the reductions in Ca^{2+} signaling protein

To further investigate the potential role of P2X7R in OC function. As show in Fig. 6A, P2X7R knockdown decreased the levels of phosphorylated PYK2 and SRC. P2X7R knockdown also disrupted the association of PYK2 and SRC by using immunoprecipitation. Similarly, after treatment with OPG, P2X7R knockdown promoted OPG-induced reduction in PYK2 and SRC phosphorylation, and disrupted the association of PYK2 with SRC. By contrast, BzATP (a P2X7R agonist) exerted the opposite effects. As shown in Fig. 6B, SEM allowed us to analyze the morphological changes of adhesion structures in mature OCs. P2X7R knockdown cells showed no obvious lamellipodia and filopodia in the periphery of cells. The data suggested that P2X7R knockdown suppressed OC podosome formation. Our study further found that P2X7R knockdown aggravated OPG-induced OC adhesion structure damage, mainly via the disappearance of the podosome. However, As show in Fig. 6C, interestingly, P2X7R knockdown also aggravated OPG-induced the reduction of osteoclast bone resorption activity. BzATP (a P2X7R agonist, 300uM) no effect cell viability Fig. s1, and exerted the opposite effects. To further reveal the effect of P2X7R on OPG-induced OC adhesion structure damage, we demonstrated the localization of PYK2/SRC and integrinαvβ3 in mature OCs after treatment with P2X7R shRNA or control shRNA. As show in Fig. 6D, PYK2/SRC and integrinαvβ3 colocalized with dense actin ring in the control shRNA group, while PYK2/SRC and integrinαvβ3 was diffusely distributed throughout the cytoplasm in the P2X7R shRNA group. These results suggested that P2X7R knockdown affected the localization of PYK2/SRC and integrinαvβ3. More importantly, P2X7R knockdown aggravated the OPG-induced abnormal colocalization of PYK2/SRC and integrinαvβ3 in mature OCs. BzATP (a P2X7R agonist) had the opposite effects. Therefore, these results showed that P2X7R knockdown aggravated OPG-induced OC adhesion structure damage.

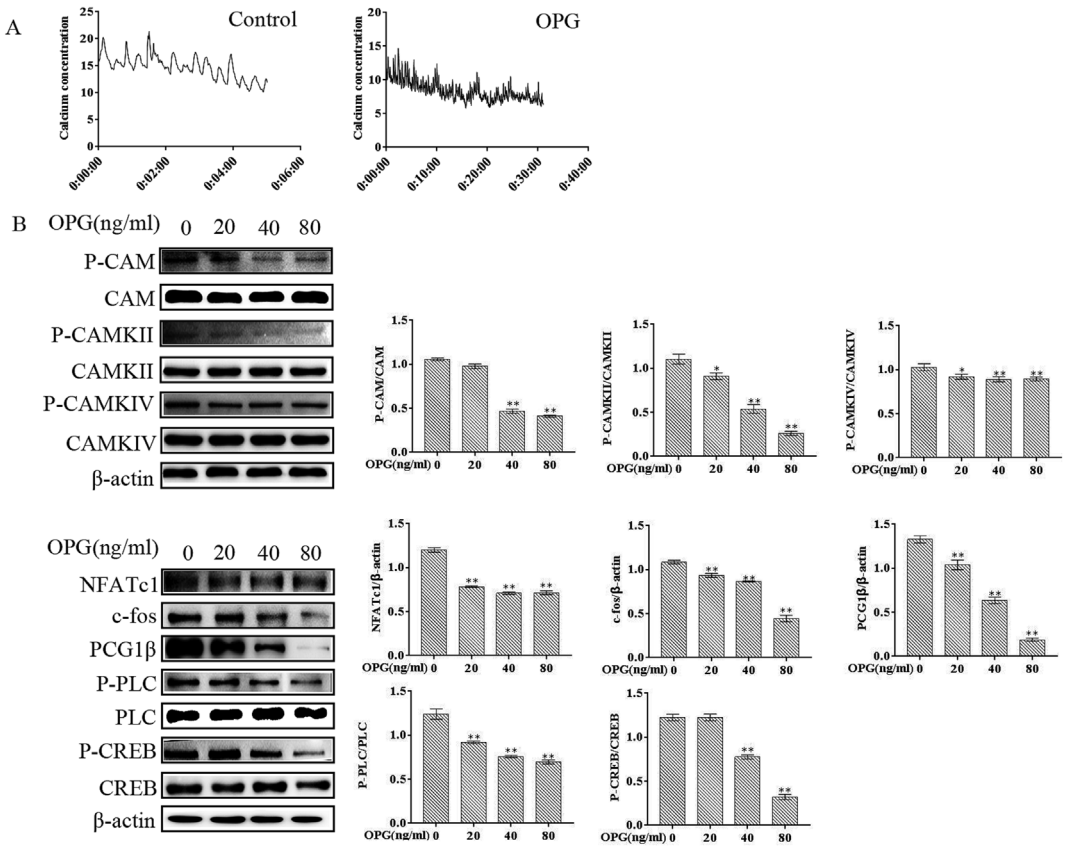

Fig. 2. OPG reduced RANKL-induced calcium concentration and inhibited phosphorylation of Ca^{2+} signaling-related proteins. BMMs were plated and cultured on specialized cell culture dishes for laser confocal microscopy and in 6-well plates in the presence of M-CSF (30 ng/mL) and RANKL (60 ng/ml) for 5 days. (A) Osteoclasts were incubated with Fluo-3 (5 μM) dye before analysis using laser confocal microscopy, followed by 80 ng/ml OPG for another 30 min before being examined using confocal microscopy. The results shown are tracings of a typical experiment with similar results observed in three separate experiments. (B) Osteoclasts were treated with OPG (0, 20, 40, and 80 ng/ml), lysed, and subjected to western blotting to determine the levels of Ca^{2+} signaling-related proteins, $^{**}p < 0.05$ *vs.* the control. Each blot is representative of at least three replicate experiments.

To examine the effect of P2X7R on OPG-induced Ca^{2+} signaling pathways. As shown in Fig. 6E, the result showed P2X7R knockdown significantly inhibited the phosphorylation of Ca^{2+} signaling protein, including CAM, CAMKII, CAMKIV, CREB, and PLC. Meanwhile, the levels of PCG1, NFATc1, and c-Fos were also significantly reduced. However, after treatment with OPG, P2X7R knockdown significantly promoted the OPG-induced reduction in Ca^{2+} signaling proteins. By contrast, BzATP (a P2X7R agonist) exerted opposite effects. Therefore, P2X7R-mediated Ca^{2+} signaling plays an essential role in regulating osteoclast adhesion structure.

4. Discussion

Recently, we reported that OPG disrupts the peripheral adhesion structure of OCs by modulating PYK2 and SRC activation [19]. We also found that OPG induces podosome disassembly in OCs through calcium, ERK, and P38 MAPK signaling pathways. However, ATP/P2X7R-dependent influx of Ca^{2+} accelerates NF-κB activation, followed by OC formation [20]. In the present study, BMMs were transfected with a P2X7 shRNA in the presence of M-CSF and RANKL. P2X7R knockdown suppressed RANKL-stimulated OC differentiation and aggravated OPG-induced OC adhesion structure damage. Therefore, P2X7R may play an important role in regulating OC function.

Ca^{2+} signaling pathways play a pivotal role in RANKL-induced OC differentiation. In this study, we found that OPG significantly reduced intracellular calcium concentration in mature OCs, and inhibited the phosphorylation of calmodulin/calmodulin kinase. These data are consistent with our hypothesis that OPG decreases RANKL-induced calmodulin/calmodulin kinase phosphorylation levels by reduced calcium concentration. Nevertheless, the precise molecular mechanisms of OC differentiation have been clarified in recent studies [21,22]. They showed that RANKL evokes Ca^{2+} oscillation, leading to Ca^{2+}-CAM-calcineurin-mediated activation of NFATc1 and c-Fos. However, the transcriptional activity of NFATc1 is maintained via CREB pathway. Therefore, CREB pathway is also crucial for OC differentiation and function. In addition, CREB activation induced expression of PCG1β, which stimulates mitochondrial biogenesis and regulates osteoclast function [23]. Endoplasmic reticulum (ER) as another source of Ca^{2+} also plays an important role in osteoclast differentiation. The extracellular stimuli or ATP activate the plasma membrane receptor (P2X7), which induced the phospholipase C(PLC) activation. PLC can activate inositol 1,3,5-triphosphate (IP3) and IP3 induces calcium release from ER. The release of Ca^{2+} from ER is the major reason of calcium oscillation that results activation of NFATc1, which to regulate OC function. However, In the present study, we demonstrated that OPG decreased the RANKL-induced expression of NFATc1, c-Fos, and PCG1β, and inhibited the phosphorylation of CREB/PLC. Moreover, this work confirmed the results of our previous study at the molecular level. In this study, we further demonstrated that OPG impaired OC adhesion structures. However, Ca^{2+}/CREB/PLC/PCG1 signaling not only involved in regulating OC differentiation, also may mediate OC adhesion function. Therefore, Ca^{2+} mediated CREB/PLC/PCG1 signaling play an important role on OPG-induced OC adhesion damage, however, OPG also may be considered as a novel therapeutic to preventive osteoporosis.

However, PYK2 and SRC are both required for OC cytoskeletal

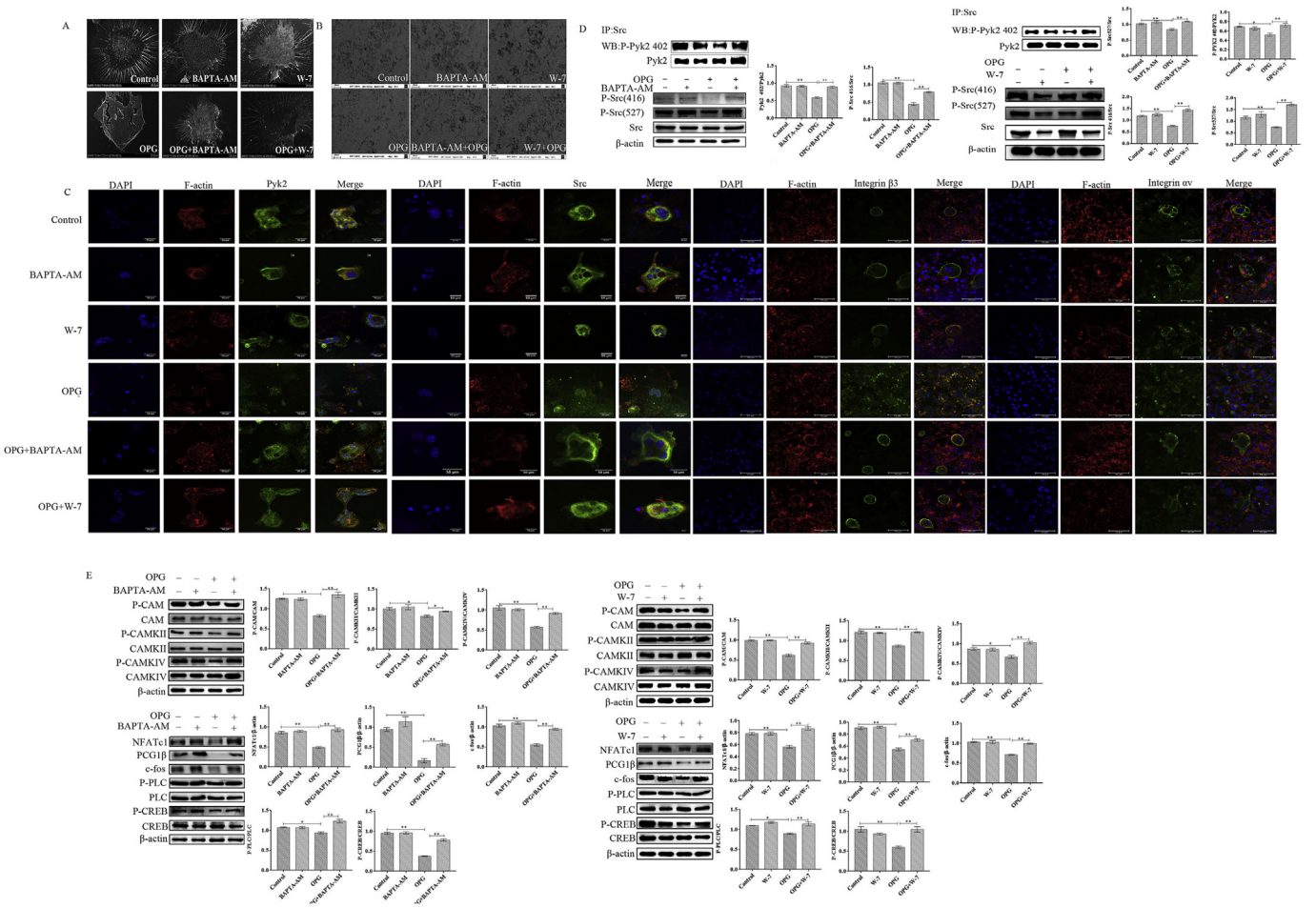

Fig. 3. BAPTA-AM/W-7 relieved OPG-induced osteoclast adhesion damage and Ca^{2+} signaling protein phosphorylation level. (A) Osteoclasts were cultured on glass coverslips and pretreated with BAPTA-AM (5 μM)/W-7 (10 μM) for 30 min, followed by 80 ng/ml OPG for another 30 min. Osteoclast adhesion structures were observed using SEM. (B) Osteoclasts were pretreated with BAPTA-AM (5 μM)/W-7 (10 μM) for 30 min, followed by 80 ng/ml OPG for 2d, osteoclast bone resorption activity were observed by SEM. (C) Osteoclasts were pretreated with BAPTA-AM (5 μM)/W-7 (10 μM) for 30 min, followed by 80 ng/ml OPG for another 30 min, fixed and stained for DAPI, F-actin, PYK2/SRC and integrin αvβ3, and examined using confocal immunofluorescence microscopy.(D) Osteoclasts were pretreated with BAPTA-AM/W-7 for 30 min, followed by 80 ng/ml OPG for another 30 min the phosphorylation of PYK2 and SRC were determined using western blotting. PYK2 and SRC complexes were analyzed using co-immunoprecipitation. $^{**}p < 0.05$ *vs.* the control, $^{**}p < 0.05$ *vs.* the co-treated control. Scale bar = 50 μm. (E) Osteoclasts were pretreated with BAPTA-AM (5 μM)/W-7 (10 μM) for 30 min, followed by 80 ng/ml OPG for another 30 min Ca^{2+} signaling protein phosphorylation level were analyzed by western blotting $^{**}p < 0.05$ *vs.* the control, $^{**}p < 0.05$ *vs.* the co-treated control. All experiments were repeated at least three times.

organization. PYK2 tyrosine phosphorylation depends on SRC kinase activity and is stimulated by SRC binding to the autophosphorylated site Tyr402 on PYK2 [24]. Usually, an equilibrium exists between the inactive and active forms of SRC, which is strictly regulated and favors the inactive bound conformation. The dormant form of the enzyme is destabilized by dephosphorylation of Tyr 527 and by phosphorylation of the activation loop at Tyr 416 [25]. In the present study, OPG significantly inhibited SRC phosphorylation, including SRC PY416 and PY527, and reduced the phosphorylation at Tyr402 on PYK2. This suggested that OPG disrupted the equilibrium between PYK2 and SRC. Previously, PYK2 was identified as a Ca^{2+}-dependent tyrosine kinase in OCs, and its phosphorylation is stimulated by an increase in Ca^{2+} [26]. However, BAPTA-AM has been widely used as a tool to study the role of calcium in a variety of cell functions, BAPTA-AM partially reversed the effects of OPG on PYK2-PY402, SRC-PY527, and SRC-PY416 levels. This suggested that OPG inhibited phosphorylation of PYK2 and SRC via calcium signaling. Calmodulin is a versatile protein that regulates Ca^{2+} homeostasis [27]. It has been implicated in OC differentiation, function, and survival. Ca^{2+} regulates calmodulin-dependent kinase II and Ca^{2+}/calmodulin-dependent kinase IV, which are critical for OC differentiation [28]. The antagonist W-7 has been widely used to study the role of calmodulin [29]. To the best of our knowledge, this is the first report to implicate calmodulin in the regulation of OC adhesion structures. W-7 partially restored the OPG-induced reduction in the phosphorylation of key Ca^{2+}-related proteins, and prevented the OPG-induced disruption of OC adhesion structures. These results further indicated that OPG disrupted relationship PYK2 and SRC through Ca^{2+} signaling, which plays a critical role on OPG-induced osteoclast adhesion structure damage. In addition, Osteoclasts can adhere to bone matrix where they come into unique podosomes structure. Podosomes are arrayed as a specific belt, which include many adhesive protein complex. It is well known that integrin αvβ3 is expressed by osteoclast and is required for cell-matrix adhesion and cell-cell interactions. Our date indicated that BAPTA-AM and W-7 changed the distribution of intergrin αvβ3 from a diffuse distribution to the periphery of the cells, PYK2 and SRC showed same effect. However, this results likely by modulating PYK2 and SRC phosphorylation. Meanwhile, the intact adhesion structure is the premise of osteoclast bone resorption. Our study indicated that BAPTA-AM and W-7 recovered the bone resorption activity by OPG-induced. Therefore, in the current study, we demonstrated that the OPG-induced adhesive structure damage via Ca^{2+} signaling is key pathway for osteoclast function.

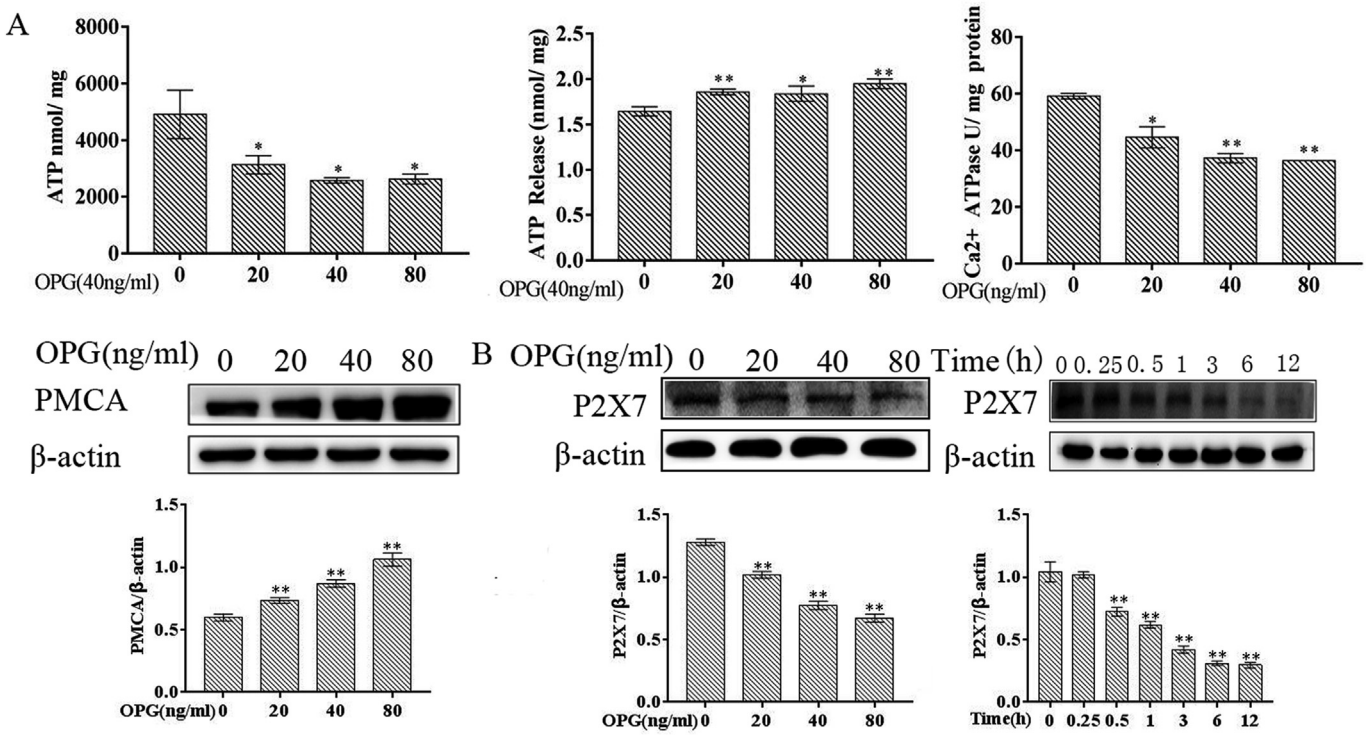

Fig. 4. OPG increases ATP release and the expression of Ca^{2+}-ATPase, and reduces the Ca^{2+}-ATPase activity and the expression of P2X7. BMMs were cultured on 6-well plates in the presence of RANKL (60 ng/ml) and M-CSF (30 ng/ml) for 5 days. Osteoclasts were treated with OPG (0, 20, 40, and 80 ng/ml) for 30 min. (A) Osteoclasts were lysed, and ATP and Ca^{2+}-ATPase were measured using ELISA kits. The level of PMCA was analyzed using western blotting. (B) Osteoclasts were treated with OPG (0, 20, 40, and 80 ng/ml) for 30 min, and OPG 80 ng/ml for different times (0, 0.25, 0.5, 1, 3, 6, and 12 h). The level of P2X7 was analyzed using western blotting, $^{**}p < 0.05$ *vs.* the control. Each results is representative of at least three replicate experiments.

Recently, the P2X7 receptor, which has a complex role in OC function, has been implicated in cell fusion. In this study, we further investigated the role of P2X7R in mature OCs. Pellegatti et al. [30]. proposed that P2X7R is absolutely required for fusion of OC via the P2X7R-dependent release of ATP, which is then broken down to adenosine with the subsequent activation of adenosine receptors, resulting in cell fusion. We also found that P2X7R knockdown significantly inhibited OC formation. This is consistent with previous reports that treatment with P2X7R antagonists (AZ15d and KN62) significantly inhibited the formation of functional OCs [31]. However, there are several ways in which ATP can be released from cells. Brandao-Burch et a. l [32] found that P2X7R-specific antagonists block ATP release from OBs by between 25 and 80%, suggesting that not all ATP is released via the P2X7R. In our research, we found that P2X7R-knockdown decreased the intracellular ATP levels; meanwhile, OPG also significantly reduced the intracellular ATP level in dose-dependent manner. Our results indicated that OPG impaired the OC adhesion structures, resulting in extensive ATP release from the damaged cells. However, although P2X7R is involved in this process, other pathways could play an important role in regulating ATP release, such as gap junctions and vesicular exocytosis. Our results showed that Ca^{2+} ATPase activity was significantly inhibited by OPG in dose-dependent manner; OPG obviously increased the expression of PMCA. However, Ca^{2+}ATPase activity and expression of PMCA show different response to OPG. A possible explanation for this discrepancy is that OPG reduced calcium concentration by Ca^{2+}ATPase channel, osteoclast may increase the expression of PMCA to maintain calcium homeostasis via compensatory mechanism. Previously, Kim et al. [33] indicated that high levels of PMCA caused efflux of intracellular Ca^{2+}, reduced Ca^{2+} oscillations, and limited NFATc1 activity. Other research has shown that nerve injury disrupts Ca^{2+} signaling, which lead to upregulation of PMCA [34]. However, our data suggested that OPG affects PMCA expression and activity to suppress Ca^{2+} oscillation, subsequently inhibiting NFATc1 activity. Armstrong et al. [35] reported that P2X7R-mediated Ca^{2+} increase resulted in the activation of key signaling molecules, such as NFATc1. This is consistent with our results, and suggested that P2X7R knockdown markedly enhanced the PMCA expression level, and significantly inhibited Ca^{2+} ATPase activity. In short, P2X7R knockdown increased PMCA expression by decreasing Ca^{2+} oscillations, which affected NFATc1 activity. Therefore, P2X7R-mediated Ca^{2+} signaling might play an important role in OC function by regulating PMCA.

In the current study, the exact physiological function of the P2X7R in OC adhesion structures is still unclear. A recent study provided evidence that P2X7R activation in rabbit OCs caused Ca^{2+} influx [36]. Additionally, blockade or absence of P2X7R inhibits the propagation of intercellular calcium signaling between OBs and OCs in human bone marrow derived cells. In the present study, we confirmed the role of P2X7R in calcium signaling, indicating that P2X7R knockdown significantly inhibited the phosphorylation of Ca^{2+} signaling-related proteins, and downregulated the expression of osteoclastic functional markers, including NFATc1, c-Fos, and PCG1β. These findings are consistent with those of Hwang et al. [37] who showed that siRNA-induced P2X7R knockdown in RAW 264.7 cells downregulated the expression of OC functional genes including those encoding NFATc1, Cathepsin K, and TRAP. Therefore, our findings indicated that P2X7R regulates OC function though Ca^{2+} signaling. P2X7R activation is also thought to be important in the differentiation and survival of OCs in both a paracrine and autocrine manner. Stimulus by a P2X7R-specific agonist induced membrane depolarization in OCs [38] and a subsequent increase in intracellular [Ca^{2+}]. A P2X7R-mediated Ca^{2+} increase would result in the activation of key signaling molecules, such as PKC, NF-κB, and NFATc1, which are dependent on increased cytosolic Ca^{2+} and regulate an array of OC differentiation-related genes. Hazama et al. [39] reported that treatment of human OCs with either BzATP or high concentrations of ATP increased bone resorption *in vitro*. In the present study, we also found that BzATP significantly changed the

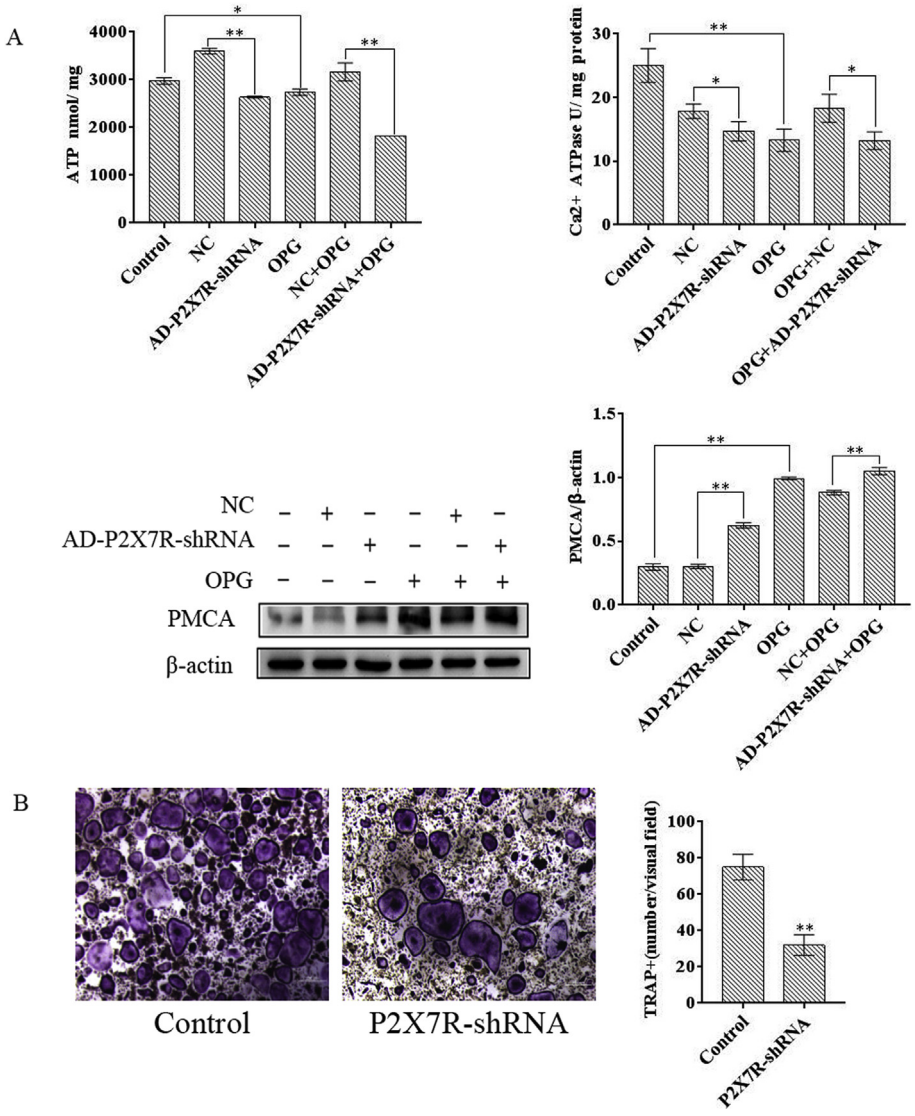

Fig. 5. P2X7 knockdown reduced intracellular ATP levels and Ca^{2+}-ATPase activity, and inhibited osteoclast differentiation. BMMs were treated with adenovirus-mediated P2X7R-shRNA and then cultured in the presence of RANKL and M-CSF. (A) ATP and Ca^{2+}-ATPase were measured using ELISA kits, and the level of PMCA was quantified using western blotting. (B) BMMs were cultured in the presence of RANKL and M-CSF for 2 days, and treated with adenovirus-mediated P2X7R-shRNA. After 8 h, the medium was changed to fresh α-MEM for 48 h. Cells were fixed and stained for TRAP. Magnification × 100; scale bar = 200 μM.

levels of key proteins in OPG-induced OCs, including NFATc1 and c-Fos. By contrast, abolishing P2X7R activity interfered with Ca^{2+} signaling activities, thereby influencing OC function by affecting the formation of the ruffled border, and subsequently bone resorption. As is well-known that integrin αvβ3 is expressed by osteoclast and is required for cell-matrix adhesion and cell-cell interactions. PYK2 was found in the sealing zone, and its phosphorylation correlates with the formation of the sealing zone and with OC bone resorption. PYK2-null OCs were unable to transform podosome clusters into a podosome belt at the cell periphery, resulting in impaired bone resorption. In *Src* (−/−)OCs, PYK2 tyrosine phosphorylation and its kinase activity were markedly reduced, which suggested that the adhesion-dependent phosphorylation of tyrosine in PYK2 is mediated by c-SRC in OCs *in vivo* [40]. The finding is consistent with our results: P2X7R knockdown significantly deteriorated OPG-induced adhesive structure damage, including to inhibit PYK2 and SRC activation, and disturbed the distribution of PYK2/SRC and intergrin αvβ, ultimately, severely affected osteoclast bone resorption activity. In conclusion, the P2X7R-mediated Ca^{2+} signaling pathway may play a critical role in osteoclasts adhesion structure and bone resorption.

In summary, we demonstrated that OPG impaired OC adhesion structures through the P2X7R–Ca^{2+} dependent PYK2/SRC signaling pathway Fig. 7. Our study elucidated the role of P2X7R in OPG-induced OC adhesion structure damage and suggests novel therapeutic targets to treat bone metabolism disease.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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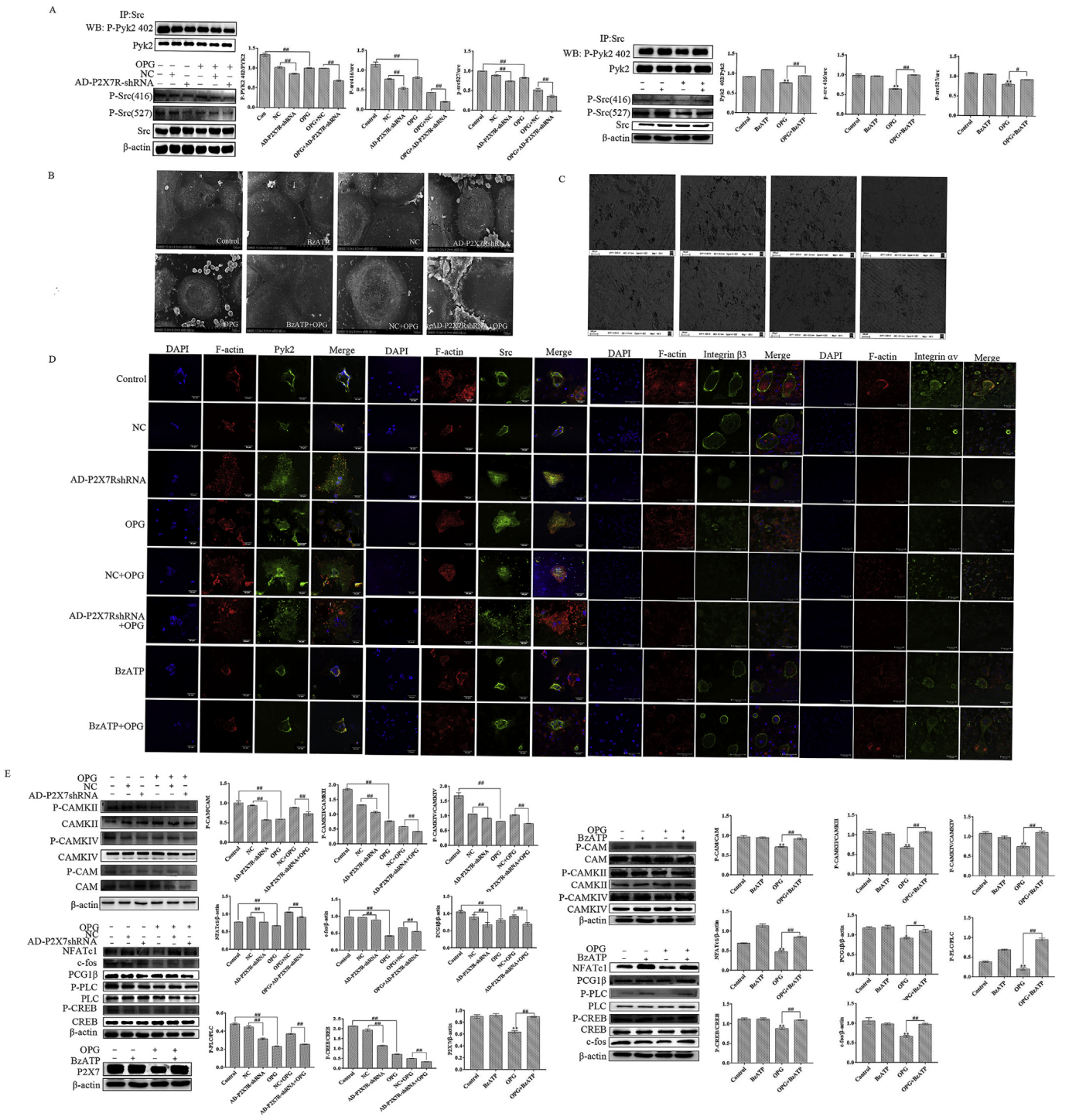

Fig. 6. P2X7R knockdown aggravated OPG-induced OC adhesion structure damage and promoted OPG-induced the reductions in Ca^{2+} signaling protein. (A) BMMS were cultured in the presence of RANKL and M-CSF for 2 days, and treated with adenovirus-mediated P2X7R-shRNA. After 8 h, the medium was changed to fresh α-MEM for 48 h. The cells were then treated with 80 ng/ml OPG for another 30 min. Osteoclasts were pretreated with BzATP (300 μM) for 3 h; followed by OPG (80 ng/ml) for another 30 min. The cells were lysed and analyzed using western blotting and co-immunoprecipitation. (B) Osteoclast adhesion structures were observed using SEM. (C) BMMS were cultured in the presence of RANKL and M-CSF for 2 days, and treated with adenovirus-mediated P2X7R-shRNA. After 8 h, the medium was changed to fresh α-MEM for 48 h. The cells were then treated with 80 ng/ml OPG for 2d. Osteoclasts were pretreated with BzATP (300 μM) for 3 h; followed by OPG (80 ng/ml) for another 2d. osteoclast bone resorption activity were observed by SEM. (D) BMMs were plated and cultured on bone slices, and then treated with adenovirus-mediated P2X7R-shRNA. After 8 h, the medium was changed to fresh α-MEM for 48 h. Osteoclasts were pretreated with BzATP (300 μM) for 3 h, followed by OPG (80 ng/ml) for another 30 min, fixed and stained for DAPI, F-actin, PYK2/SRC and integrin αvβ3, and examined using confocal immunofluorescence microscopy. Scale bar = 50 μm. (E) BMMS were cultured in the presence of RANKL and M-CSF for 2 days, and treated with adenovirus-mediated P2X7R-shRNA. After 8 h, the medium was changed to fresh α-MEM for 48 h, and then the cells were treated with 80 ng/ml OPG for another 30 min. Osteoclasts were pretreated with BzATP (300 μM) for 3 h, followed by OPG (80 ng/ml) for another 30 min. The cells were lysed and the levels of Ca^{2+} signaling-related protein were quantified using western blotting. All experiments were repeated at least three times. All experiments were repeated at least three times.

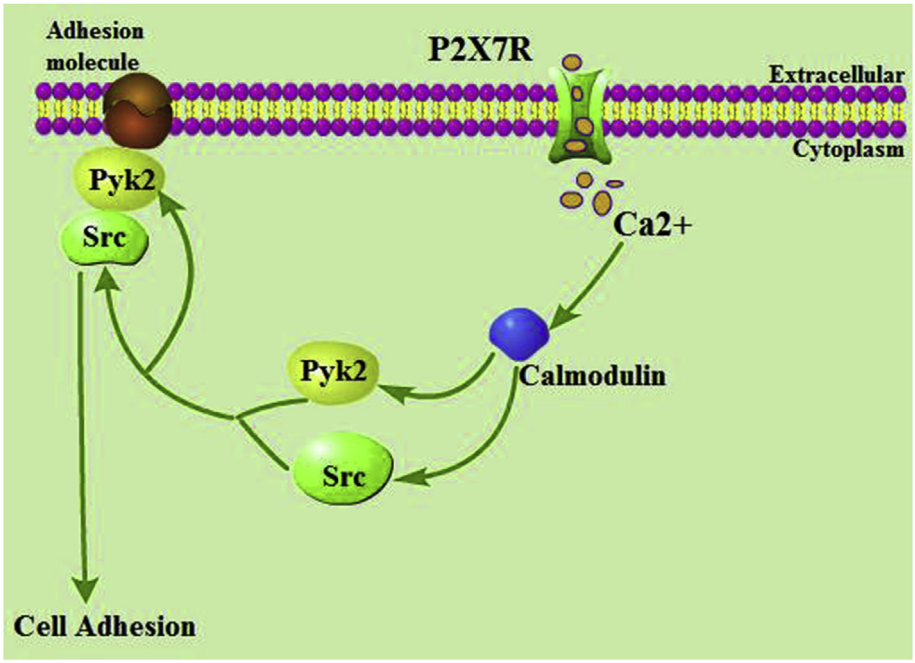

Fig. 7. Schematic rendition of P2X7-Ca^{2+}-PYK2/SRC signaling in osteoclast adhesive structure damage. The P2X7 activation increased intracellular calcium concentration and activated calcium related protein, which inhibited protein tyrosine kinase 2 (PYK2) and SRC phosphorylation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yexcr.2019.111555.

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