Randomized Trial

P2X7 Receptor-Induced Bone Cancer Pain by Regulating Microglial Activity via NLRP3/IL-1β Signaling

Ping Wu, MD[1], Xiaoqi Wu, MSc[1], Guohua Zhou,MSc[2], Yin Wang, BA[3], Xiaojun Liu, BA[3], Run Lv, MSc[4], YanSong Liu, BA[3], and Qingping Wen, MD[1]

From: [1]Department of Anesthesiology, The First Affiliated Hospital of Dalian Medical University, Dalian, China; [2]Department of Anesthesiology, Ningbo First Hospital, Ningbo, China; [3]Anesthesiology Department, Dalian Medical University, Dalian, China; [4]Department of Anesthesiology, The First Hospital of Hebei Medical University,Shijiazhuang China

Address Correspondence: Qingping Wen, MD Department of Anesthesiology The First Affiliated Hospital of Dalian Medical University Dalian 116000, China E-mail: qingping_wen@yahoo.com

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Background: Bone cancer pain (BCP) is the most severe and intractable type of cancer pain. Emerging evidence has demonstrated that activated microglia in the spinal cord could release a series of neurotoxic substances to stimulate neurons and form neuronal sensitization. The P2X7 receptor (P2X7R) is a nonselective ATP-gated ion channel predominantly present in microglia in the spinal cord as the key modulator of microglial activity. However, the specific effect and underlying molecular mechanism of P2X7R in BCP have not yet been elucidated.

Objectives: This study aimed at investigating whether P2X7R-induced BCP by regulating microglial activity through NLRP3/IL-1β signaling involvement in BCP.

Study Design: Controlled animal study.

Methods: A BCP animal model was established by injecting Walker-256 breast cancer cells into the tibia of female rats. Fifty percent paw withdrawal thresholds (50% PWTs), number of spontaneous flinches (NSF), and limb use scores were used to evaluate behavior in rats.

P2X7R inhibitor brilliant blue G (BBG) was used to assess the role of P2X7R in BCP-induced NLRP3 inflammasome activation. Western blot, RT-PCR, and immunofluorescence were used for quantitative comparison. In vitro, BV2 cells were treated with lipopolysaccharide (LPS) and BzATP, in the presence or absence of P2X7 siRNA, with nigericin (an agonist of the NLRP3 inflammasome) to further study the mechanism of P2X7R regulate NLRP3/IL-1β signaling.

Results: The inhibition of spinal P2X7R with BBG could effectively inhibit BCP due to suppressing the expression of NF-κB p-p65, NLRP3 inflammasome formation, and downstream pain factors IL-1β. Furthermore, P2X7 siRNA could reduce microglial activity, the nuclear translocation of NF-κB, and the synthesis of NLRP3 and IL-1β in BV2 cells. In addition, nigericin partially reversed the ameliorating effect of P2X7 siRNA on BV2 cells induced by LPS and BzATP.

Limitations: BBG could relieve BCP but not improve the destruction of bone, which may be related to the specificity of inoculated cells. Further mechanisms should be investigated.

Conclusion: These findings suggest that targeting the microglial P2X7R activated NLRP3/IL-1β signaling pathway could serve as a potential strategy for BCP treatment.

Key words: P2X7 receptor, NLRP3, interleukin 1β, brilliant blue G

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Cancer pain is a common complication in patients with advanced stage cancer, and it affects approximately one-third of cancer patients (1). Bone cancer pain (BCP) is the most severe and intractable type of cancer pain, which can seriously affect patients' quality of life and impose a heavy

social burden (2). To date, the available BCP treatment strategies are inadequate due to either limited analgesia efficiency or unacceptable side effects (3). Therefore, researchers should continue to carry out in-depth studies to elucidate the underlying molecular mechanisms of BCP.

Microglia serve as the resident immune cells in the spinal cord that modulate the process of chronic pain (4). Under the condition of peripheral nerve injury or inflammation, microglia at the spinal dorsal horn can be activated and release a series of inflammatory cytokines (5). Subsequently, these inflammatory factors activate the neighboring neurons, thereby resulting in pain maintenance and sensitization (6). Several studies have shown that BCP may involve the activation of microglia (7). Therefore, this calls for urgent identification of more precise targets for microglia. The P2X7 receptor (P2X7R), an ATP-gated nonselective active channel, is predominately present in microglia in the spinal cord (8). Accumulating evidence suggests that P2X7R activation will form non-selective ion channels, among them K+ efflux, which modulates the expression of NLRP3 inflammasome and transcription factors NF-κB to further promote IL-1β release (9,10). Notably, spinal cord IL-1β is a crucial pain-causing factor by activating nociceptors or promoting glial-neuron crosstalk (11). A recent study has demonstrated that the expression of NLRP3 in the microglia was upregulated in the BCP model. The application of NLRP3-specific inhibitor MCC950 effectively alleviates pain by reducing IL-1β in the dorsal horn (12). In our previous study (13), we detected elevated IL-1β in BCP rats, which led to the hypothesis that P2X7R promotes BCP and is dependent on NLRP3 inflammasome and IL-1β processing.

This study aimed at investigating the mechanisms of P2X7R promotes microglia activation in the spinal cord in BCP rats. We also hypothesized that the P2X7R-regulated NLRP3/IL-1β signaling might be involved in this process.

Methods

Experimental Animals

Female Sprague Dawley (SD) rats weighing 180-220g were purchased from the Institute of Genome Engineered Animal Models for Human Diseases (Dalian, China) and were housed under a specific-pathogen-free (SPF) grade environment at the Dalian Medical University. The rats were kept in sterile plastic cages at room temperature (22 ± 1°C) with a 12-hour dark/light cycle and were provided with sufficient food and clean water. This study was approved by the Ethics Committee on Animal Research of Dalian Medical University.

Cell Culture

Walker 256 Mammary Gland Carcinoma Cells

The Walker 256 mammary gland carcinoma cell line was donated by Prof. Changsheng Dong (Shanghai Research Institute of Traditional Chinese Medicine, China). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% L-glutamine, and 2% penicillin/streptomycin. Next, the cells were separated from the culture flask using 0.25% trypsin, centrifuged at 1000 rpm for 10 min, and resuspended in phosphate buffered saline (PBS) for subsequent injection preparation.

BV2 Cells

Mouse BV2 cell lines were purchased from Procell Life Science & Technology Co., Ltd (Wuhan, China). BV2 cells were cultured in DMEM: F12 (1:1) medium containing Glutamax and supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin in a humidified atmosphere (37°C and 5% CO_2).

Cellular Experiments

In vitro study of microglia was performed using the BV2 cell lines. Briefly, BV2 cells were transfected with NC siRNA or P2X7 siRNA (5′-GCAAGUUGUCAAAGGCCAATT-3′ and 5′- UUGGCCUUUGACAACUUGCTT -3′) using the Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions (Table 1). To determine the effect of P2X7R, the cells were divided into 4 groups: Control + NC siRNA group, Control + P2X7 siRNA group, lipopolysaccharide (LPS) + BzATP + NC siRNA group, and LPS + BzATP + P2X7 siRNA group. After transfection for 48 h, the experimental groups were stimulated for 2 h using 1ug/mL LPS, and the culture medium was discarded. Cells were then cultured in a fresh medium supplemented with BzATP (300μM). The control group received PBS instead of LPS and BzATP.

To determine the role of NLRP3 in the P2X7R-mediated microglia activation induced by LPS and BzATP + LPS, BV2 cells were split into 4 groups: Control group, LPS + BzATP group, LPS + BzATP + P2X7 siRNA group, and LPS + BzATP + P2X7-siRNA + Nigericin group. Nigericin (InvivoGen, 10μM) treatment was administered for 15 min after the BzATP challenge.

Establishment of the BCP Rat Model

BCP rat model was established according to a previously described protocol (13). Tumor cells were extracted from the ascitic fluid of rats that had received Walker 256 mammary gland carcinoma cells by intraperitoneal injection. Next, cells were resuspended in an appropriate volume of PBS to obtain the final injection concentration (5×107 cells/mL). After anesthesia, the intramedullary cavity of the left tibia was slowly injected with 10 μL of PBS containing Walker 256 breast cancer cells (about 5×105) in the BCP group, and the same volume of PBS was injected into the sham group.

Table 1. *Antibody used in immunofluorescence.*

Antibody	Company	Catalog Number	Application	Dilution
anti-P2X7R	Invitrogen	PA5-77665	IF	1:200
anti-Iba1	Servicebio	GB12105	IF	1:200
anti-GFAP	Servicebio	GB12096	IF	1:200
anti-NeuN	Proteintech	66836-1-Ig	IF	1:200
Cy3 conjugated Goat Anti-mouse IgG	Servicebio	GB21301	IF	1:300
Alexa Fluor® 488-conjugated Goat Anti-Rabbit IgG	Servicebio	GB25303	IF	1:400
anti-NF-κB p65	Proteintech	10745-1-AP	IF	1:100
CoraLite594-conjugated Goat Anti-Rabbit IgG	Proteintech	SA00013-4	IF	1:200

Drug Administration

Female SD rats were randomly divided into 4 groups: sham + NS group, sham + BBG group, BCP + NS group, and BCP + BBG group. Rats in the sham + BBG group and BCP + BBG group were intraperitoneally injected with brilliant blue G (BBG, APExBIO, C5579, 50 mg/kg, 10 mg/mL), a P2X7 specific inhibitor (14). On the other hand, rats in sham + NS and BCP + NS groups were injected with the same volume of saline solution. The treatments were administered once every day from the time the rat showed nociceptive behavior to the day of sacrifice. Notably, treatments were administered from the 7th day to the 21st day.

Nociceptive Behavioral Assays

All pain behavioral tests were performed by an individual who was blinded to the treatment groups. Rats were allowed to acclimatize in the testing room for at least 30 min before the tests began. The behavioral tests were performed one day before the operation (-1) and on the 4th, 7th, 11th, 14th, 17th, and 21st days after the operation.

50% Paw Withdrawal Threshold (50% PWT)

Mechanical allodynia was assessed by measuring the PWT using the von Frey test. During the measurement, the rat was placed in a special plexiglass grid with a grid at the bottom. A series of von Frey filaments were used to stimulate the skin of the middle sole of the rat's operation side. Next, 50% PWT was calculated according to the rat's foot withdrawal response introduced by Dixon (13).

Number of Spontaneous Flinches (NSF)

Rats were housed in individual plexiglass cages. The number of spontaneous withdrawals of the hindlimb on the operated side of the rat within 2 min was recorded, and the number of times indicated nociceptive behavior. Each rat was tested 5 times with 10 min between each test, and the average value was taken as the final NSF result. 5.3 Score of Ambulatory Pain

Rats were placed on a large glass plate and scored according to the degree of limb use during spontaneous activities. The scores were as follows: 0 = complete lack of use, 1 = partial nonuse, 2 = limping and guarding, 3 = limping, and 4 = normal walking (15).

Radiology

Rats were anesthetized by intraperitoneal injection of sodium pentobarbital and placed in front of the x-ray source (RayNova Pet DR). Next, the tibia on the operated side was photographed, and the National Institutes of Health (NIH) ImageJ software version 1.47 (NIH, Bethesda, MD) was used to analyze the scan results. The following standards were used to assess the extent of bone destruction: 0, normal bone structure without destruction; 1, small (1 to 3) radiolucent lesions in the proximal epiphysis close to the site of the injection; 2, increased number (3 to 6) of radiolucent lesions; 3, loss of medullary bone plus erosion of the cortical bone; 4, full thickness unicortical bone loss; and 5, full thickness bicortical bone loss and displaced fractures.

Immunofluorescence (IF)

Spinal Cord Immunofluorescence

Spinal cord specimens were isolated from the sacrificed rats, post-fixed, and dehydrated. The paraffin-

embedded samples were then cut into 8-um sections for subsequent immunofluorescence staining. Next, antigen retrieval was performed after washing in distilled water according to the manufacturer's instructions. Sections were then blocked using 10% donkey serum at room temperature for 1 h. Subsequently, sections were incubated with the primary antibodies: anti-P2X7R, anti-GFAP, anti-Iba1, and anti-NeuNfor 12 h at 4°C. Next, the sections were washed 3 times with PBS and incubated with the secondary antibody at room temperature for 1 h in the dark. Cell nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI). Finally, the sections were mounted with an aqueous antifade mounting medium and analyzed using a fluorescence microscope (NIKON Eclipse Ci, Japan).

Cellular Immunofluorescence

After the intervention of BV2 cells, the culture medium was removed, 4% paraformaldehyde was added to fix the cells for 15 min after PBS washing, 0.3% Triton X-100 was permeated for 5 min, the serum was blocked for 30 min after PBS washing, and incubated with primary antibody:anti-NF-κB p65 at 4°C overnight. After washing with PBS 3 times, CoraLite594 – conjugated Goat Anti-Rabbit IgG was incubated in the dark for 1 h at room temperature. After PBS cleaning, it was sealed with an anti-fluorescence quencher containing DAPI and analyzed by fluorescence microscope and confocal microscope. The antibodies used in immunofluorescence are shown in Supplementary Table 1.

Western Blot Analysis

RIPA buffer was used to extract the total protein of the L4-L6 segment of spinal cord tissues and BV2 cells. The protein concentration was quantified using the BCA method, and equal amounts of proteins were resolved using SDS-PAGE gel electrophoresis. Proteins were then transferred to the PVDF membrane and blocked with a 5% blocking solution at room temperature for 1 h. Next, membranes were incubated with an appropriately diluted primary antibody overnight at 4°C. Subsequently, membranes were washed 3 times with TBST and incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody (1:1000) for 1 h at room temperature. Finally, the protein bands were detected using the ECL procedure and evaluated using Image J software. The antibodies used in this experiment are shown in Supplementary Table 2.

Quantitative Real-Time Polymerase Chain Reaction (RT-PCR)

The total RNA of BV2 cells was extracted using Trizol reagent, and the RNA integrity and quantity were determined using RNA 6000 Nano assay. Next, cDNA was synthesized using EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech) according to the manufacturer's instructions. Subsequently, RT-PCR was performed using SYBR Select Master Mix (Life Technology), and sequences of the primers used are shown in Table 1. Relative messenger RNA (mRNA) expression was normalized to GAPDH mRNA, and the ΔΔCt method was used to calculate the relative fold change. The primer sequences used in this experiment are shown in Supplementary Table 3.

Enzyme-Linked Immunosorbent Assay (ELISA)

IL-1β in serum was determined by using ELISA assay kits (Elabscience). Experiments were carried out according to the manufacturer's instructions. The absorbance value was acquired at 490 nm.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA). Normally distributed data are expressed as mean ± SD. Between-group differences were determined using one-way or two-way ANOVA. P value < 0.05 was considered statistically significant.

Results

1. The BCP model was successfully established

X-ray results showed in the BCP group, bone destruction was observed on the 14th day, and the tibia was severely damaged by the 21st day. However, the tibia of rats in the sham group did not show any bone destruction (Fig. 1B). The 50% PWT level and the score of ambulatory pain of rats in the BCP group decreased significantly on days 7, 11, 14, 17, and 21 after surgery ($P < 0.05$) and was significantly lower than the sham group ($P < 0.05$) (Fig. 1D,1F). Concerning the NSF, in the BCP group, the NSF level showed an increasing trend, and it was significantly higher than that of the sham group on days 7, 11, 14, 17, and 21 after surgery ($P < 0.05$) (Fig. 1E). The above results indicated that the BCP model was established successfully.

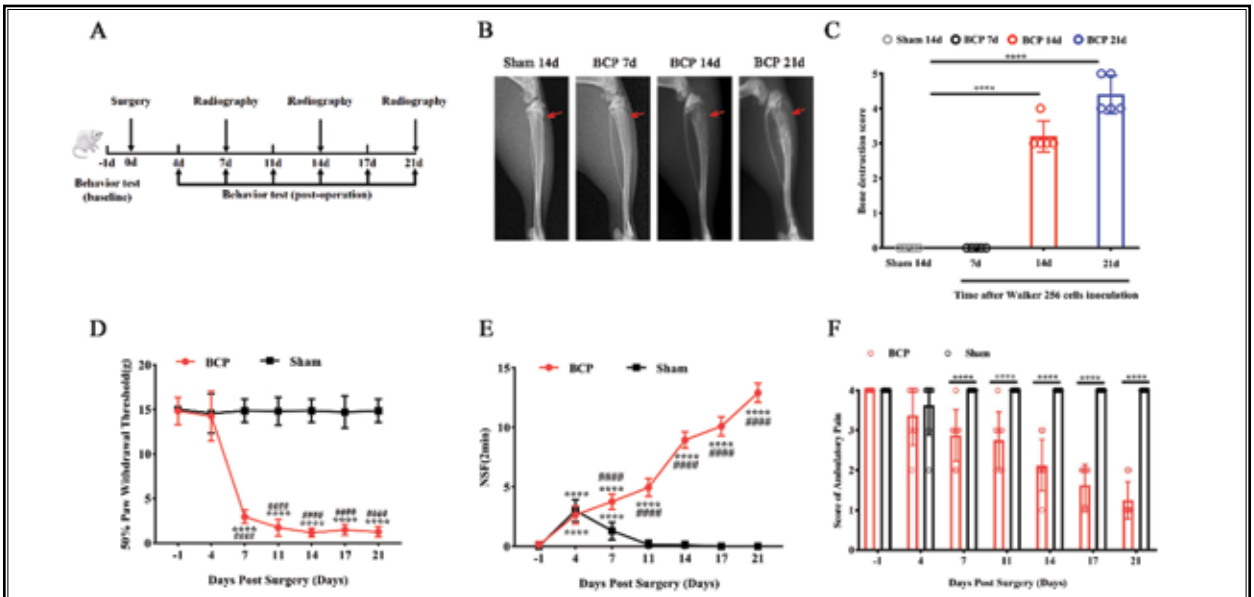

Fig. 1. *The establishment of breast cancer-induced bone metastasis model in rats. (A) The design of animal experiments. (B) Radiographs of the tibia after Walker 256 cells inoculation. The red arrow represents the damage of the proximal cortical bone. (C) Bone destruction score after Walker 256 cells inoculation. Data were presented as mean ± SD (n = 8 per group). ****P < 0.0001 versus sham 14 d. (D) 50% Paw withdrawal threshold (50% PWT) was examined on baseline (1 day before surgery) and postoperative days (POD) 4, 7, 11, 14, 17, 21. Data were presented as mean ± SD (n = 8 per group). ****P < 0.0001 versus baseline; ####P < 0.0001 versus Sham group. (E) Number of spontaneous flinches (NSF) were examined on baseline (1 day before surgery) and POD 4, 7, 11, 14, 17, 21. Data were presented as mean ± SD (n = 8 per group). ****P < 0.0001 versus baseline; ####P < 0.0001 versus Sham group. (F) Scores of ambulatory pain were examined on baseline (1 day before surgery) and POD 4, 7, 11, 14, 17, and 21. Data were presented as mean ± SD (n = 8 per group). ****P < 0.0001 versus sham 14 d.*

2. The expression of Iba-1 and P2X7R increases during BCP development

In this study, western blot analysis was used to determine the relative expression of Iba-1 in the L4-L6 spinal cord. Results showed that the protein expression of Iba-1(a specific marker of microglia activation) and the expression of P2X7R were significantly increased on the 14th and 21st days after surgery in the BCP group compared to the sham group ($P < 0.05$) (Fig. 2). In addition, our previous research showed that P2X7R was nearly completely co-localized with microglia-specific marker Iba-1, but there was no co-localization with GFAP (astrocyte-specific marker) or NeuN (neuron-specific marker) (16). These results indicate that P2X7R was significantly increased, and microglia were activated in the BCP model.

3. BBG can effectively relieve BCP and reduce activation of microglia

To ascertain the role of P2X7R in the pathogenesis of BCP, a P2X7R-specific antagonist, BBG, was used to treat BCP rats. Results showed that the 50% PWT and the score of ambulatory pain in the BCP group treated with BBG increased significantly on days 7, 11, 14, 17, and 21 after surgery compared to rats in the BCP group treated with NS ($P < 0.05$) (Fig. 3A,3C). In the BCP group, NSF showed an upward trend on days 7, 11, 14, 17, and 21 after surgery, but the values were significantly lower in the BBG treatment group than in the NS treatment group ($P < 0.05$) (Fig. 3B). The above results indicated that BBG can effectively relieve BCP. Since the previous results have shown that P2X7R activation can promote the activation of microglia, we used immunofluorescence to determine the effect of BBG treatment on microglia. It was found that the expression levels of Iba1 were significantly lower in the BCP + BBG group than in the BCP + NS group, which indicated that BBG can significantly reduce the activation of microglia in the BCP (Fig. 3D).

4. BBG relieves BCP through the NF-κB/NLRP3/IL-1β signaling pathway

Next, western blot analysis was conducted to

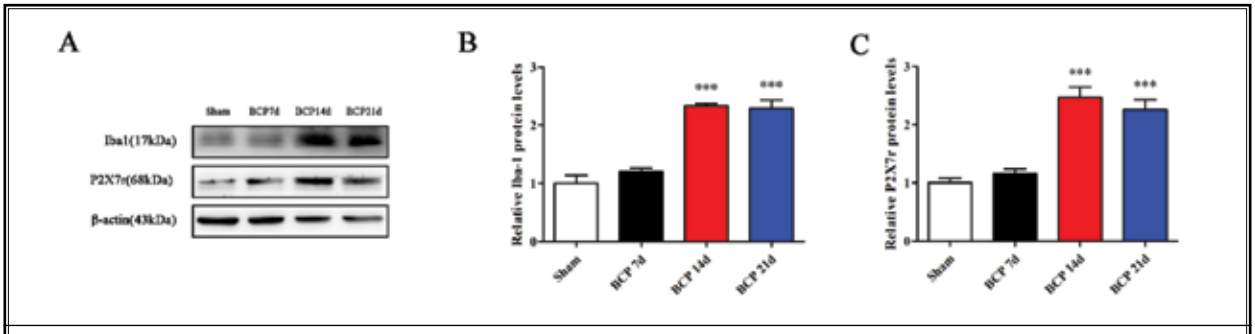

Fig. 2. *The expression of Iba-1 and P2X7R have elevated in the BCP group. (A) Representative western blotting bands of Iba-1 and P2X7R in the Sham group and BCP group on POD 7, 14, 21. (B) Quantitative data for analysis showed the protein expression levels of Iba-1 in the Sham group and BCP group on POD 7, 14, and 21. β-actin was used as an internal control. Data were presented as mean ± SD (n = 4 per group). ***P < 0.001 versus Sham group. (C) Quantitative data for analysis showed the protein expression levels of P2X7R in the Sham group and BCP group on POD 7, 14, and 21. β-actin was used as an internal control. Data were presented as mean ± SD (n = 4 per group). ***P < 0.001 versus Sham group.*

determine how the increased P2X7R expression contributes to the inflammatory responses in the spinal cord. As expected, results showed that the expressions of NF-κB p-p65, NLRP3, ASC, caspase-1, and IL-1β were significantly increased in the BCP group ($P < 0.05$). However, the levels of the above-mentioned proteins were significantly lower in the BCP + BBG treatment roup than in the BCP + NS treatment group ($P < 0.05$) (Fig. 4).

5. P2X7 siRNA inhibits LPS and BzATP-stimulated inflammatory reactions in BV2 microglial cells through the NLRP3 inflammasome

The BV2 cell line has a similar response to LPS and BzATP co-stimulation as primary microglia, which has been widely used in cell experiments related to microglia activation in vitro (17,23). Western blot analysis was used to evaluate the efficiency of transfection (Fig. S1). Immunofluorescence showed that LPS and BzATP treatment induced the translocation of NF-κB p65 from cytoplasm to nucleus, and P2X7 siRNA inhibited NF-κB p65 nuclear translocation (Fig. 5A). Western blot showed that the expression levels of NLRP3, ASC, and caspase-1 were higher in BV2 cells treated with LPS and BzATP than in BV2 cells in the PBS group. Results also showed that P2X7 siRNA can significantly reduce the expression levels of the above-mentioned proteins in BV2 cells treated with LPS and BzATP ($P < 0.05$) (Fig. 5B-E). In addition, P2X7 siRNA can effectively reduce the expression level of IL-1β in LPS, and BzATP-treated BV2 cells ($P < 0.05$) (Fig. 6F,G).

6. The NLRP3 signaling pathway was downstream of P2X7R in LPS and BzATP-stimulated BV2 cells

To further determine whether the NLRP3 signaling pathway was downstream of P2X7R, P2X7 siRNA was co-treated with nigericin (10 μM), an agonist of the inflammasome (18). Western blot results showed that nigericin can significantly upregulate the expression level of NLRP3, ASC, and caspase-1 level in BV2 cells treated with LPS and BzATP compared to P2X7 siRNA ($P < 0.05$) (Fig. 6A-E). In addition, nigericin could reverse the P2X7 siRNA-elicited changes in IL-1β in LPS and BzATP-exposed BV2 cells ($P < 0.05$) (Fig. 6F,G).

Discussion

Our study confirmed and expanded previous evidence on the study of P2X7R-mediated BCP. P2X7R-specific antagonist BBG could effectively inhibit BCP via suppressing the expression of NF-κB/NLRP3/IL-1β. The spinal cord microglia P2X7 siRNA could reduce NF-κB nuclear translocation, NLRP3 inflammasome, and IL-1β formation in BV2 cells. Here, we added the inflammasome-specific agonist nigericin could reverse the P2X7 siRNA-elicited changes in IL-1β. Our findings suggest that spinal P2X7R-dependent signaling-induced NLRP3 inflammasome activation in microglia contributes to the development and maintenance of BCP. Inhibiting P2X7R/NLRP3/IL-1β signaling could be a potential strategy to relieve BCP.

Microglia activation in the BCP model has been well documented (19), with results showing a significant increase in the expression of Iba-1 in the spinal cord. Yang et al reported that minocycline, an inhibitor of microglia activation, decreased the expression of Iba-1 protein

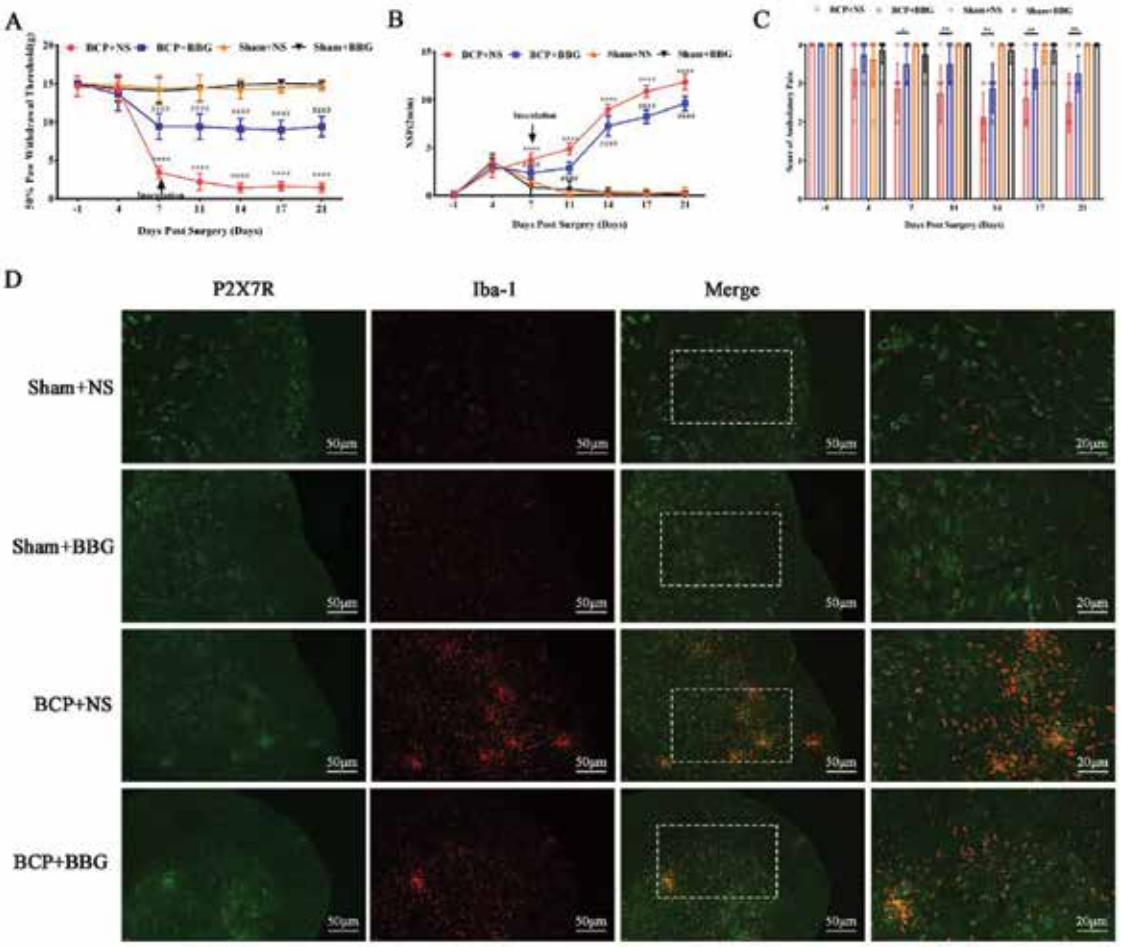

Fig. 3. *BBG can effectively relieve BCP and reduce the activation of microglia. (A) 50% PWT in the Sham + NS, Sham + BBG, BCP + NS, and BCP + BBG groups were examined on baseline (1 day before surgery) and POD 4, 7, 11, and 14. Data were presented as mean ± SD (n = 8 per group). ****P < 0.0001 versus Sham + NS group, ####P < 0.0001 versus BCP + NS group. (B) NSF in the Sham + NS, Sham + BBG, BCP + NS, and BCP + BBG groups was examined on baseline (1 day before surgery) and POD 4, 7, 11, and 14. Data were presented as mean ± SD (n = 8 per group). ****P < 0.0001versus Sham + NS group, ####P < 0.0001 versus BCP + NS group. (C) Scores of ambulatory pain in the Sham + NS, Sham + BBG, BCP + NS, and BCP + BBG groups were examined on baseline (1 day before surgery) and POD 4, 7, 11, and 14. Data were presented as mean ± SD (n = 8 per group). *P < 0.05 versus BCP + NS group.**P < 0.01 versus BCP + NS group. (D) Co-immunostaining of P2X7R(green) and Iba-1 (microglial specific marker, red) in the spinal dorsal horn of Sham + NS, Sham + BBG, BCP + NS, and BCP + BBG group.*

level and alleviated mechanical allodynia of BCP rats (20). In this study, a significant increase in Iba-1 cells was observed in the L4-L6 ipsilaterally spinal cord of BCP rats, indicating microglial activation following BCP.

Accumulating evidence suggests that the P2X7 receptor (P2X7R) is involved in a variety of chronic pain conditions, including inflammatory and neuropathic states, and is also involved in the development of morphine tolerance (21,22). Our study showed that the upregulation of P2X7R expression and the activation of microglia were consistent with the progress of BCP. Stable BCP was observed on the 7th day, and we subsequently administered the P2X7R antagonist BBG until the end of the experiment. It was found that BBG was effective in alleviating BCP until the end of the 21-day study. These results suggest that P2X7R is involved in the whole process of BCP initiation and maintenance, and this result is consistent with Falk et al (23). It is

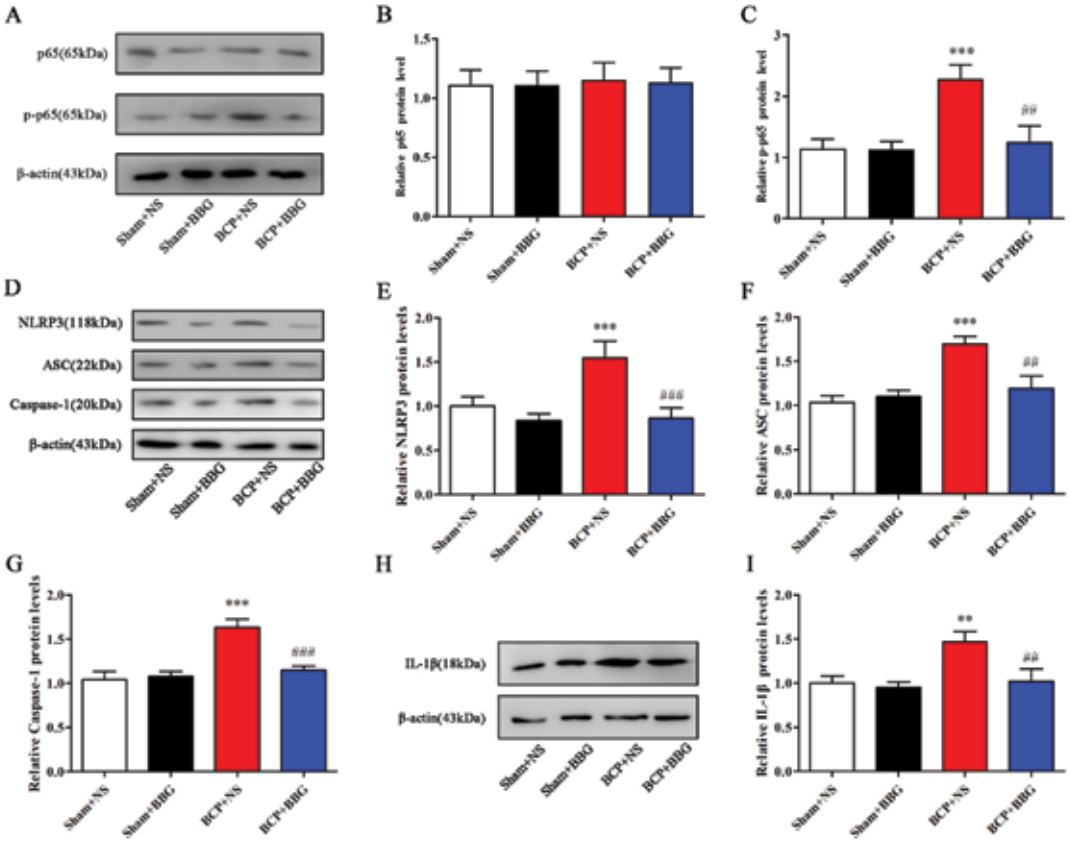

Fig. 4. *BBG reduce the NF-κB/NLRP3/IL-1β signaling pathway. (A)-(C) Representative western blotting bands and quantitative analysis showed the protein expression levels of NF-κB p-p65, p65 in the Sham + NS, Sham + BBG, BCP + NS, and BCP + BBG groups. β-actin was used as an internal control. Data were presented as mean ± SD (n = 4 per group). ***P < 0.001 versus Sham + NS group; ##P < 0.01 versus BCP + NS group. (D)-(G) Representative western blotting bands and quantitative analysis showed the protein expression levels of NLRP3, ASC, and caspase-1 in the Sham + NS, Sham + BBG, BCP + NS, and BCP + BBG group. β-actin was used as an internal control. Data were presented as mean ± SD (n = 4 per group). ***P < 0.001 versus Sham + NS group; ##P < 0.01 versus BCP + NS group; ###P < 0.001 versus BCP + NS group. (H) and (I) Representative western blotting bands and quantitative analysis showed the protein expression levels of IL-1β in the Sham + NS, Sham + BBG, BCP + NS, and BCP + BBG groups. β-actin was used as an internal control. Data were presented as mean ± SD (n = 4 per group). **P < 0.01 versus Sham + NS group; ##P < 0.01 versus BCP + NS group.*

worth thinking that in the early stage of BCP, microglia did not activate significantly on the 7th day, but BBG could alleviate the pain effectively. Obviously, microglia activation and pain sensitization are not completely synchronized; the specific mechanism needs to be further investigated.

Extracellular ATP-induced activation of P2X7R directly triggers K+ extravasation, which is thought to be responsible for activation and co-localization of NLRP3 (24). According to a previous study, P2X7R and NLRP3 can be co-localized by confocal microscopy, and co-immunoprecipitated in both rat microglia and peritoneal macrophages (25). Grace et al reported that the P2X7/NLRP3 signaling pathway may be involved in morphine-induced pain sensitization in the sciatic chronic constriction injury model (26). All these lines of evidence indicated that the regulation of inflammatory responses by P2X7R is partially dependent on the NLRP3 inflammasome.

A large body of evidence indicates in the chronic pain process, pro-inflammatory cytokines released from the spinal cord could bind to TLRs and cytokines recep-

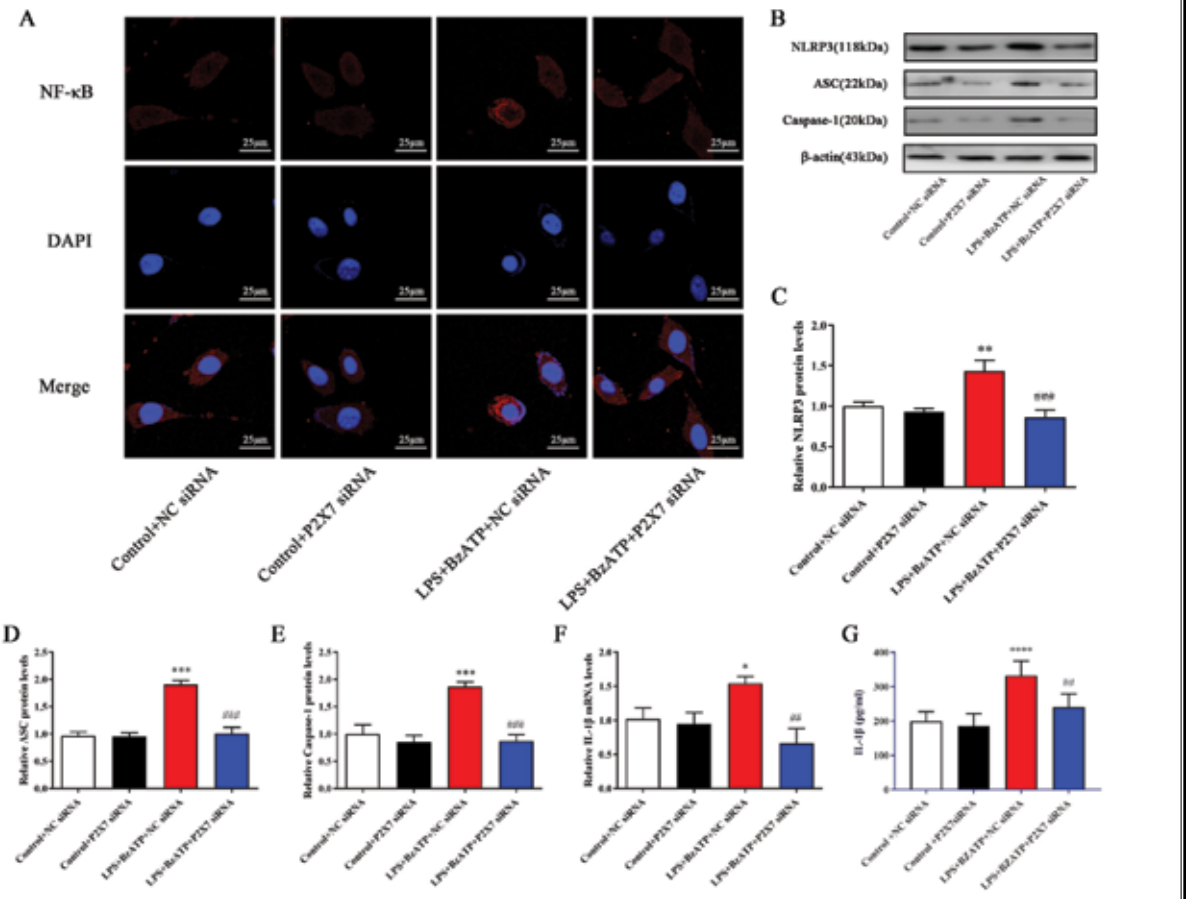

Fig. 5. *P2X7 siRNA inhibits NF-κB /NLRP3 mediated inflammatory response. (A) Representative immunofluorescence image of NF-κB (red) and PAPI (blue) in the Control + NC siRNA, Control + P2X7 siRNA, LPS + BzATP + NC siRNA, andLPS + BzATP + P2X7 siRNA group. (B)-(E) Representative western blotting bands and quantitative analysis showed the protein expression levels of NLRP3, ASC, and caspase-1 in the Control + NC siRNA, Control + P2X7 siRNA, LPS + BzATP + NC siRNA, and LPS + BzATP + P2X7 siRNA group. β-actin was used as an internal control. Data were presented as mean ± SD (n = 4 per group). **P < 0.01 versus Control + NCsiRNA group; ***P < 0.001 versus Control + NC siRNA group; ###P < 0.001 versus LPS + BzATP + NC siRNA group. . (F) Relative mRNA expression levels of IL-1β in the Control+NC siRNA, Control+P2X7 siRNA, LPS+BzATP+NC siRNA, and LPS+BzATP+P2X7 siRNA group. GAPDH was used as an internal control. *P < 0.05 versus Control+NC siRNA group; ##P < 0.01 versus LPS+BzATP+NC siRNA group. (G) ELISA analysis of IL-1β levels. Data were presented as mean ± SD (n = 4 per group). ****P < 0.0001versus Control+NC siRNA group; ##P < 0.01 versus LPS+BzATP+NC siRNA group.*

tors further to activate the NF-κB signaling (27). This process results in NLRP3 inflammasome corpuscles and pro-IL-1β synthesis (28). In a recent study, Wang et al reported that spinal TLR4 or P2X7R-dependent signaling-induced NLRP3 inflammasome activation in microglia contributes to the development of morphine-induced antinociceptive tolerance (29). In addition, Wu et al reported that inhibition of the P2X7 purinergic receptor ameliorates cardiac fibrosis by suppressing the NLRP3/IL-1β pathway (30). Herein, our results showed that the upregulated P2X7 in the BCP model induces BCP by activating NF-κB and NLRP3 to promote the release of IL-1β, and administration of BBG can significantly downregulate the expression of NF-κB and NLRP3 also reduce the secretion of IL-1β. This phenomenon was also observed in the activated microglia model. To further verify the regulatory mechanism of P2X7R and NF-κB / NLRP3 signaling, we co-treated the activated

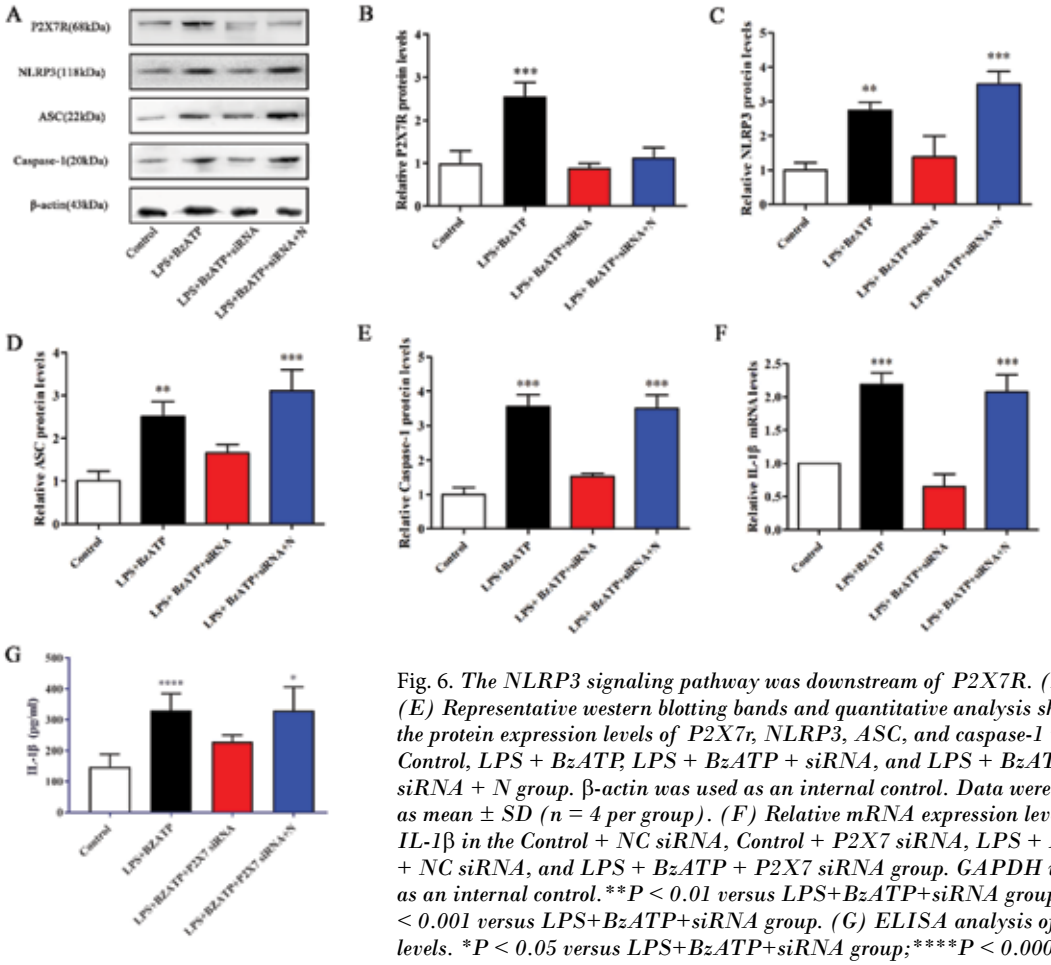

Fig. 6. *The NLRP3 signaling pathway was downstream of P2X7R. (A)-(E) Representative western blotting bands and quantitative analysis showed the protein expression levels of P2X7r, NLRP3, ASC, and caspase-1 in the Control, LPS + BzATP, LPS + BzATP + siRNA, and LPS + BzATP + siRNA + N group.* β*-actin was used as an internal control. Data were presented as mean ± SD (n = 4 per group). (F) Relative mRNA expression levels of IL-1*β *in the Control + NC siRNA, Control + P2X7 siRNA, LPS + BzATP + NC siRNA, and LPS + BzATP + P2X7 siRNA group. GAPDH was used as an internal control.**P < 0.01 versus LPS+BzATP+siRNA group; ***P < 0.001 versus LPS+BzATP+siRNA group. (G) ELISA analysis of IL-1*β *levels. *P < 0.05 versus LPS+BzATP+siRNA group;****P < 0.0001 versus LPS+BzATP+siRNA group*

BV2 microglial cell line with P2X7 siRNA and nigericin (an agonist of NLRP3 inflammatory). The results showed that NLRP3 inflammatory corpuscle agonists could reverse the downregulation of inflammatory corpuscle protein and the expression of downstream pain-promoting factor IL-1β caused by P2X7 siRNA. Some previous research has discussed the role of P2X7R in BCP, but to the best of our knowledge, this is the first study to confirm that P2X7R promotes BCP through the NF-κB /NLRP3 signaling in microglia.

Limitations

Numerous studies have shown that ATP released by the tumor microenvironment regulates tumor cell growth by activating P2X7R in tumor tissue (31). However, as a promoter of inflammatory cytokine release, P2X7R has not been reported on the relationship between tumor microenvironment and cancer pain. In our experiment, we used Walker256 breast cancer cells to establish a BCP model in rats. The x-ray results showed that BBG could not improve the destruction of bone (additional materials). We speculated that it may be related to the specificity of inoculated cells.

Further mechanisms should be investigated.

Conclusions

In conclusion, this study has shown that upregulation of P2X7R mediates microglial activation in the spinal dorsal horn, thereby contributing to the development and maintenance of BCP. P2X7R antagonist BBG could effectively alleviate cancer-induced bone pain. Moreover, the study provides supportive evidence

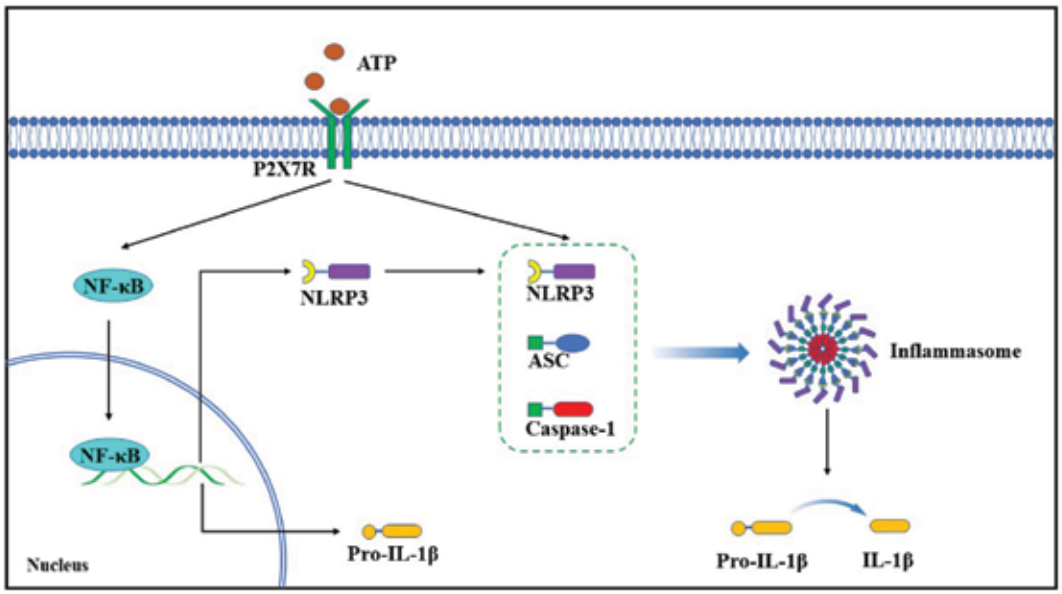

Fig. 7. *Signal pathway of P2X7R/NF-κB/NLRP3/IL-1β. ATP activates P2X7R, promotes NF-κB activation and nuclear translocation, promotes NLRP3 and pro-IL-1β transcription, NLRP3 forms inflammasome with ASC and caspase-1, and then promotes formation of IL-1β.*

that inhibiting P2X7R expression significantly reduced microglia activation via the NF-κB/NLRP3/IL-1β pathway both in vivo and in vitro. Therefore, our finding suggests that targeting the microglial P2X7R activated NF-κB/NLRP3/IL-1β signaling pathway might be a potential strategy for BCP treatment.

Supplementary material available at www.painphysicianjournal.com

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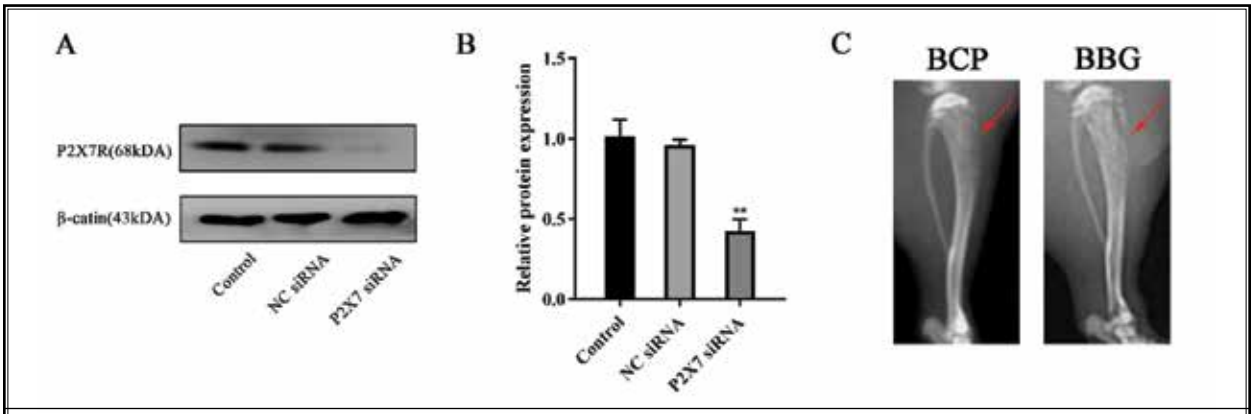

Fig. S1. *Additional materials. (A) and (B) Representative western blotting bands and quantitative analysis showed the protein expression levels of P2X7R in the Control, NC siRNA, and P2X7 siRNA groups. β-actin was used as an internal control. Data were presented as mean ± SD (n = 4 per group). **P < 0.01 versus NC siRNA group. (C) Radiographs of the tibia after Walker 256 cells inoculation in BCP group and BBG group. The red arrow represents the damage of the proximal cortical bone.*

Supplementary Table 1. *Antibody used in immunofluorescence*

Antibody	Company	Catalog Number	Application	Dilution
anti-P2X7R	Invitrogen	PA5-77665	IF	1:200
anti-Iba1	Servicebio	GB12105	IF	1:200
anti-GFAP	Servicebio	GB12096	IF	1:200
anti-NeuN	Proteintech	66836-1-Ig	IF	1:200
Cy3 conjugated Goat anti-mouse IgG	Servicebio	GB21301	IF	1:300
Alexa Fluor® 488-conjugated Goat Anti-Rabbit IgG	Servicebio	GB25303	IF	1:400
anti-NF-κB p65	Proteintech	10745-1-AP	IF	1:100
CoraLite594-conjugated Goat Anti-Rabbit IgG	Proteintech	SA00013-4	IF	1:200

Supplementary Table 2. *Antibody used in western blot analysis*

Antibody	Company	Catalog Number	Application	Dilution
Anti-Iba1	Abcam	Ab178846	WB	1:1000
Anti-P2X7R	Abcam	Ab109054	WB	1:1000
Anti-NLRP3	Abcam	Ab263899	WB	1:1000
Anti-ASC/ TMS1	ABclonal Technology	A1170	WB	1:1000
Anti-caspase-1	ABclonal Technology	A0964	WB	1:1000
Anti-IL-1β	Proteintech	16806-1-AP	WB	1:800
Anti-β-actin	Abcam	ab115777	WB	1:1000

Supplementary Table 3. *Primer sequences used for real-time polymerase chain reaction analysis*

Gene name	Forward 5'-3'	Reverse 5'-3'
IL-1β	CTTTCCCGTGGACCTTCCA	CTCGGAGCCTGTAGTGCAGTT
GAPDH	CCAAGGTCATCCATGACAAC	TCCACAGTCTTCTGAGTGGC

IL-1β interleukin 1β, GAPDH glyceradehyde-3 phosphate dehydrogenase