A740003 Can Reverse Chondrocyte Apoptosis and Prevent Extracellular Matrix Degradation by NF-KB Pathway in Facet Joint Osteoarthritis

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Abstract

Facet joint osteoarthritis (FJOA) is one of the common causes of low back pain, but the molecular mechanism is still unclear. Previous studies have found that P2X7 receptor has been proved to play an important role in skeletal and joint diseases. The purpose of this study was to explore the role of A740003, selective P2X7R antagonist, in the development of FJOA. Our study found that A740003 can inhibit the expression of P2X7R in OA chondrocytes. It can lead to anti-inflammatory and anti-apoptotic effects in primary chondrocytes by IL-1 β /BzATP. Our results imply that decreased P2X7R can reverse chondrocyte apoptosis and prevent extracellular matrix degradation by NF-KB pathway. Moreover, in our present work, we found that A740003 inhibit the aberrantly activation of NF-KB pathway by preventing the activated P65 translocation to nucleus. Our results indicate that P2X7R is a therapeutic target for the treatment of FJOA, and that A740003 could be a therapeutic candidate for this clinical application.

Introduction

Low back pain is a common pain syndrome which causes are very complex, including nerve root compression, spinal stenosis and others^[1]. In addition, previous studies have shown that facet joint osteoarthritis (FJOA) is also a common complaint of patients in clinic, its features include loss of articular cartilage integrity, synovitis and subchondral bone remodeling^[2, 3]. Chondrocytes are primary cells in articular cartilage which can regulate osteoarthritis through a variety of biological events. Chondrocyte apoptosis is the first and best characterized type of OA. A number of studies have shown that modulating the apoptotic-related pathways could improve the outcome of osteoarthritis^[4]. Besides, it can synthesise ECM components, which are composed mainly of type II collagen (Collagen II) and aggrecans, and play a critical role in maintaining the dynamic equilibrium between ECM anabolism and catabolism^[5, 6]. Therefore, a better understanding of the molecular mechanisms and functions of proteins related to the pathological process of chondrocyte degeneration is necessary for the treatment of lumbar facet joint osteoarthritis.

ATP is a non selective agonist, including 7 kinds of ionic purine receptor P2X and 8 kinds of metabolic purine receptor P2Y. As an excitatory neurotransmitter/modulator, ATP is widely present in the nervous system and performs a variety of physiological functions^[7-9]. Among them, the purine receptor P2X7 is widely distributed in vascular endothelial cells, red blood cells, immune cells, myocardial tissue, skeletal tissue, and muscle tissue^[10], highly selective to express in the small-diameter sensory neurons related to nociception, plays an important role in the modulation of nociceptive information. Previous studies have found that P2X7 receptor has been proved to play an important role in in skeletal and joint diseases by regulating the balance between osteoclasts and osteoblasts^[11, 12]. In osteoclast, the activation of P2X7 receptor can promote the calcium signaling pathway between osteoclast and osteoblast. In addition, it can induce the fusion of mononuclear precursors osteoclast and the formation of multinucleated osteoclast^[13]. In osteoblast, activation of P2X7 receptor also stimulates its proliferation and bone deposition^[14]. In knee osteoarthritis, inhibition of P2X7 can significantly alleviate the inflammatory effect

of chondrocytes and the degradation of cartilage matrix, which is dependent of NF-KB pathway^[15, 16]. However, the role of P2X7 in lumbar facet joint osteoarthritis is still unclear, which provides us with a new idea for the treatment of FJOA.

In this study, we investigate the expression of P2X7R in FJOA patients and primary chondrocytes. We further explain the role of A740003, a selective P2X7R antagonist, in the development of FJOA. We try to demonstrate the potential effect of A740003 on chondrocytes and elucidate its underlying mechanisms. Our results show A740003 inhibit P2X7R in IL-1 β stimulated chondrocytes. Moreover, it can reverse reverse chondrocyte apoptosis and prevent extracellular matrix degradation by NF-KB pathway which induced by P2X7R. This suggests that A740003, as a key factor for osteoarthritis, has great therapeutic potential to our clinical treatment of FJOA, Likewise A740003 can be a therapeutic candidate for this clinical application.

Materials And Methods

Antibodies and reagents

IL-1 β was obtained from Peprotech. A740003 (a P2X7R selective antagonist) and helenalin [a nuclear factor- κ B (NF- κ B) signaling pathway inhibitor] were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Abcam. Antibodies against p-P65, P65 were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against P2X7R, MMP13, COL2, GAPDH were obtained from abcam. Bax, Bcl-2 and cleaved caspase 3 antibody were obtained from Proteintech. Other reagents were obtained from Sigma unless noted otherwise.

Specimens

Human facet joint cartilage originally were obtained from patients with FJOA who underwent surgery in our hospital. They were divided into two groups: control group (n=5) and FJOA group (n=5). Informed consent was obtained from all individual participants included in the study. Each lumbar segment was examined by computed tomography (CT). After obtaining International Ethical Principles approval and patient consent, FJ tissues were harvested and the cartilage was visually graded for degeneration from grade 0 (normal), 1-2 (early degeneration), 3-4 (advanced degeneration) according to the Weishuapt grading system^[17].

Histological analysis

FJ tissues were surgically excised from the surgical patient and were fixed immediately in 4% PFA for 48 h and decalcified for 14 days. Following dehydration and embedded in paraffin wax, a portion of tissue sections were cut for hematoxylin and eosin (H&E) staining. The other were used for immunohistochemical analysis. According to the manufacturer's protocol, the sections were incubated with anti-P2X7 antibody (1:200), anti-COL2 antibody (1:200), then incubated with enzyme-labeled goat anti-mouse/rabbit IgG secondary antibody. Tissue sections were observed with an upright microscope

(Nikon, Japan). The level of cartilage degeneration was measured by the Osteoarthritis Research Society International (OARSI) scores^[18].

Chondrocyte isolation and culture

According to Muhammad 's protocol, primary chondrocytes were extracted from the articular cartilage mentioned above for further identification, chondrocytes between passage 0 and passage 2 were used for all experiments^[19]. Stimulus include IL-1 β (10ng/ml) A740003(0, 20,50 μ M) , BzATP (50 μ M) and helenalin(1 μ M).

Cell viability assay

Cell viability was performed by CCK-8 kit (Dojindo,Japan). According to the protocol, chondrocytes were firstly pre seeded in 96 well plates and stimulated with different stimulants (IL-1 β /A740003), then CCK-8 dye was added into each well, the absorbance was tested multifunctional microplate detector (BioTEK, USA).

ELISA

After IL-1 β stimulation, the amount of TNF-a and IL-6 from cell supernatants were measured according to the manufacturer's manual(R&D systems). Each independent experiment were performed in triplicate.

Flow cytometry

Flow Cytometry was performed in consistent with manufacturer's manual (BD Biosciences, cat. no. 556421). Briefly, cells were collected after different treatments, and the chondrocytes were incubated with Annexin V-PE and 7-AAD in the dark at room temperature, then analyzed by flow cytometry(BD FACSArialI).

Nuclear extract preparation

After resuspended in a hypotonic buffer (Beyotime, China, cat. no. P0027), primary chondrocytes were incubated at 4 °C for 30 min. They would undergo 1%Triton X-100 and vortexed vigorously for a 10 min preincubation on ice. After centrifugation for 1 min, 10 μ l of nuclear extraction buffer (Beyotime, China, cat. no. P0027) was used to resuspend. The pellets were stirred for 30 min at 4 °C followed by centrifugation for 5 min. Then the supernatants were collected as the nuclear extracts for Western blotting.

Western blotting

Total protein of chondrocytes was extracted with the cell lysis buffer. After loaded onto the SDS-PAGE gels (10–12%), PVDF membranes were transferred . The membranes were blocked with 5% skimmed milk at room temperature for 2 h and incubated with respective first primary antibodies at 4 °C overnight. The incubated primary antibodies were as follows: anti-P2X7R antibody (anti-rabbit,1:500), anti-Bax antibody

(anti-rabbit,1:200), anti-Bcl2 antibody (anti-rabbit,1:200), anti-cleaved caspase 3 antibody (anti-mouse, 1:200), anti-MMP13 antibody (anti-rabbit, 1:500), anti-COL2 antibody (anti-rabbit, 1:500), anti-P65 antibody (Anti-rabbit, 1:500), anti-P-P65 antibody (anti-rabbit, 1:500), anti-GAPDH antibody (anti-rabbit, 1:1000).The respective secondary antibody were incubated for 2h. After washing in TBST, the protein was visualized using ultra-sensitive ECL kit (Bio-Rad).

Immunofluorescence staining

The chondrocytes were cultured (2×10^4 cells/coverslip) in 24-well plates and treated with respective stimulation:IL-1 β (20 ng/ml) and(or) A740003 for 36 h.s. Then incubated with primary antibodies against MMP13(1:200), COL2(1:200), P65(1:200), followed with Alexa Fluor 488-conjugated anti-IgG second-ary antibodies and Alexa Fluor 647-conjugated anti-IgG for 2 h and stained with DAPI for 7 min. Samples were examined with an upright microscope (Nikon, Japan).

Statistical analysis

All data were analyzed using the GraphPad Prism 7 software (GraphPad Software, Inc., La Jolla, CA, USA). One-way ANOVA followed by the Tukey's test were used for statistical analysis. All data were presented as $P < 0.05$, $P < 0.01$ value was considered statistically significant.

Results

P2X7 receptor, consistent with osteoarthritis marker, is mainly expressed in degenerative cartilage

There is overwhelming evidence to indicate that COL-II decreased in degenerative cartilage, and they are often regarded as markers of osteoarthritis. As shown in fig1a, CT shows that the degenerative facet joint has a larger osteophyte around the joint capsule, and there is a vacuum sign in the joint space. H&E staining indicates that the surface of the joint space was eroded to form multiple fissures, and the chondrocytes in the deep layer were hypertrophic where many nuclei were shrunk. OARSI score also verify the results(fig1b). Immunohistochemical analysis shows that P2X7 receptor was up-regulated in the FJOA degeneration group (fig1a, e), and the extracellular matrix-related proteins, the expression of COL-II was decreased(fig1a, d).Together, these results suggested a potential role of P2X7 receptor in FJOA.

A740003, a selective P2X7 receptor antagonist, reverse the apoptosis of chondrocytes and inhibit the release of pro-inflammatory factor

We used A740003, a selective P2X7 receptor antagonist, to inhibit P2X7R with various concentrations. First of all, we extracted primary chondrocytes from the cartilage tissue of patients undergoing surgery, as shown in fig2a. Aggrecan, the marker of chondrocyte, was expressed in chondrocytes. Passage 0-2 primary chondrocytes were used to following experiments. And we noted that A740003 concentrations up to 20 μ M showed significant anti-apoptotic effects on IL-1 β treated-chondrocytes(fig2 b-c), 50 μ M A74003 concentration showed better effects. Compare to the control group, higher expression of IL-6, TNF- α , were

noted in IL-1 β group, and it can be reversed by the antagonist in 50 μ M, indicating the associated inflammatory response may relate to the apoptosis(fig2 d-e).

Inhibition of P2X7R by A740003 decreases apoptosis in chondrocytes

Since A74003 showed that it could reverse chondrocyte apoptosis in CCK-8 test, we verified the effect of A74003 on the expression of P2X7R and apoptosis related pathways by Western blot. Results confirmed that A740003 can reduce the expression of P2X7, Bax and Cleaved-caspase3 and raise the level of Bcl-2(fig3 a-b). Besides, we performed flow cytometry to detect the anti-apoptotic effect of A740003. Fig3c-d showed that IL-1 β can induce the apoptosis of chondrocytes compared the control group. However, the rise can be inhibited by A740003. These findings suggested that the A740003 might have a protective effect on the inflammation and chondrocyte apoptosis.

The anti-apoptotic effects of A74003 was modulated by NF-KB signaling in chondrocytes

It is mentioned in the above literature that the apoptosis of chondrocyte is accompanied by abnormal activation of NF-kB signaling pathway. We found IL-1 β caused the increased expression of p-p65, however, pretreated with different concentrations of A74003 can reduce the aberrantly expression(fig4 a-b). Furthermore,western blot show that helenalin(selective NF-KB signaling inhibitor), can abolish the pro-apoptotic effects of BzATP(strongly selective P2X7R agonist)(fig4 c-d). This phenomenon reveals that A74003 plays an anti apoptotic role by inhibiting the abnormal activation of NF-kB signaling pathway after inhibiting P2X7R.

A74003 can inhibit the translocation of p65 into nucleus after BzATP stimulation

The expression of downstream transcription factors and the release of inflammatory factors caused by p65 entry into the nucleus are considered to be the main mechanism of abnormal activation of NF-kB. As shown in fig5a-b, BzATP cause the increased expression of nuclear marker Histone-H3, A740003 partially supposed the effect of P65. The cytoplasm marker of A-tublin showed the opposite result. The immunofluorescence also indicated that translocation of p65 into the nucleus was weakened when A740003 was performed (fig. 5c).

A740003 decreased the ECM catabolic activity by NF-KB signaling in IL-1 β /BzATP-treated chondrocytes

We tested the effect of A740003 on IL-1 β -induced metabolic activity of ECM in chondrocytes using western blot analysis. As shown in fig.6a-b, IL-1 β induced the increase of MMP-13 and decrease of collegan II were reversed by A740003, immunofluorescence results show the similar expression in fig6g. Furthermore, the western blot in fig6c-f shows that the degradation of ECM were dependent on NF-KB signaling. All these indicate that A74003 plays an anti ECM catabolic activity by inhibiting the abnormal activation of NF-kB signaling pathway after inhibiting P2X7R.

Discussion

Facet joint degeneration(FJD) is a debilitating disease causing chronic back pain, stiffness and physical disability which may severely hurt patients' health^[20]. Risk factors of FJD induced chronic back pain has drawn increasing attention in recent years. It is obvious that inflammation, obesity and spinal instability are closely associated to the FJD^[21]. When OA occurs in articular cartilage, it is often accompanied by degradation of cartilage matrix, release of pro-inflammatory factors and apoptosis of chondrocytes^[22]. In view of this, many researchers have focused on whether there is such a therapeutic target in recent years, which can target the above several pathogenesis. However, the development of targeted therapy remains a major clinical challenge.

In recent years, the research of P2 purinergic receptors in bone metabolism has attracted much attention^[23]. P2 purinergic receptors family include P2X ion channel receptors family (p2x1-7) and P2Y family G protein coupled receptors (P2Y1, P2Y2, P2Y4, P2Y6, p2y11-14)^[24]. P2 purinergic receptors have attracted extensive attention in the fields of inflammation, cardiovascular disease and bone metabolic disease. Extracellular nucleotides can act on many types of cells and participate in functional regulation through them. Mountain of studies have shown that P2X7 receptor activation is related to many physiological and pathological processes, including autophagy, aging and metabolic diseases^[25]. In addition, evidence shows that adenosine triphosphate (ATP) can be used as an important local messenger in the regulation of bone and cartilage function^[26]. Studies have reported that P2X7 receptor can be expressed in osteoblasts, osteoclasts, osteocytes and synoviocytes^[11, 27]. It is the diversity of this expression that P2X7 receptor is widely involved in bone metabolism diseases, including osteoarthritis, osteoporosis, bone cancer, stress fracture and et al^[28].

In previous studies, Hu et al has already elaborated that P2X7R play an crucial role in the occurrence and maintenance of osteoarthritis. It can cause the release of inflammatory cytokine and degradation of extracellular matrix^[16]. We also support this by primary chondrocytes which were extracted from the patients of lumbar facet joint osteoarthritis. In current study, P2X7R was sharply increased in the cartilage tissue of FJD patients, which was also confirmed by the primary chondrocytes experiments.

Subsequently, western blot analysis and flow cytometry experiments showed that A74003 can effectively reverse P2X7 receptor dependent chondrocyte apoptosis. Further study show that this increase was through the NF- κ B signaling pathway. When P2X7 receptor was stimulated by extensive stimulus including ATP, inflammation, injury and oxidative stress, P2X7 receptor was activated rapidly, it promotes *P65* nuclear localization and enhances *P65* binding to its downstream target genes. The release of inflammatory cytokines and the degradation of extracellular matrix followed induce the persist apoptosis of chondrocytes. A74003 can reverse this effect by significantly inhibiting the aberrant activation of P2X7 receptor.

A740003, like a438079 and a839977, belongs to the second generation of specific P2X7 receptor antagonist. They display differences in chemical structure, species selectivity and specificity with traditional first generation P2X7 antagonists such as KN-62, PPADS, brilliant blue G (BBG)^[29]. Up to now, many in vitro experiments were still performed by the first generation of P2X7 receptor inhibitors, and the

development and utilization of the second generation has broad prospects. Studies have shown that A438079 can effectively reduce hind foot swelling and related pathological changes in the rat model of arthritis. Besides, the reduction of inflammatory cytokines was also observed in the cell model^[30]. Our experiment revealed that A74003, as a selective P2X7 inhibitor, can effectively reduce the release of inflammatory factors, alleviate the degradation of extracellular matrix and reverse chondrocyte apoptosis. This provides a basis for the development of related targeted drugs in the future.

Our study still has some limitations, the most significant of which is the lack of validation in rodent models. Different from knee arthritis, there is a lack of authoritative experiments to verify the pain caused by lumbar facet degeneration. In addition, there are no agreement on the destruction of lumbar facet capsule when constructing rodent models. Some believe that this kind of simulated back pain is more likely to be associated with the injury of lumbar facet capsule, which is different from degenerative lumbar disease to a certain extent. Therefore, our next focus is to develop a more valuable animal model to better explain the pathological mechanism of low back pain. However, it is undeniable that chondrocyte apoptosis is accompanied by the whole process of chondrocyte inflammation, A74003 is still an important targeted therapeutic drug with broad prospect.

In conclusion, in this study, A74003—a selective P2X7 receptor antagonist, was identified not only as a regulator which may alleviate *IL-1 β* induced apoptosis of chondrocytes, but that it can also decrease the expression of MMP-13 and NF- κ B signaling. All these indicate that A74003 may serve as a potential therapeutic target for the management of FJOA-related cartilage degenerative diseases.

Declarations

AUTHOR CONTRIBUTION

Pengfei Xue, Guanyin Wu, Jinhuricha and Jiawei Jiang performed the experiments; Mo Zhang, Chu chen and Chao Gui analyzed and interpreted the data; Guofeng Bao and Guanhua Xu wrote the manuscript; Zhiming Cui designed the experiments, wrote and revised the manuscript. All authors reviewed the results and approved the manuscript.

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DATA A VAILABILITY

The datasets generated/analyzed during the current study are available.

Ethics Approval The current study was performed with the approval of the Ethics Committee of the second affiliated hospital of nantong university. All animal experiments were approved by the Animal

Care and Use Committee of Nantong University and conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Extensive efforts were made to ensure minimal suffering of the included animals.

Conflict of Interest The authors declare no competing interests.

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Figures

Figure 1

P2X7 receptor, consistent with osteoarthritis marker, is mainly expressed in degenerative cartilage

a CT and H&E staining and immunohistochemical staining of degenerative fact joint and control group show the P2X7 and COL-II expression in each group(scale bar 50x). b box shows OARSI score between the two group, significant differences are indicated as * $P < 0.05$, $n = 5$. c-e boxes shows the expression of P2X7 and COL-II between the two group, significant differences are indicated as * $P < 0.05$, $n = 5$.

Figure 2

A740003, a selective P2X7 receptor antagonist, reverse the apoptosis of chondrocytes and inhibit the release of pro-inflammatory factor

a The immunofluorescence staining of primary chondrocytes shows positive Aggrecan immunoreactivity. b-c CCK-8 assays of A740003-pretreated chondrocytes stimulated by IL-1β show the

anti-apoptotic effects . d-e ELISA indicate that IL-1 β cause the sharp increase of TNF-a/IL-6, and A740003 can inhibit the release of above pro-inflammatory factor during various stimulations. Columns represent mean $\pm$ SD, Significant differences between the treatment and control groups are indicated as *P < 0.05, **P< 0.01, n=5

Figure 3

Inhibition of P2X7R by A740003 decreases apoptosis in chondrocytes

a Representative western blots of P2X7R, Bax, Bcl-2 and cleaved-caspase 3 in each group. b Quantification data of P2X7R, Bax, Bcl-2 and cleaved-caspase 3 in each group. c Analysis of chondrocyte

apoptosis in chondrocytes by flow cytometry assay. Columns represent mean $\pm$ SD, Significant differences between the groups are indicated as * $P < 0.05$, ** $P < 0.01$, $n=5$.

Figure 4

The anti-apoptotic effects of A74003 was modulated by NF-KB signaling in chondrocytes.

a, b Representative western blots and quantification data of p-P65 and P65 in each group. c-d Representative western blots and quantification data of p-P65, P65, Bax, Bcl-2, and cleaved-caspase 3 in

each group. Columns represent mean $\pm$ SD, significant differences between groups are indicated as * $P < 0.05$, ** $P < 0.01$, $n=5$.

Figure 5

A74003 can inhibit the translocation of p65 into nucleus after BzATP stimulation

a,b Representative western blots and quantification data of P65 in nucleus or cytoplasm in each group. c Translocation of cytoplasmic and nuclear NF-kB p65 in the chondrocytes, assessed by immunofluorescence staining (scale bar = 50μm). Columns represent mean ± SD, Significant differences between the treatment and control groups are indicated as *P < 0.05, n=5.

Figure 6

A740003 decreased the ECM catabolic activity by NF-KB signaling in IL-1 β /BzATP-treated chondrocytes

a, b Representative western blots and quantification data of MMP-3 and collegan-II in each group. c-f Representative western blots and quantification data of p-P65, P65, MMP-3 and collegan-II in each group. g-h Immunofluorescence staining of MMP-3 and collegan-II proteins in each group (scale bar: 50 μ m). Columns represent mean $\pm$ SD, Significant differences between the groups are indicated as *P< 0.05, **P< 0.01, n=5.