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Rhein inhibits ATP-triggered inflammatory responses in rheumatoid rat fibroblast-like synoviocytes

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic and systemic inflammatory disorder, which may lead to joint disabilities. So far the pathogenesis of RA remains largely undetermined, and there are still no potent drugs for clinical treatment. Rhein, a natural bioactive anthraquinone derivative, exhibited significant anti-inflammatory activities demonstrated by previous studies. Here we aimed to investigate the effects of rhein on ATP-induced inflammation responses in fibroblast-like synoviocytes isolated from a rat model of collagen induced arthritis (CIA). Our results showed that ATP triggered rapid cytosolic calcium concentration ($[Ca^{2+}]_c$) increase depending on extracellular Ca^{2+} entry. Given the major P2 subtypes expressed in rat synoviocytes were $P2X_4$ and $P2Y_2$ receptors, ATP-elicited calcium entry should be mainly resulted from activating $P2X_4$. Interestingly, rhein could effectively block the ATP-induced $[Ca^{2+}]_c$ increases in a dose-dependent manner. Besides, rhein also suppressed the production of intracellular reactive oxygen species (ROS) induced by ATP in synoviocytes that was resulted from $P2X_4$-mediated Ca^{2+} entry. Brilliant blue G (BBG), which can block $P2X_4$ receptor at high concentration, showed similar suppressive effects on above responses. Furthermore, in lipopolysaccharide-primed cells, application of ATP synergistically promoted the gene expression of cyclooxygenase-2, interleukin-6 and matrix metalloproteinase-9. Both rhein and BBG attenuated these inflammatory gene expressions enhanced by ATP. Above data together suggested a potential anti-arthritic role of rhein by inhibiting ATP-induced $[Ca^{2+}]_c$ increase, ROS production and inflammatory gene expression targeting $P2X_4$ in CIA rat synoviocytes, which will provide a novel insight in the therapy of RA.

1. Introduction

Rheumatoid arthritis (RA), a type of chronic systemic autoimmune disease, is prominently marked by inflammatory synovitis and progressive joint destruction [1]. However, there is still no specific drug for RA clinical treatment up to date [2]. The pathogenesis of RA primarily shows an inhomogeneous distribution of immune cells in the synovial lining [3] and production of diverse pro-inflammatory cytokines [4]. These cytokines thereby activate fibroblast-like synoviocytes, which play pivotal roles in joint erosion by hyperplasia, or further producing inflammatory mediators such as prostaglandin E2 (PGE2) and interleukin (IL)-6 that perpetuate inflammation [5], as well as matrix-degrading proteases that contribute to cartilage and bone destruction [6]. Therefore, synoviocytes act as key effector cells in RA progression and new agents targeting them will potentially improve current therapies.

Rhein (4, 5-dihydroxyanthraquinone-2-carboxylic acid) is a natural bioactive anthraquinone derivative extensively found in traditional Chinese herbs such as rhubarb, *Polygonum multiflorum* and aloe [7]. Previous studies have demonstrated that rhein possesses a series pharmacological activities including anti-inflammation [7], anti-tumor [8], and anti-angiogenesis [9]. Particularly, diacerein, a purified metabolic precursor of rhein, has long been used in the clinical treatment of osteoarthritis (OA) [10]. Furthermore, it has been reported that rhein/diacerein could alleviate the severity of joint inflammatory responses and bone destruction in adjuvant-induced arthritis rats [11–13], which proposed a promising application of rhein in the therapy of not only OA but also RA.

Recently, emerging evidences indicated that the anti-inflammation mechanisms of rhein may involve the regulation of receptor activity and corresponding signaling transduction [14,15]. During inflammatory processes, large amount of nucleotides including ATP, ADP and UTP liberated from apoptotic cells and acted as signal molecules by

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interacting with membrane purinergic P2 receptors [16]. P2 receptors can be subdivided into two distinct families: ligand-gated ion channel P2X and G-protein coupled P2Y receptors, both of which are closely associated with mediating inflammation [17–19]. For instance, activation of P2X subtypes $P2X_7$ and $P2X_4$ can promote the production of pro-inflammatory cytokine IL-1β in macrophages and OA synoviocytes, respectively [20,21]. Nowadays, great efforts have been made to discover specific P2 antagonists for new therapeutic strategies against RA and other inflammatory diseases [22,23].

In this study, we intended to determine the effects of rhein on ATP-induced inflammatory responses including intracellular calcium mobilization, reactive oxygen species (ROS) production and inflammatory gene expressions in isolated collagen induced arthritis (CIA) rat fibroblast-like synoviocytes and explore the underlying molecular mechanism. Our results provide compelling evidences for the inhibitory effects of rhein on these ATP-induced inflammatory responses via blockade of $P2X_4$ receptors in CIA rat synoviocytes.

2. Materials and methods

2.1. Ethics statement

The animal protocol in this study conformed to the Guide for the Care and Use of Laboratory Animals (*the Guide*, NRC 2011), and it was also approved by the Institutional Animal Care and Use Committee at Nankai University (Approval ID 201009080081).

2.2. Animals and reagents

Healthy male Wistar rats with body weight of 200 ± 50 g were obtained from Institute of Health and Environmental Medicine, Academy of Military Medical Sciences (Tianjin, China, Certification Number: SCXK 2016-0006). DMEM and fetal calf serum (FCS) were purchased from Gibco (USA) and HyClone (USA), respectively. Fura-2/AM was from Biotium (USA). Superoxide dismutase (SOD) and catalase (CAT) were purchased from Beyotime Institute of Biotechnology (Haimen, China). Freeze-dried BCG vaccine was from Shanghai Institute of Biological Products (China). The rest of reagents, including rhein, ATP, BzATP, collagen type II, Freund's incomplete adjuvant, collagenase II, lipopolysaccharide (LPS), dihydroethidium (DHE), ethidium bromide (EB), BBG (brilliant blue G), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethylsulfoxide (DMSO) were from Sigma (USA).

2.3. Establishment of collagen-induced arthritis (CIA) rat models and culture of fibroblast-like synovial cells

The establishment of CIA rat models was followed the method described previously [24]. Twenty healthy rats were used for the induction of CIA. Firstly, freeze-dried BCG vaccine and Freund's incomplete adjuvant were homogenized at a 1:2.5 ratio to prepare the Freund's complete adjuvant. Then, collagen type II (CII) and Freund's complete adjuvant were homogenized at a 1:1 ratio, with sufficient emulsification to make the final concentration of CII to 2 mg/ml. Each rat was injected intradermally at multiple sites on the back with a total of 0.3 ml of the emulsion (day 0). Seven days later (day 7), this immunization protocol was repeated. After the initial injection of 15–20 days, 11 rats began to show inflammatory response characterized by red and swollen ankles. By day 21 the inflammatory response has reached its peak, and 30–40 days later there was significant joint pathology. Then, the isolation and culture of synovial fibroblasts were performed as reported [25]. Briefly, one of the rat with arthritis was sacrificed and its hind limbs were excised. After that, the articular synovial membranes were carefully separated, minced in D-Hank's buffer, and digested in 0.2% collagenase II for 3 h at 37 °C in serum-free DMEM. The cell suspension was centrifuged at 300*g* for 10 min, and the isolated synovial cells were resuspended and cultured in DMEM supplemented with 10% FCS in a humidified incubator with 5% CO_2 at 37 °C. Cultured cells were subjected to a minimum of 6 passages to obtain a pure culture.

2.4. Reverse transcription and quantitative real-time PCR

Total RNA from rat synoviocytes was isolated using RNAprep pure Cell/Bacteria Kit (Tiangen Biotech, China) following the manufacturer's instructions. The RNA (1 μg) was subjected to reverse transcription (RT) using the PrimeScript™RT Master Mix (Takara, Japan). The reaction mixtures were incubated at 45 °C for 30 min, 99 °C for 5 min to inactivate the enzyme, and then chilled on ice for 5 min. Subsequently, the product of RT reaction (1 μl) was amplified using a GoTaq PCR Core system (Promega, USA) in a total volume of 50 μl PCR buffer containing Green Master Mix (25 μl), sense primer (100 pM) and antisense primer (100 pM). The reaction mixtures were preheated to 95 °C for 2 min followed by 40 thermal cycles in a PCR machine (MJMini™, BIO-RAD, USA). For each cycle, denaturation was at 95 °C for 30 s, annealing at 63.5 °C for 30 s, and extension at 72 °C for 1 min. Quantitative real-time PCR (qRT-PCR) was conducted with SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, Japan). Results were analyzed using the ΔΔCt method and expressed as the fold changes in gene expression after normalization. Sequences of gene-specific primers (Table S1) were designed using the Primer 6 (Premier Biosoft, USA) and evaluated with Oligo 7 (Molecular Biology Insights, USA).

2.5. Measurement of cytoplasmic Ca^{2+} concentrations ($[Ca^{2+}]_c$)

$[Ca^{2+}]_c$ was measured by Ca^{2+} imaging with ratiometric fluorescent Ca^{2+} indicator fura-2 as previously described [25]. Synoviocytes were treated with indicated agents in prior to load with 5 μM fura-2/AM in HBSS for 1 h at room temperature in dark. After a gentle washing step, cells were bathed in fresh HBSS solution for monitoring the changes in $[Ca^{2+}]_c$ triggered by ATP/BzATP. The calcium imaging system was built on an inverted fluorescence microscope (Olympus IX51), and alternately excited at 340 nm and 380 nm with a Lambda 10-2 Sutter (Sutter Instrument, USA). Fluorescence images (filtered at 515 nm ± 25 nm) were acquired by a CCD camera (CoolSNAP fx digital monochrome detector system, Roper Scientific Inc.) and analyzed with MetaFluor (Universal Imaging Corporation, USA). $[Ca^{2+}]_c$ was represented by the ratio of fluorescence intensity at 340 nm/fluorescence intensity at 380 nm (F340/F380). At least three independent experiments were done for each condition. One curve of calcium changes was plotted as the representation of other similar traces.

2.6. Detection of intracellular reactive oxygen species (ROS)

After treatment with indicated reagents, synoviocytes were incubated with 5 μM dihydroethidium (DHE), a reduced form of ethidium bromide (EB), in HBSS for 30 min at 37 °C in dark. Then, the cells were rinsed twice with HBSS and observed by a fluorescence microscope at the excitation wavelength of 488 nm and emission wavelength of 610 nm with a 20× objective. The intracellular ROS level is represented by the fluorescent intensity of DHE.

2.7. Immunofluorescence for $P2X_4$ and $P2X_7$

Synoviocytes were seeded on confocal dishes (Corning, USA) at 2×10^4 cells per well, incubated overnight before immunostaining. After fixing with 4% paraformaldehyde and permeabilizing with 0.1% Triton X-100 for 15 min consecutively, the samples were incubated in blocking solution (5% BSA in PBS) for 1 h to block non-specific protein-protein interactions. Then, the cells were incubated with a polyclonal antibody against rat $P2X_4$ or $P2X_7$ (Alomone labs, Israel) at 1:200 dilutions overnight at 4 °C. After washing in washing buffer (0.2% BSA

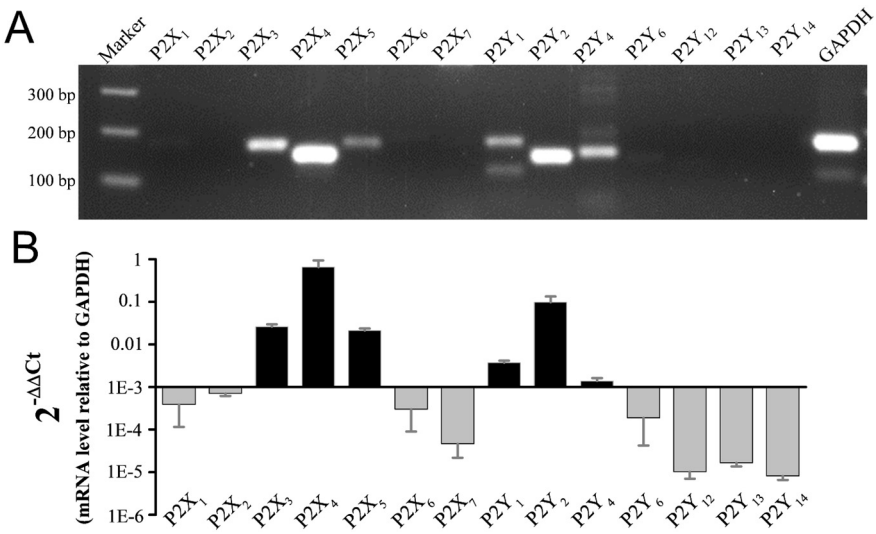

Fig. 1. The expression of P2 receptors in CIA rat synoviocytes. (A) Gene expressions of P2 receptors in rat synoviocytes at mRNA level analyzed by RT-PCR and shown in a gel. The lanes in the gel were organized as follows: Marker (standardized DNA sequences from 100 bp to 300 bp), $P2X_{1,2,3,4,5,6,7}$ & $P2Y_{1,2,4,6,12,13,14}$ (experimental group with primers directed towards the rat P2 mRNA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, housekeeping gene, positive control). (B) Quantitative statistical results of real time PCR shown as the fold changes of gene expression by normalizing to GAPDH (GAPDH group was taken as 1, $n = 3$ for each group).

and 0.1% Triton X-100 in PBS) for three times, the cells were incubated with the secondary antibody Alexa Fluor® 647 goat anti-rabbit IgG (H + L) (ThermoFisher, USA) at 1:400 dilutions for 1 h at room temperature. DAPI (2 μM) was used to stain the cell nuclei for 5 min. Finally, after washing with PBS for three times, images were collected by illuminating with 647 nm or 385 nm light and detecting emission at 699 nm or 465 nm on a Nikon Eclipse Ti-E inverted fluorescence microscope using a 40× oil-immersion objective.

2.8. Western blotting for $P2X_4$ and $P2X_7$

Synoviocytes were seeded in a 25 cm^2 flask (~2 × 10^6 cells) and incubated overnight in DMEM with 10% FBS at 37 °C. Total protein lysates were isolated by RIPA (Beyotime, China) and the concentration of protein was determined using a BCA assay kit (Beyotime, China). Aliquots (20 μg/lane) were separated by 8% SDS-PAGE and transferred to a nitrocellulose membrane, then blocked non-specific binding sites with 5% skimmed milk at room temperature for 1 h, immunoblotted with anti-$P2X_4$ or $P2X_7$ antibody (1:200, Alomone labs, Israel) as well as anti-β-actin antibody (1:20,000, Proteintech, USA) overnight at 4 °C, followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:1000; Beyotime, China). Finally, the ECL detection reagent (Proteintech, USA) was used for visualization in Tanon 5200 MultiImage System.

2.9. Dye uptake assay

Synoviocytes were seeded in 6-well plates (Corning, USA) at 1 × 10^6/well and incubated in fresh culture medium for 4 h. Following treatment with indicated concentrations of rhein for 20 min, the cells were exposed to 5 mM ATP for 10 min in the presence of 6 μM EB. Dye uptake positive cells were identified by illuminating with 488 nm light and detecting emission at 633 nm using an Olympus IX51 inverted fluorescence microscope with a 20× objective.

2.10. Cell viability assay

Cell viability was tested by MTT assay. Briefly, synoviocytes were seeded into 96-well plate at ~1 × 10^4 cells/well and incubated overnight before treated with or without test agents for the indicated time. MTT solutions was added into each well, and then incubated for 4 h at a final concentration of 10 μg/ml before the end of such treatments. The culture medium was aspirated and replaced with 100 μl lysis solution (50% DMSO and 50% ethanol). The absorbance at 570 nm (A_{570}) for each well was determined by an ELISA reader (Bio-Rad Imark Microplate Reader). The percentage of living cells was calculated as $A_{570,\ test}/A_{570,\ control}$.

2.11. Statistical analysis

All data are presented as mean ± standard deviation (SD) from three independent experiments. The statistical comparison between two groups was carried out using Student's *t*-test (Origin 8.5), and the analysis for multiple groups was done using Dunnett's test (SPSS 18.0, one-way ANOVA). $P < 0.05$ was considered to be statistically significant. The values of half maximal inhibitory concentration (IC_{50}) were derived by the dose-response curve fitting with the Hill1 equation (Origin 8.5): $Y = START + (END - START)\frac{x^n}{IC_{50}{}^n + x^n}$, in which Y is the response ratio, x is the concentration of rhein and n is the Hill coefficient.

3. Results

3.1. Expression of P2 receptor subtypes in CIA rat synoviocytes

We examined the expression of different subtypes of P2 receptors in primary synoviocytes isolated from a CIA rat model using RT-PCR. The total RNA from synoviocytes was reverse-transcribed and subjected to PCR amplification using the previously cloned specific primers for rat P2 receptor mRNA (Table S1). As shown in Fig. 1A, rat synoviocytes co-express multiple subtypes of P2 receptors, including $P2X_{3,4,5}$ and $P2Y_{1,2,4}$ receptors. The quantitative mRNA levels of these P2 subtypes were further evaluated by real time PCR using the ΔΔCt method. Data showed that the gene expressions were 2.61 ± 0.34% for $P2X_3$, 65.52 ± 22.64% for $P2X_4$, 2.10 ± 0.28% for $P2X_5$, 0.37 ± 0.11% for $P2Y_1$, 9.74 ± 1.37% for $P2Y_2$, and 0.13 ± 0.003% for $P2Y_4$ relative to GAPDH, respectively (Fig. 1B). Therefore, these results clearly indicated that the predominant P2 subtypes expressed in rat synoviocytes were $P2X_4$ and $P2Y_2$ receptors, involving slight expression of $P2X_{3,5}$ and $P2Y_{1,4}$ whereas almost excluding $P2X_{1,2,6,7}$ and $P2Y_{6,12,13,14}$.

3.2. Rhein inhibited ATP-triggered Ca^{2+} entry in rheumatoid rat synoviocytes via antagonizing $P2X_4$ receptors

Then, we tested the influence of rhein on the ATP-induced Ca^{2+} response in rat synoviocytes. As expected, ATP (1 mM) triggered a rapid single-peak $[Ca^{2+}]_c$ increase (Fig. 2Aa). This Ca^{2+} response was significantly inhibited by removal of extracellular calcium using EGTA, suggesting that ATP-elicited increase in $[Ca^{2+}]_c$ was mainly dependent on extracellular Ca^{2+} entry (Fig. 2Ab and C). According to the mRNA

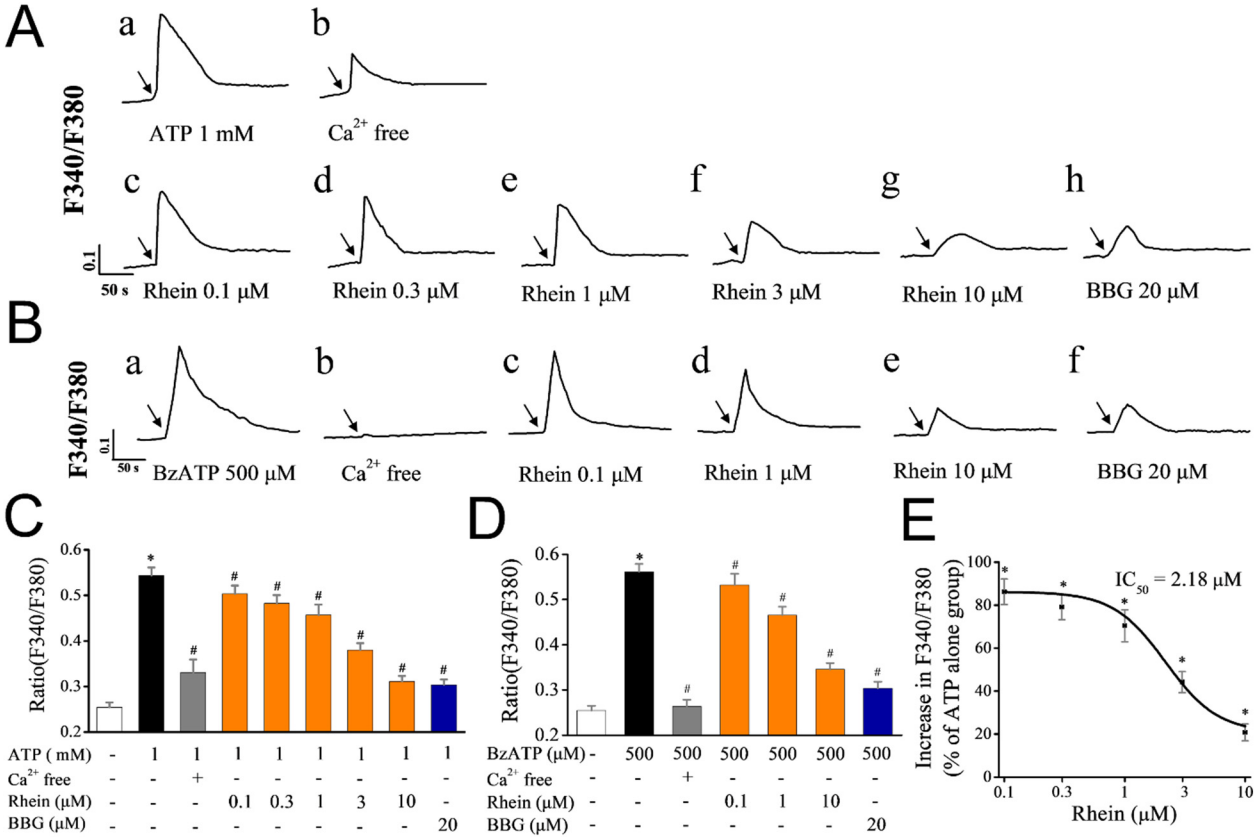

Fig. 2. Rhein inhibited the ATP/BzATP-induced $[Ca^{2+}]_c$ increases in rat synoviocytes. (A) Representative $[Ca^{2+}]_c$ traces for stimulating synoviocytes with ATP (1 mM) in Ca^{2+} containing solution (a), Ca^{2+} free solution (b) or after pretreatment with rhein (0.1, 0.3, 1, 3, 10 μM) (c–g) as well as BBG (20 μM) (h). (B) Typical $[Ca^{2+}]_c$ profiles for stimulating synoviocytes with BzATP (0.5 mM) in Ca^{2+} containing solution (a, control), Ca^{2+} free solution (b) or after pretreatment with rhein (0.1, 1, 10 μM) (c–e) as well as BBG (20 μM) (f). Arrows indicated the application of ATP or BzATP. (C and D) Summary of the peak values in F340/F380 ratio after application of ATP (C) or BzATP (D) from various experiments shown in (A and B). *, $P < 0.05$, compared to control group; #, $P < 0.05$, compared to ATP/BzATP alone group ($n = 15$ cells for each case). (E) Statistic data of the relative percentage of increase in F340/F380 ratio after application of ATP were plotted against the dose of rhein, with the smooth curve represented the fit to the Hill1 equation (ATP alone group was taken as 100%). *, $P < 0.05$ compared with ATP alone group (n = 15 cells for each case).

expression results, $P2X_4$ and $P2Y_2$ receptors were the major P2 subtypes expressed in rat synoviocytes (Fig. 1). Thus, ATP-elicited Ca^{2+} entry should be mostly resulted from the opening of ligand-gated $P2X_4$ receptor ion channel. Rhein was found to robustly inhibit ATP-triggered $[Ca^{2+}]_c$ increase (Fig. 2Ac–g and C). The inhibition was dependent on the concentration of rhein with an IC_{50} of 2.18 μM (Fig. 2E). To further verify the roles of rhein on $P2X_4$ receptors, we also evaluated the ATP analog 2′,3′-O-(benzoyl-4-benzoyl)-ATP (BzATP), a non-selective P2X receptor agonist [26]. It was shown that BzATP (0.5 mM) resulted in a rise in $[Ca^{2+}]_c$ completely through extracellular Ca^{2+} entry in synoviocytes (Fig. 2Ba,b and D). Similarly, rhein inhibited BzATP-induced increase in $[Ca^{2+}]_c$ (Fig. 2Bc–e and D). Besides, as reported, brilliant blue G (BBG) can block rat $P2X_4$ receptor with $IC_{50} > 10$ μM [27]. Thus, we also performed parallel experiments utilizing BBG (20 μM) as a positive control. BBG exhibited similar inhibitory effects on ATP- or BzATP-induced increases in $[Ca^{2+}]_c$ to those of rhein, respectively (Fig. 2Ah, Bf, C and D), strengthening the involvement of $P2X_4$ in $[Ca^{2+}]_c$ increase triggered by ATP/BzATP. These results together suggested an efficient inhibitory effect of rhein on $P2X_4$-mediated extracellular Ca^{2+} entry in rat synoviocytes.

3.3. Rhein suppressed ATP-evoked ROS production in rat synoviocytes

We further examined the effects of rhein on ATP/BzATP-evoked intracellular ROS production, a key intracellular signaling component associated with various inflammatory responses by activating different signaling pathways in RA [28]. As shown in Figs. 3 and S1, stimulation with ATP (1 mM) or BzATP (0.5 mM) for 30 min induced augmented intracellular ROS generation in synoviocytes comparing with control (cells treated with vehicle solution). ATP-evoked ROS accumulation was abolished in calcium-free solution (Fig. 3Ac and B), indicating a potential role of calcium entry mediated by $P2X_4$. In contrast, rhein (10 μM) or BBG (20 μM) alone had little influences on ROS production (data not shown). Pre-incubation with rhein (0.1, 0.3, 1, 3, 10 μM) or BBG (20 μM) for 3 h effectively suppressed the ROS production evoked by ATP, respectively (Fig. 3A and B). The IC_{50} of rhein on ATP-induced ROS was 1.05 μM (Fig. 3C). Rhein also blocked BzATP-induced ROS generation in a concentration-dependent manner (Fig. S1). Besides, ROS generation was inhibited by pretreatment with two typical ROS scavengers, superoxide dismutase (SOD, 100 U/ml) and catalase (CAT, 100 U/ml), as positive controls (Fig. 3Ae,f and B). Taken together, these results suggested a suppressive effect of rhein on ATP/BzATP-induced ROS production, which might be controlled by $P2X_4$-mediated Ca^{2+} entry.

3.4. Rhein inhibited ATP-induced inflammatory gene expression in LPS-primed synoviocytes

Since the pathological processes of RA are mediated by a number of cytokines and MMPs [4–6], we finally evaluated the gene expression levels of several related inflammatory mediators including prostaglandin-endoperoxide synthase 2 (also known as cyclooxygenase-2 or

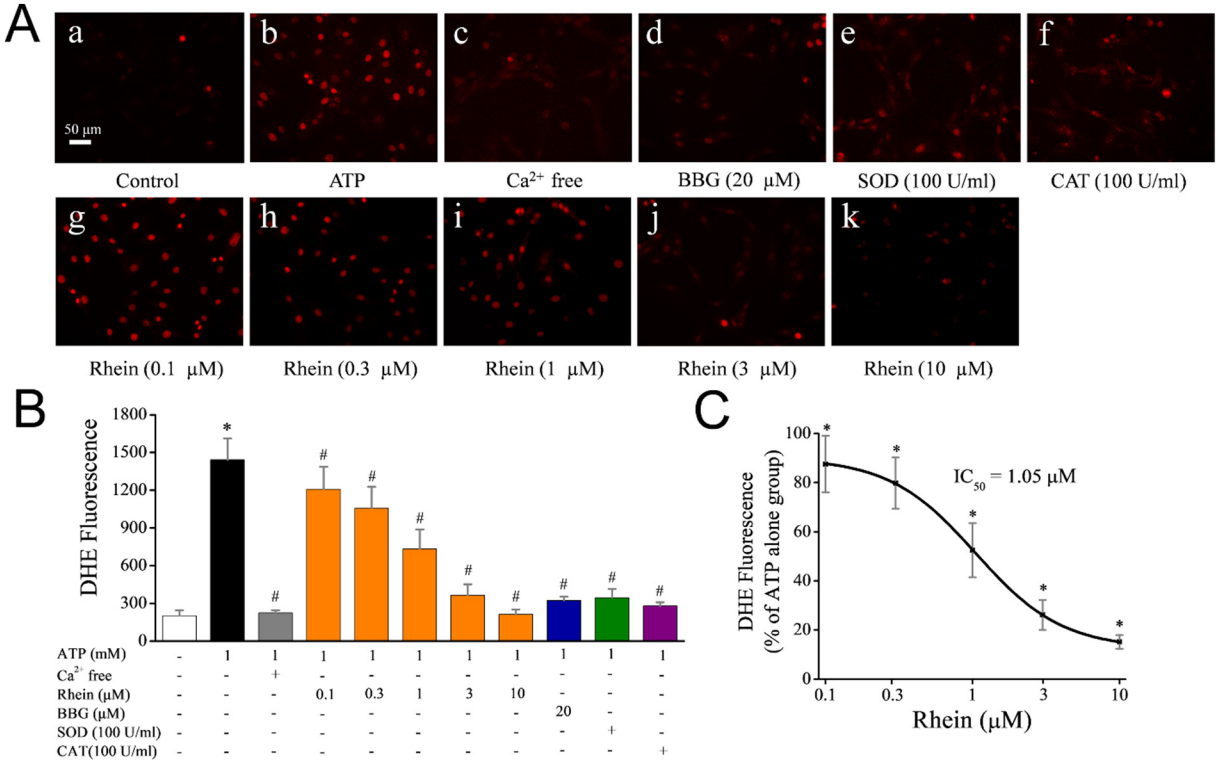

Fig. 3. Rhein attenuated ATP-induced ROS production in rat synoviocytes. (A) Dihydroethidium (DHE) fluorescence images for stimulating synoviocytes with ATP (1 mM) for 30 min after pretreatment with rhein (0.1, 0.3, 1, 3, 10 μM), BBG (20 μM), SOD (100 U/ml) and CAT (100 U/ml) for 3 h. (B) Statistic data for DHE fluorescence intensity from three independent experiments. *, $P < 0.05$, compared to control group; #, $P < 0.05$, compared to ATP alone group ($n = 90$ cells for each case). (C) Summary of the relative percentage of fluorescence intensity in rhein group (ATP alone group was taken as 100%). The smooth curve represents the fit to the Hill1 equation with an IC_{50} of 1.05 μM. *, $P < 0.05$, compared with ATP alone group.

COX-2), interleukin-6 (IL-6) and matrix metalloproteinase-9 (MMP-9) by qRT-PCR. As previously suggested [29], the cell cultures were more responsive to ATP after LPS priming. Therefore, synoviocytes were pretreated with 5 μg/ml LPS for 5 h before ATP treatment in following experiments. As shown in Fig. 4, the mRNA expressions of all these genes were enhanced in LPS-activated synoviocytes. After stimulation with ATP (1 mM) for additional 2 h in the presence of LPS, the levels of these three genes were further promoted (Fig. 4A, D and G). Pretreatment with rhein together with LPS dose-dependently downregulated the levels of these inflammatory genes with an IC_{50} of 1.38 μM for COX-2 (Fig. 4B), 1.55 μM for IL-6 (Fig. 4E) and 2.97 μM for MMP-9 (Fig. 4H), respectively. Furthermore, the levels of these genes were also attenuated by BBG (Fig. 4C, F and I), implying the involvement of $P2X_4$. Collectively, these data indicated that rhein reduced ATP-induced inflammatory gene expression by inhibiting the activation of $P2X_4$ receptors.

3.5. ATP had little effect on membrane permeability and cell viability in rat synoviocytes

As reported, prolonged stimulation with high concentration of ATP will lead to pore formation and cell death in many cell types such as macrophages and microglia, which were hallmark responses associated with $P2X_7$ receptor activation [30]. Despite the trace mRNA level of $P2X_7$ in RA rat synoviocytes, we still checked these effects because of the prominent contribution of $P2X_7$ to inflammation [17–20]. However, stimulation with ATP (5 mM) for 30 min had little effect on the cell membrane permeability examined by EB uptake in synoviocytes compared with HEK293 cells expressing rat $P2X_7$ receptors (a generous gift from Dr. Lin-Hua Jiang) (Fig. 5A). Moreover, the cell viability was not influenced after exposure to ATP (1, 2, 5 mM) for 24 h by MTT assay (Fig. 5B). These results excluded the participation of $P2X_7$. Besides of the quantitative RT-PCR data, western blotting (Fig. 5C) and immunofluorescence (Fig. 5D) results also revealed little $P2X_7$ receptor expression at protein level in synoviocytes, corroborating that above responses were mostly attributed to $P2X_4$ rather than $P2X_7$ receptors.

4. Discussion

Rhein, one of the effective constituents in traditional Chinese herb rhubarb, exerts pleiotropic pharmacological activities particularly anti-inflammation [7–9,13–15]. Clinical applications have validated the remarkable curative effects of rhein in inflammatory diseases such as osteoarthritis (OA) and diabetic nephropathy (DN) [10,31]. To date, there has been significant progress in understanding the mechanisms underlying the anti-inflammation ability of rhein at the cellular level. It was found that rhein could attenuate the synthesis and secretion of IL-1β, tumor necrosis factor (TNF)-α as well as nitric oxide through inhibition of nuclear factor-κB (NF-κB) signaling in OA chondrocytes and macrophages [32–36]. Rhein could also down-regulate expression of matrix metalloproteinases by suppressing mitogen-activated protein kinase (MAPK) pathways in OA chondrocytes [37,38]. Additionally, rhein was reported to decrease ROS generation in monocytes and macrophages [39,40], as well as reducing the proliferation of OA synoviocytes and chondrocytes [41].

In spite of abundant researches regarding anti-OA properties of rhein, the probable curative effects of rhein on RA still remain to be elucidated. Like other inflammatory diseases, the pathological process of RA is accompanied with increased concentration of extracellular ATP [42]. Therefore, in the current study, we firstly examined the effects of ATP on fibroblast-like synoviocytes, the key effector cells in RA pathogenesis and progression [43]. It was shown that ATP robustly triggered increase of $[Ca^{2+}]_c$ (Fig. 2) and resulted in intracellular ROS accumulation (Fig. 3) in rat synoviocytes derived from a CIA rat model,

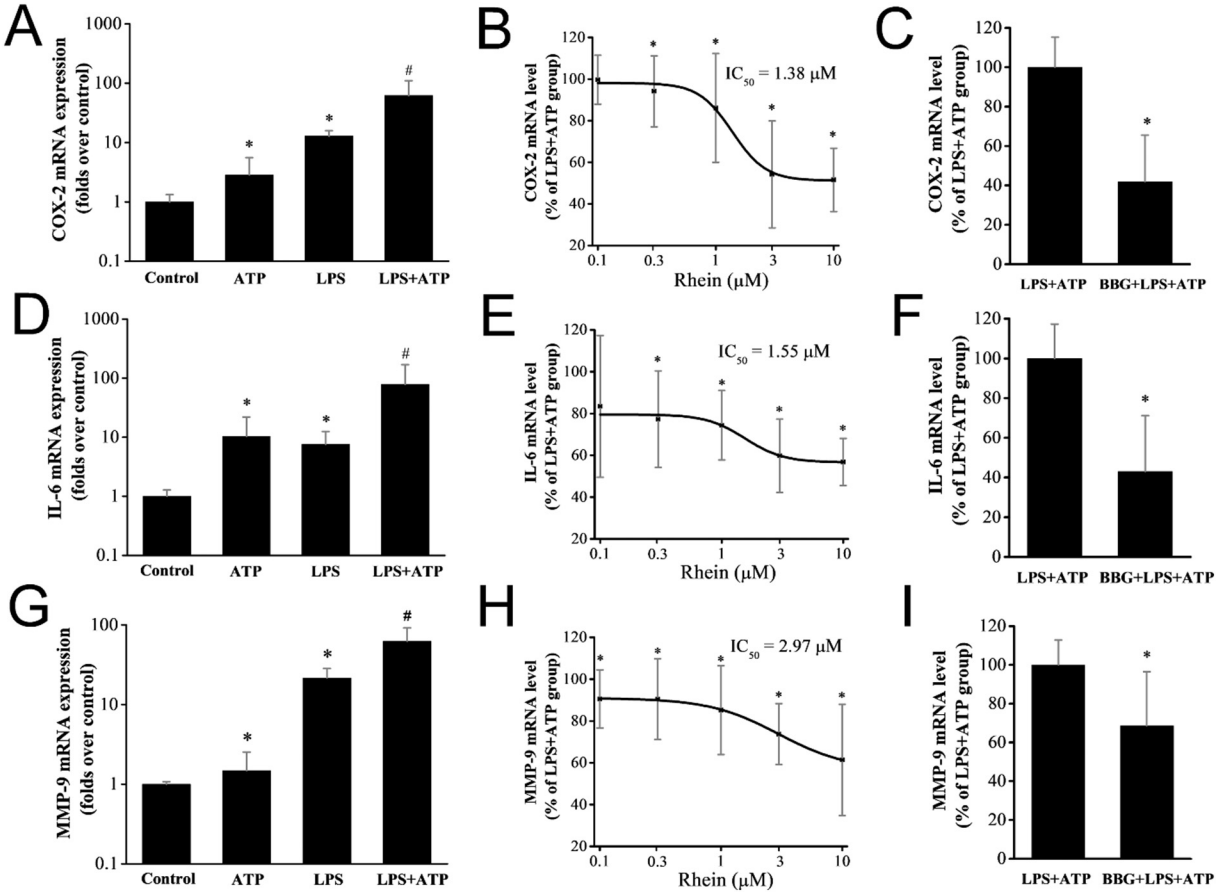

Fig. 4. Rhein decreased ATP-upregulated inflammatory gene expression in LPS-primed rat synoviocytes. Synoviocytes were pretreated with LPS (5 μg/ml) alone or in presence of rhein (0.1, 0.3, 1, 3, 10 μM) and BBG (20 μM) for 5 h simultaneously, then stimulated with ATP (1 mM) for additional 2 h. (A, D, G) Statistic data of the fold changes in gene expression of COX-2 (A), IL-6 (D) and MMP-9 (G) in control group, ATP alone group, LPS alone group and LPS + ATP group (normalized to control group, which was taken as 1). *, $P < 0.05$, compared to control group; #, $P < 0.05$, compared to LPS-primed group ($n = 3$ for each group). (B, E, H) The statistical data of the fold changes in gene expression of COX-2 (B), IL-6 (E) and MMP-9 (H) in rhein + LPS + ATP group (LPS + ATP group was taken as 1). *, $P < 0.05$, compared to LPS + ATP group ($n = 3$ for each group). The smooth curve represents the fit to the Hill1 equation with an IC_{50} of 1.38 μM, 1.55 μM and 2.97 μM, respectively. (C, F, I) Summary of the fold changes in gene expression of COX-2 (C), IL-6 (F) and MMP-9 (I) in BBG + LPS + ATP group (LPS + ATP group was taken as 1). *, $P < 0.05$, compared to LPS + ATP group ($n = 3$ for each group).

both of which were dependent on extracellular Ca^{2+}. Furthermore, ATP potently enhanced the inflammatory gene expression of COX-2, IL-6 and MMP-9 in LPS-activated synoviocytes (Fig. 4). Subsequently, it was revealed that rhein suppressed ATP-triggered $[Ca^{2+}]_c$ increase in a concentration-dependent manner, with an IC_{50} of 2.18 μM (Fig. 2). Rhein also dose-dependently reduced the Ca^{2+}-associated ROS production in response to ATP, with an IC_{50} of 1.05 μM (Fig. 3). Moreover, rhein suppressed the ATP-enhanced gene expression of COX-2, IL-6 and MMP-9 from LPS-activated synoviocytes, with an IC_{50} of 1.38 μM, 1.55 μM, 2.97 μM, respectively (Fig. 4). These findings provided strong evidences that rhein exerted anti-inflammatory effects through the inhibition of Ca^{2+} mobilization, ROS generation and inflammatory gene expression in rat synoviocytes. Therefore, it may have potential therapeutic value for the treatment of RA. Actually, in our preliminary in vivo experiments, we found that the paw swelling in a CIA rat model was significantly alleviated by treatment with rhein (Fig. S2). Besides, there have also been other laboratories examined the therapeutic effects of rhein/diacerein as well as other rhubarb anthraquinone derivatives including emodin and aloe-emodin on RA animal models or RA patients [11–13,44–46]. Results from these documents demonstrated many beneficial effects of rhein such as suppression of synovial hyperplasia, joint destruction and pro-inflammatory cytokine production, thereby corroborating the potential clinical application with these anthraquinone drugs against RA.

With respect to the molecular target of rhein, previous work have demonstrated the inhibitory effects of rhein on the activities of membrane receptors such as Toll-like receptors [14,47], as well as protein kinases that mediate signaling pathways including MAPK, phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT), and inhibitor of NF-κB kinase (IKK) [13,15,35,38,47]. In recent years, the membrane ATP receptors, also known as purinergic P2 receptors, have been extensively reported to involve in immunity and inflammation [16–19,42], thereby may serve as candidate targets for rhein against inflammatory diseases. For instance, $P2X_4$ promotes the maturation and secretion of IL-1β via NOD-like-receptor mediated inflammasome in OA and DN [21,48]. $P2X_4$ also contributes to PGE2 released by macrophages and initiates inflammatory pain [49]. $P2X_7$ receptor-mediated release of cathepsins from macrophages is a cytokine-independent mechanism potentially involved in joint diseases [50]. Besides, $P2Y_2$ is associated with ATP-guides neutrophil chemotaxis [51], and selective induction of endothelial $P2Y_6$ nucleotide receptor plays an important role in vascular inflammation [52]. Among these P2 subtypes, $P2X_7$ appears to become focus of attention for therapy of RA due to its prominent participation in inflammatory processes [23,53,54]. Nevertheless, recent work has also demonstrated that inhibition of $P2X_4$ led to reduced articular inflammation and erosive progression in CIA rat models [55,56], which shed a new light for targeting $P2X_4$ receptors in RA.

In this study, despite the expressions of multiple P2 subtypes

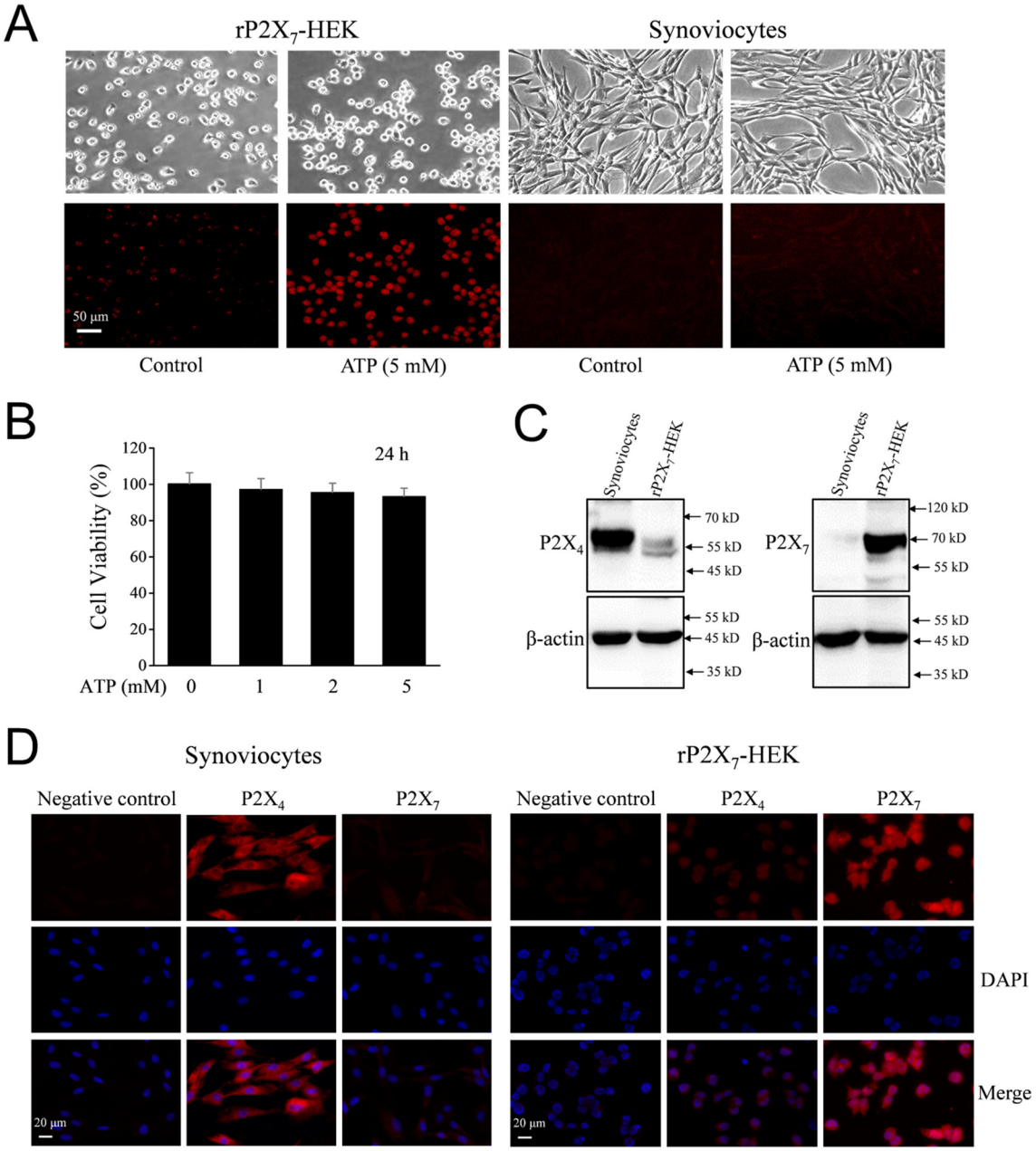

Fig. 5. The influences of long exposure to ATP on ethidium bromide dye uptake and cell survival of rat synoviocytes. (A) Representative phase contrast images visualizing all cells (upper panel) and fluorescent images showing EB dye uptake positive cells (lower panel) in HEK293 cells expressing rat $P2X_7$ receptors ($rP2X_7$-HEK293, a generous gift from Dr. Lin-Hua Jiang in Institute of Membrane and Systems Biology, Faculty of Biological Sciences, University of Leeds, Leeds, UK) and rat synoviocytes in response to ATP (5 mM) for 30 min, respectively. (B) Synoviocyte viability in control cells (no ATP) and cells exposed to ATP (1, 2, 5 mM) for 24 h, respectively. *, $P < 0.05$ compared to control group (n = 3 for each case). There was no significant difference between treated cells and control cells. (C) Western-blot assay indicates the expression of $P2X_4$ and $P2X_7$ receptors in rat synoviocytes and $rP2X_7$-HEK293 cells respectively. (D) Immunolocalization of $P2X_4$ and $P2X_7$ receptors in rat synoviocytes and $rP2X_7$-HEK293 cells. Rat synoviocytes and $rP2X_7$-HEK293 cells labeled with isotype-matched non-specific antibody (normal rabbit IgG, negative control), anti-$P2X_4$ antibody, or anti-$P2X_7$ antibody (red) and DAPI (blue, showed the nuclei) were imaged simultaneously (upper and middle panel). The merge images of each group were showed in the bottom panel. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

including $P2X_{3,4,5}$ and $P2Y_{1,2,4}$, $P2X_7$ receptors that predominantly existed in macrophages [57], were almost undetectable in CIA rat synoviocytes (Figs. 1 and 5). Our quantitative results revealed that $P2X_4$ receptors were the most highly expressed P2X cation-selective receptor channels in rat synoviocytes (Fig. 1). Therefore, ATP-triggered extracellular Ca^{2+} entry and downstream Ca^{2+}-dependent ROS production were mainly mediated by opening $P2X_4$ channels (Figs. 2 and 3). Furthermore, our experiment with $P2X_4$ antagonist BBG and/or $P2X_4$ agonist BzATP confirmed that $P2X_4$ was responsible for ATP-induced $[Ca^{2+}]_c$ increase (Fig. 2), ROS production (Figs. 3 and S1), as well as inflammatory gene expression (Fig. 4). Besides, we excluded the involvement of $P2X_7$, which was considered to be the most relevant receptor subtype in numerous pro-inflammatory responses of myelomonocytic cells [17–20,40], by detection of $P2X_7$-mediated hallmark responses described in other cell types [30] (Fig. 5). These data together suggested that the suppressive effects of rhein on ATP-induced inflammatory responses was resulted from the antagonism of $P2X_4$ rather than $P2X_7$ in rat synoviocytes.

Although our results provided evidences for the inhibition of $P2X_4$ receptors by rhein, the deep molecular interaction mechanisms of the receptors with rhein was still undetermined. It has been established that other rhubarb anthraquinone derivatives especially emodin can directly combine with some receptors or protein kinases, and block their activities via a competitive occupy at the ATP binding site [58–60]. For instance, the analysis of crystal structure of casein kinase 2 (CK2) in complex with emodin reveals the molecular interaction details [61,62]. Emodin enters the nucleotide binding site of the enzyme, filling a hydrophobic pocket. Interestingly, the position occupied by emodin in the hydrophobic pocket is deeper than ATP. The replacement of ATP by emodin induces conformational modifications in the catalytic site of CK2 [61,62]. Since each kinase or receptor possesses its unique three-dimensional structure, different kinds of anthraquinone molecules should exhibit distinct affinity, efficiency and specificity on particular kinase or receptor in different cell types. Therefore, it's probably rhein actioned in a similar way to competitive occupy the ATP binding site of P2 receptors in synoviocytes due to the similar structure and characteristics to that of emodin. Nevertheless more definitive molecular mechanisms need for further researching.

In summary, our present study clearly demonstrated that rhein inhibited several ATP-induced inflammation responses including $[Ca^{2+}]_c$ increase, ROS production and inflammatory gene expression of MMP-9, COX-2 and IL-6 in CIA rat synoviocytes. The molecular mechanism may be attributed to the inhibition of membrane $P2X_4$ receptors. Our findings provide a reasonable support for applying rhein as a potential preventive or cure for RA. In addition, the identity of molecular target will give a novel insight for developing other therapeutic drugs against RA targeting $P2X_4$ receptors.

Author contributions

LP and JX conceived and supervised the study; FH and LP designed the experiments; FH, DZ and WP performed the experiments; FH, IL and ZX analyzed data; FH and PL wrote the manuscript.

Declaration of Competing Interest

The authors state that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.105780.

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