Galangin protects human rheumatoid arthritis fibroblast-like synoviocytes via suppression of the NF-κB/NLRP3 pathway

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Abstract. Rheumatoid arthritis (RA) is a chronic autoimmune disease that significantly affects patient quality of life. Galangin is an extract with multiple health benefits, including anti-oxidative, anti-proliferative, immunoprotective and cardioprotective effects. However, to the best of the authors' knowledge, no detailed studies have investigated its regulatory effects on the nuclear factor (NF)-κB/NLR family pyrin domain containing 3 (NLRP3) signaling pathway. The present study aimed to investigate the protective mechanism of galangin in RA fibroblast-like synoviocytes with regards to the NF-κB/NLRP3 signaling pathway. Human RA fibroblast-like synovium cells (RAFSCs) were treated with lipopolysaccharide (LPS) to induce inflammation. The levels of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-18, inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, prostaglandin E2 (PGE_2), and nitric oxide (NO) were measured by enzyme-linked immunosorbent assay or western blotting in the absence or presence of different concentrations of galangin. Superoxide dismutase (SOD) activity and malondialdehyde (MDA) content were additionally evaluated. Furthermore, factors involved in the NF-κB/NLRP3 pathway, including NLRP3, apoptosis-associated speck-like protein containing A, IL-1β, pro-caspase-1, caspase-1, phosphorylated (p)-NF-κB inhibitor α and p-NF-κB, were assessed by western blotting. The results revealed that LPS significantly stimulated IL-1β, TNF-α, IL-18, PGE_2, NO, iNOS, COX-2 and NF-κB/NLRP3 factor expression, compared with the control. SOD activity was reduced. Pre-treatment with galangin significantly attenuated the effects of LPS, and galangin was demonstrated to have effective anti-oxidative properties. In conclusion, galangin protected RAFSCs through downregulation of the NF-κB/NLRP3 signaling pathway. These findings suggested that galangin may provide a novel direction for the development of RA therapies in the future.

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Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disorder. It affects the joints, to eventually result in joint deformities (1), and influences other parts of the body; symptoms include a low red blood cell count, and inflammation around the lungs and heart (2). Age is a risk factor for RA, and RA is considered a global health risk as the aging population increases in a number of countries (3). At present, RA treatments predominantly focus on reducing pain and inflammation to improve patient quality of life (4).

Fibroblast-like synoviocytes are highly specialized cells in the joint synovial membrane, which are considered to be involved in the pathogenesis of RA (5). The synovial membrane is located between the joint capsule and the joint cavity, which reduces friction between the joint cartilage and supplies nutrients to the surrounding cartilage (6). Fibroblast-like synoviocytes are one of the two principal cell types in the intima of the synovial membrane, and are essential for internal joint homeostasis (7). During RA progression, the physiology of fibroblast-like synoviocytes is markedly altered; contact inhibition properties are lost and excessive proliferation occurs. Furthermore, these cells begin to secrete numerous pro-inflammatory cytokines, including interleukin (IL)-1β, tumor necrosis factor (TNF)-α and IL-18, which creates an inflammatory environment in the synovium and contributes to the destruction of the joint (5,8).

The nuclear factor (NF)-κB/NLR family pyrin domain containing 3 (NLRP3) signaling pathway is the central hub of the inflammatory response, which mediates the transcription of a large number of pro-inflammatory genes, including TNF-α, IL-1β, IL-6 and IL-18 (9,10). Overactivation of the NF-κB pathway results in multiple inflammatory diseases, including RA (11). Inhibition of NF-κB activity markedly ameliorates the symptoms of RA (12).

Galangin is a natural flavonoid extracted from the roots of *Alpinia officinarum*. In South Africa, this traditional herb is used to treat infection (13). Galangin has exhibited multiple beneficial properties, including anti-oxidative, anti-proliferative, immunoprotective and cardioprotective effects (14). Among

these, the anti-inflammatory properties of galangin have gained the attention of researchers in the RA field (15-17).

As the potential of galangin in treating RA has previously been demonstrated (18,19), the present study attempted to investigate the mechanisms underlying the protective effects of galangin in RA fibroblast-like synoviocytes.

Materials and methods

Cell line and reagents. Primary human RA fibroblast-like synovium cells (RAFSCs; cat. no. JNO17-929) were purchased from Guangzhou Jennio Biotechnology Co., Ltd. (Guangzhou, China). Galangin was purchased from Sichuan Weikeqi Biological Technology Co., Ltd. (Chengdu, China). Lipopolysaccharide (LPS) was obtained from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Primary antibodies against inducible nitric oxide synthase (iNOS; cat. no. ab3523) and cyclooxygenase (COX)-2 (cat. no. ab15191) were purchased, including anti-NLRP3 (cat. no. ab4207; all Abcam, Cambridge, UK), anti-apoptosis-associated speck-like protein containing A (ASC; cat. no. sc-22514-R), anti-IL-1β (cat. no. sc-1250), anti-pro-caspase-1 (cat. no. sc-514), anti-caspase-1 (cat. no. sc-56036), anti-phosphorylated (p)-NF-κB inhibitor α (IκBα; cat. no. sc-8404), anti-IκBα (cat. no. sc-847), anti-p-NF-κb (cat. no. sc-101749) and anti-NF-κB (cat. no. sc-109; all Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and fluorescent-labeled (SABC-DyLight 488) secondary antibodies (cat. no. SA1033) were purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China).

Cell culture. RAFSCs were cultured in complete medium [Dulbecco's modified Eagle's medium (DMEM); Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA] containing 10% fetal calf serum (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells in the logarithmic growth phase were used. Primary keratinocytes were incubated in DMEM for 24 h at 37˚C in the dark with 1 mg/ml freshly made alanine, and *Propionibacterium acnes (P. acnes)* at a ratio of 50:1 (*P. acnes*: keratinocytes) for starvation. Following starvation, cells were cultured with LPS and different doses of galangin. Nothing was added to the control group. LPS (1 μg/ml) in combination with galangin (0, 1, 5 and 10 ng/ml) was added to each group. Cells were incubated for 24 h at 37˚C and subsequently collected for further experimentation. All cells were adherent.

ELISA. RAFSCs were homogenized in PBS and centrifuged at 1,000 x g for 15 min at 4˚C. Supernatant (1 ml) was subjected to ELISA detection of IL-1β, TNF-α, IL-18, prostaglandin (PG) E_2, and nitric oxide (NO) levels using the Quantikine ELISA kit (cat. no. DHD00B; R&D Systems, Inc., Minneapolis, MN, USA) and the PGE_2 ELISA Correlate EIA™ kit (cat. no. ab133021; Abcam), according to the manufacturers' protocols. The absorbance of the samples at 450 nm was detected using a microplate reader (Thermo Fisher Scientific, Inc.).

Measurement of superoxide dismutase (SOD) activity and malondialdehyde (MDA) content. In order to investigate the antioxidant effect of galangin, SOD activity and MDA content in RAFSCs was measured. At the end of incubation for 24 h at 37˚C, the cell solution was centrifuged at 1,000 x g for 15 min at 4˚C. Culture supernatant and lysate was collected. SOD activity and MDA content was measured using a Cell MDA assay kit purchased from Nanjing Jiancheng Bioengineering Institute (cat. no. A003-1; Nanjing, China), according to the manufacturer's protocol. The absorbance of SOD and MDA was detected using a microplate reader (Thermo Fisher Scientific, Inc.) at 550 and 450 nm, respectively.

Western blot analysis. Total protein was extracted from RAFSCs using 1% SDS lysis buffer (Beyotime Institute of Biotechnology, Haimen, China), and protein concentration was determined using a bicinchoninic acid protein assay. Proteins (40 μg/lane) were loaded and separated by 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Membranes were incubated with primary antibodies against NLRP3 (1:800), ASC (1:1,000), IL-1β (1:900), pro-caspase-1 (1:1,000), caspase-1 (1:1,000), p-IκBα (1:1,200), IκBα (1:900), anti-p-NF-κB (1:1,000), anti-NF-κB (1:1,000), anti-β-actin (1:2,000; cat. no. ab8227; Abcam), anti-iNOS (1:800) and anti-COX-2 (1:900) at 4˚C overnight, followed by incubation with HRP-conjugated goat anti-rabbit IgG and fluorescent-labeled secondary antibodies (1:10,000; Wuhan Boster Biological Technology, Ltd.) at 37˚C for 2 h. Enhanced chemiluminescence (Beyotime Institute of Biotechnology) solution was added and the blots were exposed on X-ray films in a dark room. Images were subsequently captured, and Bandscan software version 5.0 (Glyko, Inc.; BioMarin Pharmaceutical, Inc., Novato, CA, USA) was used for analysis of the gel images.

Statistical analysis. All data are expressed as the mean ± standard deviation (n=6), and statistical analysis was performed with SPSS 11.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance followed by Tukey's post-hoc test was used to determine significant differences between multiple groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Galangin inhibits LPS-induced IL-1β, TNF-α, IL-18, PGE_2 and NO expression levels in RAFSCs. The effects of galangin on the expression of IL-1β, TNF-α and IL-18 in RAFSCs were determined by ELISA. As presented in Table I, the expression of these cytokines was strongly induced by LPS ($P<0.05$), with an increase of 4-7 fold. However, when cells were pre-treated with galangin and LPS, IL-18, IL-1β and TNF-α expression was significantly inhibited ($P<0.05$; Table I). When cells were treated with 10 ng/ml galangin, IL-1β and TNF-α expression was comparable to the control group; IL-18 expression was additionally markedly inhibited, compared with the LPS only group. These findings suggested that galangin inhibited LPS-induced IL-1β, TNF-α and IL-18 expression in RAFSCs in a concentration-dependent manner, highlighting its potential anti-inflammatory effects.

Levels of PGE_2 and NO in the RAFSC culture supernatant were additionally detected by ELISA, in order to investigate

Table I. Expression levels of IL-1β, TNF-α, IL-18, PGE2 and NO in rheumatoid arthritis fibroblast-like synovium cells treated with LPS and/or galangin.

	Marker				
Group	IL-1β	TNF-α	IL-18	PGE2, ng/ml	NO, μmol/ml
Control	65.21±3.12[a-d]	29.47±1.28[a-d]	35.47±9.64[a-d]	0.11±0.08[a-d]	35.74±3.56[a,b]
LPS only	279.36±20.78[b-d]	167.34±32.15[b-d]	234.97±21.45[b-d]	2.51±0.15[b-d]	298.09±37.45[b-d]
LPS + 1 ng/ml galangin	135.27±12.34[a,c,d]	82.39±19.85[a,c,d]	135.65±11.67[a,c,d]	1.45±0.26[a,c,d]	68.17±28.57[a,c,d]
LPS + 5 ng/ml galangin	93.74±9.85[a,b,d]	61.46±14.61[a,b,d]	85.76±9.73[a,b,d]	1.02±0.32[a,b,d]	37.12±9.78[a,b]
LPS + 10 ng/ml galangin	76.51±15.67[a-c]	39.27±5.8[a-c]	62.21±4.38[a-c]	0.39±0.18[a-c]	34.37±7.62[a,b]

Each value represents the mean ± standard deviation (n=6). [a]P<0.05 vs. LPS only group; [b]P<0.05 vs. LPS + galangin (1 ng/ml); [c]P<0.05 vs. galangin (5 ng/ml); [d]P<0.05 vs. galangin (10 ng/ml). IL-1β, interleukin; TNF-α, tumor necrosis factor-α; PGE2, prostaglandin E2; NO, nitric oxide; LPS, lipopolysaccharide.

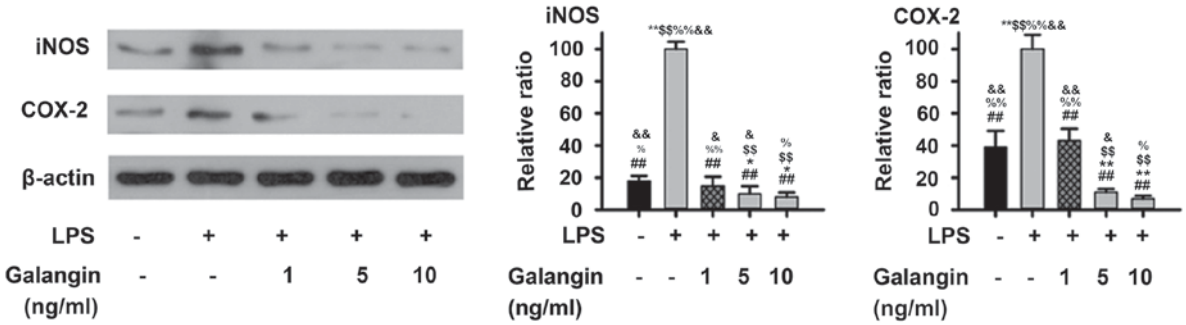

Figure 1. Expression levels of iNOS and COX-2 in RAFSCs treated with LPS and/or galangin. In the LPS group, cells were treated with 1 μg/ml LPS only. In the galangin groups, cells were treated with 1 μg/ml LPS and galangin at 1, 5 or 10 ng/ml. The LPS group had significantly elevated levels of iNOS and COX-2 compared with the control group. Cells treated with galangin had significantly lower levels, compared with the LPS group. [*]P<0.05, [**]P<0.01 vs. control group; [##]P<0.01 vs. LPS group; [$$]P<0.01 vs. LPS + galangin (1 ng/ml); [%]P<0.05, [%%]P<0.01 vs. LPS + galangin (5 ng/ml); [&]P<0.05, [&&]P<0.01 vs. LPS + galangin (10 ng/ml). iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase 2; RAFSCs, rheumatoid arthritis fibroblast-like synovium cells; LPS, lipopolysaccharide.

whether galangin regulated LPS-induced NO and PGE_2 production. As presented in Table I, PGE_2 and NO levels were significantly increased by LPS, compared with the control group (P<0.05). Furthermore, PGE_2 and NO levels were suppressed with galangin treatment (P<0.05 vs. LPS only group). These results demonstrated that galangin inhibited the LPS-induced expression of PGE_2 and NO in RAFSCs.

Galangin inhibits the LPS-induced expression of iNOS and COX-2 in RAFSCs. Considering that galangin inhibited NO and PGE_2 production, western blot analysis was performed in order to examine whether these inhibitory effects were associated with iNOS and COX-2 regulation in RAFSCs. As presented in Fig. 1, LPS significantly increased the expression of iNOS and COX-2 (P<0.01). Pre-treatment with 1 ng/ml galangin decreased iNOS and COX-2 expression (P<0.05), which was not significantly different when compared with the control (P>0.05). At a dose of 5 or 10 ng/ml, the iNOS and COX-2 expression levels decreased below those of the control group (P<0.05). Therefore, these results demonstrated that galangin inhibited LPS-induced expressions of iNOS and COX-2 in RAFSCs.

Galangin decreases SOD activity and increases MDA content. It was demonstrated that LPS significantly decreased SOD activity (P<0.05), and pre-treatment with galangin increased SOD activity (P<0.05) (Fig. 2). MDA content had an opposite tendency, with galangin decreasing the levels of MDA. These findings provided evidence that galangin exhibited antioxidative effects in RAFSCs.

Galangin suppresses the NF-κB/NLRP3 signaling pathway. The NF-κB/NLRP3 pathway, which upregulates multiple pro-inflammatory cytokines, has been considered to be a key signaling pathway in the progression of RA (12). In order to understand the underlying mechanisms of the protective effects of galangin in RAFSCs, the expression of multiple factors in the NF-κB/NLRP3 signaling pathway were measured by western blot analysis. The expression of NLRP3, ASC, IL-1β, pro-caspase-1, caspase-1, p-IκBα and p-NF-κB in RAFSCs was upregulated by LPS stimulation (P<0.05) (Fig. 3). Pre-treatment with galangin (1 ng/ml) decreased ASC, pro-caspase-1/caspase-1, p-IκBα and p-NF-κB (P<0.05) expression; however, it did not significantly decrease NLRP3 or IL-1β expression. Pre-treatment with 5 or 10 ng/ml galangin significantly attenuated the LPS-induced overexpression of all these factors (P<0.05). These results indicated that galangin may have protected RAFSCs by suppressing the NF-κB/NLRP3 signaling pathway.

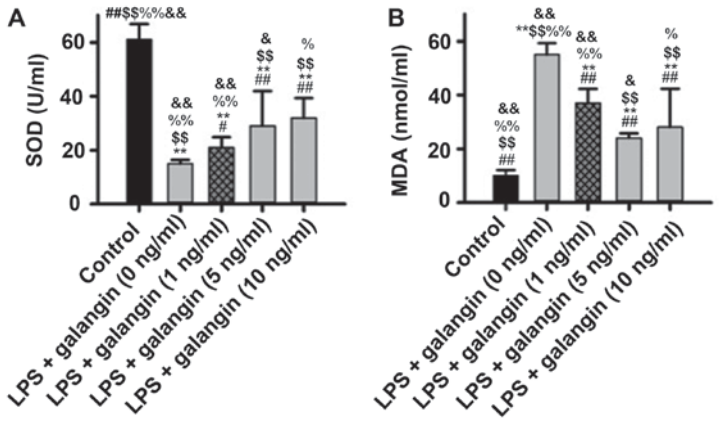

Figure 2. SOD activity and MDA content in RAFSCs treated with LPS and/or galangin. The LPS group had significantly lower (A) SOD activity and higher (B) MDA content compared with the control group, whereas the galangin groups had significantly higher SOD activity and lower MDA content compared with the LPS group. **P<0.01 vs. control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. LPS group; $^{\$\$}$P<0.01 vs. LPS + galangin (1 ng/ml); $^{\%}$P<0.05, $^{\%\%}$P<0.01 vs. LPS + galangin (5 ng/ml); $^{\&}$P<0.05, $^{\&\&}$P<0.01 vs. LPS + galangin (10 ng/ml). SOD, superoxide dismutase; MDA, malondialdehyde; RAFSCs, rheumatoid arthritis fibroblast-like synovium cells; LPS, lipopolysaccharide.

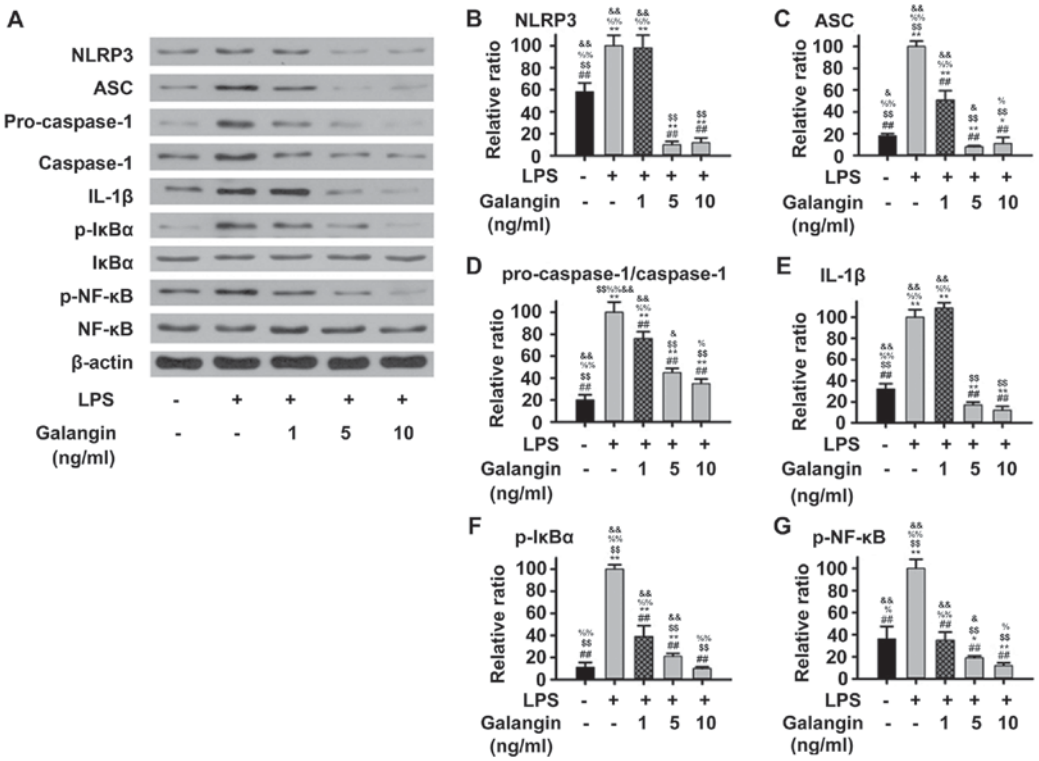

Figure 3. Expression of proteins associated with the NF-κB/NLRP3 signaling pathway in RAFSCs treated with LPS and/or galangin. (A) Western blot analysis. The LPS group had significantly elevated levels of (B) NLRP3, (C) ASC, (D) pro-caspase-1/caspase-1, (E) IL-1β, (F) p-IκBα and (G) p-NF-κB. When RAFSCs were pre-treated with 1 ng/ml galangin, expression levels of ASC, p-IκBα, p-NF-κB were significantly lower compared with the LPS group, whereas when the cells were pre-treated with 5 or 10 ng/ml galangin, the expression of all proteins was significantly lower, compared with the LPS group. *P<0.05, **P<0.01 vs. control group; $^{\#\#}$P<0.01 vs. LPS group; $^{\$\$}$P<0.01 vs. LPS + galangin (1 ng/ml); $^{\%}$P<0.05, $^{\%\%}$P<0.01 vs. LPS + galangin (5 ng/ml); $^{\&}$P<0.05, $^{\&\&}$P<0.01 vs. LPS + galangin (10 ng/ml). NF-κB, nuclear factor-κB; NLRP3, NLR family pyrin domain containing 3; RAFSCs, rheumatoid arthritis fibroblast-like synovium cells; LPS, lipopolysaccharide; ASC, apoptosis-associated speck-like protein containing A; IL-1β, interleukin-1β; p-, phosphorylated; IκBα, NF-κB inhibitor α.

Discussion

RA is a systemic immune and inflammatory disease. For the majority of patients, RA is a progressive, life-long disease that shortens life expectancy by 3-20 years (20). Unfortunately, despite extensive research, RA pathogenesis remains unclear, and there is currently no cure for this disease (21).

Bacterial LPS is capable of eliciting a strong immune response *in vitro* and *in vivo*, and is frequently used to induce symptoms of RA (22). IL-18, IL-1β and TNF-α are pro-inflammatory cytokines that have pivotal roles in RA, and are frequently used as inflammatory markers (23,24). Furthermore, a number of factors downstream of proinflammatory cytokines are responsible for creating an inflammatory environment around the joint. For example, NO is a free radical

that is generated enzymatically by the cytokine-induced iNOS pathway and contributes to the pathogenesis of arthritis (25). In addition, one tissue specific isoform of COX-2 produces PGE_2, which aggravates synovial inflammation by increasing local blood flow and vasopermeability (26). Furthermore, TNF-α and other pro-inflammatory cytokines enhance the production of COX-2 and PGE_2 (27). All these factors were considered as incentives in RA.

Therefore, the present study investigated the inhibitory effect of galangin on the LPS-induced increase in IL-1β, TNF-αIL-18, PGE_2, NO, iNOS and COX-2 expression levels in RAFSCs. The results indicated that galangin significantly inhibited the release of IL-1β, TNF-α, IL-18, PGE_2, NO into the medium, in addition to iNOS and COX-2. The observed reduction in NO production and PGE_2 release when cells were pretreated with galangin may have resulted from the transcriptional suppression of iNOS and COX-2. However, a limitation of the present study was that the effect of treatment with galangin on cell survival and apoptosis was not evaluated.

Free radicals cause damage to cellular components and contribute to the development of numerous inflammatory diseases (28). SOD is an enzyme that reduces the damage of superoxides and MDA is a marker for oxidative stress; the expression of these molecules is negatively and positively associated with RA symptoms, respectively (29). In the present study, it was revealed that galangin decreased LPS-induced cytokine expression in the RAFSC culture supernatant, and therefore may have protected cells from cytokine-induced damage. The antioxidative effects of galangin were also investigated. Galangin exhibited significant antioxidant effects, as evidenced by the increased SOD activity and lower MDA content detected. Cells treated with 1 or 5 ng/ml galangin had significant differences in expression compared with the LPS only treatment group, in SOD activity and MDA content. The increase in SOD activity suggested that galangin enhanced the antioxidant capability of the cells, while the decrease in MDA content indicated that galangin may have reduced lipid membrane oxidation by scavenging free radicals. Although SOD activity and MDA content may be sufficient to draw the conclusion that galangin had antioxidative effect on RAFSCs, *in vivo* experiments to visualize cellular ROS with molecular dye or other methods may further elucidate the function of galangin in reducing the inflammatory response. Therefore, further studies *in vivo* are required in the future.

A previous study suggested that galangin prevents osteoclastic bone destruction and osteoclastogenesis in osteoclast precursors, and additionally in collagen-induced arthritis mice, without toxicity via attenuation of TNF superfamily member 11-induced activation of the mitogen activated protein kinase (MAPK)8, MAPK14 and NF-κB signaling pathways (18). In the present study, it was determined that the protective effects of galangin in RAFSCs were likely due to NF-κB/NLRP3 signaling pathway down-regulation.

As a result, it was concluded that galangin suppressed pro-inflammatory signaling in fibroblast-like synoviocytes *in vitro*, and that inhibition of the NF-κB/NLRP3 pathway was a key mechanism in this protective effect. Therefore, galangin may provide a novel direction for the development of RA therapies in the future.

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Availability of data and materials

All data generated or analyzed during the current study are included in this published article.

Authors' contributions

QF, YG, HZ, ZW and JW were responsible for the conception and design of the study. QF, YG and HZ performed the experiments, and analyzed and interpreted the data. QF drafted the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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