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Original Article

Casticin attenuates rheumatoid arthritis through PKC-NF-κB signaling in vitro and in vivo

Ye Li, Yan Shen

Division of Immunology and Rheumatology, Huadong Hospital, Fudan University, Shanghai, P. R. China

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Abstract: Casticin, as a major effective component in Chinese herbal medicine *Vitex agnus-castus* exhibits an anti-inflammatory property to rheumatoid arthritis (RA), while the potential mechanism is unclear. We aim at investigating the mechanism of Casticin on lipopolysaccharides (LPS)-induced fibroblast-like synoviocytes (LiFLSs) inflammation model. Fibroblast-like synoviocytes (FLSs) were treated with 200 mg/ml of LPS for 24 h to establish RA-like model, LiFLSs. FLSs were pretreated with casticin (0.1-1 μM) for 30 min in the treatment groups. Quantitative real-time polymerase chain reaction (qRT-PCR) and enzyme-linked immunosorbent assays (ELISA) were used to detect mRNA and protein level of IL-10 and TNF-α. Signal proteins involved in IL-10 production were analyzed by Western blotting. Casticin significantly reversed the inhibitory effects of LPS on IL-10 expression in FLSs by activating PKC-NF-κB pathway. Besides, casticin inhibited TNF-α expression in FLSs which was induced by LPS, and this effect was markedly diminished by IL-10 neutralizing antibody. The IL-10 mediated suppression of TNF-α transcription which was demonstrated by no response to protein synthesis inhibitor cyclohexamide and no mRNA decay. Casticin inhibits TNF-α production induced by LPS in FLSs through PKC-NF-κB-IL-10 pathway, and this study also highlights the potential application of casticin in the treatment of RA.

Keywords: Rheumatoid arthritis, inflammation, casticin, PKC, NF-κB

Introduction

Rheumatoid arthritis (RA) as one of the most common chronic autoimmune disease is characterized by chronic inflammation, articular destruction and abnormal immune response [1]. Fibroblast-like synoviocytes (FLSs) are key cellular participants in RA and are crucial in initiating and driving RA in concert with inflammatory cells [2-4]. Non-steroidal anti-inflammatory drugs (NSAIDs) are the principal treatment for arthritis patients, such as indomethacin, which had been identified to reduce the expression of inflammatory factors in LiFLSs [5]. However, anti-inflammatory agents have the risk of gastrointestinal toxicity, heart failure, etc [6, 7]. Therefore, new anti-inflammatory drugs are urgently to be discovered.

Casticin, as the major active substance in *Lithospermum erythrorhyzon,* had been reported to mediate multiple pharmacological activities such as antioxidation, antiviral, cardiovascular protection, antineoplastic and anti-inflammation [8-11]. In RA, researchers had found that casticin could inhibit inflammation, regulate immunity and relieve pathological lesion of joint in a mouse RA model which induced by 2-collagen at the late period [12].

In collagen type II induced murine arthritis model, casticin was found to induce IL-10 production through a GATA-3 dependent mechanism *in vivo* [13]. IL-10 is an immunosuppressive cytokine in inflammatory responses [14]. Increasing evidence showed that IL-10 elevated in peripheral blood and synovial joint of RA patients [15-17]. Besides, Casticin was also found to inhibit TNF-α production in human macrophage and murine cells [8, 11]. TNF-α is a proinflammatory cytokine that plays a pivotal role in enhancing the inflammatory response in RA, which is abundantly presented in RA patients' serum and the arthritic synovium [18], inhibitors that specific for TNF-α have been demonstrated efficacy as monotherapy or in combination with NSAIDs in the treatment of RA. Based on these observations, we speculate

that casticin inhibits TNF-α production may IL-10, but no data has fully demonstrated this pathway.

In the present study, we further investigated the effect of casticin on IL-10 and TNF-α production in a RA-like model, LiFLs. Our results showed that casticin could induce the expression of IL-10, as well as PKC and NF-κB protein in LiFLSs. Moreover, inhibition of either PKC or NF-κB abolished the casticin-induced IL-10 production. And the induction of IL-10 further promoted the down-regulation TNF-α mRNA. This study provides novel insight into the mechanisms of casticin in anti-inflammatory, and highlights the application of casticin in the treatment of RA.

Materials and methods

Mice and RA-like model in vivo

Male Wistar rats, aged 6-8 weeks (160-180 g), were purchased from the Experimental Animal Center of Wuhan University (Wuhan, China). One week before the experiment the animals were allowed to acclimatize and the animals were maintained at room temperature (25 ± 2°C), with a 12 h light-dark cycle, relative humidity 40-70% and allowed food and water ad libitum. This experiment was approved by the Bioethics Committee of the Wuhan University (Wuhan, China), and the procedures of the experiment strictly adhered to generally accepted international rules and regulations. 40 rats were divided as 4 groups: a. control group; b. model group; c. meloxicam group (50 mg/kg, daily) and d. casticin group (2 mg/kg daily). Complete Freund's adjuvant (CFA, sigma) was prepared by suspending heat killed BCG in liquid paraffin at 10 mg/mL. The model of AA rat was induced by a single intradermal injection of 0.1 mL of CFA into the left hind metatarsal footpad of rat [19]. From day 1 to day 28 after the immunization, the RA rats were treated with casticin (2 mg/kg) oral administration does. Meloxicam (50 mg/kg) was used as a reference drug and was given by intragastric (ig) administration twice a week, the control group and RA model group were given an equal volume of the vehicle (CMC-Na) at the same time.

Establishment of RA-like model in vitro

Fibroblast-like synoviocytes (FLSs) were isolated from knees of rats. Synovia membranes were minced and digested with 1 mg/ml type II collagen (Sigma) in DMEM (Hyclone) at 37°C for 1 h in 5% CO_2. After centrifugation and washing, the cells were resuspended in DMEM supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin and 100 U/ml streptomycin (Beyotime). Non-adherent cells were removed after 24 h and adherent cells were trypsinized with 0.25% trypsin (Hyclone) at confluence and then plated in cultured flask. To obtain a homogeneous population of synoviocytes, confluent cultures from passages 3-7 were used. FLSs were verified by immunocytochemistry (BOSTER) as a homogeneous population (phenotype: > 99% vimentin and < 1% CD68, data not shown), FLSs were treated with 200 mg/ml of LPS for 24 h to establish the RA-like model, LiFLSs.

Western blot analysis

LiFLSs were lysed in RIPA [50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS] buffer containing protease inhibitor mixture (0.1% CTAB, Sigma). Cytoplasmic and nuclear extraction was performed using the protocol reported by Jobin [20]. Lysate (50 μg of protein) was separated on 5-12% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes (Millipore) using a Mini Tank Transfer System (Bio-Rad Laboratories, Hercules, CA, USA) at 200 mA for 2 h. Proteins were detected using western blotting and visualized by chemiluminescence (Pierce, SuperSignal West Pico Chemiluminescent Substrate).

Quantitative real-time polymerase chain reaction (qRT-PCR)

mRNA expression of TNF-α and IL-10 was analyzed by using the ABI 7300 real-time PCR system (Foster City, CA, USA). TNF-α primer pairs [21]: 5'-TTCTCATTCCTGCTCGTGG-3' and 5'-TTTGGTGGTTCGCCTCCT-3'; IL-10 [22]: 5'-TGCCAAGCCTTGTCAGAAATGATCA AG-3' and 5'-GTATCCAGAGGGTCTTCAGCTTCTCTC-3'; GAPDH [23]: 5'-TGGCCTCCAAGGAGTAAGAAAC-3' and 5'-GGCCTCTCCCTCTCAGTATC-3'. The reverse transcription reaction was performed with 1 μg total RNA. cDNAs were amplified using SYBR Green Real-time PCR Master Mix (Takara) and 0.4 μmol/L of each primer pair. The reaction was carried out with an initial step at 94°C for 30 s, followed by 40 cycles of amplification

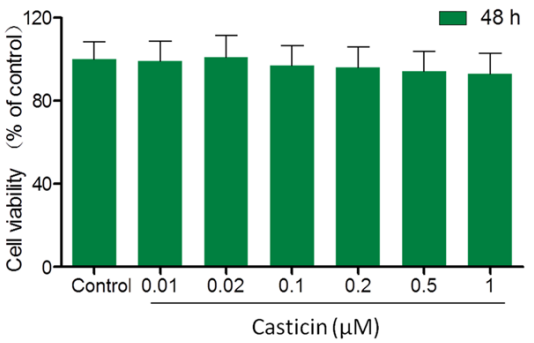

Figure 1. Effects of casticin on cell viability of FLSs. FLSs were treated with casticin (0.01-1 μM) for 48 h, and cell viability was measured by MTT assay. Data was presented as mean ± SD, n = 6. Compared with the control group, $*P < 0.05$, $**P < 0.01$.

step (94°C for 30 s, 60°C for 60 s, and 72°C for 1 min). Each sample was carried out in triplicates and the averages of the threshold cycles were used to interpolate curves using 7300 System SDS Software. Results were expressed as the relative expression to internal control GAPDH.

ELISA

Culture medium was harvested at the indicated time. The concentration of TNF-α and IL-10 in the supernatant of cultured cells were detected by sandwich enzyme-linked immunosorbent assay (ELISA) kits (Dingguo, Beijing, China) according to the manufacture's protocol.

Confocal microscopy analysis

Cells were maintained on the glass coverslips in 24-well plates for 24 h. After stimulating with casticin (0.1, 0.5 and 1 μM), the cells were fixed with ice-cold dehydrated ethanol/acetone. Then, washed with cold PBS for 10 min, and blocked with 5% normal goat serum for 30 min at room temperature, and incubated with primary anti-NF-κB p65 (1:100) overnight for 4°C. The cells were washed and then incubated with FITC-conjugated goat anti-rabbit secondary antibody for 1 h in the dark. After several additional washing steps, the coverslips were mounted in Fluoromount-G™ mounting media with DAPI, and the fluorescence was visualized using Leica confocal software.

Histological analysis

On day 28, all rats were sacrificed via anesthesia after serum collection. Hind paws and knee joints were removed from the rats for histological examination. The joints were fixed in 10% phosphate buffered formalin, decalcified in 10% EDTA for 30 days at 4°C, and then embedded in paraffin. Serial paraffin sections (5 mm) were stained with hematoxylin and eosin (H&E).

Immunohistochemistry of NF-κB

Immunohistochemistry was performed according to the streptavidin-peroxidase (Sp) method using a standard Sp Kit (JRDUN biotech, Shanghai, China). The TMA slide was incubated with monoclonal mouse anti-NF-κB p65 antibody (1:200) (Medical and Biological Laboratories, Nagoya, Japan) overnight at 4°C, and diaminobenzidine (DAB; JRDUN biotech, Shanghai, China) was used to produce a brown precipitate. The immunoreactivity was assessed blindly by two independent observers using light microscopy (Olympus BX-51 light microscope), and the image was collected by Camedia Master C-3040 digital camera [24, 25].

Statistical analysis

Results are expressed as Mean ± SD. The data were analyzed by One-way ANOVA followed by the Student-Newman-Keuls test for multiple comparisons. Results were considered statistically significant at $P < 0.05$.

Results

Casticin had no cytotoxic effect on FLSs viability

As shown in **Figure 1**, casticin treatment for 48 h at concentrations ranging from 0.01 to 1 μM had no significant cytotoxic effect on FLSs. We therefore used casticin at 0.1, 0.5 and 1 μM for further study.

Casticin induced the up-regulation of IL-10 and down-regulation of TNF-α

IL-10 as an important immunosuppressive mediator is usually down-regulated in RA. In LiFLSs, IL-10 was decreased by LPS treatment, while casticin reversed the mRNA expression of IL-10 in a dose-dependent manner (**Figure 2A**). In addition, casticin remarkably decreased the mRNA expression of TNF-α in LiFLSs (**Figure 2B**). As for the inhibitory effect on TNF-α, casticin showed similar potency with positive control indomethacin.

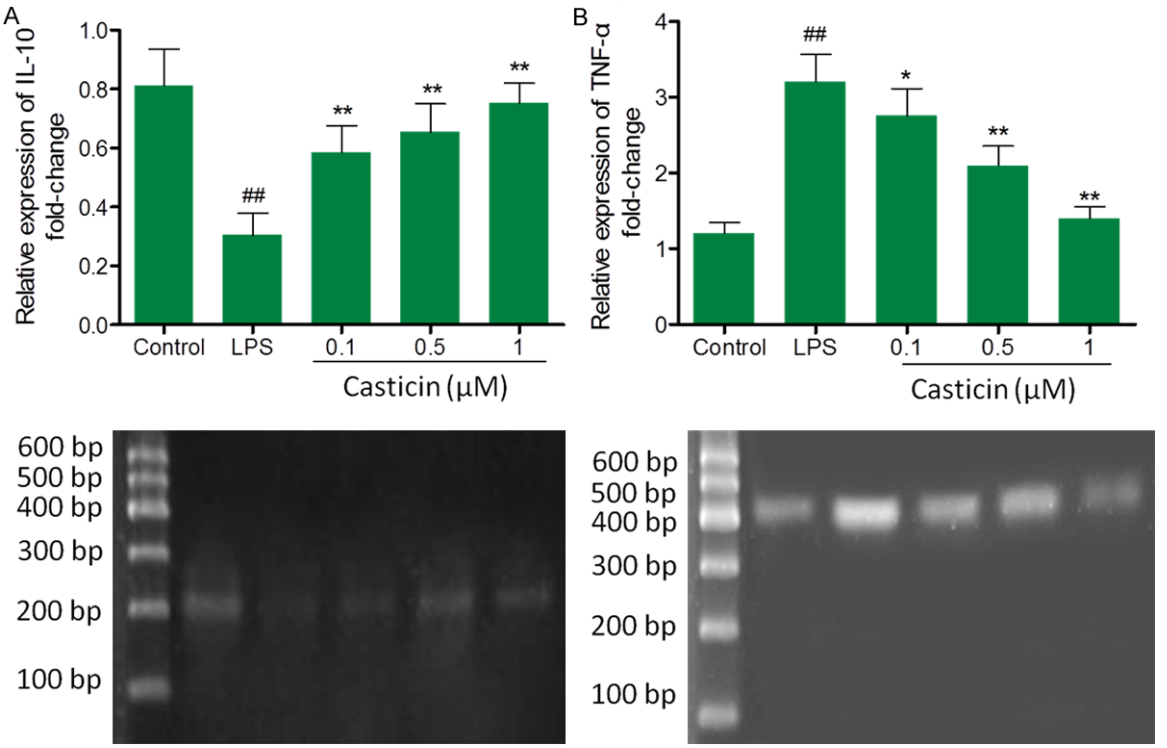

Figure 2. Effect of casticin on mRNA expression of TNF-α and IL-10 in LiFLSs. Fibroblast-like synoviocytes was treated with LPS (200 μg/ml) for 24 h to establish RA-Like model. (A) IL-10 and (B) TNF-α mRNA expression were quantified by qRT-PCR and normalized to GAPDH. Data was presented as mean ± SD, n = 6. Compared with the control group, $^{\#\#}P < 0.01$; compared with the LPS group, $^{*}P < 0.05$, $^{**}P < 0.01$.

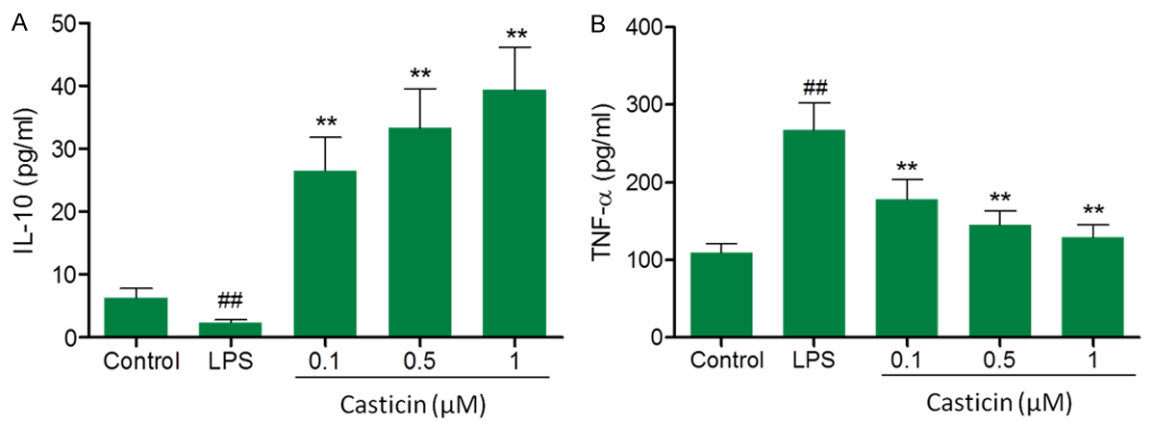

Figure 3. Effect of casticin on the protein production of TNF-α and IL-10 in LiFLSs. Experiment process same as **Figure 1**, (A) IL-10 and (B) TNF-α protein production in supernatant were quantified by ELISA. Data was presented as mean ± SD, n = 6. Compared with the control group, $^{\#\#}P < 0.01$; compared with the LPS group, $^{**}P < 0.01$.

Casticin regulated the release of IL-10 and TNF-α

IL-10 and TNF-α in the cell culture medium were also identified by ELISA. As showed in **Figure 3A**, IL-10 level was decreased significantly after LPS treatment. A notable increase was observed in casticin treated groups. As for TNF-α, LPS induced high release in the model group and casticin descended the level of TNF-α significantly (**Figure 3B**).

Casticin decreased the expressions of p-NF-κB and PKC

PKC/NF-κB signaling is considered as an important pathway in mediating inflammation response [26, 27]. PKC, NF-κB and phosphory-

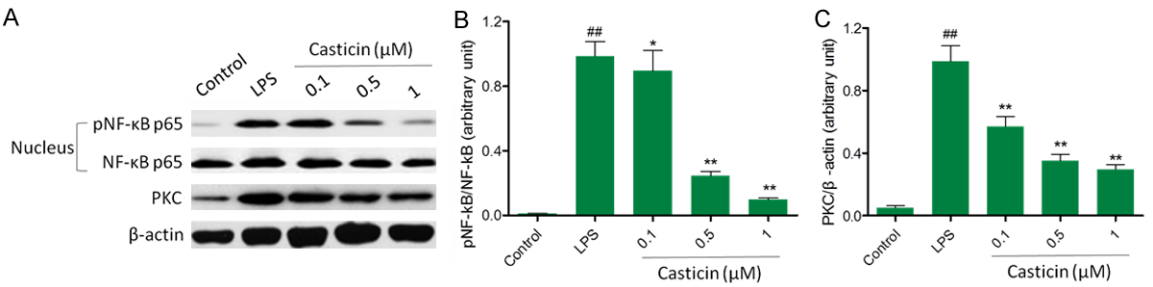

Figure 4. Effect of casticin on PKC-NF-κB signaling. A-C. LiFLSs treated with casticin were lysed for Western blot analysis at 6 h using antibodies against the PKC, NF-κB p65 and p-NF-κB p65. GAPDH was detected as control for sample loading. Data was presented as mean ± SD, n = 6. Compared with the control group, $^{\#\#}P < 0.01$; compared with the LPS group, $^{**}P < 0.01$.

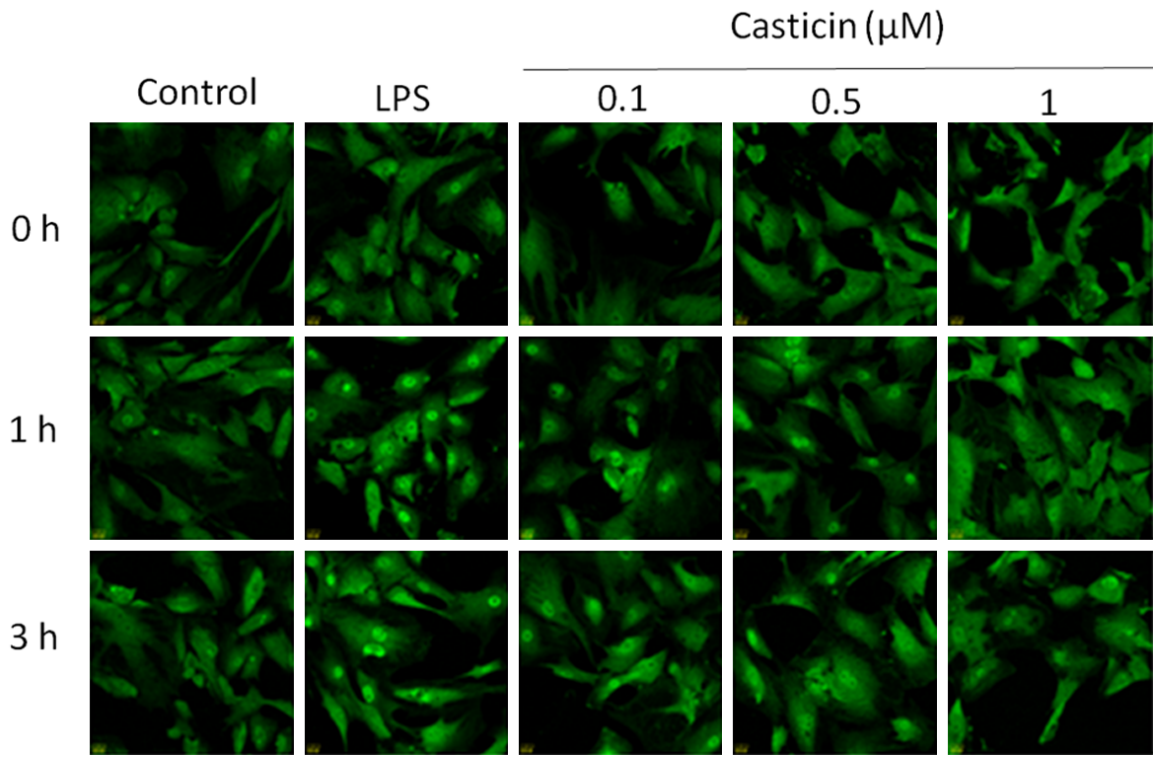

Figure 5. NF-κB subcellular localization in LiFLSs treated with casticin (×200). After treated without or with casticin (0.1, 0.5 and 1 μM), the NF-κB subcellular localization in cells were assessed by confocal microscopy analysis.

lation of NF-κB expression in nucleus were measured by western blot assay. As shown in **Figure 4A-C**, PKC and phosphorylation of NF-κB in nucleus were increased after the LPS treatment. However, casticin dramatically reduced the expression of PKC and p-NF-κB in LiFLSs.

Casticin inhibited the transfer of NF-κB

From laser confocal microscopy, LPS promoted the process of NF-κB transferring from cytoplasm to the nucleus in LPS treated group from 1 h to 3 h. After casticin treated for 1 h, a mediated decrease in NF-κB concentration in nucleus was seen (**Figure 5**). Casticin could suppress the transfer of NF-κB from cytoplasm to the nucleus in a time- and dose- dependent manner.

Casticin attenuated the RA in vivo

RA is well characterized with synovial hyperplasia, pannus formation, and cartilage and bone

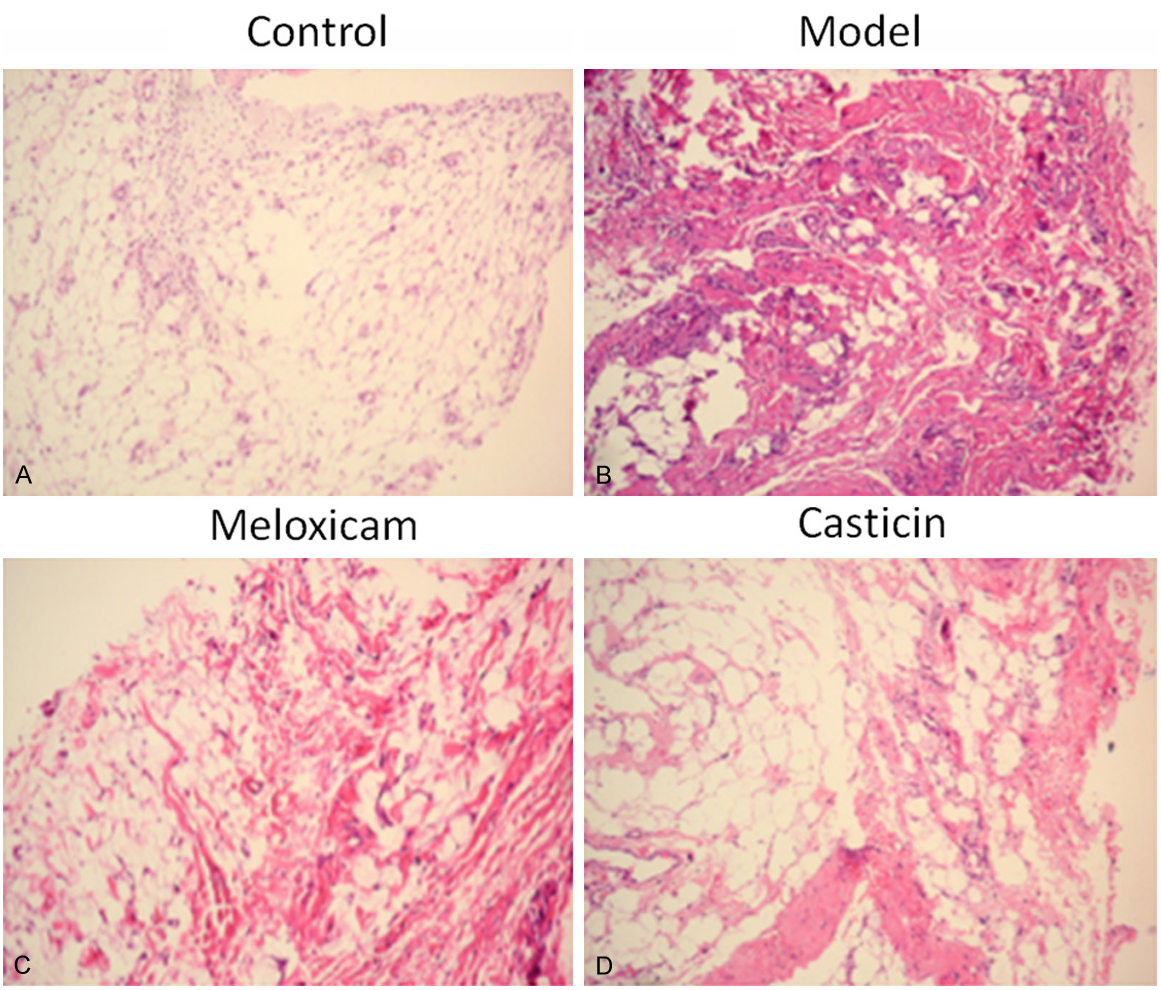

Figure 6. Pathological changes in synovium of RA-like rats. RA-like rats were treated with casticin for 28 days; all rats were sacrificed via anesthesia after serum collection. Hind paws and knee joints were removed from the rats for histological examination.

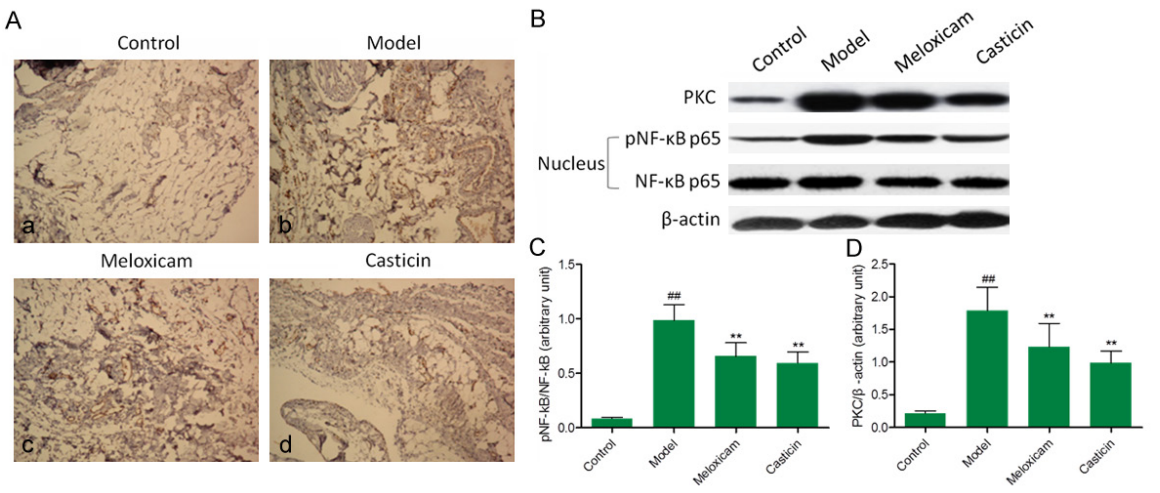

Figure 7. NF-κB expression in synovium of RA-like rats. A. Immunohistochemistry of NF-κB expression in synovium. B-D. Synovium was lysed for Western blot analysis using antibodies against the PKC, NF-κB p65 and p-NF-κB p65. GAPDH was detected as control for sample loading. Data was presented as mean ± SD, n = 6. Compared with the control group, $^{\#\#}P < 0.01$; compared with the model group, $^{**}P < 0.01$.

destruction in the joint [28, 29]. In our histopathological evaluation by H&E staining, the knee joints of the model group rats displayed notable synovial hyperplasia, inflammatory cell infiltration into the joint capacity, and partial bone destruction (**Figure 6B**). The rats administrated with 50 mg/kg of meloxicam and 3 mg/kg of casticin showed a remarkable reduction in synovial hyperplasia, inflammatory cell infiltration compared with control rats (**Figure 6C** and **6D**).

Casticin suppressed the NF-κB expression in vivo

We then measured the NF-κB p65 expression in tissue by immunohistochemistry and western blot. As shown in **Figure 7A** and **7B**, NF-κB p65 expression was up regulated in model group compared with that of control group. A mediate decrease of NF-κB p65 was observed in meloxicam and casticin group (**Figure 7A-C**). **Figure 7B** and **7D** also showed that protein level of PKC was increased in model group, while meloxincam and casticin effectively reduced the PKC expression.

Discussion

RA is characterized by the proliferation of the synovial membrane into a pannus, which includes resident fibroblast-like synoviocytes (FLSs) and infiltrating mononuclear cells capable of producing inflammatory cytokines [30]. Prompt treatment is the key to preventing joint destruction and organ damage, traditionally RA has been treated with disease modifying antirheumatic drugs, NSAID, but taking an NSAID tablet can increase the risk of serious stomach problems, thus, it is urgent to find new agents.

Casticin, as a major active chemical component isolated from the dried root of *Lithospermum erythrorhizon*, possesses numerous pharmacological properties, including anti-inflammatory and antitumor properties and promoting wound healing activity [31]. It was reported that casticin significantly inhibits the concentrations of TNF-α, IL-6 and IL-1β in bronchoalveolar lavage fluid and primary macrophage cultures induced by LPS [9, 10, 32]. In RA, casticin also exerts beneficial effects on collagen-induced arthritis (CIA), a mouse RA model, and markedly abrogating joint swelling and cartilage destruction [12, 13].

In this study, we investigated the potential beneficial effects of casticin in an RA-like model in vitro and in vivo. Our result showed that casticin significantly increased the level of anti-inflammatory cytokine IL-10. IL-10 can effectively block the production of the proinflammatory cytokines TNF-α, IL-1 and IL-8 in snivel macrophages and synoviocytes [33-35]. IL-10 was also found to inhibit TNF-α production in interferon gamma-activated macrophages [36], and has been used in the treatment of RA in clinical trials [37, 38]. In this study, we found that casticin inhibited TNF-α production through IL-10 in LiFLSs, and casticin exerted similar potency as indomethacin. These results suggested that casticin may serve as an effective candidate for the treatment of RA.

NF-κB activation normally correlated with pro-inflammatory cytokines production, like IL-6 and IL-8 [39], and mediated inflammation [40]. So far only one report about HIV transactivating Tat protein [41] found that Tat protein through at least three signaling pathways concurrently, including the classical, alternative and IKKa pathways, to induce NF-κB activation and thus promote production of IL-10. In our study, casticin markedly increased PKC expression, NF-κB production and translocation, which indicated that PCK-NF-κB signaling was the possible pathway involved in.

In summary, this study demonstrated the mechanism of casticin in treatment of RA. By using a RA-like model, we found that casticin has anti-inflammatory effects targeting on IL-10 and TNF-α, two key cytokines in the development of RA pathogenesis. Casticin inhibited TNF-α transcription by stimulating PKC-NF-κB-IL-10 pathway. Our work highlights the application of casticin in the treatment of RA in future.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yan Shen, Division of Immunology and Rheumatology, Huadong Hospi-

tal, Fudan University, Shanghai 200040, P. R. China. Tel: 86-21-62483180; Fax: 86-21-62483180; E-mail: shenyan2015121@126.com

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