Inhibitory Effect of Baicalin on Collagen-Induced Arthritis in Rats through the Nuclear Factor–κB Pathway

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ABSTRACT

This study focused on the potential therapeutic effect of baicalin on collagen-induced arthritis (CIA) in rats and the underlying mechanisms. The CIA rats were injected with baicalin (50, 100, or 200 mg/kg) once daily for 30 days. The rats were monitored for clinical severity of arthritis, and joint tissues were used for radiographic assessment and histologic examination. We quantified tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) in experimental animals and used Western blots to assess levels of protein abundance, phosphorylation, and acetylation of nuclear factor (NF)-κB p65 and sirtuin 1 (sirt1) protein expression in joint tissues. Human fibroblast-like synoviocytes from rheumatoid arthritis (HFLS-RA) were adopted in further mechanistic investigations. Baicalin intraperitoneal injection for 30 days dose-dependently blocked clinical manifestations of CIA, such as functional impairment and swollen red paws. Meanwhile, it alleviated collagen-induced joint inflammation injury and inhibited the secretion of TNF-α and IL-1β in both rat synovium and HFLS-RA. Further mechanistic investigations revealed that baicalin suppresses NF-κB p65 protein expression and phosphorylation in synovial tissue and human-derived synoviocytes. Moreover, the acetylation of NF-κB p65 was downregulated by baicalin, which negatively correlates with the baicalin-induced upregulation of sirt1 expression in the same conditions. The data indicate that CIA in rats can be alleviated by baicalin treatment via relieving joint inflammation, which is related to the suppression of synovial NF-κB p65 protein expression and the elevation of its deacetylation by sirt1.

Introduction

Rheumatoid arthritis (RA) is a common autoimmune disease characterized by chronic inflammation of synovial membranes and proliferation of the synovial lining, leading to synovial hyperplasia, vasculogenesis, cartilage and bone destruction, and joint malformation (Yang et al., 2010; Luo et al., 2011). Currently, RA affects 1% of the adult population worldwide, leading to serious loss of quality of life (Feldmann et al., 1996). Several biological agents, including tumor necrosis factor (TNF)-α inhibitors, interleukin (IL)-1 receptor antagonists, and nonsteroidal anti-inflammatory drugs, have proved clinically effective in RA patients. However, given the cost of such biologic agents and their limited efficacy in some patients (Genovese et al., 2002; Zink et al., 2005), there has been a great demand for the development of novel therapeutic agents with fewer toxic effects.

Although the etiology of RA is not completely understood, synovial cells are known to play a fundamental role in joint damage during RA. It has been clearly demonstrated that nuclear factor (NF)-κB is highly activated and involved in the pathogenesis of RA and animal models of experimental arthritis. NF-κB activation may enhance recruitment of inflammatory cells and production of proinflammatory mediators (Tsaö et al., 1997; Miagkov et al., 1998; Tuk and Firestein, 2001). IL-1β and TNF-α play critical roles in the pathophysiology of RA (Ivashkiv, 1996; Han et al., 1998; Odeh, 1998; Lee et al., 2008).

NF-κB controls the expression of gene products that affect important cellular responses, such as inflammation, immunity, cell proliferation, and apoptosis. The mammalian NF-κB protein family has five known members: p65 (RelA), p50 (NF-κB 1), p52 (NF-κB 2), Rel B, and c-Rel. The most prevalent form of NF-κB is the p65/p50 heterodimer. NF-κB is activated by specific stimuli, such as proinflammatory cytokines, and the liberated NF-κB translocates into the nucleus to regulate the expression of various target genes (Karim and Ben-Neriah, 2000; Tuk and Firestein, 2001; Lee et al., 2008). Recent studies have revealed additional posttranslational modifications of p65, including reversible phosphorylation and acetylation, which modulate NF-κB transcriptional activity on target genes (Kim et al., 2012).

An important site of phosphorylation of the p65 subunit is Ser536 (phospho-Ser536-p65), which promotes p300 recruitment

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ABBREVIATIONS: CIA, collagen-induced arthritis; ELISA, enzyme-linked immunosorbent assay; HFLS, human fibroblast-like synoviocytes; IL-1β, interleukin-1β; MTX, methotrexate; NF-κB, nuclear factor-κB; RA, rheumatoid arthritis; sirt1, sirtuin 1; TNF-α, tumor necrosis factor-α.
to the p65 complex and contributes to p65 acetylation mainly at Lys310 (K310). Acetylation of Lys310 is required for the full transcriptional activity of NF-κB (Chen et al., 2002; Lanzillotta et al., 2010; Kim et al., 2012). Activation of NF-κB depends on the balance between p65 acetylation and deacetylation.

Baicalin, whose chemical structure is shown in Fig. 1A, is a predominant flavonoid isolated from the dry root of *Scutellaria baicalensis* Georgi (Huang-Qin, a medicinal plant). It is considered to be one of the effective and safe drugs widely used in Asia for the treatment of a variety of diseases, such as brain diseases, hepatic disorders, inflammatory diseases, and so on. Furthermore, it has been reported that baicalin has multiple biologic functions, including anti-inflammatory, antioxidant, antiapoptotic, and immune regulation properties (Xu et al., 2011; Yin et al., 2011; Hou et al., 2012). There is increasing evidence to support the notion that this compound may have potential roles in anti-inflammation and immune regulation (Liu et al., 2008). Our previous studies showed that baicalin protects hippocampal neurons by upregulating the expression of brain-derived neurotrophic factor and inhibiting the expression of caspase-3 in a global ischemic gerbil model (Cao et al., 2011). Lee and Kim (2010) found that baicalin displayed an antioxidant effect in rheumatoid arthritis. Although baicalin was previously observed to inhibit NF-κB activation in acute and chronic inflammation models, no further studies of the NF-κB signaling pathway in light of the anti-inflammatory effects of baicalin in rheumatoid arthritis have been reported. Thus, the exact mechanism of its action remains to be clarified.

Regarding the experimental arthritis models, in contrast to lipopolysaccharide- or adjuvant-induced arthritis model, collagen-induced arthritis (CIA) has been widely used as a model of human rheumatoid arthritis, since the CIA model shares

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**Fig. 1.** Blocking of the development and progression of collagen-induced arthritis by treatment with baicalin. (A) Chemical structure of baicalin (C_{21}H_{18}O_{11}; molecular weight 446.4). (B) Representative examples of rat hind paws. Treatment began on day 16 after the primary immunization and continued for 30 days (to day 46). (a) Naive group: rats without CIA, received physiologic saline 0.1 ml/100 g. (b) Vehicle-treated group: rats with CIA, received physiologic saline 0.1 ml/100 g, once daily. (c) MTX-treated group: rats with CIA, received MTX 1 mg/kg every 3 days. (d–f) Baicalin-treated groups, respectively, received baicalin 50, 100, and 200 mg/kg once daily. (C) Body weight changes of the rats. (D) The severity of arthritis was evaluated by clinical arthritic scoring. (E) Hindpaw thickness changes of the rats. Data are expressed as the mean ± S.D. *P < 0.05 compared with the vehicle-treated group.
a number of clinical, immunologic, and pathologic features with RA (Shou et al., 2006). Therefore, human fibroblast-like synoviocytes (HFLS–RA) and the collagen-induced arthritis model, a well studied animal model of RA that has proved useful in the development of new therapies for RA (Brand et al., 2003; Shou et al., 2006) were adopted in this study to determine the potential therapeutic action of baicalin and identify its underlying molecular mechanism.

Materials and Methods

Animals. Female Wistar rats, 8 weeks old (180–200 g b.w.t.), were purchased from the Experimental Animal Center of Jilin University (Changchun, China). The animals were housed five per cage in a clear and ventilated environment maintained under laboratory conditions (temperature 22 ± 1°C, relative humidity 50 to 70%, and 12-hour light/dark cycle). Standard food and water were provided ad libitum throughout the experiments. Animals were acclimated to their surroundings over 5 days to eliminate the effect of stress prior to initiation of the experiments. All animal experiments were performed according to relevant international experimental animal rules and ethical guidelines.

Induction of CIA in Rats and Baicalin Treatment. CIA was induced according to the method described previously with minor modification (Du et al., 2008). In brief, bovine type II collagen (Chondrex, Redmond, WA) was dissolved at 2 mg/ml in 0.05 M acetic acid by gently stirring overnight at 4°C. Collagen solutions were emulsified with an equal volume of complete Freund’s adjuvant (Chondrex) with a homogenizer (Ronghua Instrument Manufacturing Co., JiangSu, China) on the ice water bath. We injected 0.2 ml collagen (200 mg) of the emulsion subcutaneously at the base of the tail, approximately 2 cm distal from the base. To ensure a high incidence and severity of arthritis, a booster injection was given on day 7 after initial immunization. Freund’s incomplete adjuvant (Chondrex) was applied instead of Freund’s complete adjuvant to perform the secondary immunization. We prepared the collagen–incomplete Freund’s adjuvant emulsion as described above and injected 0.1 ml (100 mg collagen) of the emulsion subcutaneously into the tail (inserting the needle at 3 cm from the base of the tail until the needle tip reached 1.5 cm from the base). In this model, the onset of arthritis in rats occurs within 1 week after the second immunization. Starting on the day after the booster injection, the rats were regularly monitored for the development and severity of paw inflammation. The primary immunization day was defined as day 0. Body weight of rats was measured using an electronic scale (Type ESJ200-4; Shenyang Longteng Electronic Co., Shenyang, China) every 5 days from the primary immunization. Hind paw thickness was measured with electronic digital calipers (Hangong Tools Manufacturing Co., Hangzhou, China) every 5 days, beginning on day 10. On day 16 after the primary immunization, rats displaying the onset of arthritis (arthritis index > 2) were randomly assigned to the following groups (n = 6): 1) vehicle-treated group: rats with CIA received physiologic saline (0.1 ml/100 g once daily i.p.); 2–4) baicalin-treated groups: rats with CIA were treated daily with intraperitoneal baicalin (Sigma-Aldrich, St. Louis, MO) with purity >95%, at the doses of 50 mg/kg (group 2), 100 mg/kg (group 3), and 200 mg/kg (group 4); 5) methylxanthate-treated group: rats with CIA were treated with methylxanthate (MTX) (Sigma-Aldrich), 1 mg/kg body weight every 3 days i.p.; and 6) naïve group (untreated controls), six normal rats without CIA received physiologic saline (0.1 ml/100 g i.p.). Treatment continued for 30 days. Baicalin was suspended in physiologic saline prior to experimentation and injected intraperitoneally.

Clinical Assessment of Arthritis. Rats were inspected daily for the onset of arthritis in the paws from the second immunization. Macroscopic signs of clinical arthritis were assessed by a qualitative clinical score every 3 days beginning on the day when arthritic signs were first visible. Each paw was scored according to the following criteria: 0, normal; 1, mild redness and swelling of ankle or wrist joints; 2, moderate redness and swelling of ankle or wrist joints; 3, severe redness and swelling of the entire paw including digits; and 4, paws with deformity or ankylosis (Alonzi et al., 1998). The maximum score for a single paw was 4 and for a single rat was 16; arthritis scores for all four paws of each rat were summed as arthritis index. In a given group, the mean arthritis score for each group was calculated as the mean of total arthritis scores of all rats within the group. Arthritis index was conducted under blinded conditions.

Radiographic Assessments. At day 46 after the first immunization (i.e., day 30 after CIA rats were treated), the rats were sacrificed via anesthesia and the hind paw was obtained from the normal and treated rats. The rats’ hind paw images were taken (MRAD-D50S RADREX-I; Toshiba Medical Manufacturing Co., Ltd., Tokyo, Japan) to observe the radiologic changes. The X-ray parameters were 40 kV, 100 mA, and 0.02 millisecond. Images were read independently in a blinded fashion, and radiologic score was assessed according to the following criteria: 0) no radiologic changes were observed; 1) mild changes, with tissue swelling and edema; 2) moderate changes, with joint erosion and disfiguration; and 3) severe changes, with bone erosion and osteophyte formation (Cai et al., 2007). The total radiologic scores were calculated from the sum of both hind paws of each rat; the maximum value was 6.

Histopathologic Assessments. For histologic analysis, the joints of hind paw were removed and fixed in 4% paraformaldehyde for at least 2 days. The joints of hind paw were then decalcified for 30 days in 10% EDTA, the decalcification liquid was changed every 4 days. Afterward, the paws were embedded in paraffin blocks, longitudinally cut into 4-μm sections using microtome. To ensure extensive evaluation of the arthritic joints, at least three serial sections were cut, the sections were then aplyed on water surface and mounted on microscope slides, and stained with H&E to study the degree of synovitis and bone erosions by microscopic evaluation in a blinded manner. Histologic scores were evaluated on the basis of infiltration of inflammatory cells, synovial hyperplasia, cartilage destruction, and bone erosions (Li et al., 2013). The rating criteria for studies on prognosis are as follows: 1) inflammatory cell infiltration: 0 = normal, 1 = mild infiltration, 2 = moderate infiltration, and 3 = severe infiltration (large number of inflammatory cells were observed); 2) synovial hyperplasia: 0 = no hyperplasia, 1 = mild hyperplasia, 2 = moderate hyperplasia, and 3 = severe hyperplasia; (3) destruction of cartilage: 0 = no destruction, 1 = mild destruction, 2 = moderate destruction, and 3 = severe destruction plus vasculogenesis; and 4) erosions of bone: 0 = no erosions, 1 = mild erosions, 2 = moderate erosions, and 3 = severe erosions (extended erosions and destruction of bone).

Cells and Culture Conditions. Human fibroblast-like synoviocytes–rheumatoid arthritis were purchased from Cell Applications, Inc. (San Diego, CA). Cells were cultured in synoviocyte growth medium (Cell Applications) supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified incubator with 5% carbon dioxide and 95% air. HFLS-RA between passages four and seven were used for subsequent experiments, during which time they were a homogeneous population of synoviocytes.

Cell Treatment. The synoviocyte growth medium was replaced with serum-free medium for an additional 24-hour culture before further treatments. HFLS-RA were suspended at 2 × 10^6 cells/ml for experiments. In the mechanism experiments, HFLS-RA treated with serum-free medium only served as the control group. HFLS-RA were treated with different concentrations of baicalin (10, 20, or 30 μM) in Dulbecco’s modified Eagle’s medium for 24 hours.

HFLS-RA Proliferation Assay by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl-2H-Tetrazolium Bromide. HFLS-RA were cultured using the above method and suspended at 2 × 10^5 cells/ml. Cells were seeded into 96-well plates (100 μl/well) and incubated for 24 hours before various concentrations of baicalin (10, 20, 30, 40, 50, or 60 μM) were added for 48 hours. According to the manufacturer’s recommendations, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide solution (0.5 mg/ml in phosphate-buffered saline) (Sigma-Aldrich) was added to each well and incubated with cells under standard conditions for 4 hours. Subsequently, the formazan
crystals in each well were dissolved with dimethylsulfoxide after the medium was removed. Finally, the optical density was measured with the enzyme-linked immunosorbent assay (ELISA) microwell reader (Bio-Rad, Hercules, CA) at 490 nm, and the results were expressed as the mean of triplicate wells.

### Measurements of TNF-α and IL-1β Levels in Serum and Culture Supernatants by ELISA.

At day 46 after the first immunization, the rats were anesthetized with an intraperitoneal injection of 18% chloral hydrate (0.3 ml/100 g). Serum samples were collected and stored at −80°C until used. To determine cytokine levels in vitro, HFLS-RA were treated with or without baicalin using the above method; supernatants were harvested from each well. TNF-α and IL-1β levels in serum and culture supernatants were measured using rat TNF-α and IL-1β ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

### Western Blotting Detection.

Western blot analysis was performed as previously described (Wang et al., 2010) for measuring NF-κB p65, phospho-NF-κB p65 (Ser536), acetyl-NF-κB p65 (Lys310), and sirtuin 1 (sirt1). Briefly, total proteins were extracted from the synovium of rats and HFLS-RA lysates, thereafter separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. The membranes were blocked in 5% skim milk in phosphate-buffered saline–Tween at room temperature for 2 hours, and probed with anti–NF-κB p65, anti–phospho-NF-κB p65, anti–acetyl-NF-κB p65, and anti-sirt1 (Cell Signaling Technology, Danvers, MA). Horseradish peroxidase–conjugated secondary antibodies (Sigma-Aldrich) and an enhanced chemiluminescence substrate kit were used in detection of specific proteins. β-Actin was used as the loading control.

### Statistical Analysis.

Data were expressed as mean ± S.D. and analyzed with GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA). Comparisons were by one-way analysis of variance with the Tukey post hoc test. *P < 0.05 was regarded as statistically significant.

### Results

#### Significant Blocking of the Progression of CIA by Treatment with Baicalin.

In this study, we used the collagen-induced arthritis model to define the therapeutic effects of baicalin. Relative to other experimental arthritis models, CIA has been widely used as a model of human rheumatoid arthritis, since the CIA model shares a number of clinical, immunologic, and pathologic features with RA (Brand et al., 2003; Shou et al., 2006).

The onset of arthritis in rats occurs within 1 week after the second immunization. Treatment began on day 16 after the primary immunization and continued until day 46. It consisted of intraperitoneal injections of 50, 100, and 200 mg/kg baicalin, 1 mg/kg methotrexate, or physiological saline. Clinical manifestations, such as functional impairment and swollen red paws, were observed (Fig. 1B). Some infected rats exhibited lusterless hair and slow body weight gain (Fig. 1C). Arthritis started to develop and worsen over time in vehicle-treated CIA rats. As shown in Fig. 1D, the therapeutic effect of baicalin was dose-dependent, baicalin (100 and 200 mg/kg once daily) markedly reduced arthritic scores in the baicalin-treated group rats compared with the vehicle-treated CIA rats, as assessed by clinical arthritis score. CIA rats treated with vehicle continued to develop severe arthritis, and reached a plateau between days 28 and 34. In contrast, CIA rats treated with baicalin showed a progressive decrease in the severity of arthritis; this difference was statistically significant (*P < 0.01) at the end of the experiment (when rats were killed), especially for rats given 200 mg/kg baicalin. A similar reduction in arthritis score was observed with 100 mg/kg baicalin and 1 mg/kg MTX in rats with CIA. Similar changes were also observed in hind paw thickness (Fig. 1E). Thus, our results suggest that baicalin can block the progression of inflammatory arthritis.

#### Significant Suppression of Collagen-Induced Joint Inflammation Injury by Treatment with Baicalin.

After 30 days of treatment, the rats were sacrificed and hind limbs were obtained from both normal and treated rats. X-ray radiographs and histologic examinations were carried out to further assess the therapeutic effects of baicalin. Under X-ray imaging conditions, soft tissue swelling, cartilage and bone destruction, joint narrowing, and bone loss were observed. As shown in Fig. 2, the rats without arthritis (naive group, Fig. 2A-a) showed normal soft tissue, joint structure, and joint space (arrow). The CIA rats treated with vehicle showed...
severe swelling of the soft tissues, bone erosion, and a narrowed joint space (Fig. 2A-b). Moderate change was observed in rats treated with baicalin 50 mg/kg, and mild change was observed in rats treated with MTX and baicalin 100 mg/kg. The X-ray films of rats in the baicalin 200 mg/kg group revealed that the soft tissue of each immunized paw was slightly swollen, and less joint destruction was evident in comparison with other treatment groups. Radiologic analysis revealed severe joint erosion in the CIA rats, as shown in Fig. 2B. The mean radiologic score in the vehicle-treated group was significantly higher than the scores for rats receiving 50 mg/kg baicalin, 100 mg/kg baicalin, or MTX. The CIA rats receiving 200 mg/kg baicalin exhibited significant protection, with the lowest scores for bone erosion. The radiographic arthritic scores indicate that 200 mg/kg baicalin markedly suppressed bone erosions and destruction of the joints.

Further evidence to support the inhibitory effects of baicalin on CIA was obtained by histopathology analysis of joints. The nonimmunized rats showed normal architecture of the joint with normal appearance of the cartilage lining, joint space, and the underlying bones, without inflammatory infiltrates in the synovial tissue (Fig. 3A-a). In vehicle-treated CIA rats, the histologic appearance of the joint was highly abnormal, with pronounced synovial hyperplasia, inflammatory cell infiltration, pannus formation, and extensive erosion changes in cartilage and bone (Fig. 3A-b). High-power views of the boxed area show pannus formation with extensive articular cartilage destruction as compared with the naive group. In contrast, CIA rats treated with baicalin were largely spared the chronic inflammation of synovial tissue. These rats showed dose-dependent, significant reductions in inflammatory cell infiltration, pannus formation, cartilage destruction, and bone erosion compared with the vehicle-treated group (Fig. 3, A and D–F). The histologic scores of CIA rats that received baicalin were significantly lower than the scores of CIA rats treated with vehicle. These data demonstrate that baicalin relieved joint inflammation injury in CIA rats.

**Baicalin Inhibits the Secretion of TNF-α and IL-1β in Rat Serum and Human Fibroblast-Like Synoviocytes.** To investigate whether baicalin modulates the inflammatory process by regulating the secretions of cytokines in vivo, we measured the serum levels of TNF-α and IL-1β in rats by ELISA. As shown in Fig. 4A, substantial increases in pro-inflammatory cytokine levels were found in the serum samples of vehicle-treated CIA rats on day 46 after primary immunization. In contrast, TNF-α levels were significantly and dose-dependently lower in baicalin-treated groups (50, 100, and 200 mg/kg) than in the vehicle-treated group (P < 0.01). In summary, these findings suggest that baicalin may exert its anti-inflammatory effect by inhibiting the production of pro-inflammatory cytokines, thereby attenuating the joint inflammation in CIA rats.

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**Fig. 3.** Therapeutic effects of baicalin on synovial inflammation and cartilage-bone destruction in rats with collagen-induced arthritis. On day 46 after primary immunization, the joints of hind paws were treated as described under Materials and Methods. (A) The sections were stained with hematoxylin and eosin to study the degree of synovitis and cartilage-bone erosions. Histologic features of representative joints are shown for each group of rats. Further magnification of the black-bordered box (top) shows the typical inflammatory injuries (bottom). Naive group (a) shows normal cartilage, bone, and synovium without inflammation. CIA rats treated with vehicle (b) showed pronounced synovial hyperplasia, inflammation, cell infiltration, cartilage destruction, bone erosion, and pannus formation. A high-power view of the boxed area shows pannus formation with extensive articular cartilage destruction. CIA rats treated with baicalin 200 mg/kg (f) exhibited well-preserved joint spaces and articular cartilage surfaces, with minimal pannus formation compared with the vehicle-treated group, indicating that the administration of baicalin directly correlated with a reduction in disease severity. Mild changes in joint pathology were seen in CIA rats treated with MTX (c) and baicalin 100 mg/kg (e). Moderate change was observed in rats treated with baicalin 50 mg/kg (d). bo, bone; ca, cartilage; sy, synovium; js, joint space; sh, synovial hyperplasia; ce, cartilage erosion; ic, inflammatory cells; pf, pannus formation. Original magnification, 10×; 20× in high-power views. (B) Histologic score was evaluated from the joints of CIA rats treated with or without baicalin on the basis of infiltration of inflammatory cells, synovial hyperplasia, pannus formation, cartilage destruction, and bone erosion. Data are expressed as mean ± S.D. (n = 6 per group). *P < 0.05 compared with vehicle-treated rats.
tissues of patients with RA. A major hallmark of HFLS-RA is the production of mediators of inflammation, which contribute to cartilage degradation and joint infiltration by immune cells (Bartok and Firestein, 2010). HFLS-RA were incubated and treated with baicalin (10, 20, and 30 μM) for 24 hours in six-well plates. Subsequently, culture supernatants were harvested from each well, and the levels of TNF-α and IL-1β were determined by ELISA. Fig. 4B demonstrates that baicalin significantly reduced the levels of TNF-α and IL-1β in a dose-dependent manner compared with the vehicle-treated group.

**Baicalin Suppresses NF-κB p65 Activation in Rat Synovium and Human Fibroblast-Like Synoviocytes.** NF-κB is an important transcription factor for the induction of various proinflammation cytokines (Han et al., 1998; Tak and Firestein, 2001). To gain insight into baicalin’s mechanism of action, we used Western blot analysis to examine whether baicalin inhibits the NF-κB signal pathway in CIA and HFLS-RA. On day 46 after primary immunization, total proteins extracted from the synovium of rats were subjected to Western blot analysis. NF-κB p65 protein abundance and NF-κB p65 (Ser536) phosphorylation were suppressed by the addition of baicalin (10, 20, 30 μM) in a dose-dependent manner (Fig. 5, A–C). Further investigation of HFLS-RA in vitro revealed that baicalin (30 μM) also inhibited NF-κB protein expression and phosphorylation (Fig. 5, D–F). Moreover, the transcription-related acetylation of NF-κB p65 was downregulated by baicalin in both the CIA model and human-derived pathologic synoviocytes. In contrast, deacetylase sirt1 expression was upregulated by baicalin in the same conditions (Fig. 6). These results indicated that baicalin exerted an anti-CIA effect via inhibiting NF-κB activation, including suppressing protein expression and lowering phosphorylation and acetylation levels.

**Discussion**

The effects of baicalin on rheumatoid arthritis have not yet been fully determined. In the current study, we demonstrated for the first time that baicalin has a therapeutic role in CIA via inhibiting the inflammation reaction in a concentration-dependent manner. The NF-κB pathway accounts for baicalin’s suppression of the inflammation reaction in CIA, and the underlying mechanism is via suppression of the phosphorylation and acetylation of NF-κB p65. Sirt1, a potent deacetylase in transcription, is involved in the deacetylation of p65 in baicalin-suppressed CIA.

Various elegant publications have shown that baicalin, a chemical that has been used in the treatment of brain diseases, hepatic disorders, and inflammatory diseases, has a potent inhibitory effect on inflammation reactions. For example, baicalin inhibits lipopolysaccharide-induced inflammation caused by endotoxic shock. It also inhibits proinflammatory cytokines and nitric oxide production, NF-κB activation, and caspase-3 activity (Liu et al., 2008). Recently, it has been reported that baicalin inhibits splenic Th17 cell population expansion in vivo, which prevents expansion of the IL-17–mediated inflammatory cascade and effectively reduces inflammatory joint injury in murine adjuvant-induced arthritis (Yang et al., 2013). All the data show that baicalin-inhibited inflammation reaction is a key therapeutic mechanism in many diseases. Therefore, in the current investigation, we sought to determine whether baicalin was able to alleviate/reverse the progression of CIA by regulating the inflammation reaction.

**Fig. 4.** Baicalin inhibits the production of TNF-α and IL-1β in sera of collagen-induced arthritis rats and the supernatants of human fibroblast-like synoviocytes–rheumatoid arthritis. (A) On day 46 after primary immunization, the rats were sacrificed and blood samples were collected, and serum levels of TNF-α and IL-1β were determined by ELISA. High-dose baicalin (200 mg/kg) reduced TNF-α and IL-1β levels significantly when compared with the vehicle-treated rats; middle-dose baicalin (100 mg/kg) and MTX (1 mg/kg) also reduced levels of TNF-α and IL-1β. Data are expressed as mean ± S.D. For in vitro studies, HFLS-RA were cultured in synoviocyte growth medium, HFLS-RA passages four to seven were used for all experiments. **P < 0.01 compared with vehicle-treated group.**

Based upon the effects of baicalin on CIA, we next sought to investigate the possible inhibitory effects of baicalin on proinflammatory cytokine production. Human fibroblast-like synoviocytes–rheumatoid arthritis were purchased from Cell Applications, Inc.; HFLS was derived from inflamed synovial...
Consistent with our hypothesis, the results showed that baicalin exerts a therapeutic role in CIA via suppressing the inflammation reaction, including alleviating redness and swelling of the ankle and decreasing the secretion of key cytokines in pathologic synovium. Radiologic and histologic analyses also revealed that baicalin significantly alleviated joint damage and blocked the progression of inflammatory arthritis. Other possible mechanisms mediated by baicalin in RA, such as its antiapoptotic role in the brain (Cao et al., 2011) and its antioxidant effect in RA (Lee and Kim, 2010), will be addressed in future studies.

The conventional inflammation reaction is mediated by many cytokines, such as TNF-α, IL-1β, IL-6, TGF-β, IL-8, IL-10, and so on. In the current study, we found that baicalin lowered circulating TNF-α and IL-1β levels in plasma in the CIA rat model. Further investigations involving the pathologic RA cell model and human fibroblast-like synoviocytes from rheumatoid arthritis showed that baicalin also decreased the secretion

![Fig. 5](image-url)

**Fig. 5.** Effects of baicalin on NF-κB signaling pathways both in vivo and in vitro. (A) For in vivo studies, on day 46 after primary immunization the rats were sacrificed and total proteins extracted from the synovium were separated by SDS-PAGE as described in Materials and Methods. Proteins were subjected to Western blot analysis for NF-κB p65 and phospho-NF-κB p65 using specific antibodies. (B and C) Quantitation of Western blots of NF-κB p65 and phospho-NF-κB p65 in synovium of rats from different groups. (D) HFLS-RA were incubated and treated with baicalin (10, 20, 30 μM) for 24 hours in six-well plates. Protein was subjected to Western blot analysis for NF-κB p65 and phospho-NF-κB p65. (E and F) Quantitation of Western blots of NF-κB p65 and phospho-NF-κB p65 in HFLS-RA from different groups. Data are expressed as mean ± S.D.; β-actin was used as the loading control. *P < 0.05 compared with the control group.

![Fig. 6](image-url)

**Fig. 6.** Effects of baicalin on NF-κB signaling pathways both in vivo and in vitro. (A) For in vivo studies, on day 46 after primary immunization the rats were sacrificed and total proteins extracted from the synovium were separated by SDS-PAGE as described in Materials and Methods. Protein samples were subjected to Western blot analysis for acetyl-NF-κB p65 and sirt1 using specific antibodies. (B and C) Quantitation of Western blots of acetyl-NF-κB p65, and sirt1 in the synovium of rats from different groups. (D) HFLS-RA were incubated and treated with baicalin (10, 20, 30 μM) for 24 hours in six-well plates. Protein samples were subjected to Western blot analysis for acetyl-NF-κB p65, and sirt1 in HFLS-RA from different groups. Data are expressed as mean ± S.D; β-actin was used as the loading control. *P < 0.05 compared with the control group.
of TNF-α and IL-1β in cell medium in vitro. All the data showed that baicalin suppressed the inflammation reaction by reducing the secretion of the key inflammatory cytokines in HFLS-RA and the CIA rat model.

It is well established that TNF-α and IL-1β, the main inflammation cytokines, exert their proinflammatory effects by interacting with their own receptors in cell membranes and regulating the NF-κB pathway. The NF-κB pathway is the key mediator of the conventional inflammation reaction. The anti-inflammatory activity of baicalin has been associated with NF-κB, as shown in various acute and chronic inflammation models; it attenuates inflammation by inhibiting NF-κB activation in Staphylococcus aureus–induced mastitis and a cigarette smoke–induced inflammatory model (Lixuan et al., 2010; Guo et al., 2013). Animal models of experimental arthritis support the notion that NF-κB activation plays a pathogenic role in the inflammation reaction. For example, increased synovial NF-κB binding precedes the development of clinical joint involvement in CIA and gradually increases during the evolution of disease (Han et al., 1998; Tak and Firestein, 2001). The important role of NF-κB in inflammation has also been shown in rats with streptococcal cell wall–induced arthritis and adjuvant-induced arthritis (Tsao et al., 1997; Mingkov et al., 1998). Consistent with these published findings, we found that baicalin suppressed the protein expression of NF-κB p65, not only in synovium in CIA rats but also in HFLS-RA. Moreover, the phosphorylation levels of NF-κB p65 were reduced by baicalin treatment in a dose-dependent manner. All the data indicate that the NF-κB pathway participates in baicalin’s inhibition of the progression of CIA.

Since the protein expression and phosphorylation of NF-κB were lowered by baicalin treatment in both HFLS-RA and the synovium in the CIA model, we sought to determine whether its transcriptional activity is affected by baicalin. It is well documented that transcriptional activation of NF-κB correlates closely with NF-κB p65 acetylation (Lys310) (Chen et al., 2002; Lanzillotta et al., 2010; Kim et al., 2012). In the current study, we found that baicalin dose-dependently reduced the acetylation levels of NF-κB p65 in synovium in CIA rats. The inhibitory effect of baicalin on p65 acetylation was observed in HFLS-RA in vitro as well. Likewise, NF-κB transcriptional activity can be inhibited by the NAD+-dependent class III histone deacetylase sirtuins. Indeed, it has been reported that sirtuin 1, the best characterized and most well studied of the sirtuins, is a potent inhibitor of NF-κB/p65 at lysine 310 (Yeung et al., 2007). Suppression of the onset and progression of collagen-induced arthritis by NF-κB is required for the development of collagen-induced arthritis. J Exp Med 187:461–468.


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