Inhibitory effect of the Baicalin on collagen-induced arthritis in rats through nuclear factor kappa B pathway

Hong-Zhi Wang, Hai-He Wang, Shi-Shun Huang, Hong Zhao, Yong-Gang Cao, Guang-Zhi Wang, Dong Wang, Zhi-Gang Wang, and Yan-Hong Liu

Department of Laboratory Diagnosis (H.-Z.W., Y.-H.L.), The Second Affiliated Hospital of Harbin Medical University, Harbin, China; Department of Laboratory Diagnosis (H.-Z.W., G.-Z.W.), Department of Respiratory Medicine (H.Z.), and Department of Orthopaedic Surgery (D.W.), The Fifth Affiliated Hospital of Harbin Medical University, Daqing, China; College of Medical Laboratory Science and Technology and the key laboratory of molecular diagnosis in laboratory medicine (H.-H.W., S.-S.H., Z.-G.W.), Department of Pharmacology (Y.-G.C.), Harbin Medical University (Daqing), Daqing, China
Running Title: Therapeutic effects of Baicalin on CIA in rats

Corresponding author:
Yanhong Liu, Professor
Department of Laboratory Diagnosis
The Second Affiliated Hospital of Harbin Medical University
246 Xuefu Road, Nangang District
Harbin, 150001, P. R. of China
Phone: (86)-451-86296362
Fax: (86)-451-86605363
E-mail: hrb_liuyanhong@126.com

ABBREVIATIONS: CIA, collagen-induced arthritis; RA, rheumatoid arthritis; NF-κB, nuclear factor-κB; Lys310, acetyl-NF-κB p65; Ser536, phospho-NF-κB p65; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; IL-1β, interleukin-1 beta; TNF-α, tumor necrosis factor-alpha; HFLS, Human Fibroblast-Like Synoviocytes.

Number of text pages: 35
Number of tables: 0
Number of figures: 6
Number of references: 34
The number of words in the Abstract: 240
The number of words in the Introduction: 714
The number of words in the Discussion: 878
Abstract

This study focused on the potential therapeutic effect of baicalin on the collagen-induced arthritis (CIA) in rat and the underlying mechanisms. The CIA rats were injected with baicalin (50 mg/kg, 100 mg/kg 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. The tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) in rats were detected. And the levels of protein abundance, phosphorylation, acetylation of nuclear factor kappa B (NF-κB) p65 and sirtuin 1 (sirt 1) protein expression in joint tissues were determined by western blotting. Human Fibroblast-Like Synoviocytes from Rheumatoid Arthritis (HFLS-RA) were adopted in further mechanistic investigation. Baicalin intraperitoneal injection for 30 days dose-dependently blocked the clinical manifestations of CIA, such as functional impairment and swollen red paws. Meanwhile, it alleviated the collagen-induced joint inflammation injury, inhibited the secretion of TNF-α and IL-1β in both rat Synovium and HFLS-RA. Further mechanistic investigation revealed that baicalin suppresses NF-κB p65 protein expression and phosphorylation in Synovium tissue and human-derived synoviocytes. Moreover, the acetylation of NF-κB p65 was downregulated by baicalin as well, which negatively correlates with the baicalin-induced upregulation of sirt1 expression in the same conditions. The data indicates that the CIA in rats could be restored by baicalin treatment via relieving joint inflammation, which is related with the suppression of
synovium 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). Although several biologic agents, including TNF-a inhibitors, IL-1 receptor antagonists and non steroidal anti-inflammatory drugs (NSAIDs) 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, but have fewer toxic effects.

Although the etiology of RA are not completely understood, synovial cells play a fundamental role in joint damage during RA. It has been clearly demonstrated that 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 (Tsao et al., 1997; Miagkov et al., 1998; Tak and Firestein, 2001). Especially interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-a), play a 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 includes 5 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 p65/p50 heterodimer. NF-κB was activated by specific stimuli, such as proinflammatory cytokines, and the liberated NF-κB translocates into nucleus to regulate the expression of various target genes (Karin and Ben-Neriah, 2000; Tak 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 p65 subunit is at serine-536 (phospho-Ser536-p65), which promotes p300 recruitment to the p65 complex and contributes to p65 acetylation mainly at lysine310 (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 (7-glucuronic acid, 5,6-dihydroxy-flavone), 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). As 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 biological functions, including anti-inflammatory,
antioxidant, anti-apoptotic and immune regulation properties (Xu et al., 2011; Yin et al., 2011; Hou et al., 2012). More recently, there is increasing evidence to support the notion that this compound may have potential role on anti-inflammation and immune regulation (Liu et al., 2008). Our previous studies showed that baicalin protects hippocampal neurons by up-regulating the expression of brain-derived neurotrophic factor (BDNF) and inhibiting the expression of caspase-3 in a global ischemic gerbil model (Cao et al., 2011). Lee, J.H et al. found that Baicalin displayed an antioxidant effect in rheumatoid arthritis (Lee and Kim, 2010). Although baicalin was previously observed to inhibit NF-κB activation in acute and chronic inflammation models, no further studies of NF-κB signaling pathway about the anti-inflammatory effects of baicalin in rheumatoid arthritis, the exact mechanism of its actions remains to be clarified.

Regarding the experimental arthritis models, in contrast to LPS- or adjuvant-induced arthritis model, collagen-induced arthritis (CIA) has been widely used as a model of human rheumatoid arthritis (RA), since the CIA model shares a number of clinical, immunological and pathological features with RA (Shou et al., 2006). Therefore, Human Fibroblast-Like Synoviocytes-Rheumatoid Arthritis (HFLS-RA) and collagen-induced arthritis (CIA) model, as a well-studied animal model of RA, which were 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 (180-200g body weight), were purchased from the Experimental Animal Center of Jilin University (Changchun, China). The animals were housed five per cage in clear and ventilated environment maintained under laboratory conditions (temperature 22 ± 1°C, relative humidity 50 – 70%, and 12 h light–dark cycle). Standard food and water were provided ad libitum throughout the experiments. Animals were acclimated to their surroundings over five 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 (CII, Chondrex, 20021) was dissolved at 2mg/ml in 0.05 M acetic acid by gently stirring overnight at 4°C. Collagen solutions was emulsified with an equal volume complete Freund’s adjuvant (CFA,Chondrex, 7001) with a homogenizer on the ice water bath. Inject 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 (IFA, Chondrex, 7002) was applied instead of Freund’s complete adjuvant to perform the secondary immunization. Prepare the collagen-IFA emulsion as described above and inject 0.1 ml (collagen: 100 mg) of the emulsion subcutaneously in the tail (insert needle at 3 cm from the base of the tail until needle tip reaches 1.5 cm from the base). In this model, the onset of arthritis in rats occurs within 1 week after the second immunization. Following the day 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, China) every 5 days from the primary immunization. Hind paw thickness was measured with electronic digital calipers (Hangzhou, China) every 5 days from day 10. On day 16 after the primary immunization, the rats in the onset of arthritis (arthritis index>2) were randomly assigned to the following groups (n=6): (1) Vehicle-treated group, rats with CIA were received physiological saline (0.1 ml/100g, once daily, i.p.); (2–4) Baicalin-treated group, rats with CIA were treated with baicalin (Sigma, with a purity >95%, 50, 100 and 200 mg/kg, once daily, i.p.), respectively; (5) Methotrexate-treated group, rats with CIA treated with methotrexate (MTX, Sigma, 1 mg/kg body weight every 3 days, i.p.). In addition, six normal rats were selected as the untreated controls. namely; (6) Naive group, rats without CIA were received physiological saline (0.1ml/100g, i.p.). Treatment continued for 30 days. Baicalin was suspended in physiological saline prior to experimentation and intraperitoneally (i.p.) injected.
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 three days beginning on the day when arthritic signs were first visible. Arthritis score in 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; 4, paws with deformity or ankylosis (Alonzi et al., 1998). Maximum score of a single paw was 4 and a single rat was 16, arthritic 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 score 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 (Toshiba Medical Manufacturing Co., Ltd., MRAD-D50S RADREX-i, Japan) to observe the radiological changes. The X-ray parameters were 40kv, 100mA and 0.02 ms. Images were read independently in a blinded fashion and radiological score was assessed according to the following criteria: 0, no radiological changes were observed; 1, mild changes: tissue swelling and oedema; 2, moderate changes: joint erosion and disfiguration; and 3,
severe changes: bone erosion and osteophyte formation (Cai et al., 2007). The total radiological scores were calculated from the sum of both hind paws of per rat, maximum value was 6.

**Histopathologic assessments**

For histologic analysis, the joints of hind paw were removed and fixed in 4% paraformaldehyde for at least two days. The joints of hind paw were then decalcified for 30 days in 10% EDTA, the decalcification liquid was changed every four days. Afterwards, the paws were embedded in paraffin blocks, longitudinally cut into 4μm sections using microtome. To ensure extensive evaluation of the arthritic joints, at least three serial sections were cut, the sections were then splayed on water surface and mounted on microscope slides, and stained with hematoxylin and eosin (H&E) to study the degree of synovitis and bone erosions by microscopic evaluation in a blinded manner. Histological score was 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; 3, severe infiltration (large number of inflammatory cells were observed); (2) Synovial hyperplasia: 0, no hyperplasia was observed; 1, mild hyperplasia; 2, moderate hyperplasia; 3, severe hyperplasia; (3) Destruction of cartilage: 0, no destruction; 1, mild destruction; 2, moderate destruction; 3, severe destruction plus vasculogenesis. (4) Erosions of bone: 0, no erosions; 1, mild erosions; 2, moderate erosions; 3, severe erosions (extended...
erosions and destruction of bone).

Cells and culture conditions

Human Fibroblast-Like Synoviocytes-Rheumatoid Arthritis (HFLS-RA) was purchased from Cell Applications Inc (San Diego, CA, USA). HFLS derived from inflamed synovial tissues of patients with RA. Cells were cultured in synoviocyte growth medium (Cell Applications) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin at 37°C in a humidified incubator with 5% carbon dioxide and 95% air. HFLS-RA between passages 4 and 7 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 h culture before further treatments. HFLS-RA were suspended at 2×10^6 cells/mL for experiment. In the mechanism experiments, HFLS-RA treated with serum-free medium only served as control group. HFLS-RA were treated with different concentrations of baikalin (10, 20 or 30 μM) in DMEM supplemented for 24 h.

HFLS-RA proliferation assay by MTT

HFLS-RA were cultured using above method and suspended at 2×10^6 cells/mL. Cells were seeded into 96-well plates (100 μL/well) and incubated for 24 h before various concentrations of baikalin (10, 20, 30, 40, 50 or 60 μM) were added for 48 h.
According to the manufacturer’s recommendations, 20 μl of MTT solution (Sigma, 0.5 mg/ml in PBS) was added in each well and incubated with cells under standard conditions for 4 h. Subsequently, the formazan crystals in each well was dissolved with dimethyl sulfoxide (DMSO) after the medium was removed. Finally, the optical density (OD) was measured with the enzyme-linked immunosorbent assay (ELISA) microwell reader (Bio-Rad, Hercules, CA, USA) at 490 nm, and the results were expressed as 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 10% chloral hydrate (0.3 ml/100g). 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 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, USA) according to the manufacturer’s instructions.

**Western blotting detections**

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 sirt1. Briefly, total proteins were extracted from the synovium of rats and
HFLS-RA lysates, thereafter separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked in 5% skim milk in phosphate buffered saline-Tween at room temperature for 2 h, and probed with anti- NF-κB p65, anti-phospho- NF-κB p65 (Ser536), anti-acetyl- NF-κB p65 (Lys310), and anti-sirt1 (Cell Signaling Technology, USA) respectively. Horseradish peroxidase-conjugated secondary antibodies (Sigma) and 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 Graph Pad Prism version 5.0 (Graph Pad software). Comparisons were by One-way ANOVA 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 (CIA) 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 (RA), since the CIA model shares a number of clinical, immunological and pathological 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 until day 46, and consisted of intraperitoneal injections of 50, 100 and 200 mg/kg baicalin, 1 mg/kg methotrexate (MTX), or physiological saline. Clinical manifestations, such as functional impairment and swollen red paws, were observed (Fig. 1B). Some infected rats were accompanied by lusterless hair and slow body weight gain (Fig. 1C).

Arthritis started to develop and worsen over time in rats 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 arthritic score. CIA rats treated with vehicle continued to develop severe arthritis, and reached a plateau between days 28 and 34. Whereas 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 200 mg/kg baicalin. A similar reduction in arthritic score was observed with 100 mg/kg baicalin and 1mg/kg MTX with rats with CIA. Similar changes was 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.**

At the end of 30 days treatments, the rats were sacrificed and the hind limbs were obtained from the normal and treated rats. X-ray radiograph and histological 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. 2A, the rat without arthritis (naive group, Fig. 2A-a) showed normal soft tissue, joint structure, joint space (arrow). The CIA rat treated with vehicle showed Severe swelling of the soft tissues and bone erosion, and a narrowed joint space (Fig. 2A-b). Moderate change was observed in rats treated with baicalin 50 mg/kg, mild change was observed in rats treated with MTX-treated and baicalin 100 mg/kg. The X-ray films of rats in baicalin 200 mg/kg groups revealed that the soft tissue of immunized paw was slightly swollen and the damage was much less in joint destruction. Radiologic analysis revealed severe joint erosion in the CIA rats, as shown in Fig. 2B. The mean radiological score in vehicle-treated group was significantly higher than those in rats receiving 50 mg/kg baicalin, 100 mg/kg baicalin, and MTX. The CIA rats receiving 200 mg/kg baicalin
exhibited significantly protection, with the lowest scores for bone erosion. The radiographic arthritic scores indicate that 200 mg/kg baicalin markedly suppressed the 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 non-immunized rats showed normal architecture of the joint with normal appearance of the cartilage lining, joint space, and the underling bones, without inflammatory infiltrates in the synovial tissue (Fig. 3A-a). Vehicle-treated CIA rats showing a highly abnormal histologic appearance of the joint, with pronounced synovial hyperplasia, inflammation cell infiltration, pannus formation, and extensive erosion changes in the cartilage and bone (Fig. 3A-b). High-power view of the boxed area showed pannus formation, with extensive articular cartilage destruction as compared with naive group. In contrast, CIA rats treated with baicalin significantly abrogated the chronic inflammation of synovial tissue and significantly reduced of inflammatory cell infiltration, pannus formation, cartilage destruction and bone erosion with dose-dependent manner compared with vehicle-treated group (Fig. 3A-d,e,f). Their histological score showed a significantly decrease compared with that of the CIA rats treated with vehicle. These data demonstrate that baicalin relieved joint inflammation injury in CIA rat.

**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 proinflammatory 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 (50mg/kg, 100mg/kg, 200mg/kg) than in vehicle-treated group (p < 0.01). In particular, at the highest dose (200mg/kg) baicalin dramatically reduced serum levels of TNF-α, furthermore, similar results were observed for IL-1β (p < 0.01).

Based upon the effects of baicalin on CIA, To next investigate the possible inhibitory effects of baicalin on proinflammatory cytokines production. Human Fibroblast-Like Synoviocytes-Rheumatoid Arthritis (HFLS-RA) was purchased from Cell Applications Inc, HFLS derived from inflamed synovial 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, 30 μM), respectively for 24 h in 6-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 decreased the levels of TNF-α and IL-1β with dose-dependent manner compared with 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 the mechanism of action following baicalin treatment, we examined whether baicalin inhibit NF-κB signal pathway in CIA and HFLS-RA by western blot analysis. 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 μM, 20 μM, 30 μM) in a dose-dependent manner (Fig. 5A-C). Further investigation in HFLS-RA in vitro reveal that baicalin (30μM) inhibited NF-κB protein expression and phosphorylation as well (Fig. 5D-F). Moreover, the transcription-related acetylation of NF-κB p65 was downregulated by baicalin in both CIA model and human-derived pathological synoviocytes. In contrast, deacetylase sirt1 expression was up-regulated 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 have the therapeutic role in CIA via inhibiting the inflammation reaction in a concentration-dependent way. NF-κB pathway accounts for baicalin-suppressed inflammation reaction in CIA, and the underlying mechanism is via suppressing 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 showed that baicalin, a chemical has been used for the treatment of brain diseases, hepatic disorders, inflammatory diseases, have the potent inhibitive effect in inflammation reaction. For instance, baicalin inhibited lipopolysaccharide (LPS)-induced inflammation caused by endotoxic shock. It inhibited pro-inflammatory cytokines and nitric oxide (NO) 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 joint inflammatory injury in murine adjuvant-induced arthritis (Yang et al., 2013). All the data showed that baicalin-inhibited inflammation reaction is a key therapeutic mechanism in many disease, and how it works in CIA. Therefore, in current investigation, baicalin were applied to determine whether it was able to alleviate/reverse the progression of CIA via regulating the inflammation reaction.
Consistant with our hypothesis, the result showed that baicalin exert its therapeutic role in CIA via suppressing the inflammation reaction, including alleviating the redness and swelling of the ankle and decreasing the secretion of key cytokines in pathological synovium. Radiologic and histologic analyses also revealed that baicalin significantly alleviated joint damage and blocked the progression of inflammatory arthritis. Other possible mechanism mediated by baicalin in RA, such as its anti-apoptotic role in brain (Cao et al., 2011) and its anti-oxidant effect in RA (Lee and Kim, 2010) will be involved in future study.

In conventional inflammation reaction, many cytokines mediated the inflammation reaction, such as TNF-α, IL-1β, IL-6, TGF-β, IL-8, IL-10 and so on. In current study, we found that baicalin lowered circulating TNF-α and IL-1β level in plasma in CIA rat model. Further mechanism investigation in pathological RA cell model, human fibroblast-like synoviocytes from rheumatoid arthritis, showed that baicalin also decreased the secretion of TNF-α and IL-1β in cell medium in vitro. All the data showed that baicalin suppressed the inflammation reaction via decrease the secretion of the key inflammatory cytokines in HFLS-RA and CIA rat model.

It is well-investigated that TNF-α and IL-1β, the main inflammation cytokines, play their proinflammatory effect via interacting with their own receptor in cell membrane and regulating NF-κB pathway. NF-κB pathway is the main key molecule mediating 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, such as baicalin attenuates inflammation by inhibiting NF-κB
activation in S. aureus-induced mastitis and 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 inflammation reaction. For instance, increased synovial NF-κB binding precedes the development of clinical joint involvement in CIA and it 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; Miagkov et al., 1998). In consistent with this publication, we found that baicalin suppressed the protein expression of NF-κB p65 not only in synovium in CIA in rat but in HFLS-RA. Meanwhile, the phosphorylation levels of NF-κB p65 was decreased by baicalin treatment in a dose-dependent way. All the data indicates that NF-κB pathway participate baicalin-inhibited progression of CIA.

Since the protein expression and phophorylation of NF-κB were lowered by baicalin treatment in both HFLS-RA and the synovium in CIA model, whether its transcriptional activity was affected by baicalin inclusion. 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 current study, we found that baicalin therapy dose-dependently decreased the acetylation levels of NF-κB p65 in synovium in CIA in rat. The inhibitive effect of baicalin in 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 (HDACs), sirtuins. Indeed, it has been reported that sirtuin 1 (sirt1), the best characterized and well-studied among sirtuins, is a potent inhibitor of NF-κB transcription through the deacetylation of p65. Sirt1 represses NF-κB gene expression by deacetylating RelA/p65 at lysine 310 (Yeung et al., 2004). In current investigation, baicalin up-regulated sirt1 expression level in both the synovium of CIA rats and HFLS-RA. All the data suggest that baicalin lowers NF-κB p65 acetylation level, and the possible mechanism is involved in inducing deacetylase sirt1 expression.

In general, the data in our paper strongly demonstrated that baicalin, an important component isolated from the dry root of Scutellaria baicalensis Georgi, have the therapeutic potential in CIA via inhibiting the inflammation reaction, and the underlying mechanism is involved in the suppression of NF-κB p65 protein expression and the elevation of its deacetylation by sirt1.
Authorship Contributions


Wrote or contributed to the writing of the manuscript: H.-Z. Wang and Z.-G. Wang.
References


Tak PP and Firestein GS (2001) NF-kappaB: a key role in inflammatory diseases. *The*


production of hydrogen peroxide and oxidative stress induced by Abeta aggregation in SH-SY5Y cells. *Neuroscience letters* 492:76-79.

Footnotes

This work was supported by the Heilongjiang Education Department, China [Grant 12541523].
Legends

Fig. 1. Blocking of the development and progression of collagen-induced arthritis (CIA) 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 until day 46, continued for 30 days. (a) Naive group, rats without CIA were received physiological saline 0.1ml/100g. (b) Vehicle-treated group, rats with CIA were received physiological saline 0.1ml/100g, once daily. (c) MTX-treated group, rats with CIA were received methotrexate (MTX) 1mg/kg, every 3 days. (d,e,f) baicalin-treated group, 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 score. E, Hind paw thickness changes of the rats. Data are expressed as the mean ± SD. *P<0.05 compared with the vehicle-treated group.

Fig. 2. Therapeutic effects of baicalin on paw swelling and bone destruction in collagen-induced arthritis (CIA) rats. On day 46 after primary immunization the rats were sacrificed and the hind limbs were obtained from the normal and treated rats. A, Representative macroradiographs of rat hind paws. Neither paw swelling nor joint erosion was observed in normal rats (a). Severe paw swelling and bone erosion were seen in CIA rats treated with vehicle (b). moderate change was observed in rats treated with baicalin 50 mg/kg (d), mild change was observed in rats treated with MTX-treated group (c) and baicalin 100 mg/kg (e). but the damage was much less in rats treated with
baicalin 200 mg/kg (f). B, The radiological scores of bone erosion in treated CIA rats were evaluated as described under materials and methods section. Data are expressed as mean ± SD (n = 6 per group). *P < 0.05, **P < 0.01 compared with the vehicle-treated rats.

Fig. 3. Therapeutic effects of baicalin on synovial inflammation and cartilage-bone destruction in collagen-induced arthritis (CIA) rats. On day 46 after primary immunization, the joints of hind paw were treated as described under materials and methods section. A, The sections were stained with hematoxylin and eosin (H&E) to study the degree of synovitis and cartilage-bone erosions, histological features of representative joints are shown for each group of rats. Further magnification of the black-bordered box (in top panel) showed the typical inflammatory injuries (bottom). Naive group (a) shows normal cartilage, bone and synovium without inflammation. In CIA rats treated with vehicle (b) showing pronounced synovial hyperplasia, inflammation cell infiltration, cartilage destruction, bone erosion and pannus formation. High-power view of the boxed area showed 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 vehicle-treated group, indicating that the administration of baicalin directly correlated with a reduction in disease severity. Mild change on joint pathology were seen in CIA rats treated with MTX-treated group (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; and ic=inflammatory cells; pf=pannus formation. (original magnification ×10; ×20 in high-power views.). B, Histological score was evaluated in the joint of CIA rat 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 ± SD (n = 6 per group). *P < 0.05 compared with the vehicle-treated rats.

Fig. 4. Baicalin inhibits the production of TNF-α and IL-1β in sera of collagen-induced arthritis (CIA) rats and the supernatants of Human Fibroblast-Like Synoviocytes-Rheumatoid Arthritis (HFLS-RA). A, On day 46 after primary immunization the rats were sacrificed and blood samples were collected, 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 compared with the vehicle-treated rats, middle-dose baicalin (100 mg/kg) and MTX (methotrexate, 1mg/kg) also decreased levels of TNF-α and IL-1β. Data are expressed as mean ± SD. *P < 0.05, **P < 0.01 compared with the vehicle-treated rats. For in vitro studies, HFLS-RA were cultured in synoviocyte growth medium, HFLS-RA passages 4 to 7 were used for all experiments. B, HFLS-RA were incubated and treated with baicalin (10, 20, 30 μM), respectively for 24h in 6-well plates. Culture supernatants were harvested from each well, and the levels of TNF-α and IL-1β were determined by ELISA. Data are expressed as mean ± SD. *P < 0.05, **P < 0.01 compared with untreated control group.

Fig. 5. Effects of baicalin on NF-κB singaling 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 of rats were separated by SDS-PAGE as described in materials and methods, protein was subjected to western blot analysis for NF-κB p65, phospho-NF-κB p65 (Ser536) using specific antibodies. B,C, Quantitation of western blots of NF-κB p65 and phospho-NF-κB p65 (Ser536) in synovium of rats from different groups. D, HFLS-RA were incubated and treated with baicalin (10, 20, 30 μM), respectively for 24h in 6-well plates. Protein was subjected to western blot analysis for NF-κB p65, phospho-NF-κB p65 (Ser536). E, F, Quantitation of western blots of NF-κB p65 and phospho-NF-κB p65 (Ser536) in HFLS-RA from different groups. Data are expressed as mean ± SD. *P < 0.05 compared with the control group; β-actin was used as the loading control.

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 of rats were separated by SDS-PAGE as described in materials and methods, protein sample was subjected to western blot analysis for acetyl-NF-κB p65 (Lys310), and sirt1 using specific antibodies. B, C, Quantitation of western blots of acetyl-NF-κB p65 (Lys310), and sirt1 in synovium of rats from different groups. D, HFLS-RA were incubated and treated with baicalin (10, 20, 30 μM), respectively for 24h in 6-well plates. Protein sample was subjected to western blot analysis for acetyl-NF-κB p65 (Lys310), and sirt1. E, F, Quantitation of western blots of acetyl-NF-κB p65 (Lys310), and sirt1 in HFLS-RA from different groups. Data are expressed as mean ± SD. *P < 0.05 compared with the control group; β-actin was used as the loading control.
Figure 1

A. Chemical structure of the compound.

B. Images of animal paws with varying degrees of inflammation:
   - a: Naive
   - b: Vehicle
   - c: MTX
   - d: Baicalin 50mg/kg
   - e: Baicalin 100mg/kg
   - f: Baicalin 200mg/kg

C. Graph showing body weight over days:
   - Naive
   - Vehicle
   - MTX
   - Baicalin 50mg/kg
   - Baicalin 100mg/kg
   - Baicalin 200mg/kg

D. Arthritic score over days:
   - Naive
   - Vehicle
   - MTX
   - Baicalin 50mg/kg
   - Baicalin 100mg/kg
   - Baicalin 200mg/kg

E. Hindpaw thickness over days:
   - Naive
   - Vehicle
   - MTX
   - Baicalin 50mg/kg
   - Baicalin 100mg/kg
   - Baicalin 200mg/kg
Figure 2

A

<table>
<thead>
<tr>
<th></th>
<th>Naive</th>
<th>Vehicle</th>
<th>MTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
</tr>
<tr>
<td>b</td>
<td><img src="image4" alt="Image" /></td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
</tr>
<tr>
<td>c</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
<td><img src="image9" alt="Image" /></td>
</tr>
<tr>
<td>d</td>
<td><img src="image10" alt="Image" /></td>
<td><img src="image11" alt="Image" /></td>
<td><img src="image12" alt="Image" /></td>
</tr>
<tr>
<td>e</td>
<td><img src="image13" alt="Image" /></td>
<td><img src="image14" alt="Image" /></td>
<td><img src="image15" alt="Image" /></td>
</tr>
<tr>
<td>f</td>
<td><img src="image16" alt="Image" /></td>
<td><img src="image17" alt="Image" /></td>
<td><img src="image18" alt="Image" /></td>
</tr>
</tbody>
</table>

Ba 50 mg/kg  Ba 100 mg/kg  Ba 200 mg/kg

B

![Bar chart](chart.png)

- Vehicle
- MTX
- Baicalin 50 mg/kg
- Baicalin 100 mg/kg
- Baicalin 200 mg/kg

Radiological score

**P < 0.01**
Figure 4

A

**Serum**

- TNF-α (pg/ml) vs. Baicalin (mg/kg)

<table>
<thead>
<tr>
<th></th>
<th>Naive</th>
<th>Vehicle</th>
<th>MTX</th>
<th>50</th>
<th>100</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td></td>
<td></td>
<td></td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>400</th>
<th>800</th>
<th>1200</th>
<th>1600</th>
</tr>
</thead>
</table>

IL-1β (pg/ml) vs. Baicalin (mg/kg)

<table>
<thead>
<tr>
<th></th>
<th>Naive</th>
<th>Vehicle</th>
<th>MTX</th>
<th>50</th>
<th>100</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td></td>
<td></td>
<td></td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>100</th>
<th>200</th>
<th>300</th>
<th>400</th>
</tr>
</thead>
</table>

B

**HFLS-RA culture supernatants**

- TNF-α (pg/ml) vs. Baicalin (μM)

<table>
<thead>
<tr>
<th></th>
<th>-</th>
<th>10</th>
<th>20</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td></td>
<td>***</td>
<td>**</td>
<td></td>
</tr>
</tbody>
</table>

|        |        |        |       |       |
|--------|--------|-------|-------|

- IL-1β (pg/ml) vs. Baicalin (μM)

<table>
<thead>
<tr>
<th></th>
<th>-</th>
<th>10</th>
<th>20</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td></td>
<td>*</td>
<td>**</td>
<td>**</td>
</tr>
</tbody>
</table>

|        |        |        |       |       |
|--------|--------|-------|-------|

**Notes:**

- **:** p < 0.01 compared to Naive group
- *: p < 0.05 compared to Naive group