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ORIGINAL ARTICLE

Baicalein Inhibits the IL-1β-Induced Inflammatory Response in Nucleus Pulposus Cells and Attenuates Disc Degeneration *In vivo*

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Abstract— Intervertebral disc degeneration (IDD) is widely considered one of the main causes of low back pain, which is a chronic progressive disease closely related to inflammation and degeneration of nucleus pulposus (NP) cells. Baicalein is a natural bioactive compound with anti-inflammatory effects in different diseases, including inhibition of the inflammatory response in chondrocytes, whose morphology and avascular supply are similar to those of NP cells. Therefore, we hypothesized that baicalein may have a therapeutic effect on IDD by suppressing the inflammatory response. *In vitro*, NP cells were pretreated with baicalein for 2 h and then incubated with IL-1β for 24 h. We found that baicalein not only inhibited the overexpression of inflammatory cytokine production, including NO, PGE2, TNF-α, and IL-6, but also suppressed the expression of COX-2 and iNOS. The IL-1β-induced overexpression of MMP13 and ADAMTS5 and degradation of aggrecan and type II collagen were reversed by baicalein in a dose-dependent manner. Mechanistically, we found that baicalein suppressed the IL-1β-induced activation of the NF-κB and MAPK pathways. Moreover, an *in vivo* study demonstrated that baicalein treatment could ameliorate IDD in a puncture-induced rat model. Thus, baicalein has great value as a potential therapeutic agent for IDD.

KEY WORDS: intervertebral disc degeneration; baicalein; inflammation; NF-κB; MAPK.

INTRODUCTION

Low back pain (LBP) is a global musculoskeletal disorder that severely affects human health and causes an enormous economic burden on society [1]. The major contributor to LBP is thought to be associated with intervertebral disc degeneration (IDD), which is implicated in more than half of LBP cases [2]. Genetic inheritance, age, inadequate metabolite transport, abnormal mechanical stress, and acute physical trauma are considered to be closely related to the pathogenesis of IDD [3]. In addition, the process of degeneration was found to first occur in the nucleus pulposus (NP) region, which is mainly composed

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of NP cells and is rich in matrix macromolecules (aggrecan and type II collagen) [4]. Although the causes of IDD are multifactorial, and a clear understanding of the pathophysiology and pathogenesis of the condition is still lacking, inflammation and the inflammatory response have been regarded as critical factors in the development of IDD. This inflammatory response has been shown to directly trigger the catabolic activities of NP cells and ultimately cause an imbalance of extracellular matrix (ECM) metabolism [5, 6]. The proinflammatory cytokine interleukin (IL)-1β has been considered to be the most important inflammatory cytokine due to its potent induction of the expression of inflammatory mediators and catabolic factors, including prostaglandin E2 (PGE2), nitric oxide (NO), a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5), and matrix metalloproteinase (MMP) 13, which results in loss of the ECM in NP cells [7, 8]. Therefore, inhibition of IL-1β or IL-1β-induced inflammation may attenuate the progression of IDD.

The nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways have been identified as master regulators of the IL-1β-mediated regulation of the inflammatory response and catabolism [9]. Once IL-1β binds to its receptor, the signal transduction cascades in the NF-κB and MAPK pathways are triggered, which leads to the activation of transcription factors and the subsequent induction of genes whose products mediate inflammation and ECM metabolism. Recently, growing evidence has shown that blocking these pathways is an effective approach for the treatment of IDD [9].

In recent years, compounds derived from natural products have become increasingly popular and have shown promising effects for the treatment of degenerative disease with fewer side effects. Baicalein is a flavonoid compound that is extracted from the plant *Scutellaria baicalensis* Georgi and has a long history in traditional Chinese medicine [10]. Baicalein has been reported to possess multiple pharmacological activities, including antioxidant [11], antimicrobial [12], and anti-inflammatory [13] effects. Previous studies reported that baicalein significantly suppressed LPS-stimulated inflammation by blocking the NF-κB pathway in cow mammary epithelial cells [14] and HBE16 airway epithelial cells [15]. Baicalein could also exert anti-neuroinflammatory effects by downregulating the MAPK and NF-κB signaling pathways to protect against rotenone-induced brain injury in rats [16]. Moreover, several studies showed that baicalein reduced the expression of catabolic factors in IL-1β-stimulated chondrocytes and ameliorated cartilage damage in a rabbit osteoarthritis model [17–19]. Interestingly, the morphology and avascular supply of NP cells are considered to be similar to those of chondrocytes, and we hypothesized that baicalein may possess the ability to attenuate the progression of IDD by protecting NP cells. Therefore, in our study, we applied IL-1β to induce inflammation in NP cells and investigated the anti-inflammatory effects of baicalein, as well as the underlying mechanism of these effects. Furthermore, the protective role of baicalein against IDD was examined in a puncture-induced rat model.

MATERIALS AND METHODS

Ethics Statement

This study was approved by the Committee of Wenzhou Medical University, and all surgical interventions, treatments, and postoperative animal care procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Chemicals

Baicalein (purity > 98%) was purchased from Nantong Feiyu Biological Technology Co., Ltd. (Nantong, China). Baicalein was dissolved in DMSO as a 100 mM stock solution and stored at − 20 °C. Further dilution was performed in cell culture medium. Primary antibodies specific for p65, IκBα, JNK, p-JNK, ERK, p-ERK, p38, and p-p38 were obtained from Cell Signaling Technologies (Beverly, MA, USA). Antibodies specific for GAPDH, INOS, COX-2, TNF-α, aggrecan, type II collagen, MMP-13, and ADAMTS-5, and fluorescein isothiocyanate-labeled and horseradish peroxidase-labeled secondary antibodies were purchased from Abcam (Cambridge, UK). The 4′, 6-diamidino-2-phenylindole (DAPI) stain was obtained from Beyotime (Shanghai, China). Recombinant human IL-1β was purchased from PeproTech (NJ, USA). Dimethylsulfoxide (DMSO) and collagenase II were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Cell culture reagents were purchased from Gibco (Grand Island, NY, USA). Griess reagent was purchased from the Beyotime Institute of Biotechnology (Shanghai, China). Enzyme-linked immunosorbent assay (ELISA) kits for PGE2 were purchased from R&D systems (Minneapolis, MN, USA).

NP Cell Culture

Gel-like NP tissue was removed from lumbar discs of male Sprague-Dawley rats that were less than 3 weeks old and had been sacrificed with an overdose of sodium pentobarbital. The NP tissue was then cut into $0.5 \times 0.5 \times 0.5$ mm^3 pieces and treated with 0.2% collagenase II for 4 h at 37 °C. This cell suspension was centrifuged at 1000 rpm for 3 min to collect the NP cells. The extracted NP cells were cultured in 75 cm^2 culture flasks with DMEM (supplemented with 10% FBS and a 1% antibiotic mixture of penicillin and streptomycin) and incubated in an atmosphere of 95% air and 5% CO_2 at 37 °C. The culture medium was changed every 2–3 days, and the cells were passaged using a 0.25% trypsin-EDTA solution when they reached 80–90% confluence. NP cells from passages two to four were used in this study.

Cell Viability Analysis

The cytotoxic effects of baicalein on NP cells were assayed by cell counting kit-8 (CCK-8; Dojindo Co., Kumamoto, Japan) according to the manufacturer's protocol. Briefly, second-passage NP cells were plated in 96-well plates (5×10^3 cells per well) for 24 h and then treated with various concentrations (5, 25, and 50 μM) of baicalein for an additional 24 or 48 h. At the indicated times, the cells were washed with phosphate-buffered saline (PBS), and 100 μl of DMEM containing 10 μl of CCK-8 solution was added to each well, followed by incubation at 37 °C for 2 h. Then, the optical density was measured at a wavelength of 450 nm with a plate reader spectrophotometer (Thermo Fisher).

Griess Reaction and ELISA

NP cells were seeded in six-well plates at a density of 4×10^5 cells per well and incubated with baicalein (5, 25, and 50 μM) for 2 h prior to IL-1β (10 ng/mL) stimulation for 24 h. The NO concentration in the culture medium was detected by the Griess reaction. In brief, 100 μL of the culture medium supernatant was mixed with the same amount of Griess reagent, followed by incubation at room temperature for 10 min, and the absorbance at 450 nm was then read using a plate reader spectrophotometer (Thermo Fisher). The PGE2 concentration in the supernatant from each sample was determined using a commercial ELISA kit (R&D Systems) according to the manufacturer's instructions.

Real-Time PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to quantify the mRNA expression of COX2 and iNOS. Total RNA was isolated using TRIzol reagent (Invitrogen) from NP cells that were treated as described above, and the concentration was determined spectrophotometrically at 260 nm (Thermo Scientific NanoDrop 2000). One microgram of total RNA was reverse transcribed to synthesize cDNA (MBI Fermentas, Germany). Then, a 10-μl total reaction volume was used for PCR amplification, including 5 μl of 2× SYBR Master Mix, 0.25 μl of each primer, and 4.5 μl of diluted cDNA. The reaction and detection were conducted in a CFX96 Real-Time PCR System (Bio-Rad Laboratories, California, USA). The cycle threshold (Ct) values were recorded and normalized based on the level of GAPDH. Data were analyzed using the $2^{-\Delta\Delta CT}$ method. The specific primers used are listed in Table 1.

Western Blot Analysis

Western blotting was performed using routine protocols. Treated NP cells were isolated using radioimmunoprecipitation assay (RIPA) buffer with 1 mM phenylmethanesulfonyl fluoride (PMSF), and the protein concentration was then measured using a BCA protein assay kit (Beyotime). Nuclear and cytoplasmic proteins were lysed using the Nuclear and Cytoplasmic Extraction Kit from Pierce (Mountain View, CA, USA) following the manufacturer's protocol. Protein aliquots (20 μg) were separated on a 12% sodium dodecyl sulfate polyacrylamide gel, transferred to a nitrocellulose membrane (Life Technologies, Gaithersburg, MD, USA), and then blocked. The membranes were incubated with primary antibodies specific for COX-2, iNOS, aggrecan, type II collagen, MMP-13, ADAMTS-5, IκBα, p65, p-ERK/ERK, p-p38/p38, p-JNK/JNK, GAPDH, and lamin B1 overnight at 4 °C, followed by subsequent incubation with the respective secondary antibodies for 2 h at room temperature. The bands were detected with the electrochemiluminescence plus reagent (Invitrogen). Finally, the signals were visualized using the ChemiDoc™ XRS Imaging System (Bio-Rad).

Immunofluorescence

NP cells were plated on slides in a six-well plate at a density of 4×10^5 per well and incubated for 24 h. The cells were pretreated with or without baicalein (50 μM) for 2 h and then stimulated with IL-1β (10 ng/ml) for 24 h. Paraformaldehyde (4%) was used to fix the cells for 15 min at

Table 1. Primer Sequences Used in qRT-PCR

Gene	Forward primer	Reverse primer
iNOS	5′-TGGGTGAAAGCGGTGTTCTT-3′	5′-TAGCGCTTCCGACTTCCTTG-3′
COX-2	5′-TCCATTTGTGAAGATTCCTGTGTTG-3′	5′-TCTCACTGGCTTATGCCGAAA-3′
TNF-α	5′-ACCACGCTCTTCTGTCTACTG-3′	5′-CTTGGTGGTTTGCTACGAC-3′
IL-6	5′-GACTTCCAGCCAGTTGCCTT-3′	5′-GCAGTGGCTGTCAACAACAT-3′
GAPDH	5′-AGACAGCCGCATCTTCTTGT-3′	5′-CTTGCCGTGGGTAGAGTCAT-3′

room temperature. After washing with PBS three times, 0.25% Triton X-100 was used to permeabilize the cells for 5 min. After blocking with 10% FBS, the cells were incubated with a primary antibody specific for p65 (1:200) overnight at 4 °C. The next day, the cells were washed with PBS, incubated with a fluorescein-conjugated goat anti-rabbit IgG antibody (diluted 1:400) for 1 h and subsequently labeled with DAPI (Invitrogen) for 1 min. Finally, three fields from each slide were chosen randomly for microscopic observation with a fluorescence microscope (Olympus Inc., Tokyo, Japan).

Surgical Procedure

Thirty-six 3-month-old male Sprague-Dawley rats were purchased from the Animal Center of the Chinese Academy of Sciences in Shanghai, China and were randomly divided into three groups (the control group, saline group, and baicalein group). All rats were anesthetized intraperitoneally with 2% (*w*/*v*) pentobarbital (40 mg/kg) and monitored by an assistant during the surgery. The saline and baicalein groups underwent the surgery to generate the IDD model. As described previously, the experimental intervertebral space (Co7/8) was located by digital palpation of the coccygeal vertebrae and was confirmed by counting the vertebrae from the sacral region in a trial radiograph. To label the experimental discs, a ring was made using a permanent marker on the skin corresponding to the sixth and ninth vertebrae. Needles (20 G, approximately 4 mm in length) were carefully inserted into the center of the disc level, perpendicular to the skin, to puncture the entire annulus fibrosus (AF) layer. All needles were rotated 360° and held in position for 30 s before extraction. Baicalein was dissolved in DMSO at a concentration of 15 mg/ml and then diluted with normal saline. After surgery, the baicalein solution was injected intraperitoneally once daily at a concentration of 20 mg/kg/day from the day of injury until the mice were euthanized. The mice in the saline group were administered an equivalent volume of saline. The rats were fed *ad libitum* and maintained under a constant temperature of 20 ± 2 °C, with a relative humidity of 50 ± 10%.

Magnetic Resonance Imaging (MRI) Assessment

MRI was evaluated at 2 time points: 4 and 8 weeks after the operation. Rats were anesthetized as described above, and the tails were straightened in a 3.0-T MRI scanner (Philips Intera Achieva 3.0 MR) to acquire images. T2-weighted sections in the sagittal plane were obtained using the device settings, as previously described. MRI interpretation was performed by a spine surgeon who was blinded to the group designation, and the follow-up time was indicated based on the Pfirrmann classification [20] (1 point = grade I; 2 points = grade II; 3 points = grade III; 4 points = grade IV; and 5 points = grade V).

Histopathologic Analysis

The rats were euthanized under pentobarbital sodium anesthesia, and discs with the adjacent vertebrae were harvested for histological evaluation. The specimens were fixed in 4% paraformaldehyde for 24 h at 4 °C and decalcified in 10% EDTA solution at 4 °C for 4 weeks. Then, gross specimens were paraffin-embedded and sectioned to a 5-mm thickness with a microtome. Slides of each disc were stained with hematoxylin and eosin (H&E). Images were captured with an optical microscope. Then, the cellularity and morphology of the NP and the AF in the sections were analyzed based on previously published grading scales [21].

Immunohistochemical Examination

The paraffin-embedded tissue sections were deparaffinized by heat, immersed in xylene, rehydrated, and washed in distilled water. Then, the endogenous peroxidase activity was blocked by placing the sections in 3% hydrogen peroxide for 10 min, and 10% normal goat serum was used to block nonspecific binding sites for 30 min at room temperature. The sections were then incubated with the primary antibody (anti-TNF-α, 1:200) overnight at

4 °C. After washing, an appropriate HRP-labeled secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA) was applied for 1 h at room temperature and further developed with diaminobenzidine (DAB) solution. Subsequently, all sections were visualized with an optical microscope. Images were captured using ImageJ software (NIH, Bethesda, USA).

Statistical Analysis

The experiments were performed at least three times. The data obtained are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism Version 5.0 software (GraphPad Software, San Diego, CA, USA). Intergroup comparisons were performed using one-way ANOVA followed by Tukey's test. Probability values of $P < 0.05$ were considered statistically significant.

RESULTS

Cytotoxic Effects of Baicalein on NP Cells

The structure of baicalein is shown in Fig. 1. To determine the cytotoxic effects of baicalein on NP cells, a CCK8 assay was performed after the cells were cultured between 24 and 48 h with complete medium in the presence of 1, 5, 25, or 50 μM baicalein. The cell cytotoxicity was calculated as a percentage of the control group. Baicalein did not affect cell proliferation and showed no obvious cytotoxic effects on NP cells after 24 or 48 h of baicalein treatment, even when the cells were exposed to 50 μM baicalein (Fig. 1). Consequently, 5, 25, or 50 μM baicalein was used in subsequent experiments.

Effects of Baicalein on iNOS, COX-2, TNF-α, IL-6, PGE2 and NO Expression in IL-1β-Stimulated NP Cells

RT-PCR was used to detect the effects of baicalein on iNOS, COX-2, TNF-α, and IL-6 production at the mRNA level. As shown in Fig. 2a, IL-1β stimulation significantly upregulated the mRNA expression of iNOS, COX-2, TNF-α, and IL-6 compared to that in the control group, and baicalein treatment reversed this upregulation in a dose-dependent manner. In addition, western blot analysis was used to determine the effect of baicalein on iNOS and COX-2 production at the protein level (Fig. 2b, c). Moreover, IL-1β-induced upregulation of endogenous NO and PGE2 production was attenuated by baicalein treatment (Fig. 2d). These data suggest that the enhanced expression of inflammatory mediators and cytokines induced by IL-1β could be suppressed by baicalein pretreatment in a dose-dependent manner.

Effects of Baicalein on IL-1β-Induced ECM Degradation

The synthesis of catabolic factors has been identified as a key player in IDD pathogenesis, which contributes to ECM degradation. The western blot results showed that NP cells exhibited a noticeable upregulation of catabolic factors, including MMP-13 and ADAMTS-5, after IL-1β treatment. In addition, the protein levels of aggrecan and type II collagen, which are considered the main components of the ECM, were decreased by IL-1β stimulation. However, all of these alterations induced by IL-1β were reversed by pretreatment with baicalein (Fig. 3).

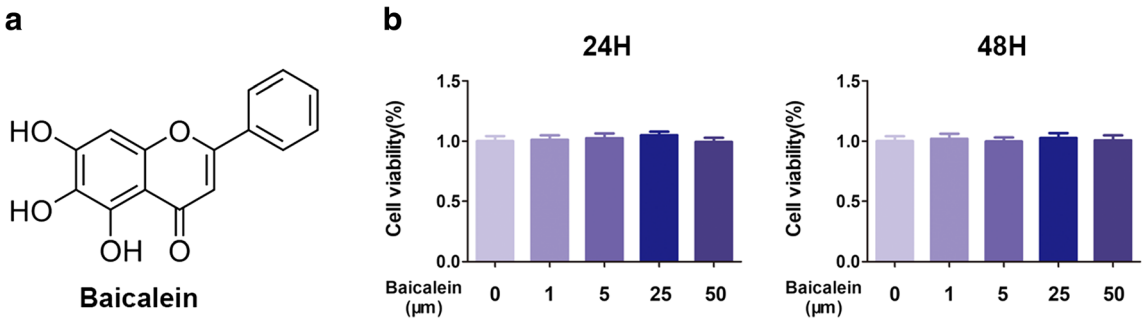

Fig. 1. Cytotoxic effects of baicalein on NP cells. **a** Chemical structure of baicalein. **b** Effects of the indicated concentrations of baicalein on the viability of NP cells at 24 and 48 h, as measured by a CCK8 assay. Data are presented as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ relative to the control group. $n = 3$.

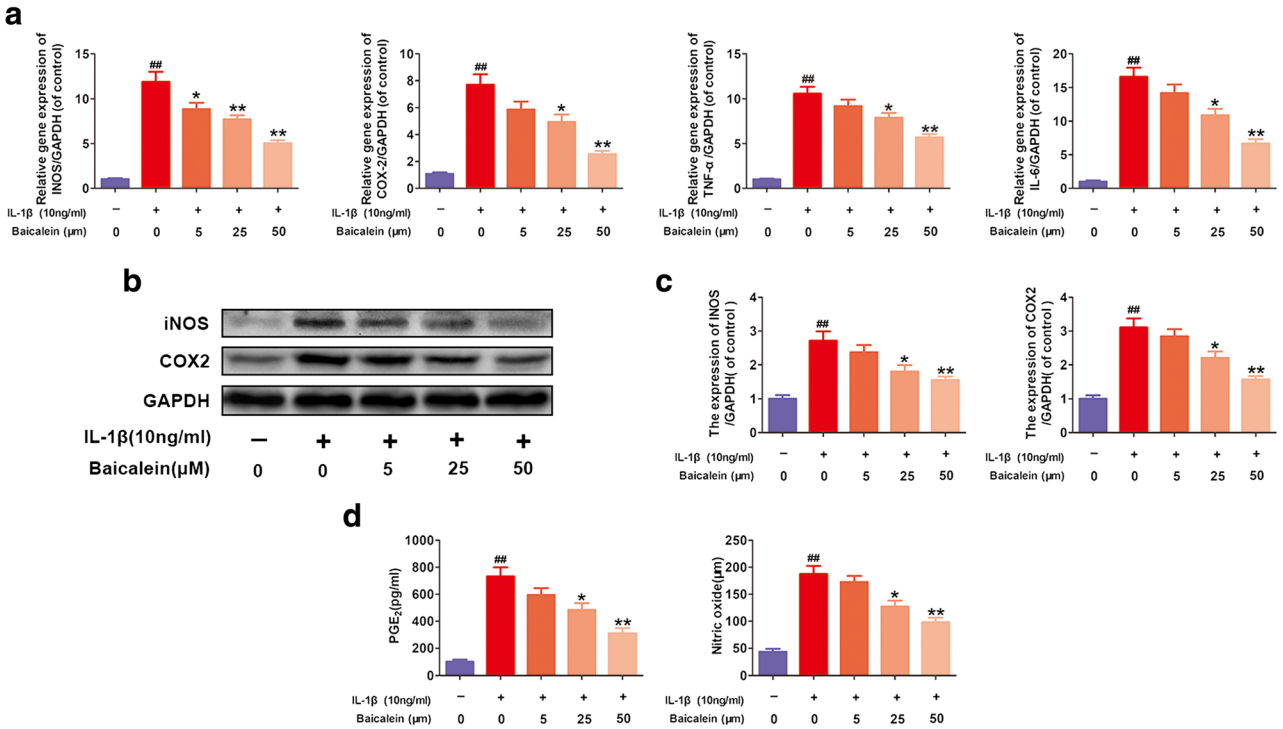

Fig. 2. Baicalein inhibits inflammatory effects in NP cells. **a** Real-time PCR analysis was performed to detect the expression of the inflammatory genes iNOS, COX-2, TNF-α, and IL-6. The expression levels of these genes were normalized to the expression of GAPDH. **b** Representative images of western blots demonstrating the effects of baicalein on iNOS and COX-2 expression induced by IL-1β. Cell extracts were prepared from NP cells pretreated with different concentrations of baicalein (5, 25, and 50 μM) for 2 h and subsequently stimulated with IL-1β for the indicated times. **c** The intensity ratios of iNOS and COX-2 relative to GAPDH. **d** The effects of baicalein on IL-1β-induced PGE2 and NO production in NP cells. Data are presented as the mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ relative to the control group. $^{*}P < 0.05$, $^{**}P < 0.01$ relative to the IL-1β-stimulated group. $n = 3$.

Effects of Baicalein on IL-1β-Induced NF-κB Activation

To further investigate the anti-inflammatory mechanism underlying the inhibitory effect of baicalein, the effects of baicalein on IL-1β-induced NF-κB translocation from the cytosol to the nucleus and IκBα degradation were detected by western blotting and immunofluorescence staining. After 2 h of IL-1β stimulation, the protein level of IκBα in the cytoplasm dramatically decreased, while the expression of p65 in the nucleus significantly increased. Baicalein treatment attenuated all of these inflammatory effects (Fig. 4a, b). Furthermore, immunofluorescence staining showed that p65 was mostly localized to the cytoplasm in unstimulated cells, while there was intense staining in the nucleus after IL-1β stimulation, indicating nuclear translocation of NF-κB. However, baicalein pretreatment significantly inhibited the translocation of the p65 subunit into the nucleus (Fig. 4c). These findings suggest that baicalein could suppress the activation of the NF-κB signaling pathway in IL-1β-stimulated NP cells.

Effects of Baicalein on IL-1β-Induced MAPK Activation in NP Cells

Because IL-1β activates MAPK signaling, we first confirmed the IL-1β-induced activation of MAPKs in NP cells. The results showed that the phosphorylation levels of JNK, p38, and ERK were upregulated after 2 h of IL-1β stimulation. However, baicalein inhibited the IL-1β-induced phosphorylation of JNK, p38, and ERK (Fig. 5).

Baicalein Ameliorates Rat IDD *In vivo*

To investigate the protective effect of baicalein on the progression of IDD *in vivo*, MRI and histologic analysis data were obtained 4 and 8 weeks after puncture in an IDD model. The T2-weighted signal intensities in the baicalein-treated group were stronger than those in the saline group, especially at 8 weeks postsurgery (Fig. 6a). Moreover, the degree of disc degeneration was determined by Pfirrmann MRI grade scores. As shown in Fig. 6b, the Pfirrmann grade scores of the baicalein-treated rats were markedly

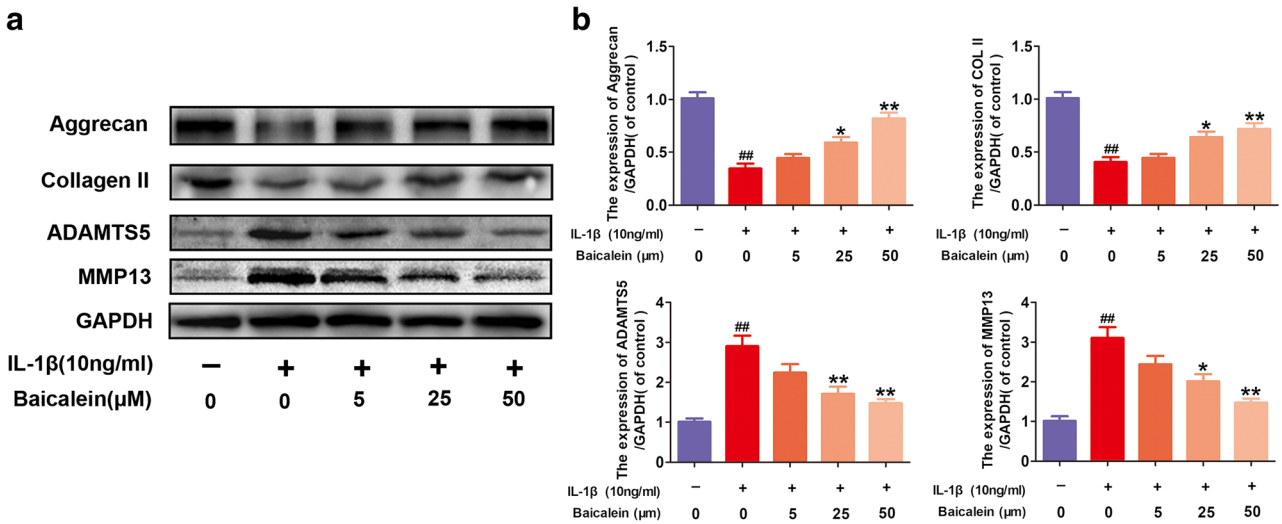

Fig. 3. Effects of baicalein on IL-1β-induced ECM degradation. **a** Representative images of western blots demonstrating the protein expression of aggrecan, type II collagen, ADAMTS-5, and MMP-13 after treatment as described in Fig. 2. **b** The intensity ratios of aggrecan, type II collagen, ADAMTS-5, and MMP-13 relative to GAPDH. Data are presented as the mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ relative to the control group. $^{*}P < 0.05$, $^{**}P < 0.01$ relative to the IL-1β-stimulated group. $n = 3$.

lower than those of the saline group, especially at 8 weeks. In addition, Fig. 6c shows representative histologic sections (H&E staining) of discs in the control group, saline group and baicalein group, which show that baicalein treatment significantly alleviated the decrease in NP tissue and the destruction of the disc structure compared to those in the saline group. In addition, the histologic scores of the baicalein group were significantly lower than those of the saline group at both week 4 and week 8 (Fig. 6d). To examine the effect of baicalein on inflammation *in vivo*, immunohistochemical staining for TNF-α was performed. The results showed that baicalein attenuated the expression of TNF-α in rat disc tissue, which was consistent with the *in vitro* results (Fig. 6e).

DISCUSSION

The intervertebral disc is an elegant structure with a gelatinous inner core (NP) that functions as a shock absorber and converts axial loads into radial forces. In the process of IDD, the balance of ECM metabolism becomes disturbed, resulting in the loss of collagen and proteoglycans (mainly aggrecan) and decreased water content in the NP [22]. The degraded ECM causes the inability to maintain intervertebral disc hydration and load absorption, which leads to inflammation in the intervertebral disc [23]. It has been reported that inflammatory cytokines can increase the expression of matrix-degrading proteases while decreasing the expression levels of matrix genes [24]. These expression changes create a vicious cycle of degeneration and further accelerate the progression of IDD. Conventional treatments, such as nonsteroidal anti-inflammatory drugs and acetaminophen, are clinically used for IDD treatment. These agents modulate inflammation and provide effective temporary relief of back pain, but they do not alter the progression of IDD, and they also result in many side effects [25, 26]. Recently, plant-derived compounds have received increased interest for the treatment of IDD because they exert anti-inflammatory effects with few side effects [27, 28]. Baicalein, the key bioactive component isolated from the root of *Scutellaria baicalensis* Georgi, has been shown to have potent anti-inflammatory activity [14, 15]. In this study, we demonstrated the inhibitory effect of baicalein on the inflammatory response in IL-1β-stimulated NP cells, as well as the underlying mechanism of inflammation (Fig. 7). Furthermore, baicalein treatment clearly prevented the progression of IDD in a puncture-induced animal model.

IL-1β is an important inflammatory cytokine in the IL-1 family that exerts strong proinflammatory effects by stimulating the production of multiple proinflammatory mediators [29, 30]. An increasing number of studies have reported that the expression of IL-1β is significantly increased in degenerative intervertebral disc tissues and cells, which suggests that IL-1β plays a critical role in the

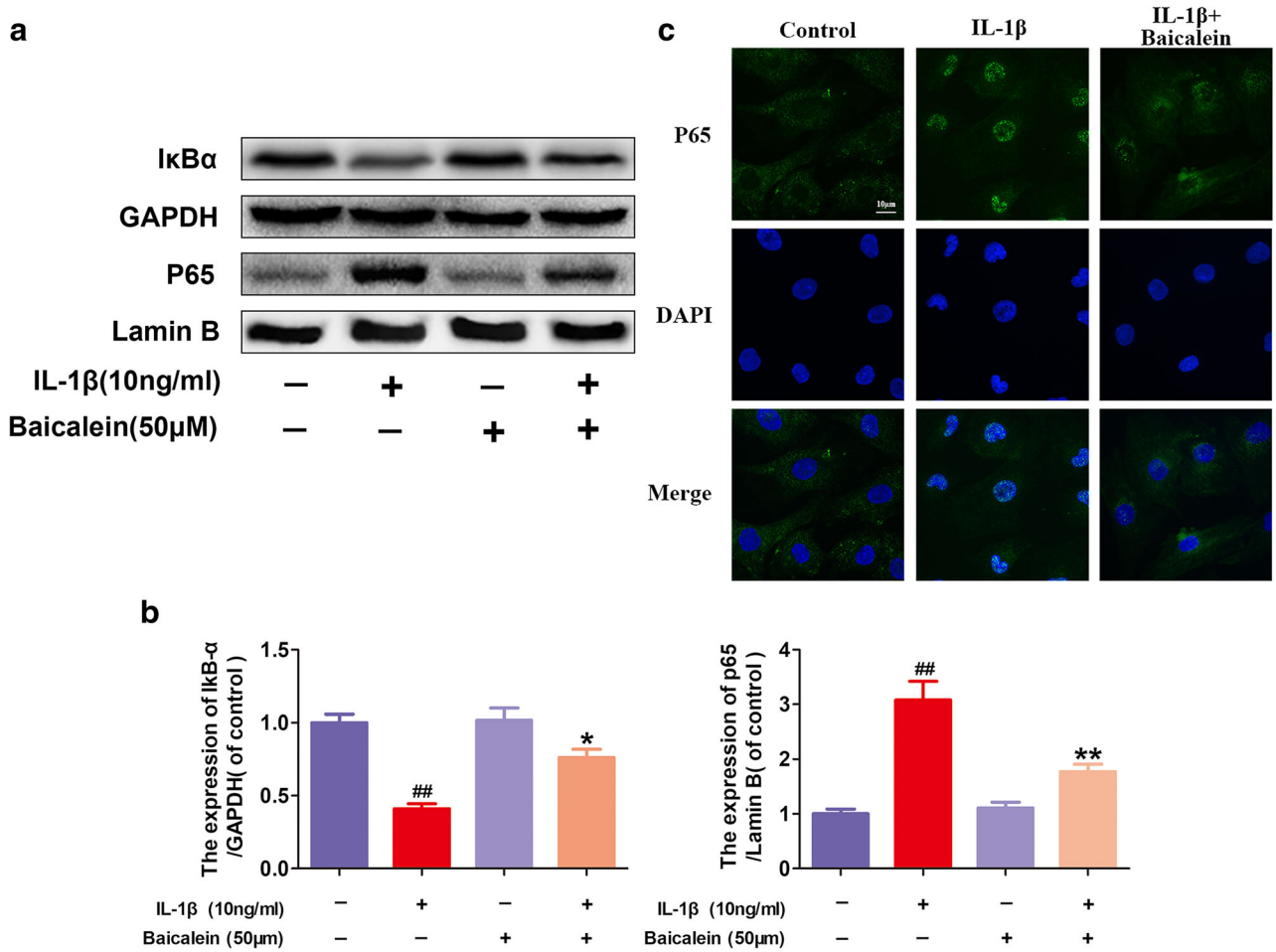

Fig. 4. Baicalein suppresses IL-1β-induced NF-κB signaling pathway activation. **a** Representative western blot images of the protein expression of IκBα in the cytoplasm and p65 in the nucleus in NP cells. Cell extracts were prepared from NP cells pretreated with baicalein (50 μM) for 2 h and subsequently stimulated with IL-1β for 2 h. **b** The intensity ratios of IκBα relative to GAPDH and p65 relative to lamin B1. **c** The nuclear translocation of p65 was detected by immunofluorescence combined with DAPI staining for nuclei. Scale bar, 10 μm. Data are presented as the mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ relative to the control group. $^{*}P < 0.05$, $^{**}P < 0.01$ relative to the IL-1β-stimulated group. $n = 3$.

progression of IDD [30]. IL-1β exerts its inflammatory effects in part by regulating the transcription of the inducible nitric oxide synthase (iNOS) gene, causing the production of high concentrations of NO [31]. NO stimulates the secretion and activation of MMPs and inhibits the synthesis of collagen and proteoglycan, resulting in ECM degradation [32]. PGE2 is another proinflammatory mediator that is synthesized in response to IL-1β stimulation; PGE2 is generated from arachidonic acid by the PGE2 synthetic enzyme COX2 and can exert many pathological effects, such as suppressing cell proliferation and inhibiting ECM synthesis [33]. Both of these effects were reported to be significantly increased with an increasing grade of degeneration, and inhibition of these proinflammatory mediators could exert protective effects during the process of IDD [34]. Collagens and proteoglycans are the main ECM components within the discs. Many studies have shown that MMPs and ADAMTSs disrupt the balance between ECM anabolism and catabolism in IDD [35, 36]. MMP-13 is a vital member of the MMP family with a strong ability to degrade type II collagen, while ADAMTS5, a primary aggrecanase of the ADAMTS family, possesses the highest specific activity for aggrecan cleavage [22, 37]. Thus, MMP13 and ADAMTS5 could be efficiently targeted to develop therapeutics for preventing and reversing IDD. In this study, we investigated the anti-inflammatory effects of baicalein in IL-1β-stimulated NP cells. We observed that after IL-1β

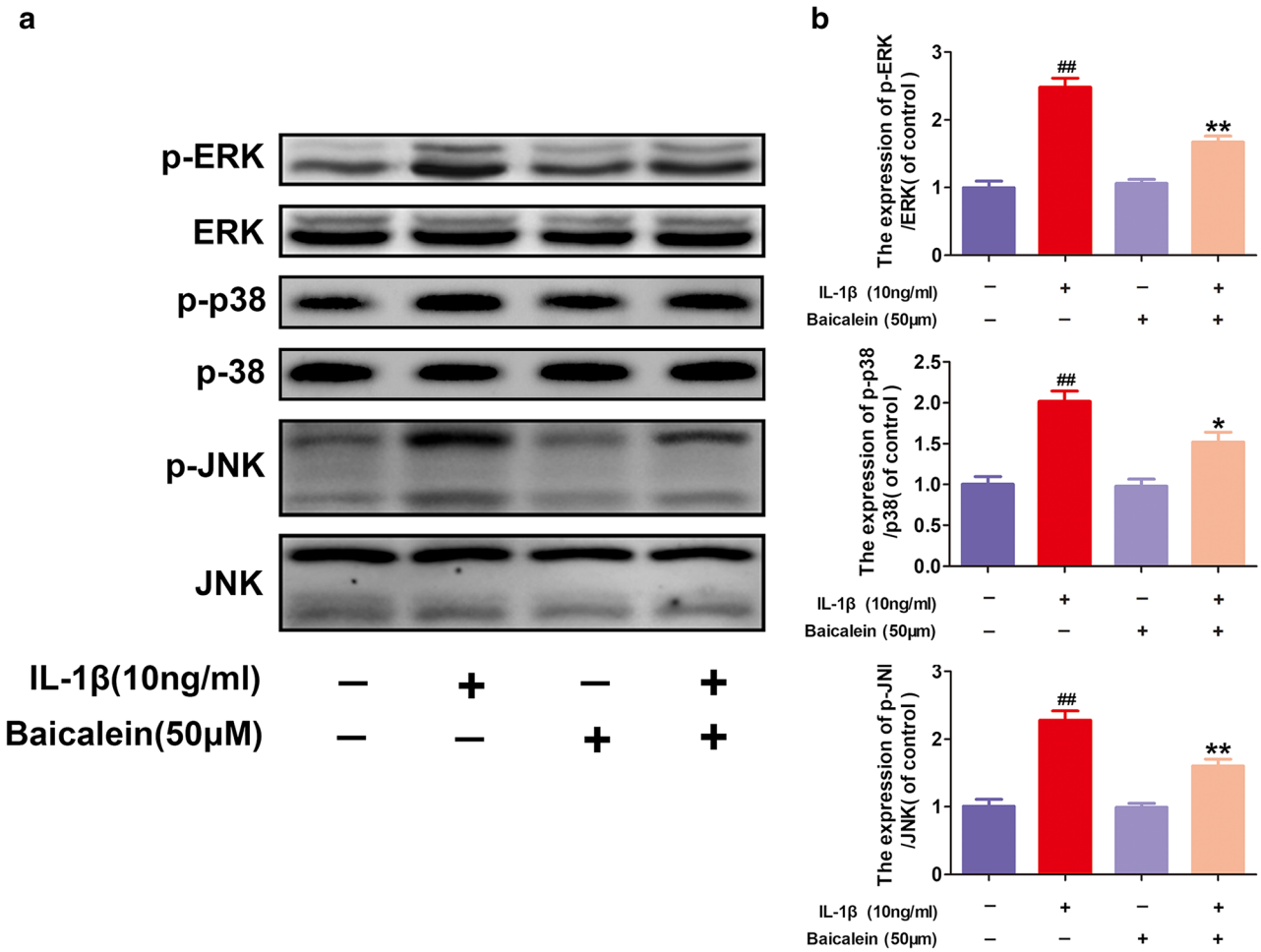

Fig. 5. Effects of baicalein on IL-1β-induced MAPK activation in NP cells. **a** Representative western blot images showing the effect of baicalein on the phosphorylation of ERK, JNK, and p38 induced by IL-1β. Cell extracts were prepared from NP cells pretreated with baicalein (50 μM) for 2 h and subsequently stimulated with IL-1β for 2 h. **b** The intensity ratios of p-ERK relative to total ERK, p-JNK relative to total JNK, and p-p38 relative to total p38. Data are presented as the mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ relative to the control group. $^{*}P < 0.05$, $^{**}P < 0.01$ relative to the IL-1β-stimulated group. $n = 3$.

stimulation in NP cells, baicalein significantly inhibited iNOS and COX-2 expression at the mRNA and protein levels, as well as NO and PGE2 production. In addition, overproduction of both MMP13 and ADAMTS-5 by IL-1β was inhibited by baicalein in a dose-dependent manner, which reversed the subsequent degradation of type II collagen and aggrecan. These data suggest that baicalein could act in a therapeutic manner in patients with IDD by alleviating inflammation.

In response to inflammatory stimuli, intracellular signaling pathways are activated that provide the signals needed to activate the production of inflammatory mediators. Two major intracellular pathways, the NF-κB and MAPK pathways, have been shown to play potentially vital roles in the regulation of the inflammatory response associated with IDD [9]. NF-κB is a protein complex composed of seven transcription factors and is normally located in the cytoplasm bound to its inhibitor IκB. NF-κB activation involves IκBα phosphorylation, which is triggered by IL-1β. Once liberated from IκB, the NF-κB p65 subunit translocates from the cytoplasm to the nucleus, where it induces gene transcription by binding to the promoters of NF-κB-responsive genes, which subsequently causes the upregulation of catabolic enzymes, inflammatory mediators, and cytokines [38, 39]. A previous study reported that baicalein significantly suppressed IL-1β-induced inflammation by blocking the NF-κB pathway in rat articular chondrocytes [18]. Similar results were found

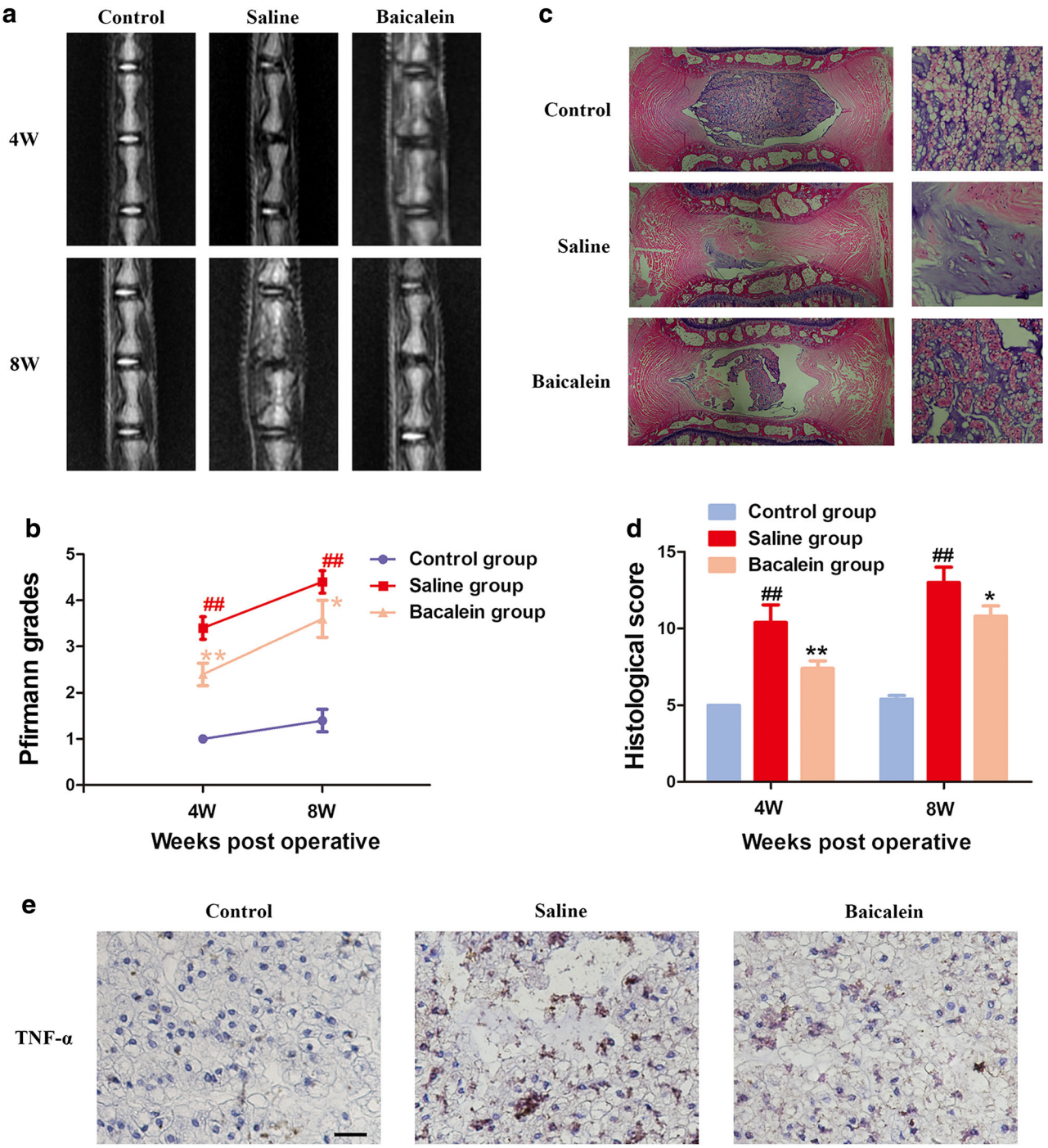

Fig. 6. Baicalein ameliorates the progression of IDD in a puncture-induced rat model *in vivo*. **a** Representative T2-weighted MRI images of rat tails with a needle-punctured disc at 4 and 8 weeks postsurgery from each group. **b** The Pfirrmann MRI grade scores in each group at week 4 and week 8 postsurgery. **c** Representative hematoxylin and eosin staining of disc samples from the different experimental groups at 4 and 8 weeks postsurgery. **d** The histological grades at week 4 and week 8 postsurgery in the three groups. Samples from 36 rats (12 in each group) were used for imaging and histopathologic analysis. **e** Immunohistochemical staining of TNF-α expression in disc samples from the different experimental groups at 4 weeks postsurgery. Scale bar, 50 μm. Data are presented as the mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ relative to the control group. $^{*}P < 0.05$, $^{**}P < 0.01$ relative to the saline group. $n = 6$.

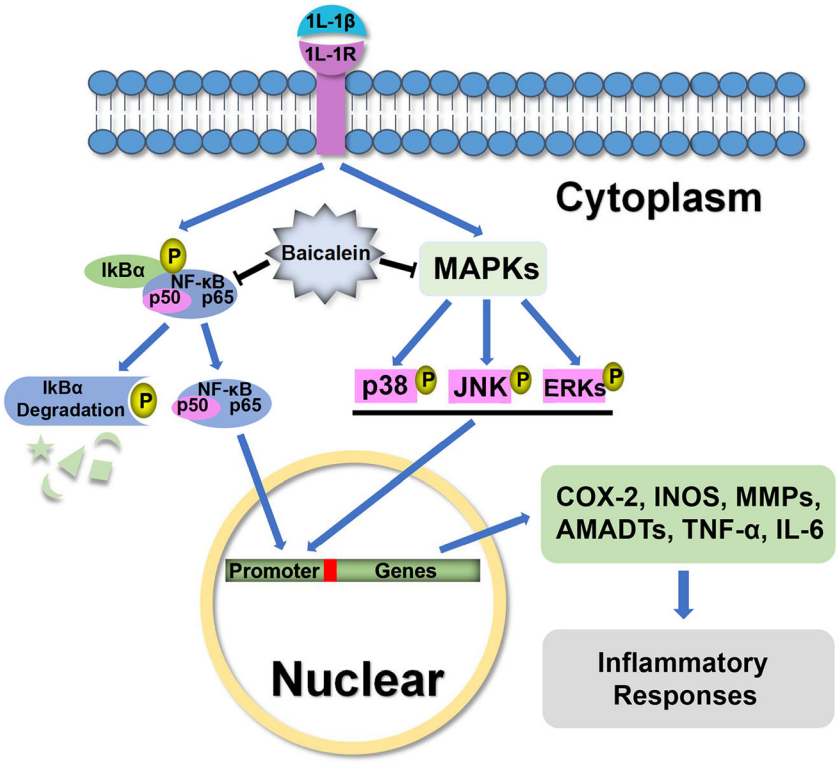

Fig. 7. Schematic model of the signaling mechanisms underlying the anti-inflammatory effects of baicalein in IL-1β-stimulated NP cells.

in our study; baicalein significantly inhibited the phosphorylation of NF-κB p65 and the degradation of IκBα in IL-1β-treated NP cells. MAPK, which belongs to a large family of serine/threonine kinases, has been implicated in the regulation of key cellular processes, including gene induction, cell proliferation, differentiation, and apoptosis, as well as inflammatory responses [40]. The extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinase (JNK), and p38 are the major subfamilies of MAPKs [41]. In recent years, MAPKs have drawn increasing attention in the progression of IDD due to their crucial role in modulating both matrix synthesis and degradation [42, 43]. Seguin et al. [42] demonstrated that the p38 and ERK signaling pathways play a role in proteoglycan metabolism, influencing the production of catabolic enzymes and inflammatory mediators, while treatment with inhibitors of p38 or ERK significantly reversed cytokine-induced ECM degradation. JNK has also been shown to regulate the production of catabolic enzymes, such as MMP1, MMP3, and MMP13 [42, 44]. Interestingly, our study also demonstrated that baicalein pretreatment significantly downregulated the IL-1β-induced phosphorylation of ERK, JNK, and p38. Taken together, these results suggest that the NF-κB and MAPK pathways are involved in the anti-inflammatory effects of baicalein on IDD (Fig. 7).

In this study, a puncture-induced rat IDD model was examined for further investigation of the therapeutic effects of baicalein *in vivo*. Compared to the saline group, the baicalein group had the appearance of stronger T2-weighted signal intensities and less severe destruction of the disc structure, indicating that baicalein could attenuate the progression of IDD *in vivo*. In addition, the increased expression of TNF-α in degenerative discs was ameliorated by baicalein administration, showing the anti-inflammatory effects of baicalein *in vivo*. Overall, this study demonstrated that baicalein may serve as a potential anti-inflammatory agent for the treatment of IDD. However, further studies are needed to investigate the detailed mechanisms and the clinical efficacy of baicalein in IDD.

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COMPLIANCE WITH ETHICAL STANDARDS

The study was performed in accordance with the Declaration of Helsinki and relevant policies in China.

Conflict of Interest. The authors declare that they have no conflicts of interest.

Ethics Statement. This study was approved by the Committee of Wenzhou Medical University, and all surgical interventions, treatments, and postoperative animal care procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

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