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Baicalin alleviates TNBS-induced colitis by inhibiting PI3K/AKT pathway activation

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Abstract. Inflammatory bowel diseases (IBDs) are chronic immunological disorders of the intestinal tract characterized by persistent inflammation. Baicalin, a type of flavonoid, has exhibited a wide range of pharmacological activities, including immunomodulation and anti-inflammation. However, little is known about the therapeutic role of baicalin in IBD. The aim of the present study was to ascertain whether baicalin could be a therapeutic drug of IBD and investigate its specific mechanisms. In the present study, the results revealed that baicalin not only significantly alleviated TNBS-induced colitis by reducing the release of IL-6, TNF-α and IL-1β and increasing the level of IL-10, but promoted the expression of tight-junction proteins ZO-1 and β-catenin, which may have been achieved by blockage of the PI3K/AKT signaling pathway. *In vitro*, the results demonstrated that baicalin clearly inhibited the release of TNF-α, IL-6 and IL-1β and promoted the expression of IL-10 in LPS-induced HT-29 cells, and significantly decreased LPS-induced HT-29 cell apoptosis by blockage of the PI3K/AKT signaling pathway. In conclusion, the present research revealed for the first time that baicalin acted as a therapeutic drug in IBD by suppression of the PI3K/AKT signaling pathway.

Introduction

Inflammatory bowel diseases (IBDs) are chronic, progressive immunologically-mediated diseases characterized by chronic inflammation of the gastrointestinal tract in genetically susceptible individuals exposed to environmental risk factors, encompassing ulcerative colitis and Crohn's disease (1-3). IBDs influence extensive populations and have been estimated to affect 1.5 million Americans, 2.2 million people in Europe, and several hundreds of thousands more worldwide, and these numbers have been increasing with worsening of the environment and changing habitats (3,4). Persistent, chronic inflammation of the gastrointestinal tract is assumed to underlie the causes of colitis-associated cancer (5), fibrosis (6), heart diseases (7) and even induces serious behavioral symptoms reflecting the effect of colitis on the central nerve system (8). The past decades have witnessed notable progress in understanding the development of IBDs, and various clinical trials aimed to interfere with IBDs have been performed, but the therapy of IBDs still remains intractable (9).

In the active state of IBDs, the intestinal epithelial barrier breaks down and cells infiltrate into the lamina propria. Cells from the innate immune system and adaptive immune system, consisting of neutrophils, monocytes, macrophages and T cells, release inflammatory cytokines (2,10) and chemokines (11,12), such as interleukin 6 (IL-6), tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), CXCL1 and CXCL9 (11). Persisting existence of inflammatory cytokines and chemokines exacerbate the apoptosis of epithelial cells leading to disruption of the epithelial barrier (13,14). In accordance with the role of inflammatory cytokines in the progression of IBDs, a great amount of research and clinical trials have been conducted (15-17), but the efficacy of anti-TNF-α was not promising and was even accompanied with side effects, such as paradoxical psoriasis (18). These challenges need to be overcome.

Baicalin (5, 6, 7-trihydroxyflavone-7-b-D-glucuronide) is a major constituent isolated from the herb Huangqin, found in the root of *Scutellaria baicalensis* Georgi (19). Baicalin has been revealed to exhibit many pharmacological activities, such as anti-inflammation (20), antitumor (21), anti-apoptosis (22) and immunomodulation (23). It has been used to treat a series of diseases, including acute myocardial infarction (24) and renal ischemia-reperfusion injury (25). It has been revealed that baicalin could ameliorate isoproterenol-induced acute myocardial infarction through the p38/MAPK pathway (20), alleviate experimental colitis through blockage of the TLR4/NF-κB pathway (26), and attenuate symptoms of experimental autoimmune encephalomyelitis via activation of the SOCS3 pathway (27), however, the molecular mechanism of baicalin in treating IBDs still remains elusive.

In the present study, the underlying mechanisms of baicalin in the treatment of IBDs were investigated in both an animal

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model of 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced rat colitis and an lipopolysaccharide (LPS)-induced HT-29 cell inflammation model. In the present study, it was revealed that baicalin reduced the levels of IL-1β, IL-6 and TNF-α and increased the expression of IL-10, and ameliorated the apoptosis of intestinal mucosal epithelial cells and promoted the expression of tight-junction proteins in the PI3K/AKT-dependent pathway *in vivo* and *in vitro*. Therefore, for the first time, our results identified the mechanism by which baicalin affects the development of colitis through the PI3K/AKT-dependent signaling pathway.

Materials and methods

Chemicals and reagents. Baicalin (purity, 95%; CAS registry no. 21967-41-9; molecular formula, C21H18O11; molecular weight, 446.36) and TNBS were obtained from Sigma-Aldrich; Merck KGaA. LPS *Escherichia coli* serotype 0111:B4 strain and LY294002 were purchased from InvivoGen, Inc. Rabbit anti-phospho-AKT (1:1,000; cat. no. 4691), anti-phospho-PI3K (1:1,000; cat. no. 17366), anti-total AKT (1:1,000; cat. no. 9272), anti-total PI3K (1:1,000; cat. no. 4255), anti-Bcl-2 (1:1,000; cat. no. 3498), anti-FasL (1:1,000; cat. no. 68405), anti-Bax (1:1,000; cat. no. 81740), anti-caspase-3 (1:1,000; cat. no. 14220), anti-caspase-9 (1:1,000; cat. no. 9508), anti-ZO-1 (1:1,000; cat. no. 13663), anti-β-Catenin (1:1,000; cat. no. 8480) and anti-GAPDH (1:1,000; cat. no. 5174) were purchased from Cell Signaling Technology, Inc.

Animals. A total of 30 Sprague-Dawley rats (aged 6-8 weeks; 15 male and 15 female; weighing 200-250 g) were purchased from the Model Animal Research Center of Nanjing University. All rats were maintained under environmentally-controlled conditions (ambient temperature, 22±2˚C; humidity 40%) in a pathogen-free facility with a 12-h light/dark cycle, and had access to water and food *ad libitum*.

All experimental procedures were performed in strict accordance with the Institutional Animal Care and Use Committee of Nanjing University of Chinese Medicine [approval no. SYXK(Su)2014-0004].

Induction of colitis. Colitis was induced by intracolonic administration of TNBS, as previously described (28). Briefly, rats that were fasted for 24 h with free access to water were lightly anesthetized with 350 mg/kg chloral hydrate, and a polyethylene catheter was inserted rectally until the splenic flexure (6-8 cm from the anus). Then, TNBS, dissolved in 50% ethanol for a dose of 100 mg/kg, 0.25 ml per rat, was administered through the catheter. After removing the catheter, the rats were held in a headfirst position for 60 sec to prevent liquid outflow.

The rats were randomly divided into the following six groups: Control group (rats received 0.9% saline.); model group (TNBS-induced colitis without treatment, TNBS group); baicalin group (TNBS-induced colitis treated with baicalin, 100 mg/kg/d, per rat by gastric lavage); LY294002 group (TNBS-induced colitis treated with LY294002, 50 μg/kg/d, per rat via intravenous (i.v.) injection); IGF-1 group (TNBS-induced colitis treated with IGF-1, 1.5 μg/kg/d, per rat via intravenous (i.v.) injection); baicalin and IGF-1 co-administration group (TNBS-induced colitis treated with baicalin, 100 mg/kg/d, per rat by gastric lavage, and IGF-1, 1.5 μg/kg/d, per rat via intravenous (i.v.) injection). The animals in each group were treated once a day for 14 days. In addition, the defecation times and shape of the feces in each group were observed. On the 15th day, the rats were sacrificed by cervical dislocation under anesthesia with a solution of ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.), and the entire colon was removed from the cecum to the anus. The length and weight of the colon were measured. Then, colonic specimens were fixed immediately in a 10% (w/v) neutral formalin solution or frozen in liquid nitrogen for further analyses.

TUNEL staining. TUNEL staining was performed to quantify cell apoptosis using a TUNEL Detection kit, according to the manufacturer's instructions. Briefly, sections were fixed with 4% paraformaldehyde at 37˚C for 20 min, immersed in the 3% H_2O_2 at 37˚C for 10 min, and dipped into 0.1% TritonX-100 on ice for 2 min. FITC-labeled dUTP (50 μl) and terminal deoxynucleotidyltransferase from the kit was added to each section and then incubated at 37˚C for 60 min, followed by incubation with 50 μl streptavidin-HRP from the kit at 37˚C for 30 min. After detection with the DAB kit for 10 min at 37˚C, sections were sealed with neutral gum. A total of six images of randomly selected visual fields were taken for each section with a fluorescence microscope (Olympus Corporation) at x400 magnification. TUNEL-positive cells in the ipsilateral hippocampus were observed and quantified with Image-Pro software (version 6.0.0.260). Brownish yellow particles in the nuclei of the cells in the hippocampal sections indicated TUNEL-positive, apoptotic cells. The apoptotic rate was calculated quantified with Image-Pro software (version 6.0.0.260; Media Cybernetics, Inc.).

Histology and immunohistochemistry. For hematoxylin and eosin (H&E) staining, colons samples were fixed in 10% neutral formalin solution for 24 h, dehydrated in increasing concentrations of ethanol, and embedded in paraffin. Thereafter, sections of tissue were cut at a thickness of 3 μm. For immunohistochemical staining, paraffin-embedded colon sections were deparaffinized, hydrated, and antigen-retrieved, and endogenous peroxidase activity was quenched by 3% H_2O_2 for 10 min. Sections were then blocked with 5% bovine serum albumin for 20 min, followed by incubation with anti-ZO-1 (cat. no. sc-33725; Santa Cruz Biotechnology, Inc.) and anti-β-catenin (1:1,000; cat. no. 8480; Cell Signaling Technology, Inc.) overnight at 4˚C and subsequently incubation with a secondary antibody IgG (1:1,000; cat. no. 3900; Cell Signaling Technology, Inc.) for 2 h. Positive cells were visualized by adding DAB to the sections. Slides were viewed with a Nikon Eclipse 80i microscope equipped with a digital camera (DS-Ri1; Nikon Corporation).

Cell culture. The moderately differentiated human colon adenocarcinoma cell line HT-29 was obtained from ATCC (HTB-38TM). Cells were cultured in McCoy's 5A Medium supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin G potassium, and 100 μg/ml streptomycin at 37˚C in a humidified atmosphere with 5% CO_2. Cells were seeded on a six-well

culture plate and were allowed to grow to 70-80% confluence in complete medium containing 10% FBS for 24 h, and then the medium was changed to serum-free medium after washing twice with serum-free medium. LPS (1 μg/ml) was used to stimulate colonic adenocarcinoma HT-29 cells to induce inflammation.

Experimental cell groups. Growth-phase HT-29 cells were divided into six groups: Normal control group (blank); model control group (LPS); baicalin; LY294002; IGF-1 and baicalin + IGF-1 group. The blank group was incubated in McCoy's 5A medium without FBS for 24 h; the medium was changed to fresh serum-free medium after washing twice with serum-free medium, and then LPS (1 mg/l) was added for 12 h. The baicalin (316 μg/ml), LY29400 (0.158 μg/ml), IGF-1 ($4.8x10^{-3}$ μg/ml) and baicalin (316 μg/ml) + IGF-1 ($4.8x10^{-3}$ μg/ml) groups were based on the stated drug interventions for 24 h.

Western blotting. After surgery, the rats were sacrificed by cervical dislocation and the colons specimens were homogenized in radioimmunoprecipitation assay lysis buffer (Sunshine Biotechnology Co., Ltd.), then the supernatants were harvested by centrifugation at 6,036 x g for 10 min at 4˚C. HT-29 cells were stimulated with different concentrations of H_2O_2 (0, 100, 200, 300, 400, 500 and 1,000 μM) for 24 h, then washed twice with PBS, and protein was extracted by adding radioimmunoprecipitation assay lysis buffer on ice for 30 min, and centrifuging at 6,036 x g for 10 min at 4˚C. Protein concentrations were determined using a bicinchoninic acid kit (Beyotime Institute of Biotechnology). Protein samples were homogenized with loading buffer and heated to 100˚C for 5 min, and 20 μg of each sample was then resolved by 10% SDS-PAGE. Proteins were transferred to a polyvinylidene difluoride membrane (EMD Millipore) and were probed with primary and secondary anti-IgG (1:1,000; cat. no. 14708; Cell Signaling Technology, Inc.). Immunoreactivity was visualized using an enhanced chemiluminescence reaction kit (OriGene Technologies, Inc.) and quantified using ImageJ 5.0 software (National Institutes of Health).

Enzyme-linked immune absorbance assay (ELISA). The amount of TNF-α, IL-6, IL-1β and IL-10 in the culture medium and in rat colon tissue extracts were measured with a commercial ELISA kit (R&D Systems, Inc.) according to the manufacturer's instructions.

Annexin V/PI binding assay. Briefly, HT-29 cells were seeded on 100-mm culture dishes to 70-80% confluence in complete medium containing 10% FBS for 24 h, and then changed to serum-free medium after washing twice with serum-free medium. Cells were treated with H_2O_2 (0, 100, 200, 300, 400, 500 and 1,000 μM) for 24 h, then harvested and washed twice, once in PBS and once in binding buffer. An Annexin V-FITC Apoptosis Detection kit (eBioscience, Inc.) was used to detect the translocation of phosphatidylserine from inner membrane to the outer leaflet of the plasma membrane. Cells were resuspended in Binding Buffer and the concentration was adjusted to 10^6/ml. FITC-conjugated Annexin V (5 μl) was added to 100 μl of the cell suspension. The tubes were gently mixed and incubated for 15 min at room temperature in the dark. The unconjugated Annexin V was removed by a wash using binding buffer, and then 5 μl of PI was added to 200 μl of the binding buffer. Flow cytometric analysis was conducted within 2 h (BD FACSCanto II; BD Biosciences), with the reagents stored between 2 and 8˚C. Immediately after use, the remaining reagents were returned to cold storage (2-8˚C).

Statistical analysis. All data examined are presented as the mean ± SEM. Statistical analysis of the data was performed using SPSS software (17.0 for Windows, IBM Inc.). Comparisons between two groups were performed using Student's t-test. Comparisons among three or more groups were performed using one-way ANOVA, followed by the Tukey test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Baicalin protects TNBS-induced colitis in rats via inhibition of PI3K/AKT pathway activation. Sprague-Dawley rats (weighing 200-250 g) were intracolonically administered TNBS to induce colitis, as previously described (28). To investigate the role of the PI3K/AKT pathway in the development of colitis, the PI3K/AKT pathway was inhibited or activated by the use of LY294002 or IGF-1. The results of western blotting revealed that LY294002 could markedly inhibit the activation of the PI3K/AKT pathway, and IGF-1 could activate the PI3K/AKT pathway by increasing the expression of phosphorylated (p)-PI3K and p-AKT (Fig. 1A). H&E staining indicated that severe mucosal injury was observed in TNBS-induced rats, characterized by increased neutrophils, epithelial cell disruption, massive bowel edema and distorted architecture of crypts, but the PI3K inhibitor LY294002 could markedly ameliorate the morphological change and histological damage of colon similar to baicalin administration. Conversely, IGF-1 administration exacerbated TNBS-induced colitis manifested by histological staining. However, the accelerated colitis syndrome induced by the combination of TNBS and IGF-1 could be suppressed by baicalin (Fig. 1B).

TNBS-induced colitis is characterized by the breakdown of the intestinal epithelial barrier, infiltration of both the innate and adaptive immune cells into the lamina propria, and these immune cells synthesize and release a host of proinflammatory and anti-inflammatory cytokines, including IL-6, IL-1β and TNF-α. Furthermore, the levels of inflammatory cytokines were detected in colitis tissues. As revealed in Fig. 1C, TNBS significantly increased the expression of IL-6, TNF-α and IL-1β and inhibited the expression of IL-10 in colon specimens, compared with the control group, however, inhibition of the PI3K/AKT pathway using LY294002 predominantly inhibited TNBS-induced inflammatory cytokine expression (IL-6, TNF-α and IL-1β) and increased the expression of IL-10, compared with the TNBS group. Furthermore, the efficacy of LY294002 in suppressing inflammatory cytokine production was equivalent to baicalin administration in the dosage of 100 mg/kg/d. By contrast, IGF-1 efficiently increased the expression of IL-6, TNF-α and IL-1β and inhibited the expression of IL-10, compared with the TNBS group. However, this tendency could be rescued after baicalin administration, compared with the IGF-1 group (Fig. 1C).

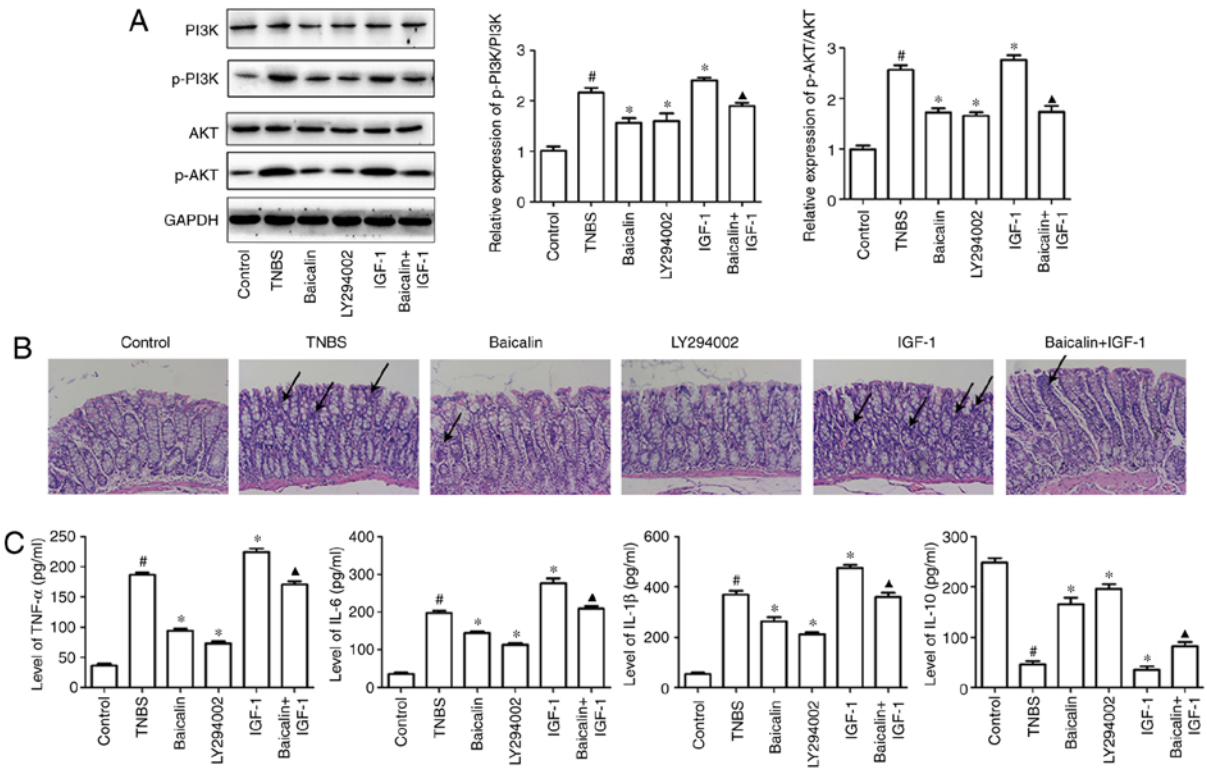

Figure 1. Baicalin protects TNBS-induced colitis in rats by inhibiting PI3K pathway activation. Colitis was induced by intracolonic administration of TNBS (100 mg/kg/d) for 14 days, then baicalin (100 mg/kg/d), LY294002 (50 μg/kg/d), IGF-1 (1.5 μg/kg/d) or IGF-1 along with baicalin were administrated for 14 days to investigate their role in TNBS-induced colitis. On the 15th day, the rats were sacrificed and colons were homogenized, and 20 μg of protein from lysates was analyzed for p-PI3K, p-AKT, PI3K and AKT expression (A) by western blotting. The other colonic specimens were fixed immediately in a 10% (w/v) neutral formalin solution for H&E staining to observe the injury of the intestine. (B) The colons of the mice in the control group exhibited a normal structure without damage. However, in the TNBS model group, the colons exhibited mucosal ulcerations and inflammatory cell infiltration, however these alterations were attenuated to varying degrees by baicalin and LY294002. In addition, IGF significantly increased inflammatory cell infiltration, however the effect could be reversed by baicalin, as indicated by the arrows. (C) The supernatants of homogenized colons were also used to perform ELISA to detect IL-6, TNF-α, IL-1β and IL-10 production. The data are representative of at least three independent experiments. Values are expressed as the mean ± SEM of at least three independent experiments. $^{\#}$P<0.05 vs. the control group; *P<0.05 vs. the TNBS group; $^{\blacktriangle}$P<0.05 vs. the IGF-1 group.

These results demonstrated that the PI3K/AKT pathway was involved in baicalin-alleviated colitis response.

Baicalin ameliorates TNBS-induced intestinal mucosal cell apoptosis through blockage of the PI3K/AKT pathway. Apoptosis of the intestinal mucosal cell is a hallmark of TNBS-induced colitis, which disrupts intestinal mucosal integrity and barrier function, and leads to other changes associated with colitis (29). Excessive inflammation is considered to be a marker of apoptosis (30). In line with the results of inflammatory cytokine production in colon tissues, intestinal mucosal cell apoptosis was then investigated using TUNEL staining. As revealed in Fig. 2A, both baicalin and LY204002 significantly inhibited intestinal mucosal cell apoptosis, but IGF-1 induced cell apoptosis, which indicated the role of the PI3K/AKT pathway in colitis development. Moreover, when baicalin was administered in TNBS and IGF-1-treated rats, intestinal mucosal cell apoptosis could be inhibited by baicalin (Fig. 2A), which confirmed that baicalin exerted the suppression partly through inhibition of the PI3K/AKT pathway.

In order to ascertain the role of the PI3K/AKT pathway in baicalin-suppressed cell apoptosis, colon tissues lysates were used to detect the pro-apoptotic and anti-apoptotic proteins by western blotting. It was revealed that baicalin or LY294002 administration significantly suppressed TNBS-induced pro-apoptotic caspase-3, caspase-9, Bax and FasL expression, but increased the expression of Bcl-2, an anti-apoptotic protein, when compared with the TNBS group. In addition, increased pro-apoptotic protein caspase-9 expression was observed with IGF-1 treatment, however this effect could be reversed by baicalin treatment (Fig. 2B). Thus, baicalin suppressed TNBS-induced cell apoptosis through inhibition of the PI3K/AKT pathway.

Baicalin protects TNBS-induced colitis by increasing mucosal tight-junction proteins through inhibition of the PI3K/AKT pathway. Integrity of the intestinal epithelial cell (IEC) barrier plays an important role in maintaining mucosal immune homeostasis. Dysregulated IEC barrier function appears to trigger and perpetuate inflammation in IBD (31). To investigate the effects of baicalin and the PI3K/AKT pathway on the integrity of the IEC barrier, colon tissues underwent immunohistochemical staining for ZO-1 and β-catenin, the marker proteins of tight-junctions. The present results revealed that baicalin and LY294002 treatment could markedly increase ZO-1 and β-catenin expression compared to the TNBS-treated model group. In addition, the use of baicalin could alleviate IGF-1-induced decrease of tight-junction proteins (Fig. 3A), which indicated that baicalin exerted the protection of the IEC barrier via inhibition of PI3K/AKT pathway activation.

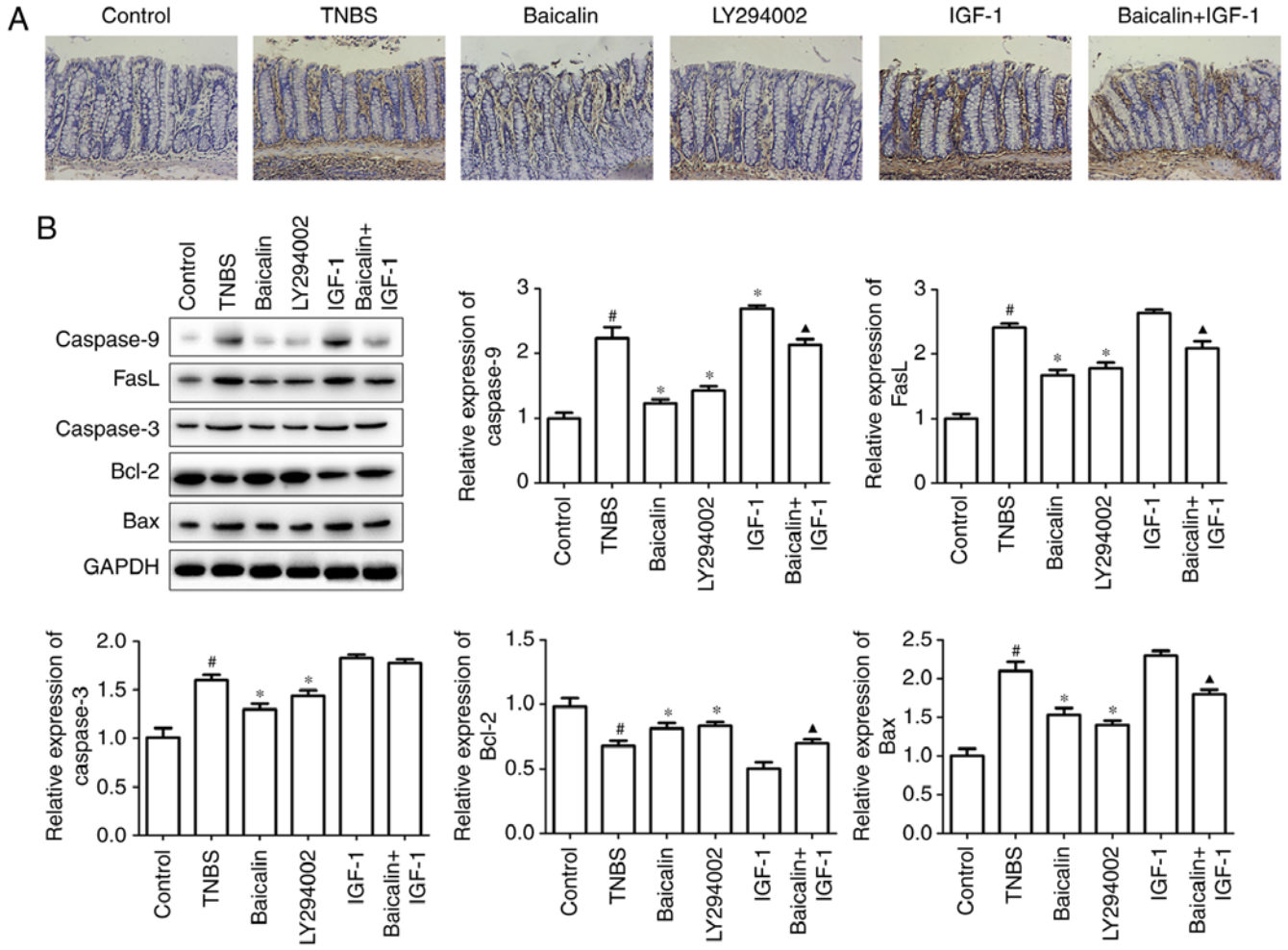

Figure 2. Baicalin ameliorates TNBS-induced intestinal mucosal cell apoptosis in PI3K/AKT-dependent manner. (A) The colon samples of rats underwent TUNEL staining to detect intestinal mucosal epithelial cell apoptosis in rats and slides were viewed with a Nikon Eclipse 80i microscope equipped with a digital camera (DS-Ri1; Nikon Corporation). (B) Western blot analyses revealed the pro-apoptotic proteins caspase-3, caspase-9, Bax, FasL and the anti-apoptotic protein Bcl-2 expression in colon lysates from the indicated group, and GAPDH was used as a loading control. #P<0.05 vs. the control group; *P<0.05 vs. the TNBS group; ▲P<0.05 vs. the IGF-1 group.

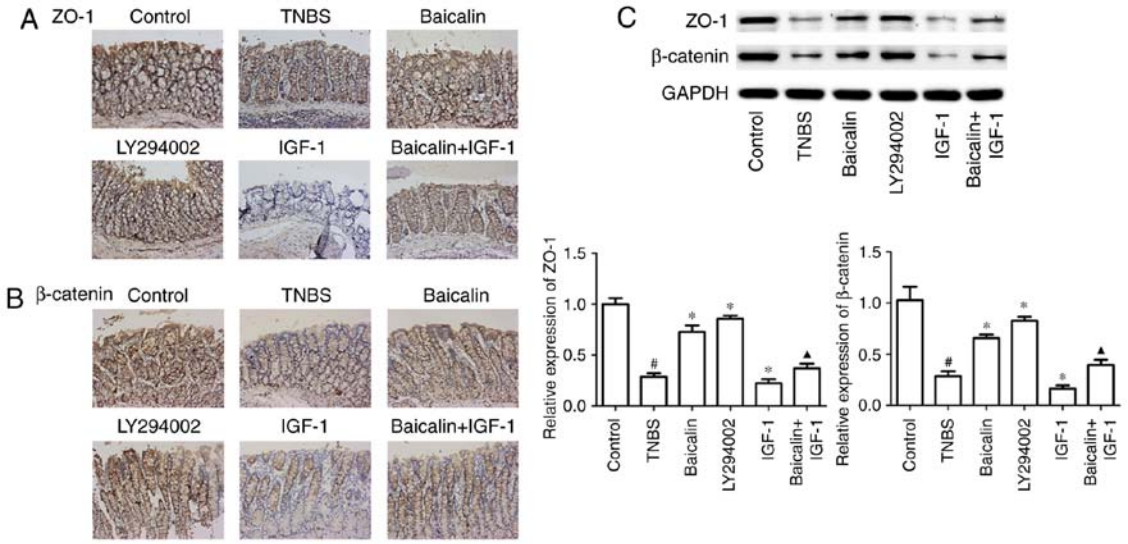

Figure 3. Baicalin protects TNBS-induced colitis by increasing mucosal tight-junction proteins in the PI3K/AKT-dependent pathway. (A and B) Immunohistochemistry was used to detect ZO-1 and β-catenin expression in rat colons from of various groups. The sections were probed with the antibodies against ZO-1 (1:50) and β-catenin (1:100). (C) Western blot analyses revealed the ZO-1 and β-catenin expression in rat colons from each indicated group. Protein (20 μg) from lysates was analyzed for ZO-1 and β-catenin, and GAPDH was used as an internal control. #P<0.05 vs. the control group; *P<0.05 vs. the TNBS group; ▲P<0.05 vs. the IGF-1 group.

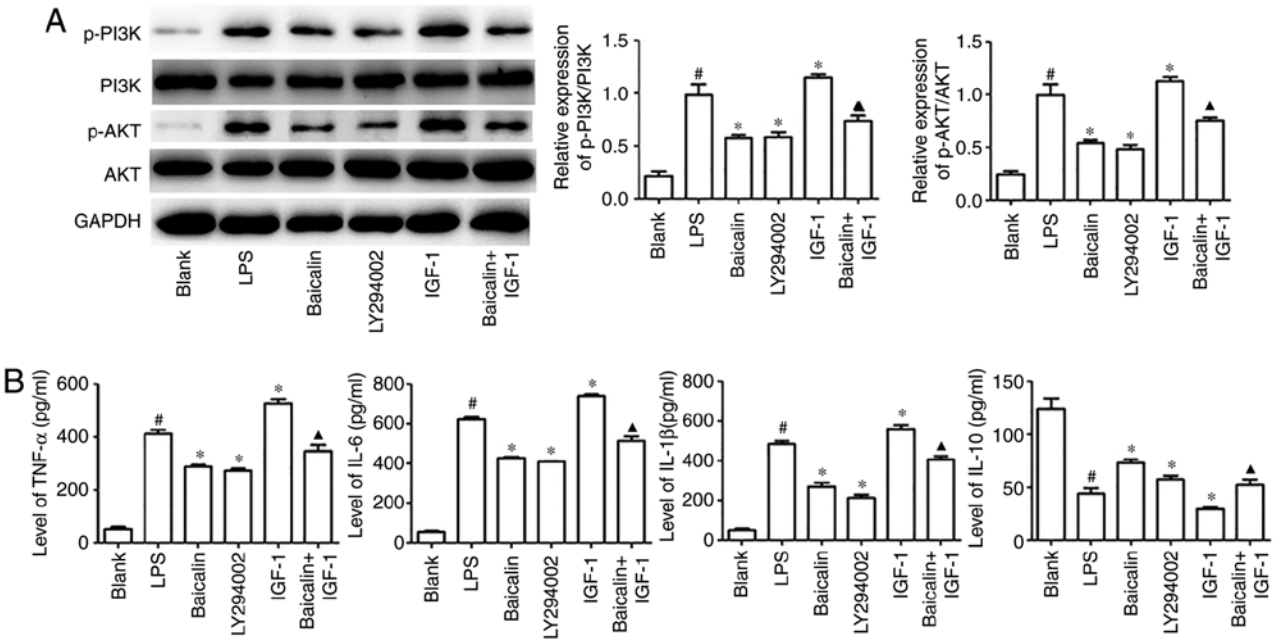

Figure 4. LPS-induced inflammation in HT-29 cells is suppressed by the use of baicalin. HT-29 cells were cultured for 24 h in complete medium, and then the medium was changed to serum-free medium after washing twice with serum-free medium. LPS (1 μg/ml) was used to stimulate HT-29 cells to induce inflammation. Then, baicalin (100 ng/ml), LY294002 (50 μM) and IGF-1 (0.2 μg/ml) were used to investigate the role of baicalin and the PI3K pathway in LPS-induced inflammation. LY294002, IGF-1 and baicalin were used to treat cells for 30 min prior to LPS stimulation. Cell lysates were used to detect representative protein expression. (A) Western blot analyses showing the expression of phosphorylated PI3K and AKT, total PI3K, AKT and GAPDH were used as loading control. (B) HT-29 cells were treated and then the supernatants were collected to detect IL-6, TNF-α, IL-1β and IL-10 concentrations by ELISA. Each column represents at least three independent experiments. Values are expressed as the mean ± SEM. $^{\#}$P<0.05 vs. the control group; *P<0.05 vs. the LPS group; $^{\blacktriangle}$P<0.05 vs. the IGF-1 group.

Furthermore, in accordance with the results of immunohistochemical staining, the results of western blotting revealed that baicalin and LY294002 increased ZO-1 and β-catenin expression, compared with the TNBS group, moreover, baicalin also reversed IGF-1-induced reduction of ZO-1 and β-catenin, compared with the TNBS group (Fig. 3C). These results indicated that baicalin protected IEC barrier integrity, which was a PI3K/AKT pathway inhibition process.

Baicalin suppresses LPS-induced inflammation in HT-29 cells. Epithelial cells are the critical first barrier for the intestine to defend itself from dangerous stimuli, and the most sensitive cells respond to hazardous substances. To study the role of baicalin in attenuating colitis, HT-29 cells were cultured, and LPS was used to induce epithelial cell injury. As revealed in Fig. 4A, when compared with the blank group, LPS induced significant activation of the PI3K/AKT pathway. However, when compared with the LPS group, baicalin markedly suppressed the levels of p-PI3K and p-AKT. Furthermore, IGF-1 was used to activate the pathway, and increased expression of p-PI3K and p-AKT was revealed compared to the LPS group. However, the influence of IGF-1 on LPS-stimulated epithelial cells could be reversed by baicalin (Fig. 4A), which indicated that baicalin was able to inhibit the PI3K/AKT pathway in HT-29 cells. Inflammatory cytokine production is the terminal result of pathway activation. Herein, baicalin significantly inhibited the LPS-induced IL-6, TNF-α and IL-1β production and promoted the expression of IL-10, and these effects were also observed with LY294002 treatment. In addition, baicalin treatment also reversed the IGF-1-induced increase of IL-6, TNF-α and IL-1β (Fig. 4B). These results indicated that baicalin ameliorated LPS-induced inflammation via the inhibition of PI3K/AKT activation in HT-29 cells.

Baicalin ameliorates apoptosis and tight-junction reduction by blockage of the PI3K/AKT pathway in HT-29 cells. To further demonstrate that baicalin could ameliorate cell death, flow cytometric analysis was employed to assess early apoptosis and late apoptosis. As presented in Fig. 5A, LPS significantly triggered colon cancer cell early apoptosis from 3.5 to 22.6% compared with the control group, whereas baicalin and LY294002 treatment decreased the early apoptosis rates from 22.6 to 10.5 and 8.33%, respectively. Furthermore, baicalin markedly attenuated the early apoptosis rate to 12.6%, which was significantly increased by IGF-1 treatment (26.2%), as compared to the control group. Notably, the results also revealed that the various treatments did not cause any significant changes in late cell apoptosis, compared with the control group, indicating that the increase of apoptosis appeared to be possibly due to early apoptosis rather than late apoptosis. Western blotting results also revealed that when compared with the LPS group, baicalin and LY294002 suppressed pro-apoptotic caspase-3, caspase-9, Bax and FasL expression, but increased the expression level of Bcl-2 in HT-29 cells (Fig. 5B). In addition, the role of baicalin on tight-junction protein expression was explored *in vitro*, and the results revealed that baicalin and LY294002 reversed LPS-induced tight-junction protein decrease. These results indicated the vital role of baicalin on alleviating inflammation, apoptosis and tight-junction reduction, and this process involved PI3K/AKT pathway inhibition.

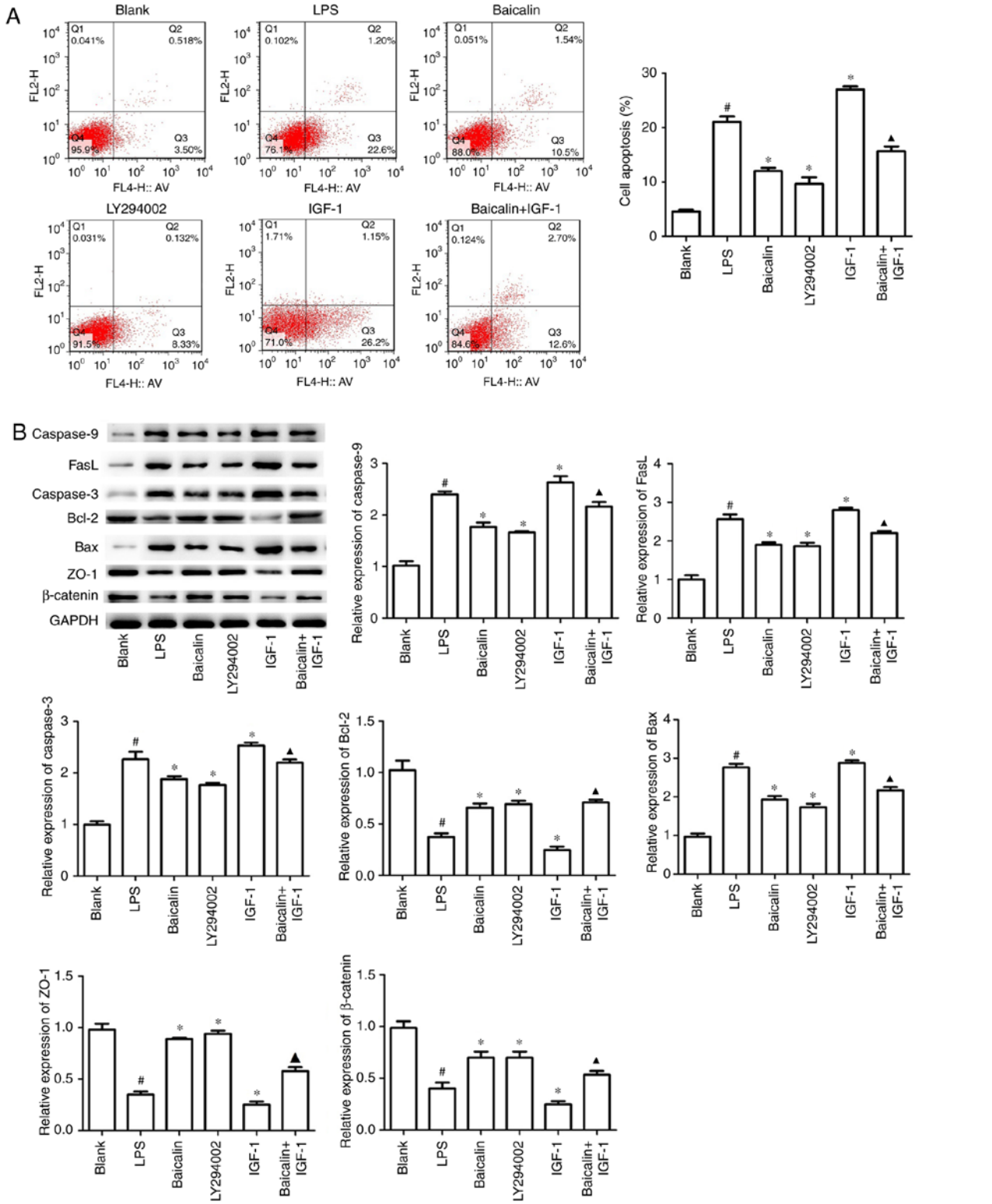

Figure 5. Baicalin inhibits the activation of the PI3K/AKT pathway in HT-29 cells to ameliorate apoptosis and tight-junction reduction. (A) HT-29 cells were harvested, and then cell apoptosis was detected using a commercial apoptosis kit and flow cytometry. (B) Western blotting was used to detect the protein expression of caspase-3, caspase-9, Bax, FasL, Bcl-2, ZO-1 and β-catenin levels using GAPDH as a loading control. #P<0.05 vs. the control group; *P<0.05 vs. the LPS group; ▲P<0.05 vs. the IGF-1 group.

Discussion

Ulcerative colitis (UC) and Crohn's disease (CD) represent the two main forms of IBDs with clear-cut clinical and histological features. UC involves the rectum and colon, whereas CD generally involves the colon and ileum. Both of them cause great pain and are costly to people and it has been estimated that 4.5 million people suffer from these diseases (32). Despite the recognized effect of IBD conventional therapies such as aminosalicylates, corticosteroids, and immunosuppressive

agents, frequent adverse effects with these agents have been documented (33). Therefore, effective and safe treatments for IBDs are urgently required. Chinese herbs have been used to treat diseases for a long time. Nowadays, the use of their active ingredients is becoming an increasingly attractive approach to deal with various inflammatory disorders. Luan *et al* revealed that 50, 100 and 200 mg/kg of baicalin could attenuate rat myocardial ischemia-reperfusion injury through the AKT/NF-kB pathway (34). In an *in vitro* HT-29 model, 50, 100, 150 and 200 μM of baicalin was used to study its role in colon cancer and the results revealed that baicalin could induce colon cancer cell apoptosis (35).

In the present study, the role of baicalin in treating TNBS-induced colitis *in vitro* and *in vivo* was investigated. The results demonstrated that baicalin alleviated the development of TNBS-induced colitis, reduced inflammation release, ameliorated intestinal mucosal cell apoptosis and increased the expression of tight-junction-associated proteins *in vivo* and *in vitro*. Notably, baicalin exerted a protective role in colitis via suppression of the PI3K/AKT pathway.

TNBS has long been used as a valuable model to explore the pathogenesis of IBDs. When TNBS is administered with ethanol, the mucosal barrier of the intestine can be broken by ethanol. TNBS is believed to haptenize colonic autologous or microbiota proteins rendering them immunogenic to the host immune system (34). The hapten-induced colitis has been characterized by dense infiltration of adaptive immune cells, predominantly $CD4^+$T cells (35), and innate immune cell-like macrophages (36). Based on inclination towards Th1 immune response which involves IL-12 and TNF-α as effector cytokines (35), baicalin was firstly investigated on inflammatory cytokine secretion. It was revealed that TNF-α, a typical Th1-type cytokine, was significantly inhibited by the use of baicalin in rat colons, which indicated that baicalin can be an effective regulator of Th1-immune response. IL-6, produced by lamina propria T cells (37) and macrophages in the intestine, has been reported to be involved in the development of IBDs (38,39). In the present study, it was reported that baicalin can also be an efficient inhibitor of IL-6 secretion.

Aberrant apoptosis of IECs is a hallmark of TNBS-induced colitis, which disrupts intestinal mucosal integrity and barrier function and leads to other changes associated with colitis (40,41). TNF-α, the main cytokine released in TNBS-induced colitis, is believed to play a vital role in the process of IEC apoptosis. Furthermore, anti-TNF-α therapies used for the treatment IBD patients were revealed to inhibit IEC apoptosis (30,42). In accordance with the present results which revealed that baicalin inhibited TNF-α production, the inhibitory effect of baicalin on IEC apoptosis was also revealed, as observed by the fact that the expression of pro-apoptotic proteins such as caspase-3, caspase-9, Bax and FasL were suppressed *in vivo* and *in vitro*, and the anti-apoptotic molecule Bcl-2 was increased. Herein, HT-29 cells were used to evaluate the role of baicalin on inflammatory bowel diseases *in vitro*. HT-29 cells have been reported in numerous studies as targets for inflammatory bowel diseases *in vitro* (43-47). In future experiments, other cell lines will be selected to study the role of baicalin on inflammatory bowel diseases.

PI3K is an intracellular enzyme that catalyzes the phosphorylation of membrane inositol lipids and is involved in many biological processes, including cell growth, differentiation and survival (48). In the present study, it was reported that baicalin significantly suppressed the TNBS- or LPS-induced phosphorylation of PI3K and AKT, which indicated that baicalin exerted the protective effect in TNBS-induced colitis partly by inhibiting PI3K/AKT pathway activation. PI3K signaling was blocked by the PI3K specific inhibitor LY294002, and it was revealed that the effect of LY294002 on the inhibition of inflammatory cytokine production and intestinal epithelial cell apoptosis was equivalent to baicalin. Numerous studies on tumors have revealed the vital role of PI3K signaling in promoting tumor cell growth and resisting apoptosis. PI3K signaling even helps tumor cells obtain the ability to immortally grow (49,50). However, in other cells except tumor cells, PI3K signaling appears to play an opposite role by promoting cell apoptosis to maintain a steady state (51). IECs are essential parts of the mucosal barrier. In order to maintain the balance and basic functions of the barrier, epithelial cells often enter the process of apoptosis upon various injury factors such as TNBS (52,53).

In conclusion, the present study demonstrated a novel mechanism in which baicalin attenuated TNBS-induced colitis via inhibition of PI3K/AKT activation, which was revealed by the decreased inflammatory cytokine levels, reduced IEC apoptosis and increased tight-junction proteins. These findings provide the first evidence of a direct link between traditional Chinese herbal ingredients and PI3K/AKT signaling in colitis, along with novel insight into the mechanisms of baicalin in treating colitis.

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Availability of data and materials

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

Authors' contributions

LeZ and HS designed the study and wrote the manuscript. PQG, YJL and LuZ performed the experiments. LuZ and JFC analyzed the data. HS supervised the work. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experimental procedures were performed in strict accordance with the Institutional Animal Care and Use Committee of Nanjing University of Chinese Medicine.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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