Biacaline Protects against TNF-α-Induced Injury by Down-Regulating miR-191a That Targets the Tight Junction Protein ZO-1 in IEC-6 Cells

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Received October 6, 2016; accepted January 6, 2017; advance publication released online January 20, 2017

Abstract

Tumor necrosis factor-alpha (TNF-α) plays an important role in the developing process of inflammatory bowel disease. Tight junction protein zonula occludens-1 (ZO-1), one of epithelial junctional proteins, maintains the permeability of intestinal barrier. The objective of this study was to investigate the mechanism of the protective effect of baicalin on TNF-α-induced injury and ZO-1 expression in intestinal epithelial cells (IECs). We found that baicalin pretreatment significantly improved cell viability and cell migration following TNF-α stimulation. miR-191a inhibitor increased the protective effect of baicalin on cell motility injured by TNF-α. In addition, miR-191a down-regulated the mRNA and protein level of its target gene ZO-1. TNF-α stimulation increased miR-191a expression, leading to the decline of ZO-1 mRNA and protein. Moreover, pretreatment with baicalin reversed TNF-α induced decrease of ZO-1 and increase of miR-191a, miR-191a inhibitor significantly enhanced ZO-1 protein expression restored by baicalin. These results indicate that baicalin exerts a protective effect on IEC-6 (rat small intestinal epithelial cells) against TNF-α-induced injury, which is at least partly via inhibiting the expression of miR-191a, thus increasing ZO-1 mRNA and protein levels.

Keywords enterocyte; tumor necrosis factor (TNF); tight junction (TJ); microRNA; anti-inflammation

Tight junctions (TJs) are junctional protein complexes and permeability barriers in epithelial cells and mainly include the proteins zonula occludens-1 (ZO-1), occludin, and claudins.1 Increasing evidence suggests that intestinal inflammation disorders are associated with defects in TJs and increased intestinal permeability.2 Tumor necrosis factor-alpha (TNF-α) is a major pro-inflammatory cytokine that increases the permeability of the intestinal epithelium and plays a central role in inflammatory bowel disease.3,4 It has been reported that the level of the TJ protein ZO-1 markedly deceases in TNF-α-induced inflammation, resulting in permeabilization of the Caco-2 monolayer.5,6

TJ proteins are partly regulated by microRNAs (miRNAs), which are a class of small, noncoding RNAs that base pair with their target mRNAs and inhibit translation.7 miRNAs regulate signaling pathways and transcription factors, thus affecting the processes of intestinal TJ synthesis.8-10 The TJ protein ZO-1 is predicted to be one of the targets of miR-191a (previous nro-miR-191) by multiple miRNA database algorithm (TargetScan, miRanda, and miRDB). It is reported that an increase in miR-191a expression leads to attenuated ZO-1 expression in vascular endothelial cells,11 and that miR-191a significantly increases in the blood of patients with Crohn’s disease when compared with that of healthy controls.12 However, the role of miR-191a in enterocyte regulation of TJs has not been elucidated.

Baicalin is an active component extracted from the traditional Chinese herb Scutellaria baicalensis and possesses extensive anti-inflammatory as well as anti-cancer properties.13-15 We have shown earlier that baicalin reversed the down-regulation of the TJ protein ZO-1 and decreased TNF-α level after lipopolysaccharide (LPS) stimulation injury of IEC-6 (rat small intestinal epithelial cells), thereby maintaining the integrity of endothelial barriers.16 However, whether baicalin has direct protective effect against TNF-α-induced injury in intestinal TJs is not known and the mechanisms that mediate the effects of baicalin on ZO-1 have not yet been investigated.

In this study, we hypothesize that baicalin might inhibit TNF-α-induced cell injury and ZO-1 depletion in intestinal epithelial cells, an action potentially mediated by down-regulation of nro-miR-191a. In order to test this hypothesis, IEC-6 cells, which are widely used to represent normal intestinal epithelial cells, were chosen as the cell model to measure the protective effects and possible mechanisms of baicalin against TNF-α-induced injury.

MATERIALS AND METHODS

Cell Culture IEC-6 cells were cultured as described previously.16 Briefly, cells were grown in complete Dulbecco’s modified Eagle’s medium (DMEM; Hyclone) at 37°C in 5% CO2 and were then subcultured after digestion with 0.25% trypsin in phosphate buffered saline (PBS).

Cell Viability Assay The effect of baicalin on the viability of TNF-α-induced IEC-6 cells was evaluated using the Cell Counting Kit-8 (CCK-8) assay kit (Dojingo, Japan). Approximately 5×103 cells per well were incubated in 96-well culture dishes for 24 h. After incubation, the cells were treated with baicalin (National Institutes for Food and Drug Control of China) at various concentrations (10, 20, 40 µg/mL) for 1 h or were left untreated. Baicalin working solution was prepared by dissolving baicalin powder in complete DMEM culture solution to achieve final concentration of 10, 20, 40 µg/mL. Both treated and untreated cells were exposed to TNF-α (400-14 peprotech) at a concentration of 50 ng/mL for 24 h. Subsequently, 10 µL CCK-8 was added to each well and incubated for 2 h. Finally, absorbance of each well was measured using
the Bio-rad Microplate Reader.

Transfection of Cells with miR-191a Mimic and Inhibitor IEC-6 cells were inoculated into 6-well culture dishes at approximately $3 \times 10^5$ cells per well in antibiotic-free medium and incubated overnight. The cells were then transfected with 20 nM miR-191a mimic, 200 nM miR-191a inhibitor, miR-mimic negative control or miR-inhibitor negative control (Biomics Biotechnologies, China) using Lipofectamine 2000 (Invitrogen, U.S.A.) according to the manufacturer’s instructions. After 4h of transfection, the medium was replaced by regular culture medium and the cells were cultured for another 24h. Total protein and cellular RNA were finally extracted for analysis.

Quantitative Real-Time RT-PCR Assays for miRNA and mRNA Total RNA was extracted with Trizol (Invitrogen). miR-191a was reverse-transcribed to cDNA using Mir-X miRNA First-Strand Synthesis Kit (TaKaRa-Clontech, Japan). ZO-1 mRNA reverse transcription was performed using PrimeScript RT reagent Kit (TaKaRa, Japan). miR-191a and ZO-1 mRNA quantitative real-time PCRs were performed using SYBR® Premix Ex TaqTM II (TaKaRa) with a BioRad CFX96™ Real-Time PCR Detection System. The reaction conditions for cDNA PCR were 95°C for 30s, followed by 40 cycles of 95°C for 5s and 60°C for 30s. The relative amount of miR-191a was normalized to the expression of U6 small nuclear RNA (snRNA). ZO-1 mRNA expression was normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences were as follows: miR-191a forward primer: 5'-CACCGGATCC AACAGCAG-3'; miR-191a reverse primer was obtained from Mir-X miRNA First-Strand Synthesis Kit (TaKaRa-Clontech); U6 forward primer: 5'-GGAACATACAGAGAGATTAGC-3', U6 reverse primer: 5'-TGGAACGTCTCCA CGAATTTCG-3'; ZO-1 forward primer: 5'-CGCTCTCTGCTCCAACCTTCCTC-3', ZO-1 reverse primer 5'-GATCTTGAATCGGTTTGATGCTG-3', GAPDH forward primer, 5'-GAAGCTGCGCGCATATG-3', GAPDH reverse primer 5'-AAGGTGAGGAATGGGATTT-3'. The relative expression levels of miR-191a and ZO-1 mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method.

Western Blot Analysis Cells were harvested and lysed in radio immunoprecipitation assay (RIPA) buffer (Keygentec, China). Electrophoresis of protein samples was performed on 4–20% polyacrylamide sodium dodecyl sulfate (SDS) gels.

Fig. 1. Cytotoxicity of TNF-α and Cytoprotective Effects of Baicalin on Viability of IEC-6 Cells

(A) Cells were treated with or without baicalin for 24h. (B) Cells were treated with or without TNF-α for 24h. (C) Model cells were treated with TNF-α; baicalin groups were pretreated with baicalin (10, 20, 40 µg/mL) for 1h before exposure to TNF-α for 24h. (D) TNF-α+baicalin+miR-191a inhibitor cells were transfected with 200 nmol/L miR-191a inhibitor before exposure to TNF-α (50 ng/mL) for 24h. TNF-α+baicalin+miR-191a inhibitor cells were transfected with 200 nmol/L miR-191a inhibitor before pretreatment with baicalin (40 µg/mL) for 1h, followed by TNF-α treatment for 24h. MiR-inhibitor control was used as negative control. *$p<0.05$, **$p<0.01$. 
transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, U.S.A.), and blocked with 5% dried milk (Amresco, U.S.A.) for 1h to prevent non-specific binding. The blocked membranes were incubated with primary rabbit anti-ZO-1 (1:1000 dilution; Santa Cruz, U.S.A.) or β-actin (1:5000 Abcam, U.S.A.) antibody overnight at 4°C. The membranes were then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (Abcam) for 1h at room temperature. Protein bands were revealed using chemiluminescence kit (Millipore). Band intensities were measured with Image-Pro Plus software.

**Immunofluorescence Analysis** IEC-6 cells were inoculated at approximately $1\times10^5$ cells per well in 12-well culture plates and incubated overnight. Cells were pretreated with baicalin (40.0 µg/mL) or were left untreated to serve as controls. The cells were then exposed to TNF-α for 24h. The cells were then fixed with 4% formaldehyde for 15min followed by blocking with 5% bovine serum albumin (BSA) for 1h at room temperature. Cells were subsequently incubated with primary antibody (rabbit anti-ZO-1; 1:200 dilution; Santa Cruz) overnight at 4°C. Cells were then incubated with secondary antibody (Cy3-labeled goat anti rabbit; 1:500 dilution; Beyotime) for 2h at room temperature. Finally, the cells were stained and observed, and the images were recorded with Image-Pro Express software and Olympus IX71 fluorescence microscope.

**Wound Healing Assay** The effect of baicalin on the migration of IEC-6 cells due to TNF-α-induced injury was evaluated by wound healing assay. Briefly, IEC-6 cells were inoculated at 5.0×10³ cells per well in 6-well culture plates and incubated overnight. Upon reaching confluence, the cell monolayer was manually scraped by a sterile 200 µL pipette tip and washed with PBS. At 0 and 24h, respectively, images of the cells were captured with an inverted microscope. The migration of cells was determined by wound closure percentage as measured by ImageJ software.

**Statistical Analysis** The experimental data were expressed as the mean±standard deviation (S.D.) and analyzed by one-way ANOVA followed by Student’s t-tests (SPSS 20.0 statistical software). Results were considered significant at $p<0.05$. All experiments were performed at least thrice for reproducibility of results.
RESULTS

Baicalin Protects Intestinal Epithelial Cells from TNF-α-Induced Injury through miR-191a

To explore the effect of baicalin on the viability of IEC-6 cells induced by TNF-α, we performed CCK-8 assay. Cells exposed only to 10–40 µg/mL baicalin did not show any obvious cytotoxic or anti-proliferation effects when compared to the untreated control group (Fig. 1A). Viability of cells exposed to increasing concentrations of TNF-α (5, 10, 30, 50 ng/mL) for 24 h was 98.26±0.73, 94.22±2.03, 84.95±1.20, and 74.94±1.78% of the control group respectively (Fig. 1B). In cells pretreated with baicalin (10, 20, 40 µg/mL) the viability rose to 82.60±2.56, 83.94±2.51 and 92.25±1.63%, respectively (Fig. 1C). What is more, viability of cells transfected with miR-191a inhibitor before exposure to TNF-α didn’t show statistical significance (Fig. 1D).

To further investigate the protective effects of baicalin in impaired intestinal epithelial cells, we introduced a wound by scraping away IEC-6 cells before exposure to TNF-α. To exclude the increased effect of baicalin on the cell viability exposed to TNF-α, we used a low concentration of TNF-α without affecting cell viability. The wound healing assay and its quantitative assessment showed that when compared to the untreated control group, baicalin alone didn’t have significant difference on cell migration, and the motility of IEC-6 cells...
was significantly inhibited after stimulation with TNF-α and that the cells were not able to migrate to the wounded area (Fig. 2). When compared to this, the migration ability of the cells pretreated with baicalin was obviously enhanced (Fig. 2). Moreover, transfected with miR-191a inhibitor before baicalin and TNF-α treatment resulted in obvious increase in the migration of IEC-6 cells (Fig. 2), thus indicating that baicalin promotes the motility of IEC-6 cells exposed to TNF-α in wound-healing, and the mechanism may be associated with miR-191a inhibition.

**TNF-α Accelerates ZO-1 Depletion via miR-191a Expression**

Recent studies have shown TNF-α to be associated with ZO-1 protein expression and localization of TJs in Madin–Darby canine kidney (MDCK) and microvascular endothelial cells. In order to explore the mechanism of TNF-α induced injury in intestinal epithelial cells, we examined the impact of TNF-α on ZO-1 in IEC-6 cells. We found that treatment with TNF-α significantly decreased ZO-1 mRNA (Fig. 3B) and protein levels (Figs. 3C, D) when compared with the control.

ZO-1 is predicted to be one of the target genes of miR-191a by multiple miRNA database algorithm (TargetScan, miRanda, and miRDB) and is confirmed to be a target of miR-191a by luciferase reporter assay in vascular endothelial cells. In order to determine whether miR-191a targets ZO-1 in intestinal epithelial cells, we transfected IEC-6 cells with miR-191a mimic (over-expression) or inhibitor (inhibition). The expression of miR-191a and ZO-1 mRNA was measured by qRT-PCR and Western blot analysis following transfection. We found that transfection with miR-191a mimic resulted in an obvious increase in miR-191a level and a concomitant decrease in ZO-1 mRNA level when compared with the miR-inhibitor negative control. Conversely, in the miR-mimic negative control, transfection with the inhibitor resulted in a reduction in miR-191a level along with an increase in ZO-1 mRNA levels (Figs. 4A, B). Western blot analysis also showed an inverse correlation between the expression of miR-191a and ZO-1 protein level in IEC-6 cells (Figs. 4C, D). These results suggest that miR-191a over-expression is sufficient to cause depletion of ZO-1 protein inside the cells. In addition, miR-191a also functions at least in part through its direct target ZO-1.

To investigate the role of miR-191a in TNF-α-induced ZO-1 depletion, IEC-6 cells were exposed to TNF-α for 24h.

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**Fig. 4. Effect of Mimic or Inhibitor of miR-191a on ZO-1 mRNA and Protein Expression in IEC-6 Cells**

Cells were transfected with 20 nM miR-191a mimic, 200 nM miR-191a inhibitor, miR-mimic negative control or miR-inhibitor negative control for 24 h. (A) Expression levels of miR-191a after transfection as measured by qRT-PCR. (B) Expression level of ZO-1 mRNA after transfection as measured by qRT-PCR. (C) Expression of ZO-1 protein after transfection as detected by Western blot analysis. *p<0.05, **p<0.01.
The results showed that treatment with TNF-α produced a 4.62±0.16 fold increase in miR-191a expression (Fig. 3A). Moreover, both ZO-1 mRNA and protein levels were reduced significantly by TNF-α when compared with the control group (Figs. 3B, C). Treatment with 50 ng/mL TNF-α for 24 h decreased ZO-1 protein expression by 64.65% when compared to the control group (Fig. 3D). We then treated IEC-6 cells with miR-191a inhibitor or its mimics before exposure to TNF-α. We found that miR-191a inhibition suppressed TNF-α-induced ZO-1 repression, both at the mRNA and protein levels (Figs. 3C, D). In contrast, TNF-α-induced ZO-1 reduction in mRNA and protein levels was aggravated by miR-191a mimics (Figs. 3C, D). These results collectively suggested that miR-191a is an important mediator in the TNF-α-induced ZO-1 depletion pathway in intestinal epithelial cells.

**Baicalin Inhibits TNF-α-Induced ZO-1 Depletion by Down-Regulating miR-191a**

We further explored whether the protective effect of baicalin against TNF-α-induced injury was due to the impact of baicalin on the above-mentioned TNF-α-miR191a–ZO-1 pathway. We tested the level of ZO-1 after pre-treatment of IEC-6 cells with baicalin. Immunofluorescence staining showed that the overall level of ZO-1 was reduced with uneven distribution in cells treated with TNF-α only, whereas in cells pretreated with baicalin before TNF-α

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**Fig. 5. Effect of Baicalin on TNF-α-Induced ZO-1 Depletion and on miR-191a Mimic**

Control IEC-6 cells were left untreated, TNF-α group was treated with TNF-α (50 ng/mL) for 24 h only, baicalin cells were treated with baicalin (40 µg/mL) for 24 h only, TNF-α+baicalin cells were pretreated with 40 µg/mL baicalin for 1 h and then exposed to TNF-α for 24 h. TNF-α+miR-191a mimic cells were transfected with 20 nmol/L miR-191a mimic before exposure to TNF-α for 24 h. TNF-α+baicalin+miR-191a mimic cells were transfected with 20 nmol/L miR-191a mimic before pre-treatment of baicalin for 1 h, followed by TNF-α treatment for 24 h. MiR-mimic control was used as negative control. (A) Relative expression of miR-191a as measured by qRT-PCR. (B) Relative expression of ZO-1 mRNA as measured by qRT-PCR. (C) Protein levels of ZO-1 as detected by Western blot analysis. β-Actin was used as a loading control. *p<0.05, **p<0.01.
treatment the ZO-1 protein was evenly distributed (Fig. 6). These results suggested that ZO-1 protein is disorganized upon TNF-α stimulation in intestinal epithelial cells. Baicalin could inhibit this effect and restore ZO-1 protein to levels similar to the control even in the presence of TNF-α.

We found baicalin pretreatment significantly reversed the effect of TNF-α on ZO-1, both at the mRNA (Fig. 3B) and protein levels (Figs. 3C, D). We speculated that this effect may be due to direct stimulation of ZO-1 expression by baicalin. In order to test this hypothesis, we examined ZO-1 expression in the absence of TNF-α treatment. IEC-6 cells treated with baicalin alone showed no significant difference in ZO-1 protein expression level when compared with the untreated control, suggesting that baicalin exerted an indirect protective effect on ZO-1 from TNF-α stimulation.

We further explored the role of miR-191a in the above described effect of baicalin on TNF-α-induced ZO-1 depletion. miR-191a level was increased to 2.45±0.14 fold by TNF-α. Pretreatment with baicalin suppressed this effect (Fig. 3A). We then transfected miR-191a inhibitor or mimic into IEC-6 cells before baicalin and TNF-α treatment. miR-191a inhibitor significantly enhanced ZO-1 protein expression that was restored by baicalin (Figs. 3B–D). In contrast, miR-191a mimic significantly suppressed the baicalin mediated reversion of ZO-1 inhibition (Figs. 5B–D). Taken together, these data suggest that baicalin inhibits TNF-α-induced ZO-1 depletion, at least partly, by down-regulating miR-191a.

DISCUSSION

Defects in TJs of the inflamed mucosa result in increased intestinal permeability, which is an important mechanism of intestinal inflammatory bowel diseases.21,22) ZO-1 protein is a structurally essential component of TJs, which works as a scaffold and constitutes a major part of TJs.21,22) Previous studies have shown that loss of ZO-1 results in a significant increase in permeability of colonic mucosa that allows endotoxins to gain access to the intestine and results in significant intestinal inflammation in the mouse model of colitis.23) In the current study, cell viability and the level of ZO-1 protein was found to obviously decrease after TNF-α treatment (Figs. 1, 3, 4). Recent research indicated that TNF-α retards intestinal epithelial cell proliferation through suppression of β-catenin/T cell factor (TCF) signaling and induces apoptosis on network-level.24) Inhibition of ZO-1 blocks the cell cycle at G0/G1 phase, ZO-1 has been shown to increase cell cycle progression, regulate cell growth and proliferation by decreased p21 nuclear localization.25) Therefore, we speculate that TNF-α-induced ZO-1 depletion may result in reduced cell viability in IEC-6 cells. Moreover, baicalin improved TNF-α-reduced cell motility, but miR-191a inhibition didn’t obviously increase cell viability (Fig. 1D), it is possible that other target genes of miR-191 contribute to the suppression of ZO-1 in the mechanisms that baicalin protects against TNF-α-reduced cell viability.

TNF-α depletes ZO-1 protein from IEC-6 cells and changes the normal structure of ZO-1, and causes ZO-1 uneven distribution and fragmentation (Fig. 6). Poritz et al. reported that ZO-1 protein is disrupted by TNF-α in MDCK cells, but quantitation of ZO-1 and occludin didn’t show any significant differences with increasing concentration of TNF-α.7) Our results showed that TNF-α leads to ZO-1 depletion in normal enterocytes. These findings imply that the impact of TNF-α on TJs varies between cell types or species. Besides, the levels of ZO-1 mRNA and protein show a huge difference between TNF-α and TNF-α+baicalin group. One possible mechanism by which miRNAs silence mRNAs is by interfering with their translation, resulting in less efficient translation of the mRNA into protein.26) Hence decrease in miR-191a may be associated with a significant reduction in protein levels but very little effect on mRNA levels. It is worth noting that baicalin failed to decrease miR-191a expression in IEC-6 cells treated with TNF-α+baicalin+miR-191a mimic. Studies have reported that baicalin suppressed the level of hypoxia-inducible factor-1 (HIF-1).27,28) HIF-1 is binding to HRE5 and initiates miR-191 transcription.29) These suggest that baicalin didn’t act on mature miR-191a, it may regulate miR-191a expression by controlling transcription factors.

Cell migration is an important feature of epithelial wound healing.30) In this study, we found TNF-α exposure significantly suppressed IEC-6 cells migration that resulted in inhibition of wound closure (Fig. 2). This potentially leads to impaired wound healing and barrier functions, an observation consistent with a recent study in Caco2 cells.31) IEC-6 cells transfected with miR-191a inhibitor moved more quickly compared with cells without transfection before TNF-α (Fig. 2), ZO-1 is the target gene of miR-191a, ZO-1 protein actively regulates cell migration, the knockdown of ZO-1 greatly inhibits wound-

![Fig. 6. Effect of Baicalin on the Distribution of ZO-1 by Immunofluorescent Staining](image_url)

Scale bars, 25 µm. Control cells were left untreated, TNF-α group was treated with TNF-α (50 ng/mL); baicalin group were treated with baicalin (40 µg/mL) for 1 h before exposure to TNF-α for 24 h.
induced cell migration and the restoration of ZO-1 improves cell motility injured by knockdown of ZO-1.\textsuperscript{3,2} In addition, we show that baicalin inhibits TNF-\(\alpha\)-induced ZO-1 depletion by down-regulating miR-191a, miR-191a inhibition enhanced the TNF-\(\alpha\)-reduced IEC-6 cells migration improved by baicalin, to sum up, we can reason that baicalin protect against TNF-\(\alpha\)-reduced cell migration in IEC-6 cells, and keep the integrity of intestinal barrier with a mechanism involving suppression of miR-191a expression.

The miR-191a used in our study is a molecule that is abnormally expressed in more than 20 cancers and inflammatory bowel disease.\textsuperscript{3,3} We show that miR-191a targets and inhibits ZO-1 protein expression in IEC-6 cells (Fig. 4). Defects in TJs were found to promote translocation of bacteria through the intestinal mucosal barrier.\textsuperscript{3,4} ZO-1 and occludin proteins, which constitute TJs maintain the integrity and permeability between enterocytes.\textsuperscript{3,5} Our findings indicate that regulated expression of miR-191a may be a potential therapeutic target for intestinal permeability disorders involving defects in the TJ barrier.

Recent investigations have confirmed the anti-inflammatory effect of baicalin.\textsuperscript{3,6–3,8} In our previous work we demonstrated that recovery from LPS induced injury, especially of ZO-1 protein, is promoted in TJs selectively by baicalin.\textsuperscript{3,6} It also decreases the production of TNF-\(\alpha\) after LPS stimulation in IEC-6 cells.\textsuperscript{3,9} Orally administered baicalin inhibits the expression of TNF-\(\alpha\) in mice injected with LPS via regulating the activation of the transcription factor nuclear factor-kappaB (NF-\(\kappa\)B).\textsuperscript{3,9} Moreover, high levels of TNF-\(\alpha\) following LPS stimulation were associated with severe loss of ZO-1 in the ileum.\textsuperscript{4,0} Our results show that baicalin significantly restored the level of ZO-1 protein and reversed the increase in miR-191a caused by TNF-\(\alpha\) (Figs. 3, 5). These observations suggest a close relationship among TNF-\(\alpha\), miR-191a and ZO-1 in TNF-\(\alpha\)-injured IEC-6 cells. Therefore, we can reasonably deduce that the protective effect of baicalin in LPS-induced injury in enterocytes may be attributed to down-regulation of TNF-\(\alpha\) and miR-191a by baicalin, thereby increasing the expression of ZO-1 in intestinal epithelial cells. As a result, baicalin could preserve the barrier function of the enterocyte.

However, there are other questions that still remain to be investigated. For instance, other relationships may exist between TNF-\(\alpha\) and ZO-1. The signal pathways involved in baicalin and TNF-\(\alpha\)-induced TJs injury warrant further research. Notch-1 signal pathway is critical for barrier function, and loss of Notch-1 expression in enterocyte is associated with dysregulated expression and localization of TJs, thus affecting the function of the intestinal epithelium.\textsuperscript{3,1} It should be noted that in this study, the effects of baicalin have been examined in vitro. In order to confirm the mechanism by which baicalin exerts its protective effect on the intestinal barrier, further tests in vivo models are needed to be performed.

In conclusion, we show that baicalin exerts a protective effect against TNF-\(\alpha\)-induced injury by down-regulating miR-191a that targets the TJ protein ZO-1 in IEC-6 cells. Our findings describe a novel anti-inflammatory mechanism of baicalin. Since an important factor in intestinal inflammation is a defect in TJs barrier, miR-191a represents a potential therapeutic target for inflammatory bowel diseases.

**Conflict of Interest** The authors declare no conflict of interest.

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