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Echinacoside alleviated LPS-induced cell apoptosis and inflammation in rat intestine epithelial cells by inhibiting the mTOR/STAT3 pathway

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ABSTRACT

Inflammatory bowel disease (IBD) is a chronic and progressive inflammatory condition of colon and small intestine. Echinacoside (ECH) is a phenylethanoid glycoside that possesses various activities, including anti-inflammatory effect. However, the role of ECH in IBD is unknown. The present study aimed to evaluate the effect of ECH on LPS-induced rat intestine epithelial cells and the potential mechanisms. The results showed that LPS inhibited cell viability in time- and dose-dependent manners. ECH treatment attenuated the inhibition effect of LPS on cell viability. ECH alleviated LPS-induced apoptosis of rat intestine epithelial cells. ECH attenuated LPS-induced secretion and mRNA expression of TNF-α and IL-6, but enhanced LPS-induced secretion and mRNA expression of IL-10 and TGF-β1 in IEC-6 cells. The mTOR/STAT3 pathway was activated by LPS, while the activation was inhibited by ECH. Rapamycin, an inhibitor of mTOR, reversed the effect of LPS on rat intestine epithelial cells. In summary, this work suggested that ECH attenuated LPS-induced inflammation and apoptosis in rat intestine epithelial cells via suppressing the mTOR/STAT3 pathway. The findings indicated that ECH might be considered as a potential strategy for the treatment of IBD.

1. Introduction

Inflammatory bowel disease (IBD) is a chronic and progressive inflammatory condition of the colon and small intestine [1,2]. Crohn's disease (CD) and ulcerative colitis (UC) are the two principal types of IBD [3]. There are various clinical symptoms of IBD, such as abdominal pain, vomiting, diarrhea, rectal bleeding and anemia [3,4]. IBD is characterized by increased cytokine production and increased apoptosis of intestinal cells, and thus results in the destruction of intestinal epithelial barrier [5]. Therefore, inhibiting inflammatory response and cell apoptosis might be useful for protecting the intestinal epithelial barrier and be beneficial for IBD treatment.

IBD is characterized by a chronic idiopathic inflammation in intestine, and many pathways associated with inflammatory response are involved in the development and progress of IBD, such as mechanistic target of rapamycin (mTOR) [6] and activator of transcription (STAT) signaling pathways [7]. It was reported that the mTOR/STAT3 pathway plays important roles in inflammation and cell apoptosis [8]. On the other hand, the mTOR/STAT3 signaling was abnormally up-regulated in the colonic epithelial cells of patients with IBD [9], and inhibition the mTOR/STAT3 pathway ameliorated IBD in a mouse model of inflammatory bowel disease [10]. Therefore, the mTOR/STAT3 pathway is a potential therapeutic target for the treatment of IBD.

Echinacoside (ECH, Fig.1) is a phenylethanoid glycoside isolated from *Cistanche* herba, which is usually used for the treatment of constipation in China [11]. It has been reported that ECH possesses various activities, including anti-oxidative [12], anti-inflammatory [13], anti-tumor effect [14]. ECH stimulates cell proliferation and reduces apoptosis of intestinal epithelial cells, indicating that ECH might be useful for the treatment of digestive tract diseases [15]. However, the role of ECH in IBD and the potential mechanism remain unknown.

In the present study, we investigated the role of ECH in LPS-induced intestinal cells and we hypothesized that ECH inhibited LPS-induced inflammation and cell apoptosis via the mTOR/STAT3 pathway.

2. Materials and methods

2.1. Reagents

ECH (purity > 98%) was obtained from Sigma (St. Louis, MO, USA). Rapamycin was purchased from Sigma and dissolved in dimethyl sulphoxide (DMSO; Sigma). Annexin V-FITC apoptosis detection kit was purchased from BD Biosciences (Franklin Lakes, NJ, USA). The caspase-3 activity assay kit was purchased from Beyotime Biotechnology

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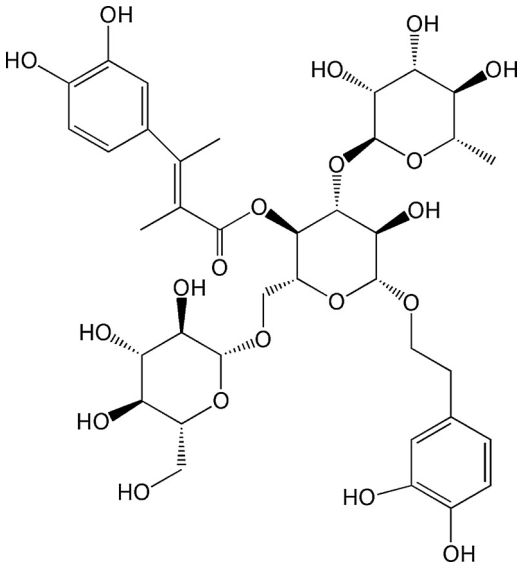

Fig. 1. The chemical structure of ECH.

(Shanghai, China). The enhanced BCA protein assay kit was purchased from Beyotime Biotechnology. The primary antibodies against phospho-mTOR (p-mTOR; Ser2448) and mTOR were obtained from Abcam (Cambridge, MA, USA). The primary antibodies against phospho-ribosomal protein S6 (p-S6; Ser235/236), ribosomal protein S6 (S6), phospho-STAT3 (p-STAT3; Tyr705), STAT3 and β-actin were obtained from Cell Signaling Technology (Beverly, MA, USA). The secondary antibody was obtained from Cell Signaling Technology. The enhanced chemiluminescence system was purchased from GE healthcare (CA, USA). The ELISA kits for determination of TNF-α, IL-6, IL-10, and TGF-β1 were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.2. Cell culture

Rat intestinal cell line IEC-6 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). IEC-6 cells were cultured in DMEM supplemented with 10% (v:v) FBS, 10 U/ml insulin, and 100 U/ml penicillin and streptomycin, and maintained at 37 °C in a 5% CO_2 environment.

2.3. MTT assay

Cell viability of IEC-6 cells was measured by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The IEC-6 cells (1×10^5/ml) were seeded into a 96-well plate and incubated under different conditions. After incubation, 20 μl MTT solution (5.0 mg/ml) was added and incubated for 4 h. The formazan crystals were dissolved by adding 150 μl DMSO. Finally, the absorbance at 490 nm was measured using an EnSpire Multimode Plate Reader (PerkinElmer, Waltham, MA, USA). The experiments were repeated thrice in three replicates.

2.4. Flow cytometry

The apoptosis rate of IEC-6 cells was detected by flow cytometry. After treatment with ECH (10 or 20 μg/ml) or rapamycin (100 nM) in the presence of LPS (l μg/ml) for 24 h, cells were collected and stained with fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide (PI) for 15 min at room temperature in the dark. Finally, cells were analyzed by flow cytometry (BD Biosciences) according to the manufacturer's instructions. The experiments were repeated three times.

2.5. Detection of caspase-3 activity

The activity of caspase-3 was measured by caspase-3 activity assay kit according to the manufacturer's instruction. Briefly, cells were collected and lysed, and the substrate Ac-DEVD-pNA (2 mM) was added and incubated at 37 °C for 2 h. Finally, the absorbance at 405 nm was measured using an EnSpire Multimode Plate Reader. The experiments were repeated three times.

2.6. Quantitative real-time RT-PCR (qRT-PCR)

Total RNA was extracted from IEC-6 cells with Trizol reagent (Invitrogen). and used for first-strand cDNA synthesis by reverse transcription with a Transcriptor First Strand cDNA Synthesis Kit (Takara, Dalian, China). The gene expression was tested by qRT-PCR using SYBR PremeScript miRNA RT-PCR kit (Takara). The expression of mRNA was normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers were as follows: TNF-α, forward 5'-ACTG AACT TCGG GGTG ATTG-3', reverse 5'-GCTT GGTG GTTT GCTA CGAC-3'; IL-6, forward 5'-GGCT AAGG ACCA AGAC CATC CAA-3', reverse 5'-TCTG ACCA CAGT GAGG AATG TCCA-3'; IL-10, forward 5'-GTTG CCAA GCCT TGTC AGAA A-3', reverse 5'-TTTC TGGG CCAT GGTT CTCT-3'; TGF-β1, forward 5'-GACC GCAA CAAC GCAA TCTA-3', reverse 5'-AGGT GTTG AGCC CTTT CCA-3'; GAPDH, forward 5'-TGAA GCAG GCAT CTGA GGG-3', reverse 5'-CGAA GGTG GAAG AGTG GGAG-3'. Relative expression levels of target mRNAs were calculated by the $2^{-\Delta\Delta Ct}$ method. The experiments were repeated three times.

2.7. Western blot

After treatment with ECH (10 or 20 μg/ml) or rapamycin (100 nM) in the presence of LPS (l μg/ml) for 24 h, cells were lysed using radioimmunoprecipitation assay buffer (Beyotime). The protein concentration was measured using BCA assay. Protein samples were separated using 12% SDS-PAGE gel and transferred onto nitrocellulose membranes. Then the non-specific bindings were blocked using 5% nonfat milk at 37 °C for 1 h. The membranes were incubated with primary antibodies against p-mTOR (Ser2448), p-S6 (Ser235/236), S6, mTOR, p-STAT3 (Tyr705), STAT3 and β-actin at 4 °C overnight, and then incubated with peroxidase-conjugated secondary antibody at 37 °C for 1 h. Finally, the bands were visualized using the enhanced chemiluminescence kit. The experiments were repeated three times.

2.8. ELISA

After treatment with ECH (10 or 20 μg/ml) or rapamycin (100 nM) in the presence of LPS (l μg/ml) for 24 h, cell culture supernatants were collected for determination of pro-inflammatory and anti-inflammatory cytokine secretion. The secretion levels of TNF-α, IL-6, IL-10, and TGF-β1 from IEC-6 cells were measured using commercial ELISA kits. The experiments were repeated thrice in three replicates.

2.9. Statistical analysis

Data are presented as mean ± standard deviation (SD). Data analysis was examined by one-way ANOVA or Student's *t* test using SPSS version 19.0. A *p* value less than 0.05 was considered statistically significant.

3. Results

3.1. LPS suppressed cell viability of IEC-6 cells

To evaluate the effect of LPS on cell viability, IEC-6 cells were incubated with LPS at a series of concentrations (0, 0.25, 0.5, 1, 2, and 4 μg/ml) for 24 h. The results in Fig. 2A showed that LPS inhibited cell