Gallic acid attenuates LPS-induced inflammation in Caco-2 cells by suppressing the activation of NF-κB/MAPK signaling pathway

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| Keywords: | gallic acid, inflammatory bowel disease, tight junction proteins, cell apoptosis, oxidative stress, NF-κB/MAPK pathway |
Figure 1

A

Cell Viability (%)

LPS (μg/mL)

B

Cell Viability (%)

LPS (μg/mL)

C

Cell Viability (%)

GA (μg/mL)

D

Cell Viability (%)

GA (μg/mL)
Figure 2

A  inos  Relative mRNA levels

B  il-6  Relative mRNA levels

C  tnf-α  Relative mRNA levels

D  il-1β  Relative mRNA levels

E  il-10  Relative mRNA levels

F  tgf-β1  Relative mRNA levels

G  tgf-β2  Relative mRNA levels

H  TLR4 and β-actin

I  Relative intensity

Legend:
- Con
- LPS
- LPS+GA (1 μg/mL)
- LPS+GA (5 μg/mL)
- LPS+GA (10 μg/mL)
Figure 3

A. Claudin-1

B. Occludin

C. ZO-1

D. Claudin-1

Occludin

ZO-1

β-actin

Con  LPS  LPS+GA (1 μg/mL)  LPS+GA (5 μg/mL)  LPS+GA (10 μg/mL)

E. Claudin-1

F. Occludin

G. ZO-1

H. DAPI  Claudin-1  Merge

Con  LPS  LPS+GA
Figure 5

A

Con

LPS

LPS+GA (1 μg/mL)

LPS+GA (5 μg/mL)

LPS+GA (10 μg/mL)

B

ROS level

fluorescence intensity

C

D

E

SOD (U/mg prot)

GSH (U/mg prot)

CAT (U/mg prot)

Legend:

- Con
- LPS
- LPS+GA (1 μg/mL)
- LPS+GA (5 μg/mL)
- LPS+GA (10 μg/mL)
Figure 6

A

p65

p-p65

Con  LPS  LPS+GA

B

IκB-α

p-IκB-α

Con  LPS  LPS+GA

**  #  **  #
Figure 7

A

p38

\[
\text{Con} \quad \text{LPS} \quad \text{LPS+GA}
\]

p-p38

B

JNK

\[
\text{Con} \quad \text{LPS} \quad \text{LPS+GA}
\]

p-JNK

C

ERK

\[
\text{Con} \quad \text{LPS} \quad \text{LPS+GA}
\]

p-ERK

\[
\text{Con} \quad \text{LPS} \quad \text{LPS+GA}
\]
Gallic acid attenuates LPS-induced inflammation in Caco-2 cells by suppressing the activation of the NF-κB/MAPK signaling pathway

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Abstract

Inflammatory bowel disease (IBD) is a chronic inflammatory disease characterized by intestinal barrier dysfunction, inflammatory synergistic effects and excessive tissue injury. Gallic acid (GA) is renowned for its remarkable biological activity, encompassing anti-inflammatory and antioxidant properties. However, the underlying mechanisms by which GA protects against intestinal inflammation have not been fully elucidated. The aim of this study is to investigate the effect of GA on the inflammation of a lipopolysaccharide (LPS)-stimulated human colon carcinoma cell line (Caco-2), on intestinal barrier dysfunction, and on the underlying molecular mechanism involved. Our findings demonstrate that 5 μg/mL GA restores the downregulation of the mRNA and protein levels of Claudin-1, Occludin, and ZO-1 and decreases the expression of inflammatory factors such as IL-6, IL-1β and TNF-α.
induced by LPS. In addition, GA exhibits a protective effect by reducing the LPS-enhanced early and late apoptotic ratios, downregulating the mRNA abundance of pro-apoptotic factors (Bax, Bad, Caspase-3, Caspase-8, and Caspase-9), and upregulating the mRNA levels of anti-apoptotic factor Bcl-2 in Caco-2 cells. GA also reduces the fluorescence intensity of reactive oxygen species increased by LPS and restores the activity of antioxidant enzymes, namely, superoxide dismutase, catalase and glutathione. More importantly, GA exerts its anti-inflammatory effects by inhibiting the LPS-induced phosphorylation of key signaling molecules in the NF-κB/MAPK pathway, including p65, IκB-α, p38, JNK, and ERK, in Caco-2 cells. Overall, our findings show that GA increases the expressions of tight junction proteins, reduces cell apoptosis, relieves oxidative stress and suppresses the activation of the NF-κB/MAPK pathway to reduce LPS-induced intestinal inflammation in Caco-2 cells, indicating that GA has potential as a therapeutic agent for intestinal inflammation.

**Keywords**: gallic acid, inflammatory bowel disease, tight junction protein, cell apoptosis, oxidative stress, NF-κB/MAPK pathway

**Introduction**

Inflammatory bowel disease (IBD), encompassing Crohn’s disease (CD) and ulcerative colitis (UC), manifests chronic inflammation and dysplasia of epithelial barrier function [1,2]. An increasing incidence of IBD has been reported worldwide. Due to shifts in diet and lifestyle, the prevalence of IBD has been steadily increasing, especially in developing countries, in recent years. [3,4]. The pathogenesis of IBD may be related to the interaction of genetic, immune, infectious and psychiatric factors [5,6]. The damage mediated by inflammation disrupts tight junction (TJ) proteins and increases paracellular permeability to macroles and antigens [7,8]. Long-
term damage to TJ proteins may lead to IBD [9,10]. Consequently, remission of IBD necessitates a decrease in inflammatory responses and reinforcement of intestinal barrier integrity [11].

*In vivo* animal models are too complex to control many gut processes alone. Thus, new treatments for IBD have been developed in a variety of cell models to simulate intestinal inflammation. Caco-2 cells have emerged as valuable tools for studying intestinal diseases [12,13]. Various drugs, such as 5-aminosalicylic acid drugs, steroids and immunosuppressants [14], have been used to dampen IBD. However, the clinical treatment efficacy is unsatisfactory, and the side effects of long-term use are serious [14]. Therefore, there is an urgent need for some mild and effective treatment alternatives. Many studies have reported that plant phenols, including sinapic acid, have garnered increased amounts of attention due to their antioxidant and anti-inflammatory properties and minimal harm to human health [15–19].

Gallic acid (GA), a plant polyphenol, is a naturally produced secondary metabolite that is present in a variety of fruits, plants, vegetables and nuts, such as strawberries, green tea and oak bark [20,21]. GA has a low molecular weight and triphenolic structure, endowing it with potent anti-inflammatory and antioxidant capabilities [22]. The phenol hydroxyl group of GA can eliminate reactive oxygen species (ROS) and interrupt the cycle of new free radical formation. These compounds act as antioxidants that inhibit the oxidation of DNA, proteins, lipids and enzymes linked to the production of free radicals [23–25]. GA has anti-inflammatory effects by reducing proinflammatory mediators, inhibiting the expressions of nuclear transcription factors and downregulating downstream inflammatory targets [26,27]. In addition to its anti-inflammatory effects, GA has been shown to have pharmacological effects on tumors, diabetes, and obesity [27–29].
Despite its proven efficacy in treating various inflammation-related diseases, the effects and mechanism of action of GA in IBD remain unexplored. Therefore, Caco-2 cells were used as \textit{in vitro} models to explore the effects of GA on the inflammatory response induced by lipopolysaccharide (LPS) and to investigate the possible underlying mechanisms involved.

**Materials and Methods**

**Reagents**

GA and LPS were obtained from Sigma-Aldrich (St Louis, USA). Assay kits for the detection of superoxide dismutase (SOD), glutathione (GSH) and catalase (CAT) were obtained from Jiangcheng Institute of Bioengineering (Nanjing, China). Antibodies against Claudin-1, Occludin, IL-1β and Toll-like receptor 4 (TLR4) were obtained from Cell Signaling Technology (Beverly, USA). NF-κB, IκB-α, p38, JNK, and ERK, as well as the abovementioned antibodies after phosphorylation, were purchased from Abcam (Cambridge, USA).

**Cell culture**

Caco-2 cells obtained from the National Collection of Authenticated Cell Cultures (Shanghai, China) were cultured in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) (Biological Industries, Kibbutz Beit Haemek, Israel) supplemented with 1% penicillin-streptomycin (Beyotime, Haimen, China) and 10% fetal bovine serum (HAKATA, Shanghai, China). The cells were subsequently replenished in 60.8 cm² cell culture dishes (BioFil, Guangzhou, China). Caco-2 cells were cultivated in an atmosphere of 5% CO₂ at 37°C. The medium was replaced every 1–2 days. Then, the
cells were subcultured at 80% confluence utilizing 0.25% trypsin-EDTA solution (Biological Industries).

**Cell viability assay**

The cytotoxicity of GA and LPS to Caco-2 cells was evaluated by using a Cell Counting Kit-8 (CCK-8) assay (Beyotime). Caco-2 cells were cultivated in 96-well plates at a density of 1–2×10⁴/well. The cells were incubated with LPS at various concentrations (1, 5, 10, 50, and 100 μg/mL) within 4 and 24 h when 70%–80% of the Caco-2 cells adhered to each well. The cells were treated with GA at different concentrations (1, 5, 10, 20, 40, and 50 μg/mL) for 24 and 48 h respectively. After that, 100 μL of serum-free DMEM supplemented with high glucose and 10% CCK8 was added to each well for a period of 1–2 h. A microplate reader was used to measure the absorbance at 450 nm.

**Immunofluorescence staining**

Caco-2 cells were inoculated on coverslips in a 24-well plate. When the preliminary dosing treatment was completed, the cell samples were fixed with 4% paraformaldehyde at 4°C overnight. In the following day, each well was rinsed with PBS and infiltrated with 1 mL of 0.1% Triton X-100/PBS for 15–20 min. Then, the cells were sluiced 3 times utilizing PBS and blocked with 5% FBS/PBS. After rinsing once again with PBS, the specimens were incubated with primary antibodies overnight at 4°C. The following day, each well was rinsed with 1% FBS/PBS 3 times and incubated with Alexa Fluor® 488-labelled secondary antibodies for 1 h. After the last rinse with 1% FBS/PBS, the Caco-2 cells were observed using a fluorescence microscope. At least 5 visual fields were randomly selected for each slide for imaging.
Quantitative reverse-transcription (qRT)-PCR

After the initial treatment, the collected cell samples were lysed, and total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, USA). The RNA extracted from the above specimens was reverse-transcribed into cDNA with reverse transcriptase. Subsequently, the cDNA was subject to qPCR. qRT-PCR analysis was performed via a CFX Connect™ Real-Time System (Bio-Rad Laboratories, Hercules, USA) with the following procedure: 1 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The total volume of every reaction was 10 μL in a 96-well plate, which included 1 μL of cDNA, 5 μL of 2× ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China), and 0.4 μL of forward and reverse primers, and was subsequently supplemented with double distilled water to 10 μL. The primers used were synthesized by Sangon Biotech (Shanghai, China) and presented in Supplementary Table S1.

Western blot analysis

RIPA lysis buffer was used for extraction of total protein from Caco-2 cells. For protein quantification in subsequent experiments, the protein concentration was detected using the Pierce BCA protein assay kit (Thermo Fisher Scientific, Shanghai, China). Then, 20–30 μg of protein was loaded and separated via SDS-PAGE for 50–60 min and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, USA). The proteins were blocked with target bands using Protein Free Rapid Blocking Buffer (Epizyme, Shanghai, China) at room temperature for 15 min, and specific primary antibodies were applied to the membranes after immunoblotting at 4°C overnight. After 3 washes with Tris-buffered
saline supplemented with Tween® 20 (TBST), all the membranes were incubated for 1 h at room temperature with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. After 3 rinses, the bands were visualized via enhanced chemiluminescence (ECL) reagent (Haoke, Hangzhou, China) and quantified via ImageJ Pro-Plus 6.0.

**Intracellular ROS concentration**

The cell suspension was homogeneously added to several 24-well plates. Once the cells had adhered to each well and reached 70%–80% confluence, the mixture was pretreated with varying concentrations of GA (1, 5, and 10 μg/mL) for a period of 24 h. Then, Caco-2 cells were stimulated with LPS for 4 h. The diluted DCFH-DA (10 μM) was added at 37°C for 30 min. The ROS detection kit (Beyotime) was used for detection. A fluorescence microscope was used to discern and photograph the slides.

**Determination of antioxidant parameters**

Kits (Jiancheng Bioengineering) were used to detect the activities of SOD, CAT and GSH via the addition of hydroxylamine, visible light and xanthopterin-oxidase-Verfahren, respectively.

**Determination of cell apoptosis**

Apoptosis in the drug-treated cells was detected by a FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, Franklin Lakes, USA). Unpolluted and sterile 6-well plates were used to inoculate Caco-2 cells, which were allowed to spread more than 70%–80% of the cells in each well. The Caco-2 cells were pretreated with GA for 24 h, and then, additional LPS stimulation was applied to the cells for 4 h. Caco-2 cells
were trypsinized, flushed twice with cold PBS and then resuspended in fixative buffer. A culture tube was filled with 100 μL of the solution. FITC-conjugated Annexin V (5 μL) and PI (5 μL) were added to the mixture, which was subsequently incubated at 37°C for 15 min in the dark. Finally, the culture tube was supplied with a total of 1 μL of energy-coupled dye solution, after which the rate of cell apoptosis was tested using flow cytometry.

**Statistical analysis**

The data were presented as the mean±SEM. All the statistical analyses were performed with GraphPad Prism version 7.0 software. Two-way ANOVA and Tukey’s test were used to examine the differences among the control and other treatment groups. A P value less than 0.05 indicated statistical significance.

**Results**

**Effects of LPS and GA on Caco-2 cell viability**

The viability of Caco-2 cells treated with LPS for 4 and 24 h at various concentrations (0, 1, 5, 10, 50, and 100 μg/mL) is shown in Figure 1A,B. The cell viability decreased when the LPS concentration was greater than 10 μg/mL, and it decreased more significantly at 4 h than at 24 h. Thus, in subsequent experiments, the cells were treated with 10 μg/mL LPS for 4 h. GA treatment at 20, 40 and 50 μg/mL for 24 and 48 h resulted in a substantial reduction in cell viability, while 1, 5, and 10 μg/mL GA exhibited no cytotoxicity to Caco-2 cells (Figure 1C,D). Therefore, we selected GA at doses of 1, 5, and 10 μg/mL for 24 h for subsequent experiments.
GA protected against the LPS-induced cellular inflammatory response in Caco-2 cells

Compared to those in the control group, the *inos*, *il-6*, *tnf-α* and *il-1β* mRNA levels in the LPS treatment group were significantly greater (Figure 2A–D). Notably, GA at concentrations of 1, 5, and 10 μg/mL effectively mitigated the LPS-induced increase in the expression of these proinflammatory genes. As shown in Figure 2E–G, LPS decreased the mRNA levels of anti-inflammatory cytokines containing *il-10*, *tgf-β1* and *tgf-β2*. GA (5 and 10 μg/mL) rescued the inhibition of these cytokines induced by LPS. The protein level of TLR4 was upregulated by LPS, and different concentrations of GA (1, 5 and 10 μg/mL) markedly inhibited the increase in TLR4 expression (Figure 2H,I). All the data revealed that 5 and 10 μg/mL GA strongly inhibited the LPS-induced inflammatory response in Caco-2 cells.

GA alleviated intestinal barrier function against LPS-induced damage

Claudin-1, Occludin, and ZO-1, crucial members of the TJ protein family, play indispensable roles in maintaining intestinal barrier function. Similarly, the mRNA levels of *Claudin-1*, *Occludin* and *ZO-1* in Caco-2 cells visibly decreased in response to LPS treatment, while 5 and 10 μg/mL GA increased the mRNA levels of these proteins in LPS-induced Caco-2 cells (Figure 3A–C). Treatment with 5 or 10 μg/mL GA markedly enhanced the protein expressions of Claudin-1 and Occludin (Figure 3E,F). However, a significant increase in the protein expression of ZO-1 was observed only with 5 μg/mL GA (Figure 3H). Consistent with the gene expression data, GA restored the LPS-induced reduction in the expressions of these TJ proteins (Figure 3D–G). Immunofluorescence staining revealed that the expression level of Claudin-1 in the LPS + GA group was prominently higher than that in the separate
LPS group (Figure 3H). These consequences implicated that GA could alleviate LPS-induced intestinal barrier dysfunction.

**GA alleviated LPS-induced Caco-2 cell apoptosis**

Compared to those in the control group, the percentages of early apoptotic cells and late apoptotic cells were elevated nearly threefold by LPS treatment. However, treatment with 5 μg/mL GA effectively reduced the percentage of apoptotic cells and restored the percentage to levels comparable to those in the control group (Figure 4A). According to the flow cytometry results, LPS induced Caco-2 cell apoptosis, and 5 μg/mL GA alleviated this apoptosis (Figure 4A). The mRNA expression levels of Caspase-3, Caspase-8, Caspase-9, Bad and Bax increased in response to LPS stimulation (Figure 4B–F), while GA counteracted the LPS-induced upregulation of these genes. The expression of genes corresponding to anti-apoptotic factors, including Bcl-2, exhibited the opposite trend (Figure 4G).

**Effects of GA on ROS levels and antioxidant parameters in Caco-2 cells**

In contrast to that in the control group, the fluorescence intensity of ROS in the LPS group dramatically increased, and this increase was alleviated by GA treatment in Caco-2 cells. Supplementation with 5 or 10 μg/mL GA dramatically reduced the fluorescence intensity of the ROS (Figure 5A). As shown in Figure 5B, LPS significantly reduced SOD activity. The addition of 5 or 10 μg/mL GA led to a significant increase in SOD activity and in CAT and GSH levels (Figure 5C–E). These results suggested that GA could alleviate oxidative stress induced by LPS in Caco-2 cells.
GA regulated intestinal inflammation by inhibiting LPS-induced NF-κB activation

To elucidate the molecular mechanism through which GA regulates inflammation, we investigated its impact on the NF-κB signaling pathway. LPS treatment obviously enhanced the phosphorylation of p65 and IκB-α; however, the phosphorylation of p65 and IκB-α was significantly decreased by pretreatment with 5 μg/mL GA for 24 h in Caco-2 cells (Figure 6). Owing to the suppressive influence of GA on the phosphorylation of p65 and IκB-α, GA may indeed reduce cellular inflammation by inhibiting the NF-κB pathway.

Effect of GA on the MAPK signaling pathway

MAPK is involved in another signaling pathway that participates in the modification of proinflammatory cytokine expression during inflammation [30]. Therefore, we next assessed the p38, JNK and ERK protein levels through western blot analysis. Our results showed that LPS stimulation markedly enhanced the phosphorylation of p38, JNK and ERK in Caco-2 cells, while 5 μg/mL GA weakened the LPS-induced phosphorylation of p38, JNK and ERK (Figure 7), indicating that MAPK was involved in the regulatory effect of GA on the inhibition of intestinal inflammation induced by LPS.

Discussion

GA, characterized by its phenolic and carboxylic acid properties, can potentially mitigate inflammation-related diseases by modulating redox status and regulating the intestinal microbiota [26,31]. In vivo studies have affirmed the anti-inflammatory effects of GA in conditions such as obesity, diabetes, and colitis [27,29,32]. Studies
have shown that its main anti-inflammatory mechanisms include suppressing the activation of factors involved in the transcription and transduction of signals, reducing the expression of inflammatory mediators and inhibiting the phosphorylation or transfer of p65-NF-κB [33–35]. In vitro studies, for example, Li et al. [35], reported that GA significantly reduced ROS in RAW264.7 macrophages induced by LPS. These findings highlighted the potent anti-inflammatory properties of GA, as evidenced by its ability to effectively inhibit the activation of the NF-κB pathway in LPS-activated macrophages, leading to a reduction in inflammatory factors such as iNOS, IL-6, and TNF-α [35]. Sripanidkulchai et al. [36] revealed that GA, as the main component of the extractive of Phyllanthus emblica Linn., could restrain the gene expression of COX-2, iNOS, and IL-6 in RAW 264.7 cells [36]. These explorations collectively underscore the potential of GA in alleviating inflammation, suggesting its applicability in the treatment of intestinal diseases. In this study, 5 μg/mL GA was shown to reduce LPS-induced inflammation through regulating TJ protein expressions, inhibiting apoptosis, reducing the generation of ROS, and suppressing the activation of the NF-κB/MAPK pathway in Caco-2 cells. These results further contribute to the growing body of evidence supporting the therapeutic potential of GA in treating intestinal inflammation.

LPS, a component of the outer wall of gram-negative bacteria cells, functions by binding to TLR4, ultimately promoting inflammation [37,38]. LPS can also disrupt the intestinal barrier by regulating inflammatory responses and reducing the expression of TJ proteins [38,39]. TJ proteins, including Claudin, Occludin and ZO-1, play pivotal roles in maintaining intestinal barrier integrity [40]. TJ proteins are critical for conserving the intestinal barrier architecture and regulating the paracellular diffusion of ions and solutes [41]. Studies have linked the decreased expression of
Claudin-1, Occludin, and ZO-1 with gastrointestinal disorders where the intestinal barrier is compromised [42,43]. The damaged intestinal barrier allows some bacteria and antigens to cross the intestinal barrier and stimulate inflammation [44]. Studies have shown that colonic biopsy of patients with IBD results in decreased expression of TJ proteins, indicating that the regulation of TJ proteins may improve intestinal barrier integrity to reduce the entry of bacteria and other substances into the gut that promote intestinal inflammation[45]. In our study, GA administration counteracted the downregulation of Claudin-1, Occludin and ZO-1 induced by LPS. These immunofluorescence results further confirmed that GA alleviated the LPS-induced reduction in Claudin-1 expression. These findings suggest that GA can potentially alleviate LPS-induced intestinal barrier dysfunction, thereby protecting the integrity of the intestinal barrier.

These data suggest that apoptosis is a key characteristic of the cellular response to LPS. Intestinal barrier damage induced by LPS was associated with increased apoptosis [46,47]. To prospect the potential mechanism through which GA repairs intestinal injury and alleviates inflammation, we further investigated the regulatory effect of GA on cell apoptosis. In the present study, the expressions of Bax, Bad, Caspase-3, Caspase-8, and Caspase-9 increased in response to LPS, and GA inhibited the upregulation of these genes induced by LPS. Moreover, GA rescued the inhibitory effect of LPS on the expression of anti-apoptotic factors such as Bcl-2. Flow cytometry revealed that the number of early and late apoptotic cells increased following LPS exposure, while GA treatment dramatically decreased the proportion of apoptotic cells. Taken together, these results indicate that GA inhibited Caco-2 cell apoptosis induced by LPS.
LPS-induced ROS production is associated with the initiation of apoptosis, and drugs that prevent LPS-induced cell apoptosis also inhibit ROS formation [46]. ROS are recognized factors involved in the pathogenesis of ulcerative colitis (UC), and antioxidants are commonly employed in UC treatment [14,48]. Naturally occurring GA has strong antioxidant activity and can inhibit apoptosis and play a defensive role in organisms [49,50]. In the present study, GA dramatically reduced the production of ROS induced by LPS. ROS accumulation can trigger NF-κB, JNK, P38 and other signaling pathways to enhance inflammation and lead to increased expressions of a series of proinflammatory mediators. Taken together, these results suggest that GA has a strong antioxidant effect and may play a protective role against LPS-induced inflammation by regulating oxidative stress.

TLR4 is an LPS reporter that is able to immediately induce inflammation through activating diverse downstream signaling pathways [51]. Patients with active ulcerative colitis often exhibit high levels of TLR4 expression in the intestinal epithelium, indicating the potential involvement of TLR4 in the progression of UC [52]. The ultimate transcription factor in the TLR4 signaling pathway is NF-κB [52]. Numerous previous explorations have confirmed that the NF-κB signaling pathway persistently plays a considerable role in the development of colitis by regulating the transcription and translation of inflammatory mediators [53]. LPS triggers IκB-α phosphorylation or ubiquitination, leading to IκB-α degradation and enabling NF-κB activation and migration from the cytoplasm to the nucleus. Subsequently, target genes of NF-κB, including IL-1β, TNF-α, and iNOS, are activated [53–55]. Pandurangan et al. [56] revealed that GA potentially plays a clinical anti-inflammatory role by restraining the activation of p65-NF-κB and IL-6/p-STAT3Y705. Zhu et al. [57] reported that GA suppressed the expression of the proinflammatory cytokines IL-1/6, TGF-β and TNF-
α and promoted the emission of the anti-inflammatory cytokine IL-4/10 through suppression of the IκB/NF-κB pathway. Most of the above studies demonstrated the impact of GA on colitis through NF-κB in mice. In our study, we investigated the effects of GA on Caco-2 cells and found that the phosphorylation of the p65 and IκB-α proteins induced by LPS was significantly downregulated after pretreatment with 5 μg/mL GA for 24 h. These results indicate that GA rescued LPS-induced inflammation through suppressing the activation of the NF-κB signaling pathway.

MAPKs are a family of serine/threonine protein kinases, including P38, JNK and ERK, that regulate gene expression, cell proliferation, differentiation and other functions and are implicated in the inflammatory response to LPS stimulation [58,59]. MAPKs also participate in regulating the transcriptional activity of the NF-κB signaling pathway. For example, P38 and JNK can induce the degradation of IκB-α [58,60]. LPS-stimulated cells can activate ERK, JNK and p38 and subsequently act on their substrates to affect the activity of various transcription factors, thus regulating the expression of diverse cytokines, including TNF, IL-6 and IL-8. Therefore, it can trigger an inflammatory response in the colon through ERK, JNK and p38 [61,62]. In previous studies, several herbal ingredients, such as Ganoderma lucidum polysaccharide, were shown to relieve colitis through the MAPK signaling pathway [63]. Consequently, we wondered whether GA also has potential therapeutic effects on these patients. In the present study, pretreatment with 5 μg/mL GA markedly inhibited the activation of JNK, ERK and p38 caused by LPS. These results indicated that GA prevents the activation of the MAPK signaling pathway stimulated by LPS in Caco-2 cells, thereby exerting anti-inflammatory effects.

In summary, this study revealed that GA significantly reduced the reduction in TJ proteins, inflammatory response, ROS production and apoptosis induced by LPS in
Caco-2 cells by inhibiting the activation of the NF-κB and MAPK pathways. Our findings revealed that GA is a promising agent for the treatment of patients with IBD.

Supplementary Data
Supplementary data are available at *Acta Biochimica et Biophysica Sinica* online.

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Conflicts of Interest
All the authors declare that they have no conflict of interest.
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Figure legends

Figure 1. The influence of different concentrations of LPS and GA on Caco-2 cell viability After different concentrations of LPS were added to 96-well plates and co-incubated with Caco-2 cells for (A) 4 h and (B) 24 h, the cell viability of the respective groups was inspected by a CCK-8 assay. Cell viability was assayed after Caco-2 cells were cultivated with GA at different concentrations for (C) 24 h and (D) 48 h. The data are presented as the mean ± SEM of 6 separate experiments. Significant differences were found between means with different letters as determined by one-way ANOVA, *P<0.05 and **P < 0.01 compared to the control. GA: gallic acid.

Figure 2. GA inhibited LPS-induced inflammation in Caco-2 cells Caco-2 cells pretreated with or without 1, 5 or 10 μg/mL GA for 24 h were exposed to 10 μg/mL LPS for an additional 4 h. qRT-PCR was utilized to measure the mRNA levels of inflammatory cytokines, such as (A) inos, (B) il-6, (C) tnf-α, (D) il-1β, (E) il-10, (F) tgf-β1, and (G) tgf-β2. The data were standardized to the mRNA expression of β-actin. (H) TLR4 protein expression was assessed via western blot analysis. (I) Relative levels of TLR4 proteins. The strips were quantified, and the values were standardized to the β-actin levels. The values are shown as the mean ± SEM, *P<0.05 and **P<0.01 contrast to the control. #P<0.05 and ##P<0.01 contrast with the LPS group. GA: gallic acid.

Figure 3. GA protected intestinal barrier function against LPS-induced damage Caco-2 cells were first treated with or without 1, 5, or 10 μg/mL GA for 24 h, followed by 10 μg/mL LPS for 4 h. RT-qPCR was used to measure the mRNA expressions of (A) Claudin-1, (B) Occludin, and (C) ZO-1, and the data were normalized to β-actin expression. (D) Western blot analysis was used to detect the expression of TJ proteins. Relative levels of (E) Claudin-1, (F) Occludin, and (G) ZO-1. (H) Immunofluorescence staining for Claudin-1 (green) and cell nuclei (blue). The values are shown as the mean ± SEM, *P<0.05 and **P<0.01 contrast with the control. #P<0.05 and ##P<0.01 contrast to the LPS group. GA: gallic acid.

Figure 4. GA alleviated LPS-induced Caco-2 cell apoptosis (A) Caco-2 cells were first treated with 5 μg/mL GA and then with 10 μg/mL LPS in 6-well plates,
subsequently stained with Annex V-FITC and analysed by flow cytometry. qRT-PCR was used to quantify the mRNA expression levels of (B) Caspase-3, (C) Caspase-8, (D) Caspase-9, (E) Bax, (F) Bad, and (G) Bcl-2. The values are presented as the mean ± SEM, n=6. *P<0.05 and **P<0.01 contrast with the control. #P<0.05 and ##P<0.01 contrast to the LPS group. GA: gallic acid.

Figure 5. GA alleviated LPS-induced cellular oxidative damage  
Fluorescence imaging showing the level of ROS after treatment with different concentrations of GA and subsequent treatment with 10 μg/mL LPS in Caco-2 cells. (B) Quantification of fluorescence images. The activity of (C) SOD, (D) GSH, and (E) CAT in Caco-2 cells. The values are shown as the mean ± SEM, n=6. *P<0.05 and **P<0.01 contrast with the control. #P<0.05 and ##P<0.01 contrast to the LPS group. GA: gallic acid.

Figure 6. GA regulated intestinal inflammation by inhibiting LPS-induced NF-κB activation  
Caco-2 cells were cultivated with 5 μg/mL GA for 24 h and then 10 μg/mL LPS for 4 h for follow-up experiments. (A) Western blot analysis was used to measure the protein expressions of p65 and p-p65. Quantitative assay of the p-p65/p65 ratio. (B) The protein expression levels of IκB-α and p-IκB-α. Quantitative analysis of the p-IκB-α/IκB-α ratio. The values are shown as the mean ± SEM, n=3. *P<0.05 and **P<0.01 contrast with the control. #P<0.05 and ##P<0.01 contrast to the LPS group. GA: gallic acid.

Figure 7. Effect of GA on the MAPK signaling pathway  
Caco-2 cells were cultivated with 5 μg/mL GA for 24 h and then incubated with 10 μg/mL LPS for 4 h for subsequent experiments. (A) Western blot analysis was used to measure the protein expressions of p38 and p-p38. Quantification of the p-p38/p38 ratio. (B) The protein expression levels of JNK and p-JNK. Quantification of the p-JNK/JNK ratio. (C) The protein expression levels of ERK and p-ERK. Quantification of the p-ERK/ERK ratio. The values are shown as the mean±SEM, n=3. *P<0.05 and **P<0.01 contrast with the control. #P<0.05 and ##P<0.01 contrast to the LPS group. GA: gallic acid.
Gallic acid (GA) has the potential as a therapeutic agent for intestinal inflammation. Here, our discoveries reveal that GA attenuates lipopolysaccharide (LPS)-induced inflammation in human colon carcinoma cell line (Caco-2) by suppressing the activation of NF-κB/MAPK signaling pathway.

(1) GA alleviates intestinal barrier function against LPS-induced damage in Caco-2 cells to protect against cellular inflammatory response.

(2) GA alleviates LPS-induced apoptosis and oxidative stress in Caco-2 cells.

(3) GA suppresses the activation of NF-κB/MAPK pathway to reduce LPS-induced intestinal inflammation in Caco-2 cells.
## Supplementary Table S1. Sequences of primers used in qRT-PCR

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For Peer Review

**GAPDH**

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Reverse  GTCTTCTGGGTGGCAGTGAT