Protective effects of 18β-glycyrrhetinic acid on LPS-induced injury in intestinal epithelial cells

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[ABSTRACT] AIM: To investigate the protective effects of 18β-glycyrrhetinic acid (GA) on lipopolysaccharide (LPS)-induced injury in the intestinal epithelial cell line (IEC-6). METHODS: IEC-6 cells were treated by LPS (100 μg·mL⁻¹) with or without GA (0, 1, 1.0 or 10 μmol·L⁻¹) for 24 h. The inflammatory cytokines in the cultured medium, TNF-α and IL-6, were measured by ELISA. The supernatant nitric oxide (NO) production was detected by nitrite assay. Reactive oxygen species (ROS) were estimated by flow cytometry. Epithelial tight junction was determined by transepithelial resistance (TER), and protein levels were measured by Western blotting. RESULTS: With GA treatment, LPS-stimulated TNF-α and IL-6 were inhibited, ROS and NO were decreased, the transepithelial permeability was recovered, ZO-1 (tight junction related protein) was reversed, the protein level of cyclooxygenase-2 (COX-2) was down-regulated, and 15-hydroxyprostaglandin dehydrogenase (PGDH) was up-regulated. CONCLUSIONS: These results suggest that GA has the protective capacity to attenuate LPS-induced injury in intestinal epithelial cells and protect the epithelial tight junction. The underlying mechanism was related to a decrease in proinflammatory factor and regulating COX-2 and PGDH.

[KEY WORDS] 18β-Glycyrrhetinic acid (GA); Anti-inflammation; Transepithelial permeability; COX-2; PGDH


1 Introduction

The intestinal mucosa is covered with a monolayer of intestinal epithelial cells (IECs). IECs play an important role in the intestinal immune system by secreting cytokines and chemokines[1]. Activation of the proinflammatory gene transcriptional program in IECs in response to challenges by bacterial products, such as LPS and inflammatory cytokines, is associated with acute and chronic intestinal inflammation[2-3].

Barrier function is highly regulated by tight junction proteins, allowing the epithelium to control transmucosal permeability to solutes, water, and electrolytes. Amongst the many components of tight junction proteins, occludin, junction adhesion molecule A (JAM-A), claudins and zonula occludens proteins (ZO-1 and ZO-2) are membrane proteins that connect adjacent cells and build the intestinal barrier[4-5]. Defects in tight junction have been implicated in the pathogenesis of a number of intestinal diseases, such as sepsis, inflammatory bowel disease (IBD) and irritable bowel syndrome[6], and bacteria-induced diarrhea[7]. LPS-induced systemic inflammation leads to functionally significant alterations in the expression of key tight junction proteins in the ileal and colonic epithelium[8]. However, the mechanisms of LPS-induced injury in the tight junction of intestinal epithelial cells is still unclear.

18β-Glycyrrhetinic acid (GA) (Fig. 1) is an aglycone of glycyrrhizin, which isolated from the licorice root (Glycyrrhiza sp.). Recent reviews have described the wide spectrum of bio-activities of glycyrrhetinic acid, such as anti-inflammatory, antiviral, hepatoprotective, antitumor, and immunomodulatory activities[9]. Previous studies have demonstrated that GA reduces the expression of proinflammatory factors, such as prostaglandin E2, histamine, and eotaxin[10]. It was also reported that GA was a scavenger of free radical species by increasing superoxide dismutase activity in atherosclerotic and
hypercholesterolemic rabbits\textsuperscript{(11)}. In previous studies in these laboratories, it was shown that GA can prevent free fatty acid-induced lipotoxicity in hepatocytes through lysosomal-mitochondrial pathways\textsuperscript{(12)}. GA exhibits the anti-inflammatory effects in some in vivo and in vitro models, although whether it can ameliorate colitis was unclear. Therefore, LPS-induced injury in intestinal epithelial cells was used to mimic the colitis caused by viruses and bacteria, and to study the underlying mechanisms of the protective effect of GA on colitis.

Fig. 1  Chemical structure of 18β-glycyrrhetinic acid (GA)

2 Materials and Methods

2.1 Materials

GA was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in DMSO. The solvent in the same composition (vehicle) was used as a control. Lipopolysaccharide (LPS, Escherichia coli O55 : B5) and insulin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit polyclonal antibody against β-actin, COX-2, PGDH, and ZO-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). ELISA kits were purchased from Mrbiotech Co., Ltd (Shanghai, China). DCFH-DA was purchased from Invitrogen Co., Ltd. Nitrite assay reagent was purchased from Nanjing Jiaocheng Bioengineering Institute (Nanjing, China).

2.2 Cell culture and treatment

IEC-6 cells were grown on polystyrene tissue culture dishes in DMEM containing 10% fetal bovine serum and 0.01 mg·mL\textsuperscript{-1} insulin at 37 °C in 5% CO\textsubscript{2}/air. IEC-6 cells were grown at a density of 5 × 10\textsuperscript{5} cells per well and cultured in a 6-well plate, and treated with various concentrations of GA in the presence of LPS (100 µg·mL\textsuperscript{-1}) for 24 h. Then the cells were harvested and incubated with 2, 7-dichlorofluorescein diacetate (DCFH-DA, Invitrogen) at 37 °C in the dark for 30 min, and washed twice with cold PBS. DCF fluorescence distribution was detected by flow cytometry on a FACScan (Becton Dickinson) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Data were analyzed by Cell Quest software.

2.4 Western blotting analysis

IEC-6 cells were cultured in a medium containing LPS with or without various concentrations of GA for 24 h to detect COX-2, PDGH and ZO-1. Proteins were extracted in lysis buffer (30 mmol·L\textsuperscript{-1} Tris, pH 7.5, 150 mmol·L\textsuperscript{-1} sodium chloride, 1 mmol·L\textsuperscript{-1} phenylmethylsulfonyl fluoride, 1 mmol·L\textsuperscript{-1} sodium orthovanadate, 1% Nonidet P-40, 10% glycerol, and phosphatase and protease inhibitors). The proteins were then separated by SDS-PAGE and electrophoretically transferred onto polyvinylidene fluoride membranes. The membranes were probed with antibodies overnight at 4 °C, and then incubated with a horse radish peroxidase- coupled secondary antibody. Detection was performed using a LumiGLO chemiluminescent substrate system (KPL, UK).

2.5 Cytokine assay

Cytokines (IL-6, IFN-α) were determined according to the instruction of ELISA kits (Becton Dickinson).

2.6 Nitrite assay

All supernatants were collected and stored at −70 °C for analysis of NO. The accumulation of NO\textsuperscript{2−}, a stable end product of NO formation, was measured in conditioned medium as an indicator of NO production. A 50 µL aliquot of cell-free conditioned medium was mixed with 50 µL Griess reagent I and 50 µL Griess reagent II (Beyotime, Haimen, China) at room temperature, and the absorbance at 540 nm was measured using a microplate reader (Beckman Coulter, Germany). The concentration of NO\textsuperscript{2−} in the samples was calculated using a standard curve of sodium nitrite.

2.7 Transepithelial electrical resistance

Transepithelial electrical resistance was measured after the cells were stimulated with LPS (100 µg·mL\textsuperscript{-1}) with or without GA at a series of concentrations (0.1, 1.0, 10 µmol·L\textsuperscript{-1}) for 24 h. Each well was measured three times and the mean calculated. The background of the control wells (wells with media only, no cells) was subtracted from the wells containing cells. The change in transepithelial electrical resistance between the upper compartment and lower compartment was calculated for each well.

2.8 Quantitative RT-PCR

RNA was extracted from cells using Trizol Reagent (Invitrogen, Carlsbad, CA). One microgram of RNA was reversely transcribed to cDNA. The primer sequences used in RT-PCR were as follows: GAPDH, 5′-AACGACCCCTTC ATTGAC-3′ and 3′-CAGCCTCATAAGCACCTG-5′, CO X-2, 5′-TACCGGACTGGATTCTAG-3′ and 3′-CTAACTG TCGGGTGGTTGAA-5′. RT-PCR was performed with the
ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) using SYBR Green I dye (Biotium, Inc., Hayward, CA), and threshold cycle numbers were obtained using ABI Prism 7000 SDS software version 1.0. Conditions for amplification were 1 cycle of 94 °C for 5 min followed by 39 cycles of 94 °C for 30 s, 59 °C for 35 s, and 72 °C for 45 s.

2.9 Statistical analysis

Data were expressed as the mean ± S.E.M. Each experiment was repeated three times. One-way ANOVA, followed by Student’s t-test, was used for the statistical analysis. The differences were considered to be statistically significant when $P < 0.05$ and $P < 0.01$.

3 Results

3.1 GA inhibited LPS-induced production of inflammatory cytokines in IEC-6 cells

IEC-6 cells were treated by LPS (100 µg·mL$^{-1}$) with or without GA at a series of concentrations (0.1, 1.0, 10 µmol·L$^{-1}$) for 24 h. The culture medium was collected, and IL-6 and TNF-α were assayed by ELISA. As shown in Fig. 2, LPS stimulation in IEC-6 cells markedly increased the levels of IL-6 and TNF-α. Against the increase, GA can inhibit IL-6 and TNF-α production. GA at 1.0 and 10 µmol·L$^{-1}$ showed a significant difference in the suppression of IL-6 and TNF-α with the LPS stimulation group ($P < 0.01$) (Fig. 2A, B).

3.2 GA prevented LPS-induced oxidative stress in IEC-6 cells

The role of oxidative stress in LPS-induced IEC-6 cells and the preventive effect of GA was examined. IEC-6 cells were treated with different concentrations of GA and stimulated with LPS (100 µg·mL$^{-1}$) simultaneously for 24 h, and then incubated with DCFH-DA for the indicated periods. The cellular ROS was determined by fluorescent DCF formation using flow cytometry. The results indicated that LPS significantly increased the cellular ROS level from 31.86% to 51.72%. Co-treatment with GA decreased LPS-induced ROS formation in a dose-dependent manner, and GA at 10 µmol·L$^{-1}$ almost completely reversed it (Fig. 3A).

**Fig. 2** GA inhibited LPS-induced production of inflammatory cytokines in IEC-6 cells. IEC-6 cells were stimulated with or without LPS (100 µg·mL$^{-1}$) in the presence of 0.1–10 µmol·L$^{-1}$ GA for 24 h, then the production of IL-6 (A) and IFN-α (B) were measured by ELISA assay. mean ± S.E.M., $n = 3$. *$P < 0.01$ vs naïve cells. # $P < 0.05$ vs LPS treatment

**Fig. 3** GA prevented oxidative stress in IEC-6 cells by LPS treatment. (A) The generation of ROS was induced by 100 µg·mL$^{-1}$ LPS in IEC-6 cells. The cells were treated in the presence of LPS with or without GA at different concentrations (0.1, 1.0, 10 µmol·L$^{-1}$) for 24 h and measured by flow cytometry with DCFH-DA staining. Data shown here was one of three different experiments with similar results. (B) The production of NO was detected after 24 h treatment. mean ± S.E.M., $n = 3$. *$P < 0.01$ vs naïve cells. # $P < 0.05$ vs LPS treatment
Then the supernatant level of NO in IEC-6 cells was detected. Against the NO production triggered by LPS (100 μg·mL⁻¹), co-treatment with various concentrations of GA showed a mild inhibition effect on the production (Fig. 3B).

3.3 GA protected the tight junction from LPS-induced injury in IEC-6 cells

Transepithelial electrical resistance was used to measure the tight junction-related barrier function. The results are shown in Fig. 4A; transepithelial electrical resistance in the naïve cells was very high, and it was reduced by more than half with LPS treatment for 24 h. The co-treatment with GA significantly recovered the level of transepithelial electrical resistance from the LPS-induced decrease in a dose-dependent manner in IEC-6 cells.

The tight junction of IEC-6 cells treated by LPS (100 μg·mL⁻¹) was then examined with or without GA at different concentrations (0.1, 1.0, 10 μmol·L⁻¹) for 24 h. The tight junction related protein ZO-1 was detected by Western blotting. LPS induced the injury of tight junction and decreased the protein level of ZO-1. GA markedly reversed the effect of LPS on ZO-1, especially at the concentration of 10 μmol·L⁻¹ (Fig. 4B).

3.4 GA regulated the expressions of COX-2 and PGDH

COX-2 and PGDH are related to inflammation regulation in many circumstances. Stimulated with LPS in IEC-6 cells, PGDH expression was decreased and at the same time COX-2 was increased. After co-treatment with GA for 24 h, COX-2 expression was down-regulated and PGDH was upregulated compared with the LPS only group, as shown in Fig. 5(A). The mRNA level of COX-2 was also decreased, as shown in Fig. 5(B).

![Fig. 4](image_url)

**Fig. 4** GA inhibited the LPS-induced change of permeability in IEC-6 cells. (A) Effects of LPS with or without varying concentrations of GA on transepithelial resistance (TER) across cultured IEC-6 cells. mean ± S.E.M., n = 3. *P < 0.05 vs naïve cells. **Fig. 5** GA regulated the expressions of COX-2 and PGDH in IEC-6 cells. IEC-6 cells were stimulated by 100 μg·ml⁻¹ LPS with or without GA for 24 h. (A) Whole cell extracts were obtained and the expressions of COX-2 and PGDH were analyzed by Western blot. Data shown here are from one of three different experiments with similar results. (B) The mRNA levels of COX-2 were measured by RT-PCR and calculated by using GAPDH as an invariant control. mean ± S.E.M., n = 3. *P < 0.01 vs naïve cells. **
4 Discussion

LPS stimulation activates nuclear factor-κB (NF-κB) via a toll-like receptor-4 (TLR-4)-mediated signaling cascade that induces inflammatory-related substances, such as TNF-α, interleukin-1β, monocyte chemotactic protein-1, PGs, and inducible NO synthase (iNOS)\textsuperscript{[14-15]}. TNF-α and IL-6 are the critical cytokines involved in inflammation, and inhibition of them is regarded as a common treatment strategy for inflammation-related diseases\textsuperscript{[16-17]}. In this study, it was found that GA had a protective effect on LPS-induced inflammation in intestinal epithelial cells by the inhibition of IL-6 and TNF-α (Fig. 2). This result coincided with the previous report concerning the anti-inflammatory properties of GA in other diseases. Several studies indicate that GA alleviates inflammatory lung disease, and that GA reduces inflammatory cytokine production via PI3K/Akt/GSK3β in macrophages\textsuperscript{[18]}.

Inflammatory disorders are often characterized by the production of significant levels of free radicals, nitrogen reactive species. In response to a pro-inflammatory stimulus, IEC-6 cells produce excessive ROS and NO\textsuperscript{[19]}. ROS act as mediators of cellular injury, and are involved in the onset of cellular damage\textsuperscript{[20]}. In this study, exposure of IEC-6 cells to LPS apparently stimulated the accumulation of intracellular ROS. And co-treatment of the cells with GA significantly attenuated LPS-induced ROS production (Fig. 3A). NO was recognized as a mediator and regulator of inflammatory responses and was produced in high amounts by iNOS in activated inflammatory cells\textsuperscript{[21]}. LPS stimulation therefore upregulates iNOS through the induction of COX-2 gene expression\textsuperscript{[22]}. The ability to decrease NO production is important in screening natural compounds for anti-inflammatory properties\textsuperscript{[23]}. Here it was found that GA reduced the production of NO in the IEC-6 cells treated by LPS (Fig. 3B).

The tight junction-related barrier in epithelia is a core aspect of the function of intestinal epithelial cells\textsuperscript{[24-25]}. Further data implicating barrier defects in disease pathogenesis is the altered expression of tight junction proteins in patients with enteritis\textsuperscript{[26]}. To investigate tight junction-related barrier function, transepithelial electrical resistance serves as a reliable tool to study its permeability in intestinal epithelium\textsuperscript{[27]}. GA dose-dependently reversed LPS-induced tight junction injury (Fig. 4A), which might be due to the inhibition of epithelial cell proinflammatory factors, and the amelioration of oxidative stress. ZO-1 is a structural and functional marker of tight junction in epithelia\textsuperscript{[25]}. In the present study, it was found that GA markedly upregulated the protein level of ZO-1 induced by LPS (Fig. 4B). These findings suggest that GA can inhibit inflammation, keep tight junctions intact and protect epithelia barrier function, which implicates a potential role of GA as a candidate for the treatment of enteritis. Therefore, the underlying mechanisms of GA was examined.

It was observed that prostaglandin levels increased in enteritis. The process of prostaglandin synthesis and degradation is mediated by COX-2 and 15-PGDH. COX-2 is responsible for prostaglandin synthesis and PGDH is the key enzyme for prostaglandin degradation. Previous studies found that COX-2 and PGDH were essential factors regulating the progression of enteritis\textsuperscript{[28]}, and lipopolysaccharide (LPS)-induced innate immune activation\textsuperscript{[29]}. Upregulation of COX-2 was involved in the disruption of tight junction proteins and blood–brain barrier integrity\textsuperscript{[30]}. In the current study, it was further found that the changes in transepithelial electrical resistance and ZO-1 expression at the intestinal epithelial tight junction were related to COX-2 expression (Figs. 4-5). This indicates that COX-2 participates in regulating tight junction in epithelia in normal physiological conditions. In these experiments, the up-regulation of COX-2 induced by injury of epithelia suggested that the high expression of COX-2 could damage the tight junction of epithelia. This conclusion did not conflict with the previous report, and it explained the role of COX-2 in the disruption of epithelia tight junction. We further detected the effect of GA on COX-2 and PGDH in LPS-treated IEC-6 cells. As the result, GA decreased the expression of COX-2 and increased that of PGDH in IEC-6 cells (Fig. 5). These results suggest that GA exhibited its protective effects on intestinal epithelial cells, at least partially, by decreasing COX-2 and increasing PGDH.

In summary, this study provided a new view of GA, a natural product, on the injury of intestinal epithelial cells. Namely, GA exerted protective effects from LPS-induced IEC-6 cell damage by inhibiting inflammation cytokine production, preventing oxidative stress, improving the tight junction, and regulating COX-2 and PGDH, which may, in the future, find use in the clinic for the amelioration of enteritis.

References

18β-甘草次酸对脂多糖诱导的肠上皮损伤的保护作用

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【摘要】目的：研究18β-甘草次酸(GA)对脂多糖诱导的IEC-6肠上皮细胞损伤的保护作用。方法：将IEC-6细胞与LPS(100 μg·mL⁻¹)和不同浓度的GA(0.1, 1.0, 10 μmol·L⁻¹)共孵24 h后，观察GA的保护作用。ELISA检测细胞因子TNF-α和IL-6的水平；亚硝酸盐分析法测量上清中NO的含量；流式细胞术检测ROS的水平；TER检测肠上皮细胞透性；Western blotting分析ZO-1、COX-2和PGDH蛋白表达水平。结果：GA可以抑制由脂多糖诱导IEC-6细胞产生的TNF-α和IL-6的水平，降低ROS和NO的产生，部分恢复肠上皮细胞的透性。显著提高紧密连接相关蛋白ZO-1的表达量，同时下调COX-2的表达量，上调PGDH的表达量。结论：GA能减轻由脂多糖诱导肠上皮细胞的损伤，并能保护肠上皮细胞的紧密连接，其中的机制和炎症因子的下调以及对COX-2和PGDH的调节有关。

【关键词】18β-甘草次酸；肠炎；上皮细胞透性；COX-2；PGDH

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