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Rhein protects against barrier disruption and inhibits inflammation in intestinal epithelial cells

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ABSTRACT

Background and aims: Intestinal epithelial barrier and intestinal inflammation play indispensable roles in the development of intestinal diseases. The major aims of the current study were to investigate the potential of rhein, a major flavonoid compound isolated from *Rheum rhabarbarum*, in the treatment of intestinal diseases and its underlying mechanisms in vitro.

Methods: The protective role of rhein on intestinal epithelial barrier was evaluated in a monolayer of IEC-6 cells stimulated by TNF-α, while the anti-inflammatory effects were investigated in an IEC-6 cell model with LPS stimulation.

Results: Rhein inhibited the increase of phenol red flux and the decrease of TEER, as well as recovered the expression and distribution of ZO-1 and weakened MLC phosphorylation, MLCK expression and NF-κB activation. Meanwhile, LPS-stimulated IL-1β and IL-6 were down-regulated, expression levels of TLR4, NLRP3 and cleaved caspase1 were weakened and NF-κB was inactivated.

Conclusions: These results suggested that rhein has potential therapeutic effects against intestinal diseases by maintaining intestinal epithelial barrier and suppressing intestinal inflammation.

1. Introduction

An intact intestinal epithelial barrier is essential for defending against the translocation of enteric pathogenic organisms, antigens and toxins and absorbing nutriment and water [1,2]. The dysfunction of intestinal epithelial barrier has been proved to be an indispensable characteristic in a series of intestinal diseases such as inflammatory bowel disease (IBD), Irritable Bowel Syndrome (IBS) [3,4]. Therefore, the maintenance of intestinal epithelial barrier is an efficient strategy for the therapy of intestinal diseases.

The intestinal physical barrier function is tightly associated with the intercellular structural integrity [5]. Tight junctions (TJs), which are located in the most apical ends of the lateral membranes of intestinal epithelial cells, play a vital role in the regulation of intestinal epithelial barrier [6,7]. The disruption of TJs could lead to the increase of the intercellular permeability and the translocation of pathogen and toxins [8]. Tumor necrosis factor (TNF)-α has been proved to participate in the destruction of intestinal epithelial barrier [9–11]. TNF-α could not only decrease the synthesis of TJs, but also destroy intestinal intercellular structural integrity by activating MLCK/p-MLC pathway [12,13]. The activation of MLCK/p-MLC pathway could result in cytoskeletal rearrangements and tight junction breakdown, in turn increase intestinal permeability [14].

What's more, intestinal inflammation has been proved to be associated with the intestinal epithelial barrier disruption [15]. LPS-induced releases of cytokines could contribute to the deterioration of intestinal inflammation [16]. Recent studies have revealed that intestinal epithelial cells could release inflammatory cytokines with LPS stimulation [17–19].

Traditional Chinese medicine is a rich source of therapeutic drugs for intestinal diseases. *Rheum rhabarbarum* has been widely used to treat enteritis, gastritis, oedema, and other diseases in traditional Chinese medicine for thousands years. Rhein (Fig. 1) is a major flavonoid compound isolated from *Rheum rhabarbarum*. Previous studies have certified that rhein has anti-oxidative and anti-inflammatory effects [20–22]. What's more, it has been reported to have a protective effect on the intestinal mucosal barrier during a series of diseases such as sepsis and IgA nephropathy [23,24]. However, the protective effects of rhein on intestinal epithelial barrier function remain unclear. This study was designed to investigate the protective effect of rhein on TNF-α-

Abbreviations: TNF-α, tumor necrosis factor α; TEER, transepithelial electrical resistance; ZO, zonula occluden; MLC, myosin light chain; MLCK, myosin light chain kinase; NF-κB, nuclear factor κB; LPS, lipopolysaccharide; IL, interleukin; NLRP, NOD-like receptor family pyrin domain-containing; TJs, tight junctions

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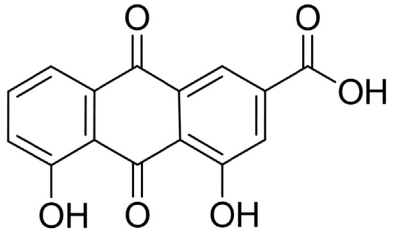

Fig. 1. Chemical structure of rhein. Chemical formula, $C_{15}H_8O_6$; molecular weight = 284.22. CAS number: 478-43-3.

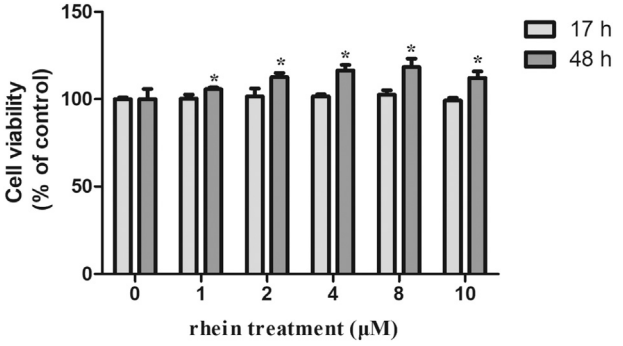

Fig. 2. Effects of rhein on the viability of IEC-6 cells. IEC-6 cells were treated with various concentrations of rhein for 17 h or 48 h, and cell viability was measured using CCK8 assay. Each value represents the mean ± SD. $_*P < 0.05$ compared with the control group.

induced damage of intestinal epithelial barrier and LPS-induced inflammation in IEC-6 cells.

2. Materials and methods

2.1. Reagents and antibodies

Rhein [> 98% high-performance liquid chromatography (HPLC) purity] was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). TNF-α was purchased from PeproTech (Rocky Hill, USA). The antibodies of anti-MLCK, anti-MLC, anti-β-tubulin were purchased from ABclonal Biotech Co., Ltd. (Wuhan, USA). Phosphorylated and non-phosphorylated forms of NF-κB p65 were purchased from ImmunoWay Biotechnology Company (Plano, TX, USA). Anti-p-MLC antibody was purchased from Bioss Biotech Co. Ltd. (Beijing, China). Anti-cleaved caspase1and anti-NLRP3 antibodies were purchased from Wanlei Biotech Co. Ltd. (Shenyang, China). The antibody of TLR4 was obtained from Abcam Biotech Co., Ltd. (Cambridge, USA). Corresponding secondary antibodies were obtained from ABclonal Biotech Co., Ltd. (Wuhan, USA). TransZol, EasyScript First-Strand cDNA Synthesis SuperMix Kit and 2 × EasyTaq PCR SuperMix were purchased from TransGen Biotech Co., Ltd. (Beijing, China). ECL reagents and BCA Protein Quantification Kit were obtained from Biyuntian BioTECH Co. Ltd. (Shanghai, China). PVDF membrane was purchased from Millipore Corp. (Bedford, MA, USA). 4′,6-Diamidino-2-phenylindole (DAPI) and lipopolysaccharide (LPS) was obtained from Solarbio Science & Technology Co., Ltd. (Beijing, China).

2.2. Cell culture

The intestinal epithelial cell line IEC-6 was purchased from American Type Culture Collection (Rockville, MD, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium, supplemented with fetal bovine serum, 50 U/ml penicillin and 50 U/ml streptomycin (all from Biological Industries, Kibbutz Beit Haemek, Israel) at 37 °C in a humidified atmosphere of 5% CO_2. For growth on filters, cells were plated on transwell filters with 0.4 μm pore size (Corning Incorporated, USA) and were grown as monolayers prior to the experiments.

2.3. Cell viability assay

Cell viability was determined by CCK8 assay. In brief, IEC-6 cells were pre-incubated with DMEM containing 5% FBS overnight in 96-well plates at a density of 2×10^4 cells per well. The cells were treated with medium containing either different concentrations of rhein for 17 h, 24 h and 48 h. According to the experimental design, treated cells in each well were added with DMEM solution containing 10% CCK8. The cells were incubated at 37 °C for 2 h. Absorbance was recorded at a wavelength of 450 nm and reference wavelength of 630 nm using a microplate reader.

2.4. Establishment of barrier injury in IEC-6 monolayers

IEC-6 cells were seeded on transwell filters at a density of 1.8×10^5 cells per filters and were grown as monolayers for 15 days. Then cells were cultured in media with varying concentrations of rhein (0, 2, 4 μM). TNF-α (50 ng/ml) was added after 12 h preincubation, and the indicated rhein concentrations were maintained throughout the duration of the experiment. After an additional 36 h incubation period, the effects on monolayers barrier were analysed according to the methods described in Sections 2.5 and 2.6.

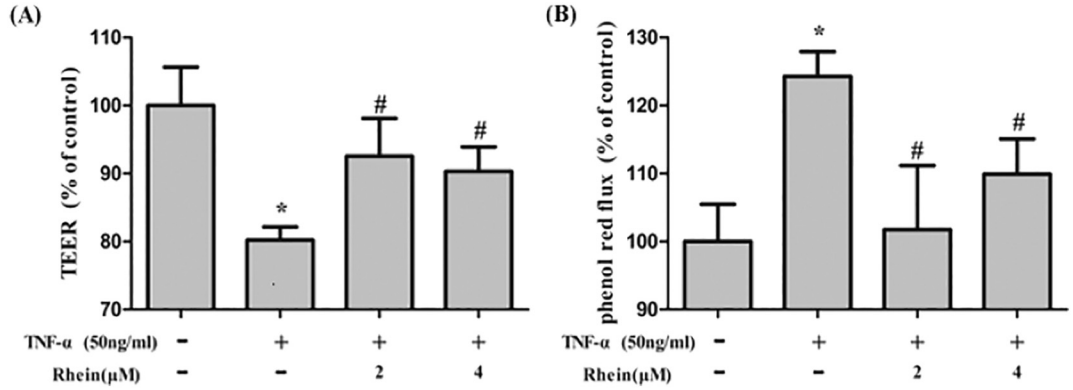

Fig. 3. Effects of rhein on TNF-α-induced decreased TEER and increased phenol red flux. Cells were cultured in media with varying concentrations of rhein (0, 2, 4uM). TNF-α (50 ng/ml) was added after 12 h preincubation, and the indicated rhein concentrations were maintained throughout the duration of the experiment. After an additional 36 h incubation period, the monolayers were analysed. TEER was measured by trans-epithelial voltohmmeter ERS-2. Automatic ELISA plate reader was used to measure phenol red flux. (A) The value of TEER. (B) The flux of phenol red. Each value represents the mean ± SD. $_*P < 0.05$ compared with the control group, $\#P < 0.05$ compared with the TNF-α-induced group.

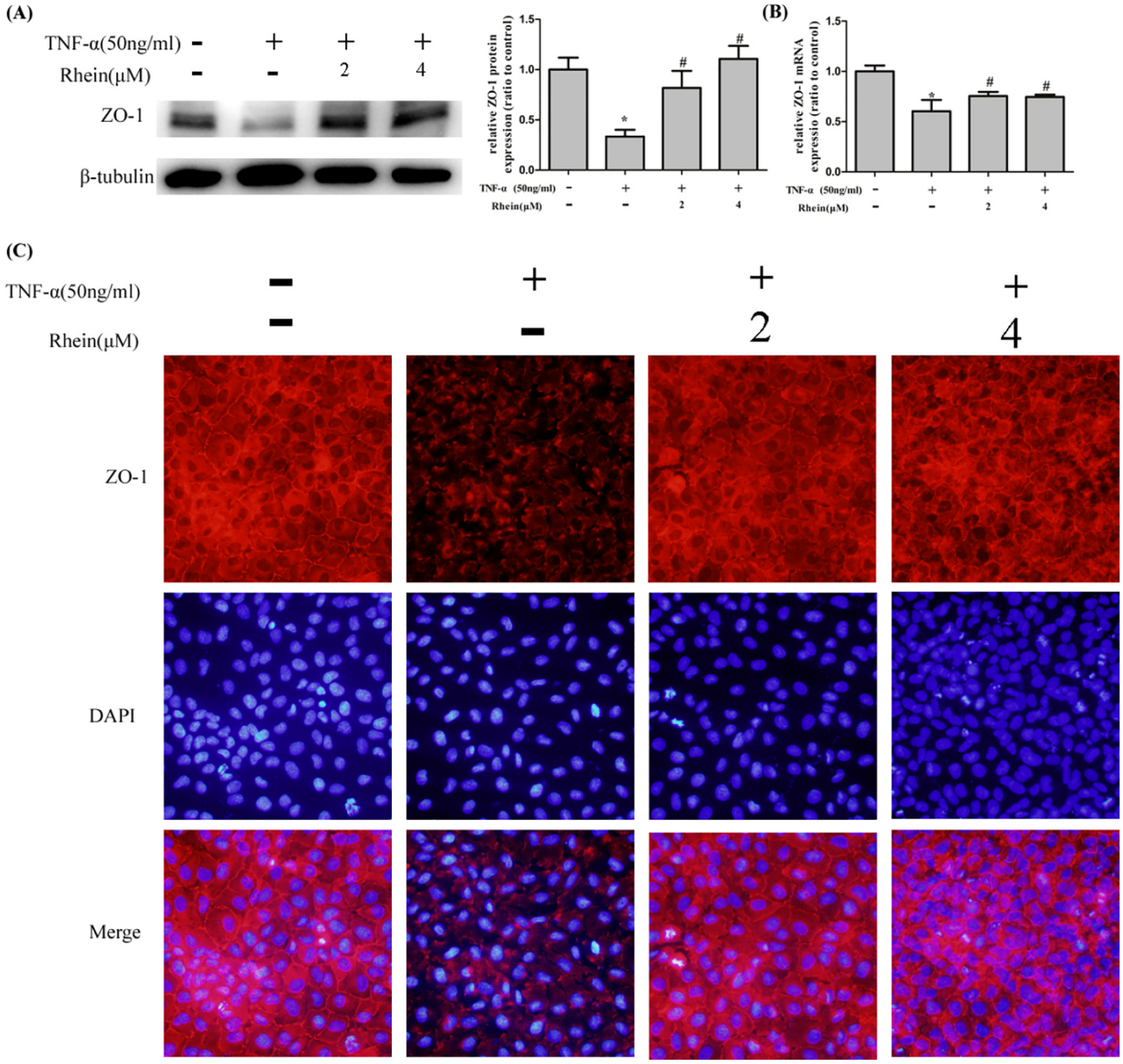

Fig. 4. Effects of rhein on decreased expression of ZO-1 protein and mRNA and altered distribution of ZO-1 protein. IEC-6 cells were cultured in media with varying concentrations of rhein (0, 2, 4 μM). TNF-α (50 ng/ml) was added after 12 h preincubation, and the indicated rhein concentrations were maintained throughout the duration of the experiment. After an additional 36 h incubation period, the cells were harvested and analysed. The level of ZO-1 protein was measured by western blotting, while the level of ZO-1 mRNA was measured by real time PCR. The distribution of ZO-1 was detected by immunofluorescence. (A) The expression level of ZO-1 protein. (B) The expression level of ZO-1 mRNA. (C) The distribution of ZO-1 protein. Each value represents the mean ± SD. $_{*}P < 0.05$ compared with the control group, $\#P < 0.05$ compared with the TNF-α-induced group.

2.5. Transepithelial electrical resistance (TEER) measurements

After the establishment of barrier injury, IEC-6 cells in transwell filters were washed with PBS for three times, following measured with an epithelial voltohmmeter ERS-2 (Merck Millipore, USA). Cells were incubated with different reagents as indicated. Each well was measured three times and the mean was calculated. The mean value of three consecutive measurements were used to calculate TEER.

2.6. Phenol red flux assays

After TEER measurements, cells were washed with HBSS for three times. Then 600 μl HBSS was added into lower chamber, while 200 μl HBSS (Hank's Balanced Salt Solution, Biological Industries, Kibbutz Beit Haemek, Israel) contained 80 μg/ml phenol red was added into upper chamber. After incubated for 6 h, the liquid in lower chamber was collected and measured to reflect phenol red flux. An automatic ELISA plate reader was used to measure the absorbance at 560 nm, and phenol red flux was expressed as a percentage relative to the control group.

2.7. Immunofluorescence assay

IEC-6 cells were seeded on glass slides in 24-well plates at a density of 1.8×10^5 cells and allowed to reach 80% confluency. Cells were cultured in media with varying concentrations of rhein (0, 2, 4uM). TNF-α (50 ng/ml) was added after 12 h preincubation, and the indicated rhein concentrations were maintained throughout the duration of the experiment. After an additional 36 h incubation period, the cells

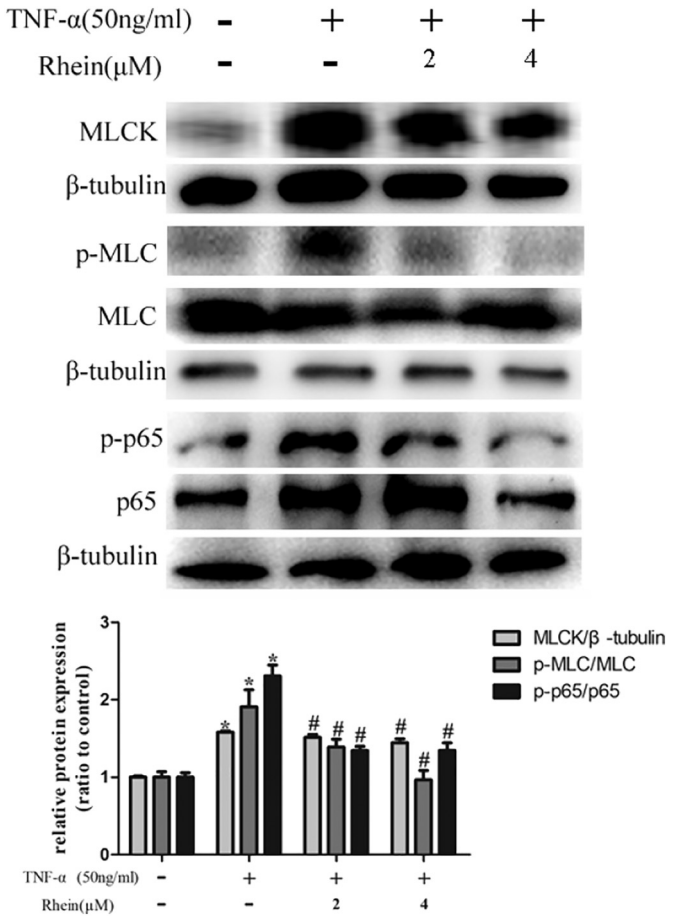

Fig. 5. Effects of rhein on TNF-α-induced NF-κB/MLCK/p-MLC activation IEC-6 cells were cultured in media with varying concentrations of rhein (0, 2, 4 μM). TNF-α (50 ng/ml) was added after 12 h preincubation, and the indicated rhein concentrations were maintained throughout the duration of the experiment. After an additional 36 h incubation period, the cells were harvested and analysed. The protein levels of NF-κB p65, NF-κB p-p65, MLCK, MLC and p-MLC were measured by western blotting. Each value represents the mean ± SD. $_{*}P < 0.05$ compared with the control group, $\#P < 0.05$ compared with TNF-α-induced group.

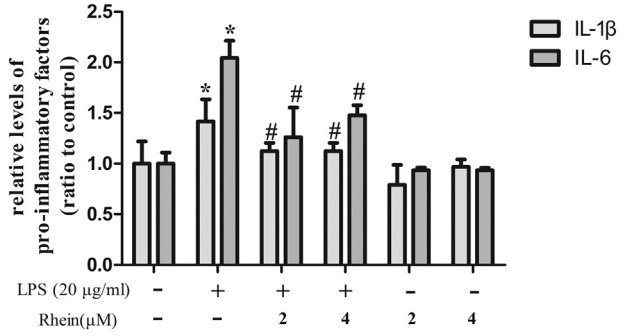

Fig. 6. Effects of rhein on LPS-induced release of IL-1β and IL-6. IEC-6 cells were treated with various concentrations of rhein for 17 h, followed by treated with LPS (20 μg/ml) accompanied with rhein or not for 4 h. The supernatant was collected to measure the levels of IL-1β and IL-6 by ELISA. Each value represents the mean ± SD. $_{*}P < 0.05$ compared with the control group, $\#P < 0.05$ compared with the LPS-induced group.

were subsequently washed with PBS for three times, fixed by 4% paraformaldehyde for 15 min at room temperature. Then the cells were rinsed with PBS and blocked by 1% bovine serum albumin (BSA) for 1 h at room temperature followed by incubation overnight at 4 °C with antibodies for ZO-1 at 1:200 a. Rinsed with PBS, the cells were incubated with goat anti-rabbit IgG conjugated to Alexa488 in the dark for 2 h at room temperature. After nuclear counterstaining of the cells with DAPI, images were captured by a fluorescence microscope. Optical density measurement for ZO-1 fluorescence used ImageJ.

2.8. Real time PCR

IEC-6 cells were seeded on glass slides in 6-well plates at a density of 1.2×10^6 cells and allowed to reach 90% confluency. Cells were then treated with TNF-α (50 ng/ml) accompanied with rhein or not for 36 h following pretreated with rhein. After that, cells' total RNA was extracted according to the manufacturer's instruction. Total RNA was reversed to cDNA by using EasyScript First-Strand cDNA Synthesis SuperMix Kit. Real-time quantitative PCR was performed using ABI Real-Time PRC detection system (ABI, USA). The expression levels of target genes were normalized to the reference gene GAPDH. The primer sequences used in RT-PCR were as follows: ZO-1 (NM_001106266.1), sense 5′-CTGAGCCCCCTAGTGATGTG-3′ and antisense 5′-TAGGGTCA CAGTGTGGCAAG-3′; GAPDH (NM_017008) sense 5′-CAACTCCCTCAA GATTGTCAGCA-3′ and antisense 5′-GGCATGGACTGTGGTCATGA-3′.

2.9. Cytokines assay

IEC-6 cells were plated on 24-well plates at a density of 2×10^5 cells per well and allowed to grow to 90% confluency. Then cells were cultured in a medium containing various concentrations of rhein (2, 4 μM) for 17 h followed by stimulated with LPS (20 μg/ml) for 4 h. The supernatant in 24-well plates was collected to measure the cytokines. The levels of cytokines (IL-1β, IL-6) were measured according to the instructions of ELISA kits.

2.10. Western blotting assay

IEC-6 cells were seeded on 6-well plates at a density of 1.2× 106 cells per well and allowed to grow to 90% confluency. Then cells were treated according to Sections 2.4 and 2.8. After stimulation, cells were collected and lysed for protein samples according to the standard protocol using RIPA lysis buffer. Protein samples which got from cell samples treated according to Section 2.4 were used to detect the levels of ZO-1, MLCK, MLC, p-MLC, p65 and p-p65, while protein samples which got from cell samples treated according to Section 2.8 was used to measure the levels of TLR4, p65, p-p65, NLRP3 and cleaved caspase1. The concentration of protein samples were quantified by a BCA Protein Quantification Kit. Equivalent amounts of protein samples were loaded onto a 12% separating gel and 5% stacking gel, then transferred onto PVDF membrane. The membrane was blocked for nonspecific binding for 120 min (3% BSA in TBST) and then the membranes were washed three times, followed by incubation overnight at 4 °C with antibodies which mentioned above. The membranes were washed three times and incubated with secondary antibodies for 90 min at room temperature, immunoblots were developed with ECL reagents. The results were normalized to β-tubulin and analysed using Image J.

2.11. Statistical analysis

All values were expressed as the mean ± SD for at least three separate experiments. The data were analysed by using one-way analysis of variance (ANOVA) followed by least significant difference (LSD) test for multiple comparisons. A value of $P < 0.05$ was considered statistically significant.

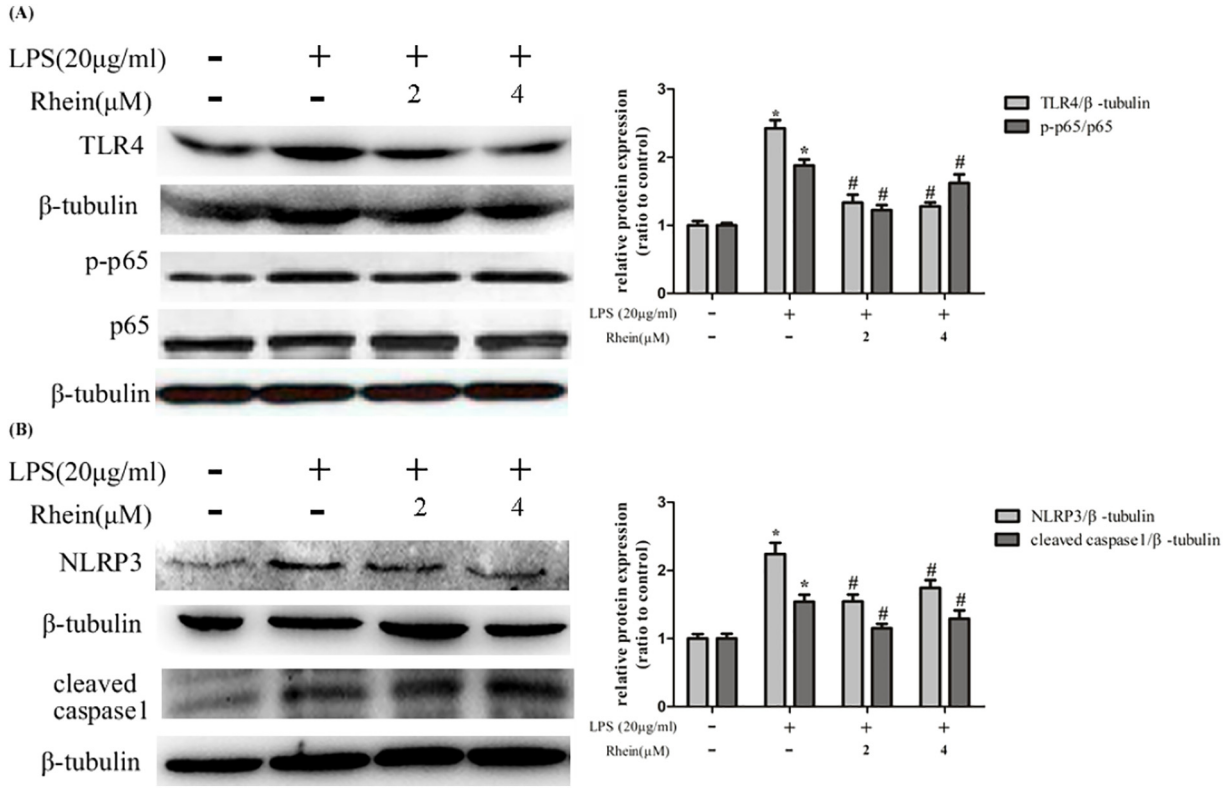

Fig. 7. Effects of rhein on TLR4/NF-κB pathway and NLRP3 inflammasome. IEC-6 cells were treated with various concentrations of rhein for 17 h, followed by treated with LPS (20 μg/ml) accompanied with rhein or not for 4 h. The protein levels of TLR4, NF-κB p65, NF-κB p-p65, NLRP3, cleaved caspase1 were measured by western blotting. (A) The expression levels of TLR4 expression and p65 phosphorylation. (B) The expression levels of NLRP3 expression and cleaved caspase1 expression. Each value represents the mean ± SD. $*P < 0.05$ compared with the control group, $\#P < 0.05$ compared with the LPS-induced group.

3. Results

3.1. The viability of IEC-6 cells stimulated by rhein

The potential cytotoxic effect of rhein on IEC-6 cells was assessed using an CCK8 assay. As shown in Fig. 2, rhein had no significant inhibitory effects on the cell viability in the cells stimulated by 1–10 μM rhein for 17, 24 and 48 h. The result implies that rhein has no inhibitory effect on IEC-6 cells viability.

3.2. The effect of rhein on TNF-α-induced decrease of TEER and increase of phenol red flux

As shown in Fig. 3, TNF-α significantly decreased the level of TEER and increased the contents of phenol red in the lower chamber ($P < 0.05$), while treatment with rhein moderated the reduced TEER and increased phenol red flux ($P < 0.05$).

3.3. The effect of rhein on TNF-α-induced down-regulation of ZO-1 mRNA and protein and distribution of ZO-1 protein

We further investigated the expressions of ZO-1 mRNA and protein in TNF-α-induced IEC-6 cells with or without rhein treatment. As shown in Fig. 4A and B, TNF-α notably inhibit the expressions of mRNA and protein levels of ZO-1 ($P < 0.05$). However, a remarkable increase in the ZO-1 mRNA and protein expressions were observed with rhein treatment ($P < 0.05$). Meanwhile, as show in Fig. 4C, TNF-α induced the rearrangement of ZO-1. However, rhein recovered the distribution of ZO-1.

3.4. Rhein inhibits TNF-α-induced NF-κB/MLCK/p-MLC activation

To elucidate the mechanism how rhein regulated intestinal epithelial barrier, we focused on NF-κB/MLCK/p-MLC pathway. As shown in Fig. 5, TNF-α significantly up-regulated protein level of MLCK and phosphorylation levels of MLC and NF-κB p65 ($P < 0.05$). In contrast, rhein treatment abolished these changes ($P < 0.05$).

3.5. Rhein inhibits LPS-induced release of inflammatory cytokines in IEC-6 cells

In order to evaluate whether rhein protected cells via inhibiting the secretion of inflammatory cytokines, we measured the IL-1β and IL-6 levels in the supernatant. As shown in Fig. 6, LPS stimulation in IEC-6 cells markedly increase the levels of IL-1β and IL-6 ($P < 0.05$), while rhein pretreatment induced a significant decrease in the release of IL-1β and IL-6 ($P < 0.05$).

3.6. Rhein inhibits the activation of TLR4/NF-κB pathways induced by LPS

As shown in Fig. 7A, The expression levels of TLR4 and phosphorylated NF-κB p65 were both notably increased induced by LPS ($P < 0.05$), while these changes were weakened following treatment with rhein ($P < 0.05$).

3.7. Rhein inhibited the expression levels of NLRP3 and cleaved caspase1 induced by LPS

As shown in Fig. 7B, the results from western blotting analysis demonstrated that LPS stimulation induced an increase in the protein levels of NLRP3 and cleaved caspase1 ($P < 0.05$). In contrast, rhein pretreatment significantly down-regulated the protein levels compared

with LPS stimulation alone ($P < 0.05$).

4. Discussion

Rhein is a major flavonoid compound isolated from *Rheum rhabarbarum*. In the current study, we demonstrated that rhein had a protective effect against intestinal epithelial barrier injury and could exert anti-inflammatory activity in intestinal epithelial cells.

Given that intestinal epithelial barrier is the central to isolating the internal milieu from the microorganism, antigens and toxins in gut, the dysfunction of intestinal epithelial barrier could contribute to the loss of homeostasis [25]. As the increased permeability is a characteristic of intestinal epithelial barrier injury [12,26,27], we first recruited TEER and phenol red flux assay to evaluate the intestinal epithelial barrier. Results showed that TNF-α stimulation for 36 h lead to a significant decrease in TEER and a notable increase in Phenol red flux assay, which meant that we established the model of intestinal epithelial barrier injury successfully. In contrast, rhein weakened these changes, indicating that rhein might have a protective potential to prevent intestinal epithelial barrier injury.

TJs play an indispensable role in the maintenance of barrier function [28]. Further data implicate that barrier dysfunction is associated with the injury of TJs [29,30]. ZO-1 is a key tight junction protein which could interact with other tight junction proteins including occludin, claudins and JAMs to take part in the assembly of TJs [31]. Previous studies also have determined that ZO-1 has been widely used to evaluate the level of intestinal epithelial barrier injury [32,33]. Therefore, our investigation focused on the expression and distribution of ZO-1. The results showed that rhein up-regulated the expression levels of ZO-1 mRNA and protein, reversed the redistribution of ZO-1 compared with TNF-α stimulation alone. Taken together, these results demonstrated that both increased expression of tight junction proteins and inhibited rearrangement of TJs might be the mechanism underlying the protective effect of rhein against intestinal epithelial barrier injury.

The current study also investigated the activation of the MLCK/p-MLC pathway, which had been proved to play an irreplaceable role in the regulation of TJs [34]. MLCK could induce the phosphorylation of MLC, in turn cause the rearrangement of TJs and the destruction of tight junction protein to increase the permeability [35,36]. Western blotting results revealed that TNF-α could promote the expression of MLCK and the phosphorylation of MLC, which are accordant with previous studies [13,37]. In contrast, rhein inhibited these changes, reflecting that rhein may inhibit the permeability increase via inhibiting MLCK/p-MLC pathway. What's more, the activation of NF-κB is involved in TNF-α-induced the activation of MLCK/p-MLC [38]. Upon activated by TNF-α, NF-κB p65 binds to the transcription element to activate the synthesis of MLCK [38]. We investigated the effects of rhein on the activation of NF-κB p65. Our results reflected that rhein prohibited the activation of NF-κB p65 induced by TNF-α. Taken together, rhein could suppress the activation of NF-κB, in turn inhibit MLCK expression and MLC phosphorylation, then block the redistribution of TJs, eventually prevent against intestinal epithelial barrier dysfunction. In addition, previous studies have also elucidated that activated NF-κB could inhibit the transcription of tight junction proteins, while inhibition of NF-κB activation could activate the synthesis of tight junction proteins [39]. It might be also the underlying mechanism how rhein exert the protective effect.

Since inflammation has been proved to play an indispensable role in the intestinal epithelial barrier dysfunction, the medicine which could prevent intestinal barrier dysfunction may exert the protective effect via its anti-inflammatory ability [40,41]. Meanwhile, the increased levels of inflammatory agents in intestinal epithelial cells play a crucial role in intestinal inflammation [42,43]. Thus, in the current study, LPS, a common stimulus to induce the release of inflammatory agents in intestinal epithelial cells [17,44], was used to evaluate the anti-inflammatory properties of rhein in IEC-6 cells. It was observed that rhein inhibit the release and expression of IL-1β and IL-6 induced by LPS, reflecting that rhein has an anti-inflammatory property. Ge et al. has also proved that rhein could inhibit the release of pro-inflammatory agents in macrophages [20].

A series of signaling molecules are involved in the regulation of the inflammatory agent expression. TLR4 is a long-sought receptor that responds to LPS [45]. After response to LPS, TLR4 could activate a series of pathways, eventually activate NF-κB to promote the production of inflammatory cytokines such as IL-6, IL-1β [45]. We investigated the effects of rhein on LPS-induced TLR4 expression and NF-κB activation. Results showed that LPS increased the levels of TLR4 protein and NF-κB phosphorylation in IEC-6 cells. However, rhein administration significantly weakened these tendencies, indicating that rhein inhibit the release of pro-inflammatory agents via down-regulating TLR4/NF-κB pathway.

NLRP3 inflammasome is a key signaling platform that recognizes that pathogenic microorganisms and sterile stimulus, in turn activate caspase1 to convert the cytokine precursor pro-IL-1β into mature and biologically active IL-1β [46,47]. The levels of NLRP3 and cleaved caspase1 were commonly recruited to evaluate the activation of NLRP3 inflammasome [48–50]. Consistently, NLRP3 expression and cleaved caspase-1 levels were elevated in stimulated cells. In contrast, rhein pretreatment significantly abrogated the increased NLRP3 and cleaved caspase-1 expression. These changes were in accordance with the change of IL-1β in the supernatant, suggesting that rhein may inhibit NLRP3 inflammasome activation to inhibit inflammation.

5. Conclusion

In conclusion, our study demonstrated that rhein has potential therapeutic effects against intestinal diseases via ameliorating the damage of intestinal epithelial barrier induced by TNF-α as well as inhibiting inflammation in LPS-stimulated intestinal epithelial cells. Rhein recovered the intestinal epithelial barrier via the inhibition of NF-κB/MLCK/p-MLC pathway, while rhein inhibited LPS-induced IL-1β and IL-6 expression via the modulation of TLR4/NF-κB pathway and NLRP3 inflammasome.

Conflict of interest

There is no conflict of interest.

Acknowledgements

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