Life Sciences 216 (2019) 168–175

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Rhein ameliorates lipopolysaccharide-induced intestinal barrier injury via modulation of Nrf2 and MAPKs

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ARTICLE INFO

Keywords:
Rhein
LPS
Intestinal barrier injury
Oxidative stress
Inflammation
MAPKs
Nrf2

ABSTRACT

Aims: In this study, we explored the underlying mechanisms of protective effects of rhein against intestinal barrier injury in a rat model, induced by intraperitoneal injection of lipopolysaccharide (LPS).

Main methods: Twenty-four male rats were assigned equally to three groups. Rats were given an oral administration of rhein (66.7 mg/kg/day) or not for three continuous days. LPS or saline were injected intraperitoneally in an hour after the last oral administration. The rats were sacrificed at 7 h after LPS or saline administration. Both blood samples and intestinal samples were collected.

Key findings: Rhein pretreatment markedly inhibited the levels of serum diamine oxidase (DAO), D-lactate (D-lac) and intestinal histological damage, significantly recovered the levels of intestinal DAO, ZO-1 and occludin. Additionally, rhein suppressed LPS-induced intestinal inflammation and oxidative stress, by decreased serum and intestinal, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and nitric oxide levels, up-regulated intestinal catalase, glutathione peroxidase (GSH-Px) activities and HO-1 expression, and down-regulated malondialdehyde (MDA) level in the small intestine. Finally, rhein inhibited JNK, p38 MAPK phosphorylation and activated Nrf2 pathway.

Significance: Rhein could exert the anti-inflammatory and anti-oxidative effects against LPS-induced intestinal barrier injury by suppressing p38 MAPK and JNK and activating Nrf2 pathway.

1. Introduction

The intestinal barrier is important as a selective barrier which could isolate the internal milieu from the microorganism, antigens and toxins in gut [1]. The dysfunction of intestinal barrier could cause the increase of intestinal mucosal permeability, induce the translocation of enteric pathogenic organisms, in turn exacerbate the injury of intestinal barrier integrity, even lead to systemic infection, multiple organs failure and septic shock, which is a common pathological progress in a series of intestinal diseases [2].

lipopolysaccharide (LPS), a component of the cell walls of Gram-negative bacteria, which plays an important role in the dysfunction of intestinal barrier, has been widely considered as a representative experimental model to evaluate intestinal barrier injury [3,4]. LPS could result in release of inflammatory mediators including tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 [5,6]. Massive evidences have revealed p38 MAPK and JNK are required for the transcription and production of various pro-inflammatory agents [7,8]. Oxidative stress is another important inducer of inflammation which involved in intestinal barrier injury [9]. Nrf2, which is also known as NF-E2-like2 (NFE2L2), is a key transcription factor which could repair injured tissues and enhance cellular defense to various oxidative stress medium including LPS [10]. Upon oxidative stress, Nrf2 activates the transcription of a sequence of antioxidant genes such as HO-1 [11]. Previous studies have demonstrated that Nrf2 plays a vital role in the recovery of intestinal barrier function [12,13].

Traditional Chinese medicines are a rich source of new therapeutic drugs for intestinal barrier. *Rheum rhabarbarum* has been widely used in traditional Chinese medicines. Rhein (Fig. 1) is a major flavonoid compound isolated from *Rheum rhabarbarum*. Both thousands years'

Abbreviations: LPS, lipopolysaccharide; DAO, diamine oxidase; D-lac, D-lactate; IL-6, interleukin 6; IL-1β, interleukin 1β; TNF-α, tumor necrosis factor α; p38 MAPK, p38 mitogen-activated protein kinase; JNK, c-Jun NH2-terminal kinase stress-activated kinase; Nrf2, nuclear factor E2 related factor 2; HO-1, heme oxygenase-1; TJ, tight junction; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; ELISA, enzyme linked immunosorbent assay; iNOS, inducible nitric oxide synthase; NO, nitric oxide; ZOs, zonula occludens

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https://doi.org/10.1016/j.lfs.2018.11.048
Received 31 August 2018; Received in revised form 20 November 2018; Accepted 21 November 2018
Available online 22 November 2018

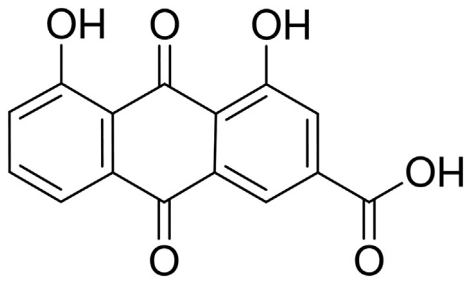

Fig. 1. Chemical structure of rhein (4, 5-dihydroxyanthraquinone-2-carboxylic acid). Chemical formula, $C_{15}H_8O_6$; molecular weight = 284.22, (CAS number: 478–43-3) [16].

usage and nearly researchers' studies have proved that rhein has widely protective roles. Rhein could decrease the level of IL-6, IL-1β and TNF-α in vitro [14]. It could also have a therapy effect against hydrogen peroxide-induced endothelial cells injury [15]. In addition, it has been proved that rhein could prevent the colon injury [16]. However, the protective effects of rhein on intestinal barrier injury need further research. In the present study, we aimed at investigating the underlying mechanisms of rhein on LPS-induced intestinal barrier injury in vivo.

2. Materials and methods

2.1. Chemicals and reagents

Rhein [> 98% high-performance liquid chromatography (HPLC) purity] was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). LPS (*Escherichia coli*, O55:B5) was purchased from Sigma-Aldrich (St. Louis, USA). Rat TNF–α, IL–1β, IL-6 ELISA kits were obtained from R&D system (Minneapolis, USA). Rat diamine oxidase (DAO) ELISA kits were obtained from Chejeter biotech LLC (Wuhan, China). Griess Reagent kit was obtained from Biyuntian Biotech (Shanghai, China). Superoxide dismutase (SOD) assay kit, catalase assay kit and glutathione peroxidase (GSH-Px) assay kit were obtained from Beyotime Biotech (Haimen, China). Malondialdehyde (MDA) assay kit and D-lactate (D-lac) assay kit were obtained from Nanjingjiancheng (Nanjing, China). The primary antibodies including ZO-1, phosphorylated and non-phosphorylated forms of JNK, p38 MAPK were purchased from Bioss Biotech Co. Ltd. (Beijing, China). The primary antibodies Occludin, GAPDH were obtained from ABclonal Biotech Co., Ltd. (Cambridge, USA). Other primary antibodies, such as Nrf2, HO-1 obtained from flarebio biotech Ltd. (Wuhan, China). SDS-PAGE, species-specific secondary antibodies and ECL were purchased from ABclonal Biotech Co. Ltd. (Cambridge, USA). All other reagents belonged to analytical grade.

2.2. Animals

Eight-week-old male SD rats, weighing 190-210 g, were provided by Laboratory Animal Center of the Academy of Military Medical Sciences (Beijing, China). The rats were maintained in controlled conditions at 25 ± 1 °C, a relative humidity of 45 ± 5% with 12 h light/dark cycles, and were access to food and water with freedom. All animals and experiments were approved by the China Agricultural University Institutional Animal Care and Use Committee. The animals were kept in isolation for at least one week before the experiments.

2.3. Animals experiments

Rats were randomly divided into three groups (8 rats in each group): control (oral administration of 0.9% saline and intraperitoneal injection of 0.9% sterile saline), LPS (oral administration of 0.9% saline and intraperitoneal injection of LPS at 8 mg/kg body weight), LPS + Rhein (oral administration of 66.7 mg/kg/day rhein and intraperitoneal injection of LPS at 8 mg/kg body mass). Each group was orally administered with rhein (66.7 mg/kg) or saline for 3 continuous days, LPS or sterile saline were injected intraperitoneally in an hour after the last oral administration, the rats were sacrificed at 7 h after LPS or sterile saline administration.

2.4. Sample collections

Seven hours after LPS or saline intraperitoneal injection, all rats were humanely sacrificed by euthanasia with an intraperitoneal injection of sodium pentobarbital. The blood samples were collected by heart punctures, and centrifuged at 12000 rpm for 10 min in order to obtain the supernatant fractions. Distal ileum of each rat was removed and rinsed with 0.9% physiological saline. About 1 cm ileal segments were rinsed with PBS and then placed in paraformaldehyde. The intestinal mucosal scrapings were scraped with sterile glass slide. After harvesting, samples were immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

2.5. Histopathology assessments

The tissues fixed in paraformaldehyde were embedded in paraffin, sliced and stained with hematoxylin and eosin (H&E). The pathological changes of intestinal tissues were observed with a light microscope.

2.6. ELISA for pro-inflammatory cytokines and DAO in serum and ileum

To measure pro-inflammatory cytokines and DAO, blood samples were collected quickly. Intestinal samples were weighed and grided at the same condition with PBS for 5 min, then centrifuged at 12000 rpm for 10 min in order to obtain the supernatant fractions. Concentration of IL-1β, TNF-α, IL-6 and DAO were measured by using ELISA kits as the manufacturers' instructions.

2.7. Analysis of nitric oxide and D-lac

The method of sample collection is in accordance with **2.6**. Serum D-lac was measured according to manufacturer's instructions. Griess reagent was used to detect nitric oxide levels in serum and tissue samples, and the steps were kept to a Griess Reagent kit.

2.8. Analysis of MDA, SOD, catalase and GSH-Px

Homogenization was carried out in RIPA lysis buffer to yield 10% w/v ileum homogenate. The homogenized tissues were centrifuged at 12000 rpm for 10 min at 4 °C. The supernatant fractions were separated and used for further biochemical assessment. The activity levels of intestinal SOD, catalase and GSH-Px and the level of tissular MDA were measured according to manufacturer's instructions.

2.9. Western blotting analysis

The tissue samples were prepared according to the standard protocol using RIPA lysis buffer (Biyuntian Biotech, Shanghai, China). The protein concentrations were quantified by a BCA Protein Quantification Kit (Biyuntian Biotech, Shanghai, China). Equivalent amounts of protein samples were loaded onto a 12% separating gel and 5% stacking gel, then transferred onto PVDF membrane (Millipore Corp., Bedford, MA, USA). The membranes were blocked for nonspecific binding for 120 min (5% BSA in TBST) and then washed three times followed by incubation overnight at 4 °C with antibodies for p-JNK, JNK, p-p38 MAPK, p38 MAPK, ZO-1, occludin, HO-1, Nrf2 and GAPDH. Then membranes were washed three times and incubated with secondary antibodies for 90 min at room temperature and detection was performed by ECL reagents. Images were analyzed by image J.

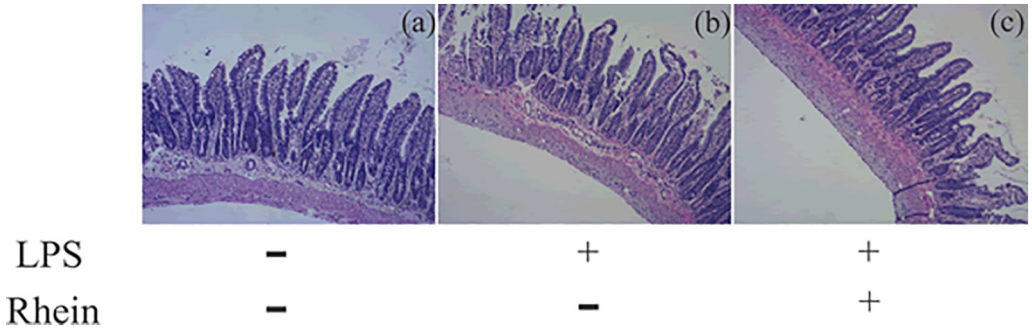

Fig. 2. Effects of rhein on histological changes in small intestine tissues in a rat model of small intestinal injury. Representative histological changes of small intestine acquired from different group.

2.10. Statistical analysis

All values were expressed as the mean ± SD for at least three separate occasions. The data were analyzed by using one-way analysis of variance (ANOVA) followed by the Student-Newman-.

Keuls test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Rhein maintained intestinal barrier integrity in rats with LPS challenge

As shown in Fig. 2, to confirm the protective role of rhein, HE staining of the intestine showed that LPS contributed to the atrophy and break of the villi, while pretreatment with rhein resulted in observably recovery in the villi compared with LPS stimulation and exhibited minimal changes in the villi similar to control group. What's more, LPS treatment resulted in a notable increase in serum DAO and D-lac compared with untreated rats ($P < 0.05$, Fig. 3A and C), pretreatment with 66.7 mg/kg rhein reduced LPS-induced the up-regulation of DAO and D-lac in serum ($P < 0.05$, Fig. 3A and C). However, DAO in small intestine was decreased notably with LPS challenge, while pretreatment with rhein alleviated this change ($P < 0.05$, Fig. 3B).

3.2. Rhein promoted the protein expression of tight junction proteins

The expression of tight junction proteins in the intestine of rats is presented in Fig. 4. Relative to the control group, LPS challenge inhibited ZO-1 and occludin in ileum ($P < 0.05$). Supplementation with rhein increased the protein expression of ZO-1 and occludin compared with rats in the LPS group ($P < 0.05$).

3.3. Rhein attenuated oxidative stress in a model of rat intestinal barrier injury

LPS challenge increased the abundance of MDA ($P < 0.05$) in the small intestinal mucosa compared with control rats. Relative to LPS-stimulated rats, supplementation with rhein decreased MDA level in the intestinal mucosa ($P < 0.05$, Fig. 5A). The enzymatic activities of catalase (Fig. 5B), SOD (Fig. 5C) and GSH-Px (Fig. 5D) were also detected, and the levels of these antioxidase activities were also inhibited with LPS challenge ($P < 0.05$). But rhein obviously enhanced the activities of GSH-px and catalase ($P < 0.05$) and had no effect on SOD activity ($P > 0.05$), compared with LPS group.

3.4. Rhein promoted Nrf2 and HO-1 expression in ileum

The changes in the expression of Nrf2 and HO-1 were also examined by western blotting (Fig. 5E). LPS stimulation obviously decreased accumulation of Nrf2 and HO-1 compared with the control group ($P < 0.05$). However, the stimulatory effect by LPS was attenuated in the rhein group ($P < 0.05$).

3.5. Rhein decreased the levels of inflammatory agents in ileum and in serum

As shown in Fig. 6A and C, the levels of IL-1β, IL-6 and TNF-α in serum and ileum were higher in the LPS group than in the control group. Rats treated with rhein prior to LPS led to a significant reduction ($P < 0.05$) in the IL-1β, IL-6 and TNF-α levels in the same time period. Meanwhile, as shown in Fig. 6B and D, rats administered with rhein had significantly lower NO levels ($P < 0.05$) in ileum and in serum than rats in LPS group.

3.6. Rhein attenuated LPS-induced JNK, p38 MAPK activation in tissues

As shown in Fig. 7, the phosphorylation of JNK and p38 MAPK in

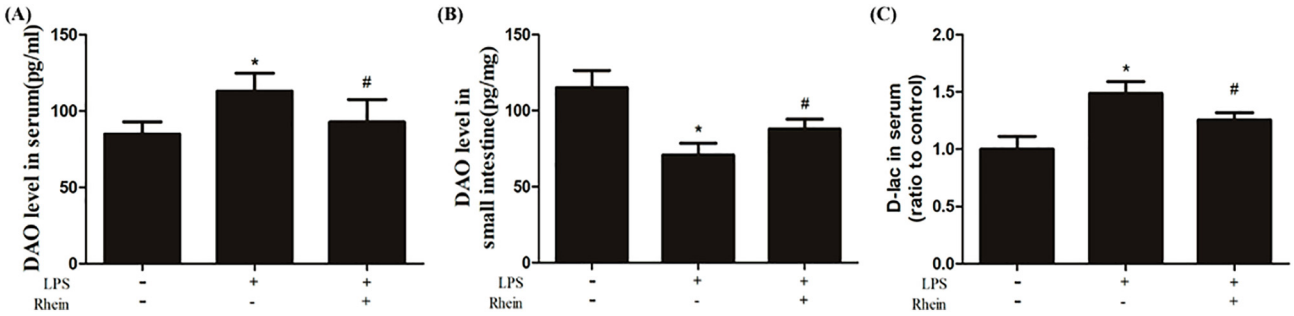

Fig. 3. Effects of rhein on (A) DAO in serum, (B) DAO in small intestine and (C) D-lac in serum. The level of DAO was measured by ELISA kit, and the level of D-lac was measured by colorimetry kit. Each value represents the mean ± SD. * $P < 0.05$ compared with the control group, # $P < 0.05$ compared with the LPS-induced group.

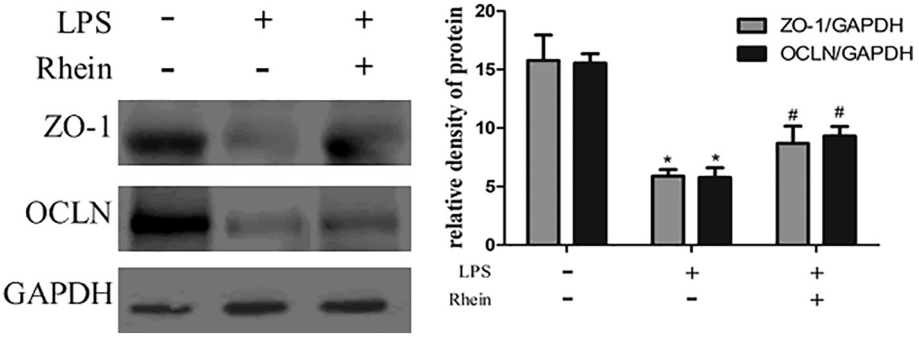

Fig. 4. Effects of rhein on the protein levels of tight junction proteins in the small intestine. Protein samples were analyzed by western blotting with specific antibodies. Each value represents the mean ± SD. *$P < 0.05$ compared with the control group, # $P < 0.05$ compared with the LPS-induced group.

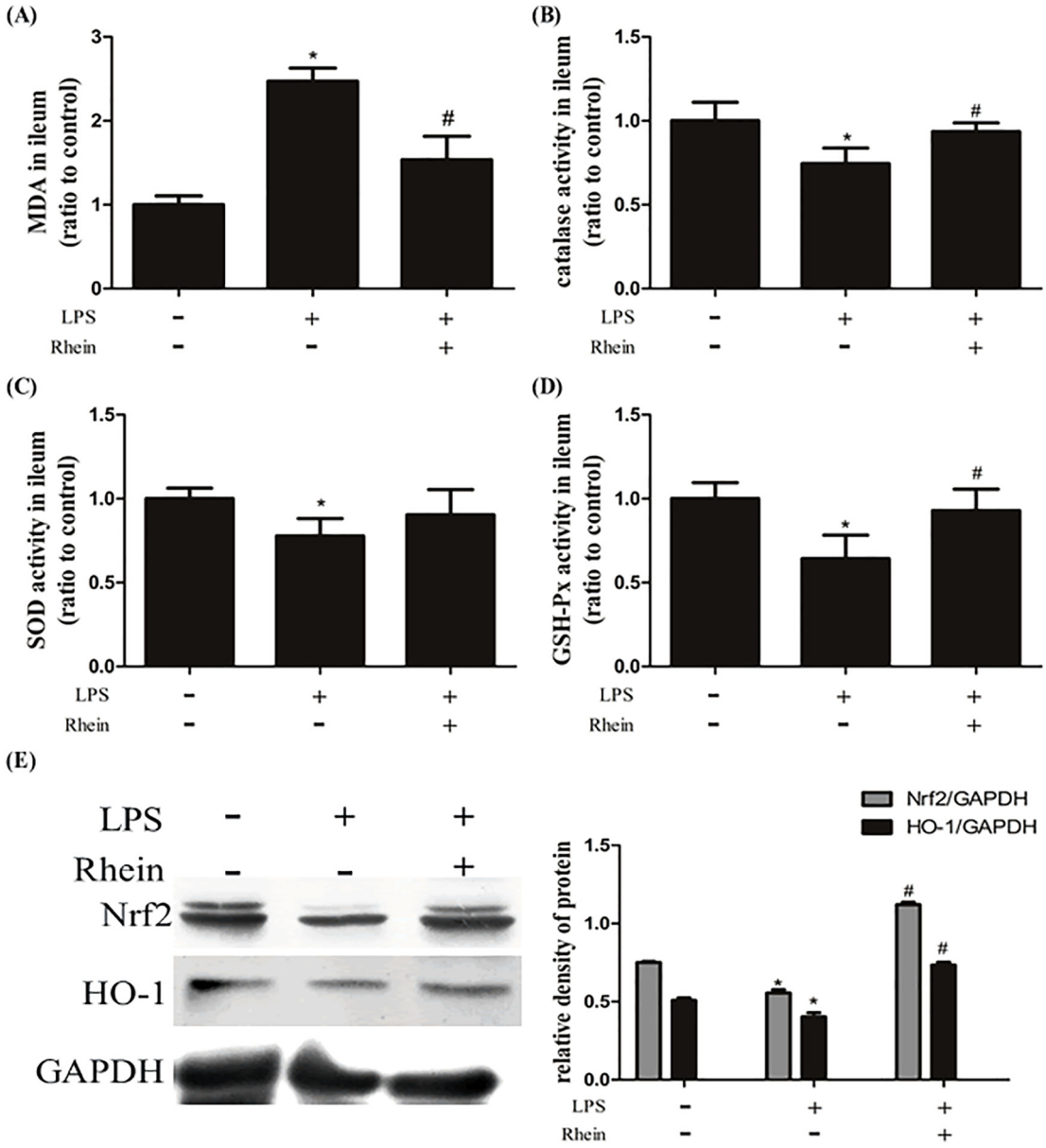

Fig. 5. Effects of treatment with rhein on (A) MDA content, (B) CAT activity, (C) SOD activity, (D) GSH-Px activity and (E) the levels of Nrf2 and HO-1 in LPS-induced intestinal barrier injury. All the data were analyzed by commercial kits. Each value represents the mean ± SD. * $P < 0.05$ compared with the control group, # $P < 0.05$ compared with the LPS-induced group.

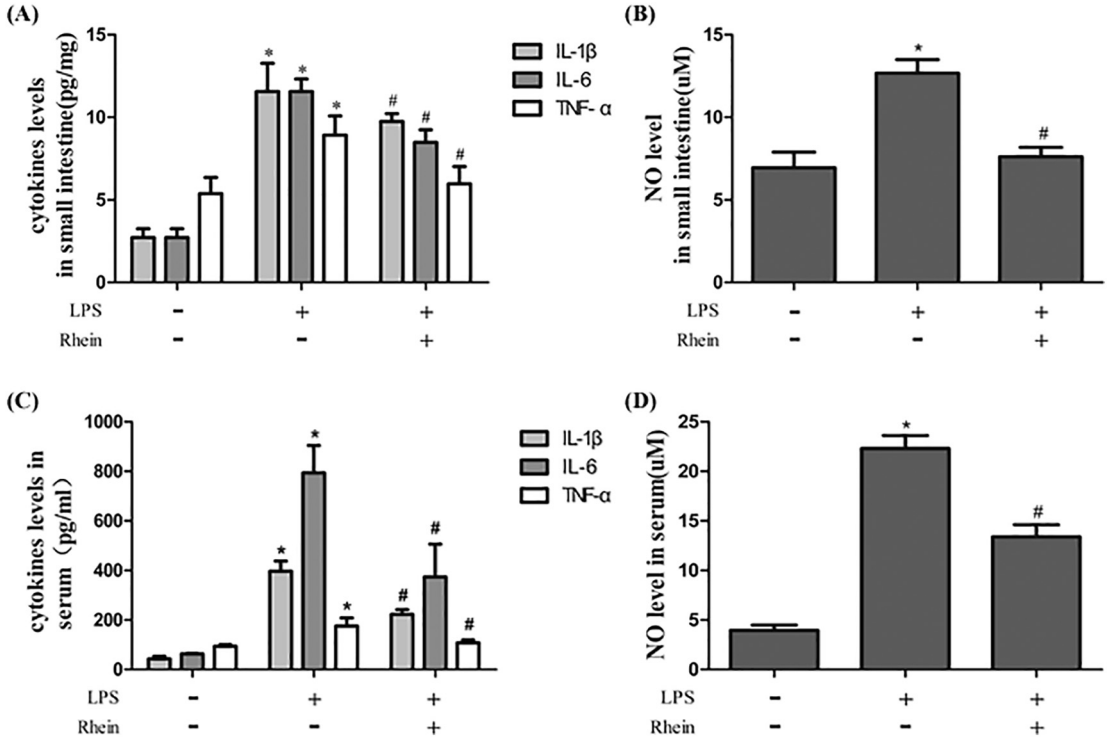

Fig. 6. Effects of rhein on the production of inflammatory mediators in a rat model of LPS-induced intestinal barrier injury. Cytokines in (A) serum and (C) small intestine were measured by ELISA kits, the level of NO in (B) serum and (D) small intestine was measured by Griess Reagent kit. Each value represents the mean ± SD. * $P < 0.05$ compared with the control group, # $P < 0.05$ compared with the LPS-induced group.

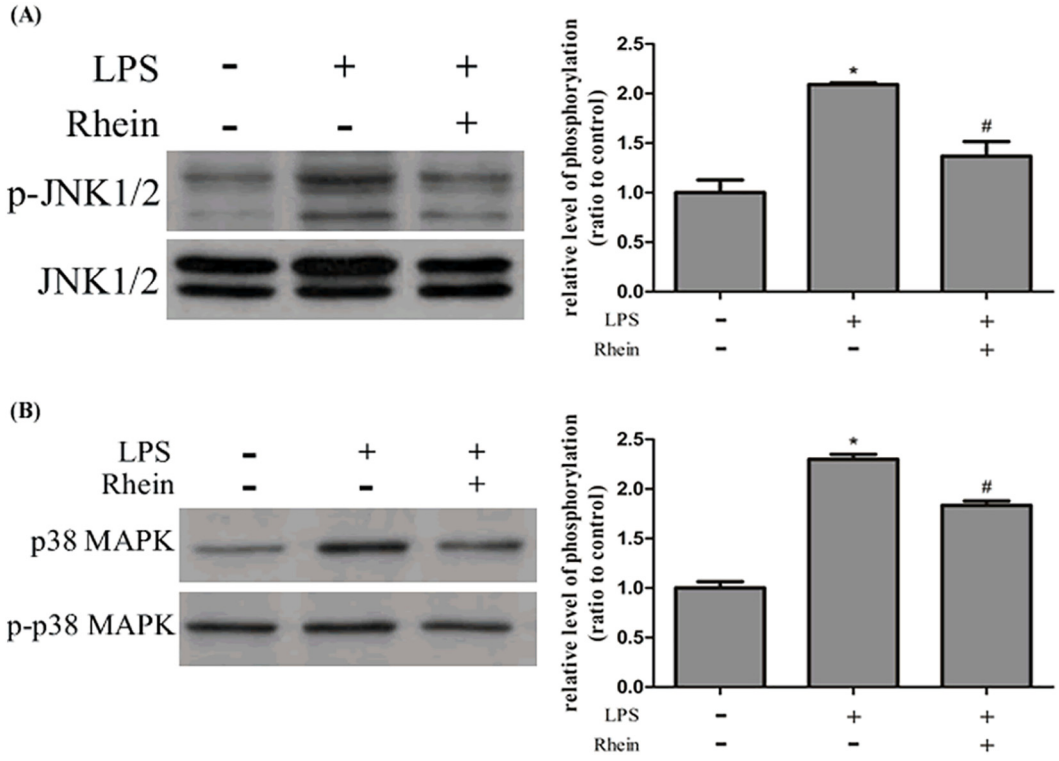

Fig. 7. Effects of treatment with rhein on (A) the phosphorylation of JNK in tissues, (B) the phosphorylation of p38 MAPK in tissues. Protein samples were analyzed by western blotting with specific antibodies. Each value represents the mean ± SD. * $P < 0.05$ compared with the control group, # $P < 0.05$ compared with the LPS-induced group.

the LPS group were obviously higher than in the control group ($P < 0.05$). However, rhein pretreatment significantly attenuated the changes ($P < 0.05$).

4. Discussion

Rhein is one of the active ingredients isolated from *Rheum rhabarbarum* and has been considered as the antioxidant and anti-inflammatory agent. In the present study, we used a rat model of intestinal barrier injury induced by LPS, a common method used in stimulating the characteristic of intestinal barrier injury [17,18], to further elucidate the potential underlying mechanisms how rhein protects the small intestinal barrier.

The previous study has proved that rhein could inhibit LPS-induced intestinal injury in colon with LPS stimulation [16]. In our study, we investigated whether rhein could attenuate the intestinal barrier injury caused by LPS. Both the morphologic alterations of intact intestinal villi and chemical markers were employed to determine the change of intestinal barrier integrity to LPS administration. The morphologic alterations of intact intestinal villi is a symbol of the intestinal barrier injury including LPS-induced injury [19,20], while DAO and D-lac, the chemical markers which are usually low in the circulation system in healthy individuals, will have a significant increase in the circulation system during the destruction of the intestinal barrier [21]. Our model showed that LPS induced broken intestinal villi in small intestine, increased tendencies in the levels of DAO and D-lac in serum and led to an evident decrease in ileal DAO level in LPS-induced group. However, all of these changes were significantly inhibited by rhein, which meant that rhein pretreatment protected intestinal barrier function.

It has been proved that tight junctions between intestinal epithelial cells play an irreplaceable role in the function of intestinal barrier [13]. Increased expressions of tight junction proteins contribute to intestinal mucosal barrier function. There are a series of tight junction proteins including claudins, occludins and ZOs, which contribute to the formation of tight junctions. Here we investigated the relative protein expression of ZO-1 and occludin, key components of tight junction that directly involved in the regulation of intestinal barrier function [22]. Results showed that LPS rapidly disrupted ZO-1 and occludin, which is accordant with previous studies [17,23]. However, rhein treatment prevented LPS-induced down-regulation of occludin and ZO-1. These results suggested that rhein might protect intestinal integrity from LPS challenge via regulating expression of tight junction proteins.

Oxidative stress can destroy essential cellular molecules such as lipids, proteins, and DNA, in turn result in a series of diseases, including intestinal barrier injury [24]. Previous reports certified that LPS exposure could lead to oxidative stress in the intestine [25]. Therefore, accelerating inhibition of oxidative stress may be a useful strategy in curing intestinal barrier injury. MDA, a product of lipid peroxidation, is acknowledged to be an index of cellular damage and excessive oxidative stress [26]. According to our results, rhein could down-regulate the level of MDA in small intestine, indicating that rhein could inhibit the oxidative stress during intestinal dysfunction.

Nrf2 is a well-known cytoprotective transcription factor which plays an irreplaceable role in the cellular defense against various oxidative stress-induced tissue injuries including intestinal barrier injury [27,28]. Once stimulated by oxidative stress, it could bind to antioxidant response element and regulate the expression anti-oxidant, phase II detoxifying enzymes, and detoxifying proteins such as HO-1, SOD, catalase and GSH-Px [29]. Based on these reports, we next investigated whether rhein worked by affecting the levels of Nrf2, HO-1, SOD, catalase and GSH-Px. HO-1, a Nrf2 target gene, has an effect on balancing redox homeostasis and inhibiting inflammatory diseases [30,31]. It could catalyze the disassociation of heme into the antioxidant biliverdin and resist oxidative damage. Many investigations have confirmed that up-regulation of HO-1 contributes to the cellular defense mechanism during oxidative stress [32–34]. Our western blotting results showed that LPS inhibited the Nrf2 and HO-1 protein levels in the rat intestine, while rhein supplement promoted Nrf2 and HO-1 expression compared with LPS stimulation independently. SOD, catalase and GSH-Px are also involved in the antioxidative resistance systems. SOD could catalyze oxygen free radicals (O^{2-}) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2), whereas hydrogen peroxide could be converted into H_2O and O_2 by catalase [35]. Another antioxidant enzyme, GSH-Px, is also an important enzyme which could catalyze the decomposition of peroxide [36]. In our study, LPS challenge decreased SOD, catalase and GSH-Px activities, there is in accordance with previous reports [23,37]. In contrast, although rhein administration had no significant effect on the level of SOD, it recovered the activities of catalase and GSH-Px. The present study revealed that Nrf2-mediated antioxidant pathway, at least partly participated in rhein-induced protective effect during intestinal barrier injury.

Previous reports have reported oxidative stress and inflammation are interoperable [33,38]. Oxidative stress damage could lead to inflammation, while inflammation usually accompanies with unbalance between oxidative system and antioxidative system [38]. Pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α play pivotal roles in the inflammatory response to pathologic stimuli including intestinal barrier injury [4,39]. TNF-α and IL-1β could not only cause damage to the intestinal barrier directly [40,41], but also trigger the synthesis of other cytokines such as IL-8 and IL-6 [42]. IL-6 has also been proved to play an important role in the occurrence and persistence of inflammatory response [43]. All of the pro-inflammatory cytokines mentioned above are believed to play a pivotal role in intestinal barrier injury, whereas inhibition of these cytokines is an effective strategy to remedy inflammatory intestinal damage [44]. In the present study, we found that pretreatment of rhein significantly attenuated the levels of pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, which suggested that rhein could inhibit the inflammatory responses.

Nitric oxide is thought to play many regulatory roles during inflammation [45]. The increase of nitric oxide contributes to intestinal barrier injury during inflammation [46]. The ability to inhibit nitric oxide production is important for medicine which could prevent the tissues from the inflammatory injury. Rhein reduced the production of nitric oxide in LPS-induced rats, which indicated a potential anti-inflammatory effect of rhein.

MAPKs (including JNK, p38 MAPK and Erk1/2) are serine–-threonine protein kinases that regulate a wide range of cellular activities including proliferation, differentiation, apoptosis, survival, inflammation, and innate immunity [47]. P38 MAPK and JNK are activated by various types of stress as well as microbial components such as bacterial lipopolysaccharide and play important roles in regulating cytokine release such as IL-6, IL-1β and TNF-α [48,49]. We investigated the effects of rhein on LPS-induced activation of JNK and p38 MAPK. Results showed that LPS injection caused the activation of JNK and p38 MAPK in small intestine, while rhein administration significantly inhibited LPS-induced the phosphorylation levels of JNK and p38 MAPK in small intestine. In addition, Nrf2-mediated HO-1 pathway was also irreplaceable in the inhibition of inflammation [50]. As mentioned above, rhein pretreatment led to the increase of Nrf2 and HO-1 expression, which may also promote to the inhibition of inflammation during intestinal barrier injury.

5. Conclusion

Our study has shown that rhein can inhibit intestinal injury with LPS challenge. The mechanisms of protective effects of rhein may be associated with the improvement of anti-inflammatory and antioxidant effects via inactivating JNK and p38 MAPK and activating Nrf2 pathway.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grant Numbers 31472228).

Conflict of interest

The authors have no conflict of interest.

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