Barbaloin Attenuates Mucosal Damage in Experimental Models of Rat Colitis by Regulating Inflammation and the AMPK Signaling Pathway

Background: Barbaloin is one of the main medicinal ingredients of aloe vera, which displays various anti-inflammatory and anti-apoptosis properties in several inflammatory and fibrotic diseases. Our study evaluated its efficacy against dextran sulfate sodium (DSS)-induced colitis in rats.

Material/Methods: Ulcerative colitis (UC) rat models were established in vivo, and after barbaloin treatment, body weight and inflammation index were measured. Additionally, the signaling mechanism by which barbaloin protects against UC was investigated using LPS-infected Caco-2 cells.

Results: Barbaloin could significantly reverse UC-induced weight loss and colon injury. Further, it could effectively increase the mRNA expression of IL-4 and IL-10 in colon tissues, while decreasing the expression of IFN-γ, IL-6, IL-1β, and TNF-α. Furthermore, it significantly enhanced UC-inhibited atresia band 1 (ZO-1), occludin, and E-cadherin, and was also found to activate the AMPK signaling pathway. Additionally, si-RAN-induced knockdown, and overexpression assay showed that barbaloin could inhibit the UC-enhanced MLCK signaling pathway by activating the AMPK signaling pathway.

Conclusions: Barbaloin can effectively inhibit inflammation and reverse epithelial barrier function to protect against UC, possibly via activation of the AMPK signaling pathway.

MeSH Keywords: AMP-Activated Protein Kinases • Anti-Inflammatory Agents, Non-Steroidal • Colitis, Ulcerative

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ANIMAL STUDY

Background

Inflammatory bowel disease (IBD) is a chronic recurrent gastrointestinal inflammatory disease. Its etiology is uncertain, and the factors that promote its pathogenesis include genetic, immunologic, microbial, and environmental factors [1]. Its 2 classical forms are Crohn’s disease (CD) and ulcerative colitis (UC) [2]. CD, like granulomatous inflammation, often leads to fistula formation, affecting the large and small intestines [3], while UC, which gives rise to colorectal cancer, is a chronic abscess confined to colon mucosa [4]. Many studies have noted that from UC to colorectal cancer, there is a malignant transformation rate of 2%, 8%, and 18% after 10, 20, and 30 years, respectively [5].

In the last 2 decades, synthetic medicines, including mesalazine, sulfasalazine, aminosalicylates, infliximab, and sulfonamides, which are reported to have some adverse effects, have been widely used clinically in UC treatment [5,6]. Infliximab-treated patients are at risk of developing infections such as coccidoid fungal disease and aspergillosis [7], and malignant transformation, severe congestive heart failure, and encephalalgia have also been observed with its clinical administration [8]. Similarly, previous studies have revealed that, in addition to liver function impairment, long-term use of aminosalicylates in UC treatment increases the risk of gastric ulcer and gastrointestinal bleeding.

Barbaloin (10-β-D-glucopyranosyl-1,8-dihydroxy-3-hydroxy-methyl-9(10H)-anthracenone), found in plants of the Liliaceae family, is the main medicinal component of aloe vera [9]. Previous studies found that it has a number of pharmacological functions, including potential antitumor activity, as well as anti-oxidant and anti-inflammatory activities [10,11]. Based on these pharmacological functions observed in ethnomedical studies, the present study was conducted to investigate whether barbaloin could protect against UC in rats, and to determine the underlying molecular mechanisms associated with this process. Thus, this study evaluated the protective effects of barbaloin against intestinal barrier changes and its pathogenesis, including changes in zonula occluden-1 (ZO-1), occludin, and E-cadherin, in DSS-induced colitis rats. Additionally, its effect on inflammation and its underlying mechanisms were investigated in vivo and in vitro.

Material and Methods

Chemicals and reagents

Dextran sulfate sodium (DSS, Mw 30 000 to 50 000 kDa) was obtained from MP Biomedicals (Solon, OH, USA), and barbaloin (purity >98%), whose chemical structure is shown in Figure 1, was obtained from J&K Scientific (Beijing, China). All chemicals used in this study were of analytical grade.

Animals

Thirty-six male Wistar rats (7–8 weeks old) weighing 160–220 g were purchased from Nanjing Qinglong Experimental Animal Center, Shanghai Institute for Biological Science, Chinese Academy of Sciences. The animals had access to standard diet pellets and filtered water, and were acclimatized for 1 week. The experiments were conducted in accordance with the guidelines for experimental animal care and use of the Second Affiliated Hospital of Soochow University, whose Ethics Committee approved this study.

DSS-induced colitis

The 36 rats were divided into 3 groups of 12 each as follows: i) Control group: rats in this group received drinking water only, and served as normal controls. ii) DSS-induced colitis group (DSS group): rats in this group were administered 3% w/v DSS (dissolved in drinking water) for 6 days, and served as the model group. iii) DSS-induced colitis plus barbaloin-treated group (DSS+Barbaloin group): rats in this group were administered 100 mg/kg barbaloin (dissolved in distilled water) for 9 days after the 6 days of DSS treatment.

Disease activity index of weight loss was measured daily from day 1 to day 15. To collect blood and colonic tissue samples, the rats in the all groups were anesthetized with an intraperitoneal injection of 4% (w/v) chloral hydrate from Sinopharm Chemical Reagent Co. (Shanghai, China), and fresh colon tissue samples collected were frozen at –80°C for further analysis.

Histopathological examination

On day 15, the rats were anesthetized, and colon tissue samples were collected (4 rats were randomly selected from
Total RNA was extracted using TRIzol reagent from Life RNA isolation and RT-quantitative polymerase chain reaction (qPCR) was performed using an ABI 7300 real-time PCR system from Applied Biosystems, Thermo Fisher Scientific, Inc. (Rochester, NY, USA). The primer sequences were as follows: IL-1β, forward, 5'-CTCACAAGCAGAGCAAGC-3' and reverse, 5'-CAGTCGCCACCATACTTGG-3'; IL-4, forward, 5'-GGTCCTCAAACCACAGCTG-3' and reverse, 5'-GCGGATGATCTCTCAAGTGA-3'; IL-6, forward, 5'-AGGATACACACCTCAACAGAC-3' and reverse, 5'-AAGTGACATCATCGTTGATCTACA-3'; IL-10, forward, 5'-ATGCTGCTGCTTACGATG-3' and reverse, 5'-CCCAGAATACCCCTTAAAAGTCCTGC-3'; IFN-γ, forward, 5'-GCTCTAGAGATTTCAACTTCTTGGCTTA-3' and reverse, 5'-TTTGCGAGCCAGGGAGCAACACATCT-3'; TNF-α, forward, 5'-ACACAGAAGCAGATGACGCGG-3' and reverse, 5'-CTATGAGGAGGGAGCCTG-3'; GAPDH, forward, 5'-GATGAGGAGGGAGCCTG-3' and reverse, 5'-GGTCCTGCTGCTTACGATG-3'. GAPDH was used as an internal control, and its Ct value was used to calculate the relative amount of mRNA copy number in each sample. The 2-ΔΔCt was calculated as the relative expression level of the target gene (ΔΔCt=Ct target gene–Ct GAPDH).

**Protein extraction and Western blotting**

Western blotting was performed as previously described [14]. Total protein concentration was detected using a BCA protein test kit from Beyotime Institute of Biotechnology (Shanghai, China). SDS-PAGE was used to separate the proteins, and 50 μg of protein was transferred onto a PVDF membrane from Millipore (Darmstadt, Germany). The membranes were blocked at 4°C with 5% bovine serum albumin (BSA) in TBST buffer, and were exposed to antibodies overnight, including ZO-1, E-cadherin, occludin, MLCK, p-AMPK, AMPK, and GAPDH antibodies, in 1% BSA. After washing with 0.1% Tween in PBS, the membranes were incubated with secondary antibodies (1: 3000) at room temperature. After a stripping procedure, GAPDH Western blotting was performed on the same membrane, and the membranes were then visualized using a non-radioactive ECL kit from Amersham Biosciences (Piscataway, NJ, USA).

**Statistical analysis**

All experiments were performed in triplicate. Quantitative data are presented as mean±SD. Mean differences between 2 groups were determined using the t test. All statistical analyses were performed using SPSS v19.0 from IBM Corp. (Armonk, NY, USA), and p values <0.05 were considered statistically significant.

Each group. Thereafter, the colon tissues were embedded in paraffin and stained with H&E for histological evaluation, as described by Wirtz et al. [12].

**Intestinal permeability**

*In vivo* intestinal epithelial permeability was evaluated as previously described [13]. The rats in each group were fasted for 12 h, and 500 mg/kg fluorescein isothiocyanate (FITC)-dextran solution was intragastrically administered the following day. After 4 h, 4% (w/v) chloral hydrate was intraperitoneally administered. Thereafter, blood samples (5 mL) were collected from the left ventricle, and serum FITC levels determined at 450 nm using a microplate fluorometer from Thermo Fisher Scientific, Inc. (Rochester, NY, USA).

**Cell model and TER**

Caco-2 cells were purchased from American Type Culture Collection (ATCC® HTB-37TM; Manassas, VA, USA) and maintained in antibiotic-free DMEM from Gibco, Inc. (Rochester, NY, USA) containing 10% FBS from Sigma-Aldrich, 100 u/ml penicillin, and 100 μg/ml streptomycin from Biochrom GmbH (Berlin, Germany). The cells were incubated at 37°C in 5% CO₂ (90% humidity), and 1 μg/mL LPS-damaged cells were treated with barbaloin. The transepithelial electrical resistance (TER) of the filter-grown Caco-2 intestinal monolayers were then measured once a day using an epithelial voltohmeter. A TER value decrease was considered to be a significant barrier dysfunction indicator.

**Cell transfection**

Cells were seeded onto 6-well plates after reaching about 60% confluence per well, and were transfected with specific siRNA from Polyplus Transfection (Illkirch, France), or c-DNA targeting AMPK with Lipofectamine 2000 from Shanghai Heng Fei Biotechnology Co. (Shanghai, China). The sequences were as follows: si-AMPK sequences, sense: 5'-GAGCCGACUAUCAAGACACUTT-3'; anti-sense: 5'-AUGUCUUUGAUGUCUGCUCTT-3'; AMPK c-DNA sequences, sense: 5'-CACAAGCTACATTCTACAGAGAGCTG-3'; anti-sense: 5'-CGCGGTATCTCCTGTTAGAATGCA-3'. After 48 h of incubation, LPS was added, and the cells were treated with barbaloin (50 μg/mL) for another 24 h. Then, the cells were collected for RNA and protein analysis.

**RNA isolation and RT-quantitative polymerase chain reaction (qPCR)**

Total RNA was extracted using TRizol reagent from Life Technologies (Copenhagen, Denmark), following the manufacturer’s protocol. RNA was reverse transcribed into cDNA with a PrimeScript Reverse Transcription (RT) Master Mix Perfect Real-Time kit, and real-time quantitative PCR was performed using an ABI 7300 real-time PCR system from Applied Biosystems, Thermo Fisher Scientific, Inc. (Rochester, NY, USA).
Results

Effects of barbaloin on DSS-influenced body weight, W/L ratio, and histological evaluation \textit{in vivo}

Rats in the DSS and DSS+Barbaloin groups exhibited marked weight loss 5 to 7 days after the initiation of the 6-day DSS treatment (Figure 2A), and after day 8, they showed body weight recovery, which was significantly greater on days 9, 10, 12, 13, and 15 in the DSS + Barbaloin group than that in the DSS group. Additionally, the colonic W/L ratio of the rats in the DSS group was markedly higher than that of the rats in the control group, whereas the ratio of the rats in DSS+Barbaloin group was significantly higher than that of the rats in the DSS group (Figure 2B). Colon histological and morphological characteristics were subsequently examined using H&E staining. In the control group, colon tissues showed normal crypt morphology and goblet cell count, as well as the absence of mucosal thickening and ulcerations (Figure 2D). In contrast, the colon tissues of rats in the DSS group exhibited superficial ulcerations, goblet cell loss, neutrophil infiltration, and goblet cell damage, indicating that they had a significantly higher histological damage score than that of the control group (Figure 2C). Additionally, relative to the colon tissue of rats in the DSS group, that of...
the rats in the DSS+Barbaloin group showed reduced neutrophil infiltration and minimal goblet cell loss; hence, a significantly lower histological damage score (Figure 2C).

The effects of barbaloin on the DSS-enhanced serum FITC-dextran in vivo

The use of FITC-dextran to evaluate barbaloin’s effects on intestinal permeability revealed that FITC-dextran level in the serum of rats in the DSS group was significantly higher than that in the control group, and the elevated serum FITC-dextran levels of the rats in the DSS+Barbaloin group were markedly reduced by barbaloin treatment (Figure 3).

Effects of barbaloin on the DSS-decreased expression of ZO-1, E-cadherin, and occludin in vivo

To investigate the involvement of epithelial tight junctions, the effect of barbaloin on tight junction-associated protein expression levels was evaluated using Western blotting, which revealed that ZO-1, E-cadherin, and occludin protein expression levels in the rats of the DSS group were significantly downregulated. Moreover, the DSS-suppressed ZO-1, occludin, and E-cadherin protein expression levels were effectively upregulated by barbaloin treatment (Figure 4).
Effects of barbaloin on the DSS-increased expression of pro-inflammatory factors in vivo

In the DSS group, IL-1β, IL-6, IFN-γ, and TNF-α mRNA expression levels in colonic tissue were significantly upregulated compared to those in the control group, whereas barbaloin treatment effectively suppressed their expression levels (Figure 5A, 5C, 5E, 5F). Moreover, IL-4 and IL-10 mRNA expression levels, which were significantly downregulated in the DSS group compared to those in the control group, were effectively upregulated by barbaloin treatment (Figure 5B, 5D).

Effects of barbaloin on the AMPKs pathway in DSS-induced colitis rats

Preliminary results showed that MLCK, p-AMPK, and AMPK protein expression levels in the DSS group were significantly upregulated. However, they were effectively downregulated by barbaloin treatment (Figure 6).

Effects of barbaloin on barrier dysfunction in Caco-2 cells

LPS-induced Caco-2 cell injury resulted in a decline in TER, which was markedly increased by barbaloin concentrations ranging between 25 and 100 μg/ml (Figure 7A). With an incubation
time ranging between 12 and 48 h, 50 μg/ml of barbaloin could significantly increase LPS-reduced TER (Figure 7B). Additionally, LPS-induced Caco-2 cell injury significantly reduced ZO-1, E-cadherin, and occludin, while barbaloin concentrations ranging between 25 and 100 μg/ml markedly increased the expression of ZO-1, E-cadherin, and occludin (Figure 7C, 7D).

**Effects of barbaloin on the regulation of AMPK/MLCK signaling pathway**

LPS-induced Caco-2 cell injury resulted in a decline in AMPK phosphorylation, while barbaloin concentrations ranging between 50 to 100 μg/mL (Figure 8A), with culture time ranging between 12 and 48 h (Figure 8B), effectively increased LPS-decreased p-AMPK in a time- and dose-dependent manner. Further experiments demonstrated that barbaloin-promoted AMPK signaling was effectively blocked by si-RNA-induced AMPK knockdown (Figure 8C), and barbaloin failed to further increase cDNA-facilitated AMPK signaling (Figure 8D). Additionally, the findings of this study suggest that LPS intervention could obviously increase MLCK signaling in Caco-2 cells. Barbaloin concentrations ranging between 25 and 100 μg/mL (Figure 9A), with culture time ranging between 12 and 48 h (Figure 9B), upregulated MLCK signaling in a time- and dose-dependent manner. As shown in Figure 9C and 9D, si-RNA-induced AMPK knockdown effectively suppressed MLCK expression, while barbaloin-induced MLCK decrease was abolished by si-RNA-induced AMPK knockdown. cDNA-induced AMPK overexpression significantly suppressed MLCK expression, while barbaloin failed to further decrease the inhibited MLCK signal.

**Discussion**

UC is characterized by serious complications and high morbidity rates, and is difficult to treat [15]. Experimental animal models of UC established by DSS administration are widely used in preclinical studies [16,17]. In the present study, significant differences in body weight, colonic W/L ratio, and histologic score were found among DSS-treated and control group rats, and barbaloin treatment could successfully reverse these disease activity indices, implying that it may be applicable in UC treatment in humans.
Various attempts have been made to recover normal immune response by administering pure natural compounds, confirmed to play protective roles in various inflammatory diseases [18]. Barbaloin is the major anthraquinone obtained from aloe leaf exudates [19,20], and previous studies noted that it plays an important role in immune regulation, intestinal moistening, and defecation [20–22]. Additionally, its use in the treatment of trauma, burns, asthma, and ulcers in folk medicine in China, due to its anti-bacterial and anti-inflammatory effects, is well known [20]. Zhang et al. [23] reported that barbaloin significantly reduced myocardial ischemia-reperfusion injury in rats by activating AMPK. Additionally, it is important to note that many studies have shown that it protects against tBHP/hemin-induced oxidative stress by promoting the sulfhydryl group and Ca^{2+}-ATPase on erythrocyte membranes [24].

UC is well known to be a disease associated with the immune system. The function of the intestinal epithelial barrier is to prevent causative agents and pathogenic antigens from penetrating the intestinal wall and destroying the immune system [25,26]. In physiological conditions, the transepithelial pathway and epithelial tight junctions contribute to intestinal epithelial permeability [27]. Nutrients can be absorbed into blood circulation via the intestinal epithelium pathway, while most of the hazardous substances are restricted due to the effects of epithelial tight junctions [28]. Additionally, previous studies have demonstrated that occludin, E-cadherin, and ZO-1 are critical regulators of intestinal barrier function, which plays important roles in maintaining intestinal permeability [29]. Although it is unknown whether intestinal mucosal barrier function is associated with intestinal inflammation, it is considered to play an important role in the pathophysiological mechanism of UC [30,31]. The present study found that barbaloin effectively inhibited DSS-downregulated tight junction protein expression, thus preventing increases in serum FITC-dextran levels. This observation demonstrates that barbaloin can restore intestinal barrier function in DSS-colitis rats by preventing tight junction protein downregulation. The underlying mechanism of this protective effect is probably associated with occludin, E-cadherin, and ZO-1 regulation.

Inflammation is a common pathologic process in UC development [32], and inflammatory factors are classified as pro-inflammatory and anti-inflammatory cytokines. Pro-inflammatory

**Figure 7.** Barbaloin protected against LPS-induced barrier function impairment in Caco-2 cells. (A) Effects of different barbaloin concentrations on LPS-decreased TER. (B) Effects of different barbaloin (50 μg/mL) treatment time points on LPS-decreased TER. (C–D) Effects of different barbaloin concentrations on LPS-decreased protein expression of ZO-1, E-cadherin, and occludin. **p<0.01 vs. the LPS(–) and Barbaloin(–) groups, and * p<0.05 and ** p<0.01 vs. the LPS(+) and Barbaloin(–) groups.
cytokines, including TNF-α, IL-1β, and IL-6, can be induced by macrophages and Th1 cells [33], while anti-inflammatory cytokines, including IL-4 and IL-10, are mainly produced by Th2 cells, and play an important role in the inflammatory reaction [34,35]. Under UC conditions, the balance between these anti-inflammatory and pro-inflammatory cytokines is upset, resulting in internal environment disorders [36,37]. The underlying mechanism of DSS-induced intestinal inflammation may be associated with mucosal damage in epithelial cells, inflammatory cytokine imbalance in the intestinal mcosa, and the induction of the excessive production of oxidative factors [38,39]. The present study found that the inflamed colon of DSS-induced colitis rats showed mucosal hyperemia, edema, increased mural thickness, and crypt distortion. Further, epithelial layer damage, goblet cell depletion with insufficient mucus production, and the transmural infiltration of inflammatory cells were clearly observed in DSS-induced colitis rats. Furthermore, the DSS-induced inflammatory changes in colon

Figure 8. Barbaloin enhanced LPS-decreased AMPK signaling in Caco-2 cells. (A) Effects of different barbaloin concentrations on LPS-suppressed AMPK signaling. (B) Effects of different barbaloin (50 μg/mL) treatment time points on LPS-suppressed AMPK signaling. (C) AMPK knockdown prevented barbaloin-enhanced AMPK phosphorylation. (D) Barbaloin failed to further increase cDNA-facilitated AMPK phosphorylation. "p<0.01 and "p<0.05 vs. the LPS(–) and Barbaloin(–) groups, and "p<0.05 and ""p<0.01 vs. the LPS(+) and Barbaloin(+) groups.

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tissues were associated with changes in intestinal permeability, increased expression of IL-1β, IL-6, IFN-γ, and TNF-α, and decreased expression of IL-4 and IL-10, unlike those observed in the control group. In this study, we found that barbaloin could effectively suppress the expression of IL-1β, IL-6, IFN-γ, and TNF-α, while increasing that of IL-4 and IL-10, compared to that in the DSS group, indicating that it can regulate inflammatory mediators, thereby suppressing an increase in inflammatory and immune responses.

Recent studies have demonstrated that the tight junctions between intestinal epithelial cells can be destroyed, thereby significantly increasing intestinal epithelium permeability under inflammatory conditions, which may cause intestinal mucosal barrier dysfunction, bacterial and toxin translocation, and excessive or imbalanced intestinal mucosal immune responses, and eventually lead to the occurrence of chronic intestinal inflammation [40,41]. When intestinal barrier function is damaged, MLCK protein expression increases, while the...
expression of ZO-1, E-cadherin, and obstructive protein (tight junction) decreases [42].

The findings of this study suggest that the mechanisms behind the protective effect of barbaloin on DSS-induced intestinal epithelial barrier dysfunction may be as follows: UC-inhibited ZO-1, occludin, and E-cadherin were significantly enhanced by barbaloin treatment, which was found to also activate the AMPK signaling pathway. Similar results were validated in LPS-induced Caco-2 cells. Barbaloin effectively promoted LPS-decreased p-AMPK and markedly suppressed the LPS-increased MLCK expression, suggesting that it probably attenuated LPS-induced intestinal barrier dysfunction via AMPK signal activation. Further, it should be noted that barbaloin could neither increase AMPK expression nor decrease LPS-increased MLCK expression, following si-RNA induced AMPK knockdown. Similarly, it could neither promote AMPK signaling nor suppress LPS-increased MLCK expression, following cDNA-induced AMPK overexpression, demonstrating that it could reverse epithelial barrier dysfunction to protect against UC, possibly by activating the AMPK signaling pathway.

This study has some limitations. Firstly, although it evaluated barbaloin’s efficacy against DSS-induced colitis in rats, these results should be supported by research in more experimental animal models, including mouse and zebrafish colitis models. Additionally, since UC is a disease with multiple etiologies, whose potential pathogenesis remains unclear, other signaling pathways in which barbaloin may be involved should be explored.

Conclusions

Conclusively, this study demonstrated that barbaloin can alleviate UC by promoting intestinal barrier function and anti-inflammatory factor production, and that the underlying mechanisms may be associated with the AMPK signaling pathway. Thus, barbaloin might contribute to the development of new pharmaceutical products for UC treatment, and further research is in progress to define the specific mechanism involved in its action in UC to aid its development as an effective drug in the future.

Conflict of interest

None.

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