Asiatic acid ameliorates dextran sulfate sodium-induced murine experimental colitis via suppressing mitochondria-mediated NLRP3 inflammasome activation

Wenjie Guo\textsuperscript{a,b,1}, Wen Liu\textsuperscript{b,1}, Biao Jin\textsuperscript{b}, Ji Geng\textsuperscript{a}, Jing Li\textsuperscript{a}, Hongquin Ding\textsuperscript{a}, Xuefeng Wu\textsuperscript{b}, Qiang Xu\textsuperscript{b,\ast,}, Yang Sun\textsuperscript{b,\ast}, Jing Gao\textsuperscript{a,\ast,\ast}

\textsuperscript{a} School of Pharmacy, Jiangsu University, 301 Xuefu Road, Zhenjiang 212013, China
\textsuperscript{b} State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, 22 Han Kou Road, Nanjing 210093, China

\textsuperscript{1} These authors contributed equally to this work.

\textsuperscript{\ast} Corresponding authors at: School of Life Sciences, Nanjing University, Nanjing 210093, China. Tel.: +86 25 88791552.
\textsuperscript{\ast,\ast} Corresponding author at: J. Gao, School of Pharmacy, Jiangsu University, Zhenjiang 212013, China. Tel.: +86 511 88791552.

E-mail addresses: molpharm@163.com (Q. Xu), yangsun@nju.edu.cn (Y. Sun), jinggao@ujs.edu.cn (J. Gao).

\textsuperscript{1} These authors contributed equally to this work.

\textbf{A R T I C L E  I N F O}

\textbf{Article history:}
Received 1 September 2014
Received in revised form 20 November 2014
Accepted 4 December 2014
Available online 15 December 2014

\textbf{Keywords:}
Asiatic acid
Colitis
NLRP3 inflammasome
IL-1β
ROS

\textbf{A B S T R A C T}

In the present study, the effect of asiatic acid, a natural triterpenoid compound, on murine experimental colitis induced by dextran sulfate sodium (DSS) and its possible mechanism were examined in vivo and vitro. Oral administration of asiatic acid dose-dependently attenuated the loss of body weight and shortening of colon length induced by DSS. The disease activity index, histopathologic scores of mucosa and myeloperoxidase activity were also significantly reduced by asiatic acid treatment. Protein and mRNA levels of DSS-induced pro-inflammatory cytokines in colon, including TNF-\textalpha, IL-1\beta, IL-6 and IFN-\gamma, were markedly suppressed by asiatic acid. At the same time, decreased activation of caspase-1 in peritoneal macrophages was detected in asiatic acid-treated mice, which suggested that the NLRP3 inflammasome activation was suppressed. In addition, we also found that asiatic acid dose-dependently inhibited IL-1β secretion, caspase-1 activation as well as inflammasome assembling in vitro. Furthermore, the mechanism of asiatic acid was related to the inhibition of mitochondrial reactive oxygen species generation and prevention of mitochondrial membrane potential collapse. Taken together, our results demonstrate the ability of asiatic acid to inhibit NLRP3 inflammasome activation and its potential usage in the treatment of inflammatory bowel diseases.

© 2014 Elsevier B.V. All rights reserved.

\textbf{1. Introduction}

Ulcerative colitis, characterized by chronic, relapsing and remitting inflammation, not only impairs patients’ quality of life, but also increases the risk of colon cancer [1,2]. The pathogenesis of ulcerative colitis is complex and may involve genetic, environmental and immunological factors. It is suggested that a breakdown at the epithelial barrier, followed by inappropriate responses to microbial products and chronic inflammation in a genetically susceptible hosts may play a key role in this disease [3]. To investigate this disease in mice, a chemical-induced model of acute colonic inflammation has been introduced by oral administration of dextran sulfate sodium (DSS) and characterized by a general inflammatory process associated with weight loss and histopathologic features that mimic some clinical demonstrations of ulcerative colitis [4].

The NLRP3 inflammasomes are multi-protein complexes which recognize unique microbial and danger components and serve as a platform for caspase-1 activation and pro-inflammatory cytokine IL-1β maturation [5,6]. NLRP3 inflammasome activation has a crucial role in host defense against infection while excessive activation will lead to various auto-inflammatory conditions [7]. Thus NLRP3 inflammasome complex may serve as a potential target for the development of novel therapeutics for patients with inflammatory related diseases [8].

Asiatic acid, a natural triterpenoid compound, is one of the active constituents of the Chinese herb \textit{Centella asiatica} which is known in both traditional Chinese medicine and Indian Ayurvedic medicine. \textit{C. asiatica} and asiatic acid show a low risk of adverse side-effects and a long history of successful use in traditional medicine which is also the constituent of other plant in the leaf like \textit{Terminalia catappa} [9]. Usually, the extract of \textit{C. asiatica} is used as a drug or tea as well as cosmetic act [10]. Previous studies have demonstrated that asiatic acid exhibits a variety of pharmacological activities including antioxidant, anti-inflammation, neuroprotective and anti-cancer effects [11–16]. In the present study, we examined the anti-colitis effect of asiatic acid and showed that asiatic acid significantly inhibited NLRP3 inflammasome activation and reduced the susceptibility to DSS-induced experimental colitis in mice.

http://dx.doi.org/10.1016/j.intimp.2014.12.009
1567-5769/© 2014 Elsevier B.V. All rights reserved.
2. Materials and methods

2.1. Mice

Six- to eight-week-old female C57BL/6 mice were purchased from the Model Animal Genetics Research Center of Nanjing University (Nanjing, China). Animal welfare and experimental procedures were carried out strictly in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, the United States) and the related ethical regulations of our university. All efforts were made to minimize animals' suffering and to reduce the number of animals used.

2.2. Reagents

Asiatic acid, phorbol myristate acetate (PMA), lipopolysaccharide (LPS) and adenosine triphosphate (ATP) were purchased from Sigma-Aldrich (St. Louis, MO). Dextran sulfate sodium (DSS, 36–50 kDa) was bought from MP Biomedical (Aurora, OH). Myeloperoxidase (MPO) activity assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). RPMI-1640 and fetal bowel serum were purchased from Life Technology (Carlsbad, CA). FLICA Caspase-1 Assay Kit was bought from Immunochemistry Technologies Company (Bloomington, USA). Caspase-1/ICE Colorimetric Assay Kit was bought from Beyotime (Nantong, China). ELISA kits for IFN-γ and human IL-1β were purchased from eBioscience (San Diego, CA). Anti-ASC was purchased from Santa Cruz (Santa Cruz, CA). FITC-anti-CD11b was purchased from Dakewe Biotech Co. Ltd (Beijing, China). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

2.3. Cell culture

Human THP-1 cells were purchased from Shanghai Institute of Cell Biology (Shanghai, China) and maintained in RPMI 1640 medium, supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomycin and 10% fetal calf serum under a humidified 5% (v/v) CO₂ atmosphere at 37 °C.

2.4. Induction of colitis and treatment

Colitis was induced in C57BL/6 mice with 2.5% DSS (molecular weight 36–50 kDa) dissolved in drinking water (days 1–7). Normal mice were given water. Vehicle control (water), asiatic acid (3, 10, 30 mg/kg) and sulfasalazine (200 mg/kg) were given orally from day 1 to day 10, respectively.

2.5. Clinical scoring and histological analysis

Body weight, stool consistency and the presence of gross blood in feces and at the anus were observed everyday. The disease activity index (DAI) was calculated by assigning well-established and validated scores. Briefly, the following parameters were used for calculation: a) diarrhea (0 points = normal, 2 points = loose stools, 4 points = watery diarrhea); b) hematochezia (0 points = no bleeding, 2, slight bleeding, 4 points, gross bleeding) [17]. At day 10 following induction of colitis, animals were sacrificed, the colon was removed and pieces of colonic tissue were used for ex vivo analysis. For histological analysis, part of the colon was fixed in 10% buffered formalin and embedded in paraffin. Sections were stained with H&E according to standard protocols. Histological scoring was performed in a blinded way by a pathologist. Histological evaluation of H&E-stained colonic sections was graded as follows: 0, no signs of inflammation; 1, low leukocyte infiltration; 2, moderate leukocyte infiltration; 3, high leukocyte infiltration, moderate fibrosis, high vascular density, thickening of the colon wall, moderate goblet cell loss, and focal loss of crypts; and 4, transmural infiltrations, massive loss of goblet cell, extensive fibrosis, and diffuse loss of crypts.

2.6. Assessment of myeloperoxidase (MPO) activity

Neutrophil infiltration into inflamed colonic mucosa was quantified by MPO activity assessment using the O-dianisidine method. Protein extracted from colonic tissue was used to assess the MPO level according to the manufacturer's instructions. The results were shown as activity units per mg tissue.

2.7. Cytokine analysis by ELISA

Colons from mice in each group were homogenated with lysis buffer to extract total protein. The homogenate was centrifuged at 12,000 g at 4 °C for 15 min. The amount of total extracted protein was determined by BCA™ protein assay kit (Pierce, Rockford, IL). The amount of IFN-γ, IL-1β, IL-6 and TNF-α in the colon homogenate was quantified by ELISA kit (Dakewe, Beijing, China).

2.8. Real-time quantitative PCR

RNA samples were reverse transcribed to cDNA and subjected to quantitative PCR, which was performed with the BioRad CFX96 Touch™ Real-Time PCR Detection System (BioRad,Ca) using iQ™SYBR® Green Supermix (BioRad,CA), and threshold cycle numbers extracted from colonic tissue was used to assess the MPO level according to the manufacturer's instructions. The results were shown as activity units per mg tissue.

2.9. Western blotting

2.10. Coimmunoprecipitation assay

For coimmunoprecipitation, cells were lysed in lysis buffer containing Triton X-100 and cell lysates were immunoprecipitated with antibody to ASC or control IgG with protein A/G-Sepharose. The beads were washed, separated by SDS-PAGE and analyzed by immunoblot with antibodies to caspase-1 and NLRP3. Protein bands were visualized using western blotting detection system.
2.11. Peritoneal macrophage purification

Peritoneal macrophages were obtained from the peritoneal cavity by PBS lavage. Cells were washed twice in PBS and suspended in RPMI-1640 medium containing 10% FCS, 10,000 U/ml penicillin and 10 mg/ml streptomycin. The macrophages suspended in culture medium were cultured in 24-well microplates for 40 min at 37 °C in a moist atmosphere of 5% CO2. Non-adherent cells were removed by washing the plate twice with PBS. The adherent macrophages were used for experiments.

2.12. FACS staining for caspase-1 activity

Spleen cells were extracted from normal, vehicle-treated and asiatic acid-treated colitis mice (30 mg/kg) at day 7 and stained with CD11b-PE and FLICA Caspase-1 (FITC) as according to the operation protocol (FLICA Caspase-1 assay Kit, Immunochemistry Technologies Company). Activation of caspase-1 in CD11b + cells was analyzed by FACS staining.

2.13. Statistical analysis

Results were expressed as mean ± SEM of three independent experiments and each experiment included triplicate sets. Data were statistically evaluated by one-way ANOVA followed by Dunnett’s test between control group and multiple dose groups. The level of significance was set at a P value of 0.05.

3. Results

3.1. Asiatic acid attenuated DSS-induced experimental colitis

In the present study, a mouse model of DSS-induced experimental colitis was used to evaluate the therapeutic effect of asiatic acid. Mice were challenged with DSS for 7 days and thus led to inflammatory conditions in the colon. It is well known that DSS induces a severe illness in mice characterized by a dramatic loss of body weight, evident rectal bleeding and diarrhea. As shown in Fig. 1A, compared with vehicle-treated group, administration of asiatic acid prevented the reduction of body weights. Asiatic acid also significantly reduced the disease activity index (DAI), a clinical parameter reflecting the severity of weight loss, rectal bleeding and stool consistency (Fig. 1B). DSS typically causes colonic shortening while such change was also improved by 10 and 30 mg/kg of asiatic acid and 200 mg/kg of sulfate (Fig. 1C and D). Histological analysis showed distortion of crypts, loss of goblet cells, infiltration of mononuclear cells, and severe mucosal damage in the colon specimens of colitis mice (Fig. 2A). The results of standard pathological examination in mice showed much improvement in pathological changes in mice treated with 10 and 30 mg/kg of asiatic acid and 200 mg/kg of sulfate (Fig. 2B). The myeloperoxidase (MPO) activity in colons

Fig. 1. Asiatic acid treatment improved DSS-induced colitis in mice. (A–D) Mice were given 2.5% DSS in drinking water for 7 days, then provided with water for another 3 days before sacrificed. Asiatic acid was given via i.g. from day 1 to day 11. (A) Loss of basal body weight of each group during the disease process. (B) Disease activity index (DAI) was calculated. (C) Macroscopic appearances and (D) the length of colons from each group of mice were measured. Data are presented as means ± SEM (n = 6 per group). In (A) and (B), *P < 0.05, **P < 0.01 vs. DSS-treated alone group at the same day. In (D), ***P < 0.01 vs. DSS-treated alone group.
from asiatic acid-treated mice was also lower than that of the vehicle-treated group (Fig. 2C).

3.2 Asiatic acid regulated the cytokine profiles in colons of mice with DSS-induced colitis

To determine the effect of asiatic acid on cytokine production in mice with DSS-induced colitis, cytokine expression in colons at both mRNA and protein levels were measured in parallel following induction of colitis. Total RNA of colons were extracted and analyzed for cytokine mRNA expression using quantitative RT-PCR method. As shown in Fig. 3, the mRNA expressions of IFN-γ, TNF-α, IL-1β, and IL-6 were remarkably increased after DSS challenge. Asiatic acid significantly inhibited the elevated expression of these cytokines after DSS challenge (Fig. 3A). Moreover, we analyzed the cytokine levels in colonic homogenated protein from each group via ELISA analysis. Administration of asiatic acid to mice significantly down-regulated the inflammatory cytokines at protein level (Fig. 3B).

3.3 Asiatic acid reduced cleaved caspase-1 expression of peritoneal macrophages in DSS-induced colitis mice

To further investigate the mechanism of protection from colitis by asiatic acid, we examined the regulation of caspase-1 activation in murine peritoneal macrophages in vivo from each group. We observed that expression of activated caspase-1 (caspase-1 p10) was significantly elevated in macrophages from vehicle-treated DSS mice. In contrast, little cleaved caspase-1 activation was detected in either Asiatic...
Fig. 4. Asiatic acid inhibited NLRP3 inflammasome activation in mice with DSS-induced colitis. (A) Peritoneal macrophages were isolated from normal, vehicle-treated and asiatic acid-treated colitis mice (30 mg/kg) at day 7. Caspase-1 activation in peritoneal macrophages was examined by western blot. (B) Spleen cells were extracted from normal, vehicle-treated and asiatic acid-treated colitis mice (30 mg/kg) at day 7 and caspase-1 activation in CD11b+ cells was analyzed by FACS staining. Data shown here are representative of three different experiments. Data are presented as means ± SEM (n = 6 per group). *P < 0.05, **P < 0.01 vs. DSS-treated group.

Fig. 5. Asiatic acid inhibited IL-1β processing via inactivation of NLRP3 inflammasome. (A) LPS-primed THP-1 cells were treated with asiatic acid (15, 30, 60 μM) for 1 h, followed by 1 h incubation of 5 mM ATP. Released IL-1β in the supernatant was analyzed by ELISA. (B) Protein levels of pro-caspase-1, cleaved caspase-1, ASC and NLRP3 were determined by Western blot. (C) Caspase-1 activity was measured. (D) LPS-primed THP-1 cells were treated with asiatic acid (15, 30, 60 μM) for 1 h, followed by 1 h incubation of 5 mM ATP. Proteins were isolated and immunoprecipitated with an Ab against ASC. *P < 0.05, **P < 0.01 vs. LPS + ATP-treated group.
3.4. Asiatic acid inhibited the activation of NLRP3 inflammasome in vitro

IL-1β was processed as an inactive cytoplasmic precursor (pro-IL-1β) which has to be cleaved by caspase-1 to produce the mature active form. We examined the ability of asiatic acid to inhibit the activation of pro-IL-1β by NLRP3 inflammasome. As shown in Fig. 5A, asiatic acid exhibited a concentration-dependent inhibition of IL-1β secretion from lipopolysaccharide (LPS)-treated human mononcytic THP-1 cells by the ELISA assay without affecting the survival of macrophages (data not shown). Recent evidence indicates that a caspase recruitment domain-containing protein called ASC binds pro-caspase-1 (p45) to induce the autocleavage of pro-caspase-1 to produce the mature active form (p10 and p20). Consistently, our results showed that activation of caspase-1 (as indicated by the presence of the cleaved form and enzyme activity) was significantly inhibited by asiatic acid (Fig. 5B and C). Furthermore, immunoprecipitation analysis showed that the process of NLRP3 inflammasome formation was also interrupted by asiatic acid (Fig. 5D).

3.5. Asiatic acid inhibited ROS generation and mitochondria dysfunction

It is reported that in the presence of signal I (NF-κB signaling), the NLRP3 inflammasome is activated by mitochondrial signaling which licensed production of IL-1β. NLRP3 secondary signal activators such as ATP induce mitochondrial dysfunction, resulting in the elevation of ROS which then activates the NLRP3 inflammasome [18]. As shown in Fig. 6, ATP treatment caused mitochondrial damage, as demonstrated by membrane potential collapse (JC-1 staining, Fig. 6A) and ROS generation (Mitosox staining, Fig. 6B). Asiatic acid treatment significantly scavenged ROS and restored the mitochondria membrane potential (Fig. 6A and B).

4. Discussion

Ulcerative colitis is a chronic inflammatory disorder in the gastrointestinal tract. It has a high prevalence worldwide and is a well-established risk factor of colorectal cancer. Generally, ulcerative colitis is treated as an autoimmune disease. Treatments including anti-inflammatory drugs, immunosuppressant, and biological therapy targeting specific components of the immune response have been proved effective in controlling the symptoms. However, they also have potential side effects including steroid dependence [19] or serious infections [20,21]. Therefore, therapeutic options and approaches for ulcerative colitis need to be developed. We report herein that the small molecule asiatic acid protects mice against DSS-induced colitis by reducing IL-1β release from macrophages via NLRP3 inflammasome inhibition.

As we know, IL-1β is a proinflammatory cytokine mainly produced by activated macrophages and monocytes, and it is likely to be essential in the early phase of the inflammatory cascade leading to the inflamed colon. High levels of the proinflammatory cytokine IL-1β are detected in cases of active colitis and are correlated with the severity of inflammation [5,22–26]. Of note, IL-1β modulates the functions of dendritic cells, macrophages, neutrophils, as well as the differentiation of Th17 cells [27,28]. It is now well-established that IL-1β is translated as an inactive 31-kDa precursor (pro-IL-1β) after toll like receptor stimulation (signaling I), and this precursor is cleaved to its activated 17-kDa form by the NLRP3 inflammasome-activated caspase-1. And mechanism study has proved that NLRP3/ASC/caspase-1-mediated maturation of IL-1β is essential for experimental colitis induced by DSS and NLRP3−/− mice developed a less severe colitis than wild-type mice [29–32]. On the other hand, there are some reports that showed that the NLRP3 inflammasome protected against loss of epithelial integrity and mortality during experimental colitis [33]. In addition, Liu et al. demonstrated the protective role of inflammasomes in the host defense against citrobacter rodentium infection [34]. Our in vivo experiments here showed that treatment with asiatic acid significantly reduced the expression of IL-1β and prevented the activation of caspase-1 in peritoneal macrophage cells of mice with DSS-induced colitis. Meanwhile, the in vitro study suggested that asiatic acid could inhibit NLRP3/ASC/Caspase-1 complex formation thus suppressing the activation of caspase-1. We hypothesized that the beneficial effect of asiatic acid on DSS-induced colitis might be attributed to its inhibition of inflammasome activation. At the same time our previous work has confirmed that some compounds can prevent the colitis by hampering NLRP3 inflammasome activation [35,36].

NLRP3 inflammasome is a cytosolic multiprotein complex and is increasingly being recognized because of their clinical importance in autoimmune, infectious and metabolic diseases [37,38]. Previous studies have suggested that reactive oxygen species (ROS) derived from damaged mitochondria is the major factor that activate NLRP3 inflammasome [39–41]. Here we showed that asiatic acid treatment effectively prevented ATP-induced collapse of the mitochondrial membrane potential and ROS generation. These results coincided with our previous study that asiatic acid can directly interact with mitochondria and prevent the open of

---

Fig. 6. Asiatic acid treatment reduced mitochondrial ROS generation and prevented mitochondrial membrane potential collapse. THP-1 cells were treated with asiatic acid (15, 30, 60 μM) for 1 h, followed by 1 h incubation of 5 mM ATP and then stained with MitoSOX (A) or JC-1 (B) and analyzed by FACS. Data shown here are representative of three different experiments. Data are presented as means ± SEM (n = 3). *P < 0.05, **P < 0.01 vs. ATP-treated group.
mitochondria permeability transition pore [15,42]. Now we are wondering the exact mechanism on how asiatic acid works on the mitochondria permeability transition pore.

In conclusion, our work explored a novel therapeutic strategy for ulcerative colitis by targeting NLRP3 inflammasome with asiatic acid. Administration of asiatic acid significantly attenuated DSS-induced experimental colitis in mice. The mechanism of asiatic acid’s action involved inhibition of ROS–NLRP3–caspase-1–IL-1β cascade in macrophages. Our results collectively suggest that asiatic acid acts as an effective candidate compound for the treatment of ulcerative colitis.

Acknowledgments

This work was supported by the Natural Science Foundation of China (no. 81402938, 81373400 and 91229109), the Natural Science Foundation of Jiangsu Province (nos. BK2012710 and BK2014575), the Grant of Jiangsu University (no. 13JDG064) and the Graduate Research and Innovation Project in Jiangsu Province (no. 1293000504).

References