Norisoboldine, a natural aryl hydrocarbon receptor agonist, alleviates TNBS-induced colitis in mice, by inhibiting the activation of NLRP3 inflammasome

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[ABSTRACT] Although the etiology of inflammatory bowel disease is still uncertain, increasing evidence indicates that the excessive activation of NLRP3 inflammasome plays a major role. Norisoboldine (NOR), an alkaloid isolated from Radix Linderae, has previously been demonstrated to inhibit inflammation and IL-1β production. The present study was to examine the effect of NOR on colitis and the underlying mechanism related to NLRP3 inflammasome activation. Our results showed that NOR alleviated colitis symptom in mice induced by 2, 4, 6-trinitrobenzene sulfonic acid (TNBS). Moreover, it significantly reduced expressions of cleaved IL-1β, NLRP3 and cleaved Caspase-1 but not ASC in colons of mice. In THP-1 cells, NOR suppressed the expressions of NLRP3, cleaved Caspase-1 and cleaved IL-1β but not ASC induced by lipopolysaccharide (LPS) and adenosine triphosphate (ATP). Furthermore, NOR could activate aryl hydrocarbon receptor (AhR) in THP-1 cells, inducing CYP1A1 mRNA expression, and promoting dissociation of AhR/HSP90 complexes, association of AhR and ARNT, AhR nuclear translocation, XRE reporter activity and binding activity of AhR/ARNT/XRE. Both siAhR and α-naphthoflavone (α-NF) markedly diminished the inhibition of NOR on NLRP3 inflammasome activation. In addition, NOR elevated Nrf2 level and reduced ROS level in LPS- and ATP-stimulated THP-1 cells, which was reversed by either siAhR or α-NF treatment. Finally, correlations between activation of AhR and attenuation of colitis, inhibition of NLRP3 inflammasome activation and up-regulation of Nrf2 level were validated in mice with TNBS-induced colitis. Taken together, NOR ameliorated TNBS-induced colitis in mice through inhibiting NLRP3 inflammasome activation via regulating AhR/Nrf2/ROS signaling pathway.

[KEY WORDS] Norisoboldine; Inflammatory bowel disease; NLRP3 inflammasome; Aryl hydrocarbon receptor

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Introduction

Inflammatory bowel disease (IBD) is characterized by severe pain, diarrhea and bleeding, which not only impairs patients’ quality of life but also contributes to the risk of colon cancer [1]. IBD comprises of multiple types of gastrointestinal inflammatory diseases, and Crohn’s disease (CD) and ulcerative colitis (UC) constitute the most common forms [2]. Although the exact pathogenesis is still unclear, a breakdown of the epithelial barrier, followed by inappropriate responses to microbial products and chronic inflammation in a genetically susceptible host, plays a key role in the onset and progression of IBD [3]. At present, anti-inflammatory and immunomodulatory agents, such as 5-aminosalicylic acid (5-ASA), are the predominant therapeutic remedies for IBD [4].

IL-1β, a pro-inflammatory cytokine that is mainly produced by activated macrophages and monocytes, is likely to be essential in the early phase of the inflammatory cascade leading to colitis [5]. High level of IL-1β is found in active colitis, and is well correlated with the severity degree of inflammation [6]. It is known that the production of IL-1β in various cells is regulated by the activation of NLRP3 inflammasome [7]. The NLRP3 inflammasome is a multi-protein complex, which can recognize unique microbial and danger
components and serve as a platform for caspase-1 activation and maturation of IL-1β [8]. The activation of NLRP3 inflammasome plays a crucial role in host defense against infection, while its excessive activation will lead to various inflammatory conditions [9]. Thus, inhibition of NLRP3 inflammasome activation is substantially beneficial for the therapy of IBD.

Radix Linderae is the dry root of Lindera aggregate (Sims) Kosterm. (L. strychnifolia Vill), which is used to treat acute and chronic colitis in traditional Chinese medicines. Norisoboldine (NOR), the main isoquinoline alkaloid ingredient of Radix Linderae, has previously been demonstrated to have anti-inflammatory and immune-regulatory effects [10-11]. In our previous studies, NOR was shown to reduce IL-1β production in lipopolysaccharide (LPS)-stimulated RAW264.7 cells at the concentrations of 3, 10, and 30 μmol·L⁻¹, and decreased the serum level of IL-1β in rats with collagen-induced arthritis at the doses of 7.5, 15, and 30 mg·kg⁻¹ [12-13]. Furthermore, many isoquinoline alkaloids could inhibit NLRP3 inflammasome activation. Berberine suppresses TXNIP-mediated NLRP3 inflammasome activation in monosodiumurate (MSU) crystal-stimulated RAW 264.7 macrophages through the up-regulation of Nrf2 transcription factor [14]. Cepharanthine reduces NLRP3 expression in diabetic kidneys of rats [15]. Sinomenine exerts a neuroprotective effect in mice with ischemic stroke by inhibiting NLRP3 inflammasome activation via the AMPK pathway [16]. In the present study, we examined the protective effect of NOR on 2, 4, 6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in mice and explored the underlying mechanism through the regulation of NLRP3 inflammasome activation.

Materials and Methods

Chemicals and reagents

NOR (purity > 98%) was isolated from Radix Linderae. Its structure was identified by 1H and 13C NMR, and its purity was tested by high-performance liquid chromatography by Prof. CHOU Gui-Xin [4]; 5-ASA sustained release granules were purchased from Ipsen Pharma (Ethypharm, Houdan, France); TNBS, α-naphthoflavone (α-NF, purity > 95%), 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT), phorbol myristate acetate (PMA), LPS, adenosine triphosphate (ATP) and 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD, 10 μg·mL⁻¹) were purchased from Sigma-Aldrich (St. Louis, MO, USA); Myeloperoxidase (MPO) activity assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China); Fetal bovine serum (FBS) was purchased from PAA Laboratories GmbH (Morningside, Linz, Australia); siAhR and pGL3-XRE reporter gene plasmid were purchased from Genechem (Shanghai, China); NLRP3, cleaved Caspase-1, NF-E2-related factor 2 (Nrf2), AhR, ARNT and HSP90 antibodies were purchased from Cell Signaling Technology, Inc (Beverly, MA, USA); cleaved IL-1β and β-actin antibodies were purchased from Santa Cruz Bio-technology (Delaware, CA, USA); Annexin V-FITC apoptosis detection kit was purchased from Beyotime Biotechnology (Shanghai, China); TRIZol reagent was purchased from Invitrogen (California, CA, USA); HiScript™ QRTSuperMix was purchased from Vazyme Biotech Co., Ltd. (Piscataway, New Jersey, USA); HiFi™ qPCR SYBR® Green Master Mix was purchased from Yeasen Biotech Company Limited (Shanghai, China); Enhanced chemiluminescent (ECL) plus reagent kit was purchased from Servicebio Company (Wuhan, Hubei, China).

Animals

Male BALB/c mice, 6-8-weeks-old, were obtained from the Comparative Medicine of Yangzhou University (Yangzhou, China). The animal experiment was conducted in Jiangning Campus of China Pharmaceutical University, approved by the Animal Ethics Committee of China Pharmaceutical University [SYXX (SU) 2013-0012]. All animals were housed in the cages (290 mm × 178 mm × 160 mm) under a 12 h light/12 h dark cycle (lights on from 7 am to 7 pm) with controlled room temperature (about 25 °C) and humidity (50%-65%) and allowed access to a diet of standard laboratory chow and tap water ad libitum. The efforts were made to minimize the animals’ suffering and to reduce the number of animals used.

TNBS-induced colitis and administration of NOR

To induce colitis, the mice were lightly anesthetized with ether. TNBS (2 mg) dissolved in 40% ethanol was slowly administered into the lumen of colons by using a 3.5F catheter [17-18]. Then, the mice were kept at a vertical position for 1 min and randomly divided into the following groups: a) Normal group, TNBS group, NOR (20 and 40 mg·kg⁻¹) groups and 5-ASA (200 mg·kg⁻¹) group; and b) Normal group, TNBS group, NOR (40 mg·kg⁻¹) group, α-NF (30 mg·kg⁻¹) group, NOR + α-NF group, and TCDD (25 μg·kg⁻¹) group. NOR and 5-ASA were orally administered with a volume of 0.2 mL from Day 0 to 7; α-NF was intraperitoneally injected with a volume of 0.2 mL from Day 0 to 7; TCDD was intraperitoneally injected with a volume of 0.2 mL only on Day 0. In addition, the mice in normal and TNBS groups were administered with an equal volume of vehicle. The body weight, bleeding and diarrhea of mice were daily measured and recorded from Day 0 to 7.

Colon length and histological scoring

On Day 7, the mice were sacrificed with excessive anesthesia of ether, the colons were removed and photographed, and colon length was measured. The colons were fixed in paraffin, and stained with hematoxylin and eosin (H&E) for histological evaluation. The histological scoring was based on three parameters as described below: a) severity of inflammation: 0 = no inflammation; 1 = mild; 2 = moderate; 3 = severe; b) depth of inflammatory involvement: 0 = no inflammation; 1 = mucosa; 2 = mucosa and submucosa; 3 = transmural; and c) crypt damage: 0 = intact crypts; 1 = loss of the basal one-third; 2 = loss of the basal two-thirds; 3 = entire crypt...
loss but intact epithelial surface; 4 = entire crypt loss and intact epithelial surface with erosion. The histological score was calculated by adding the three evaluations and given a maximal score of 10 [19-20].

Myeloperoxidase (MPO) activity

The MPO activity in the colon tissues of mice with TNBS-induced colitis was detected using a kit according to the manufacturer’s instructions (Jiancheng; Nanjing, Jiangsu, China) [21-22].

Quantitative real-time PCR (Q-PCR)

The sample preparations were as follows: 1) THP-1 cells (1 × 10^5 cells/mL) were seeded into the cell culture flasks or 6-well plates, and pre-treated with PMA (50 ng/mL–1) for 3 h. Then, they were incubated with NOR (3, 10, 30 μmol·L–1), siAhR, α-NF (1 μmol·L–1), NOR (30 μmol·L–1) + siAhR, NOR (30 μmol·L–1) + α-NF (1 μmol·L–1) or TCDD (5 nmol·L–1) for 24 h, and then activated with LPS (2 μg·mL–1) for 3 h and ATP (5 mmol·L–1) for 30 min; and 2) On Day 7, the mice with TNBS-induced colitis were sacrificed with excess anesthesia of ether, and the colons were collected, and washed with PBS thrice. The total RNA in the colon tissues or cells was extracted using TRIzol reagent according to the manufacturer’s instructions. The RNA purity and concentration were determined by measuring and comparing the absorbance at 260 nm and 280 nm. The total RNA (2 μg) samples were reversely transcribed by using HiScript™ QRTSuperMix (Vazyme Biotech; Piscataway, New Jersey, USA). The real-time PCR was performed by using Hieff™ qPCR SYBR® Green Master Mix in a MyiQ2 Detection System (Bio-Rad Laboratories; Hercules, CA, USA). The reaction conditions were as follows: denaturation step of 95 °C for 5 min followed by 40 cycles of amplification and quantification steps of 95 °C for 10 s and 60 °C for 30 s. The primer sequences used in the present study were as follows: IL-1β forward: 5′-AGTTGACGGACCCCAAAAG-3’, reverse: 5′-CTTCTCCACGACCAATGA-3’; IL-6 forward: 5′-CCGAGAGGACCTCAGAG-3’, reverse: 5′-ATTTCCAGATTCGCCAG-3’; TNF-α forward: 5′-AGGCACCCACAAAAAGAT-3’, reverse: 5′-CAGTAGACAGAAAGGCGTGTTG-3’; CYP1A1 (mouse) forward: 5′-GACTTCCAACCTCTTTTCCA-3’, reverse: 5′-GGGTTCCTCCCCACAGTCAG-3’; CYP1A1 (human) forward: 5′-TGTCACTGGTGCATTGCT-3’, reverse: 5′-ATTCAAGGGAGGTTGGGTA-G-3’; and GAPDH forward: 5′-GACCATTTGGAAGGCCACAT-3’, reverse: 5′-CAAGAGGTTCAAAAAACATCG-3’.

Cell culture

The human monocytic cell line THP-1 cells were purchased from the Shanghai Institute of Cell Biology (Shanghai, China), and cultured with RPMI-1640 containing 10% FBS at 37 °C under 5% CO2 condition (Thermo Fisher; Waltham, MA, USA). The cells were passaged every 2–4 days and were used through the 10th passage. They were matured into macrophages by culturing with PMA (50 ng·mL–1) for 3 h. Subsequently, the activated THP-1 cells were used in subsequent experiments unless otherwise indicated.

Cell viability and apoptosis analyses

The viability of THP-1 cells was evaluated by using MTT assay. Briefly, THP-1 cells (1 × 10⁵ cells/mL) were seeded into 96-well plates and incubated with NOR (0, 1, 3, 10, 30, 60, and 100 μmol·L–1) for 20 h. 20 μL of MTT solution (5 mg·mL–1 dissolved in PBS) was added to each well and incubated for another 4 h. Finally, the supernatants were removed, and 150 μL of DMSO was added to each well to dissolve the formazan crystals. The absorbance was measured at 570 nm.

The apoptosis of THP-1 cells was determined using an Annexin V-FITC apoptosis detection kit (Beyotime Biotechnology; Shanghai, China). THP-1 cells were treated with NOR (0, 1, 3, 10, 30, 60, and 100 μmol·L–1) for 24 h, collected in tubes, and centrifuged at 1 000 r·min–1 for 5 min. The supernatants were discarded, and the cells were washed with PBS thrice. Then, 195 μL of Binding Buffer, 5 μL of Annexin-V-FITC and 5 μL of PI were added to tubes. The tubes were then mixed and incubated for 20 min at room temperature in the dark. Subsequently, apoptotic cells were detected and analyzed by flow cytometry (BD Pharmingen; San Diego, CA, USA).

Western blotting and coimmunoprecipitation assays

The procedure of sample preparation was the same as described in Q-PCR assay. Then, the samples were lysed using NP40 buffer (Beyotime, Shanghai, China) containing protease inhibitors on ice for 15 min. After being centrifuged at 12 000 g for another 15 min, the supernatant was collected. The protein content of each sample was determined by Bradford method.

For immunoblotting assay, 20 μg of proteins from each sample was run on 10% SDS-PAGE and electrotransferred onto PVDF membranes. Then, the membranes were incubated with different primary antibodies for NLRP3, cleaved Caspase-1, cleaved IL-1β, ASC, AhR and Nrf2 at 4 °C overnight. After being washed with PBS thrice, the membranes were treated with HRP-conjugated secondary antibody for 2 h at room temperature. Finally, the signals were detected by using ECL reagent [23-24]. The electrophoresis instrument and film transfer apparatus used in this experiment were purchased from Bio-Rad Laboratories (Hercules, CA, USA).

For co-immunoprecipitation assay, the cell lysates were incubated with anti-AhR antibody at 4 °C overnight, and then incubated with Protein A/G Plus-agarose at 4 °C for another 4 h. After being washed with PBST, the proteins were immune-blotted with anti-HSP90 or anti-ARNT antibody.

Electrophoretic mobility shift assay (EMSA)

The THP-1 cells (1 × 10⁵ cells/mL) were seeded into cell culture flasks, and treated with PMA (50 ng·mL–1) for 3 h. Then, they were incubated with NOR (30 μmol·L–1), siAhR, α-NF (1 μmol·L–1), NOR (30 μmol·L–1) + siAhR, NOR (30 μmol·L–1) + α-NF (1 μmol·L–1) or TCDD (5 nmol·L–1) for 24 h, and activated with LPS (2 μg·mL–1) for 3 h and ATP.
fractionated on a gel electrophoresis at 10 V cm\(^{-1}\) for 3 h and ATP (5 mmol L\(^{-1}\)). Subsequently, the cells were harvested into 1.5-mL EP tubes and washed with PBS twice. Subsequently, protein-DNA complex (10 μL) was fractionated on a gel electrophoresis at 10 V cm\(^{-1}\) for 1 h at 4 °C. Then, they were transferred to a nylon membrane. For super-shift assay, the nuclear extracts were incubated with anti-AhR antibody for 30 min at 37 °C before the complexes were analyzed by EMSA. The bands on nylon membranes were visualized using film exposure with ECL reagent.

**Immunofluorescence assay**

The level of ROS was detected using the DCFH-DA fluorescence assay. Briefly, TPH-1 cells (1 × 10\(^5\) cells/mL) were seeded into 6-well plates and cultured overnight. After being treated with corresponding concentrations of test samples, they were harvested into 1.5-mL EP tubes and washed with PBS twice. Subsequently, the cells were stained with 500 μL of ROS detection solution at 37 °C for 20 min, and analyzed by using Olympus IX51 (Olympus; Tokyo, Japan).

**Transfection**

The THP-1 cells were transfected with lentivirus-mediated siAhR for 72 h according to the manufacturer’s protocols (Genechem; Shanghai, China). Briefly, the cells were seeded into 6-well plates at 40% confluence on the day before transfection. For viral infection, titrated viral stocks were suitably diluted in complete medium to obtain the desired multiplicity of infection (MOI), which was then added to each plate. Then, the expression of AhR was detected to assess the infection efficiency at 72 h after infection [25].

**Luciferase reporter assay**

The THP-1 cells were seeded into 24-well plates, and treated with pGL3-XRE reporter gene plasmid by lipofectamine 2000 (at a final concentration of 50 mmol L\(^{-1}\)) for 24 h. After transfection, the cells were pre-treated with corresponding concentrations of test samples, activated with LPS (2 μg·mL\(^{-1}\)) for 3 h and ATP (5 mmol L\(^{-1}\)) for 30 min, and lysed. The lysates were assayed for luciferase activity by using the luciferase assay system (Beyotime; Shanghai, China) with a microplate reader (Thermo Fisher; Waltham, MA, USA) [26].

**Statistical analysis**

The data were presented as means ± SEM. Significant differences of data using inhibitor or siRNA were assessed through two-way analysis of variance (ANOVA) followed by a post hoc Tukey’s test; significant differences of other data were assessed through one-way ANOVA followed by a post hoc Tukey’s test. \(P\) values less than 0.05 (\(P < 0.05\)) were considered statistically significant.

**Results**

**NOR alleviates TNBS-induced colitis in mice**

To test the anti-colitis effect of NOR, experimental colitis was induced in BALB/c mice by TNBS administration, and the clinical signs and symptoms, such as body weight loss, bleeding, and diarrhea, and survival rate were observed and recorded. TNBS treatments led to reduced survival rate and increased persistent body weight loss, NOR (20 and 40 mg·kg\(^{-1}\)) and 5-ASA (200 mg·kg\(^{-1}\)) showed significant protective effects (Figs. 1A and B).

The colon length is an indirect and reproducible morphological indicator for the severity of inflammation in colon. Significant shortening of colons was observed in the mice with TNBS-induced colitis, which was reversed by NOR (20 and 40 mg·kg\(^{-1}\)) markedly (Fig. 1C). In addition, clinical activity score and MPO activity were also significantly reduced by NOR (20 and 40 mg·kg\(^{-1}\)) and 5-ASA (200 mg·kg\(^{-1}\)) (Figs. 1D and E). H&E stain analysis further indicated that severe damage of crypts, loss of goblet cells, infiltration of mononuclear cells, and even formation of serious ulcers in the colons of the mice with TNBS-induced colitis, and NOR (40 mg·kg\(^{-1}\)) and 5-ASA (200 mg·kg\(^{-1}\)) significantly ameliorated these pathological changes (Fig. 1F).

**NOR reduces the expressions of pro-inflammatory cytokines and the activation of NLRP3 inflammasome in the colons of mice with TNBS-induced colitis**

Accumulative evidence indicates that persistent inflammation occupies an important position in the occurrence and development of IBD and impairs intestinal mucosa barrier. IL-1β, an important pro-inflammatory cytokine, has been proven to be essential in the early phase of the inflammatory cascade of colitis. As shown in Fig.2A, the mRNA level of IL-1β in the colons of colitis mice was obviously increased, and NOR (20 and 40 mg·kg\(^{-1}\)) and 5-ASA (200 mg·kg\(^{-1}\)) showed obvious reduction. Furthermore, the mRNA expressions of IL-6 and TNF-α were also inhibited by NOR (40 mg·kg\(^{-1}\)) treatment, but the inhibitory potency was relatively weaker.

It is generally accepted that the production of IL-1β in multiple kinds of cells is regulated by NLRP3 inflammasome. We further carried out in-depth research from the angle of NLRP3 inflammasome activation. The results showed that NOR (20 and 40 mg·kg\(^{-1}\)) significantly inhibited the protein expressions of NLRP3, cleaved Caspase-1, and cleaved IL-1β, but not ASC in the colons of TNBS-induced colitis mice (Fig. 2B).

**NOR does not affect the vitality and apoptosis of THP-1 cells**

Then, a series of in vitro experiments were performed. To exclude the interference of cytotoxic action, the effect of NOR on the vitality and apoptosis of THP-1 cells was detected by using MTT and Annexin V/PI assay, respectively. Results showed that NOR (0, 1, 3, 10, and 30 μmol·L\(^{-1}\)) treatment for 24 h did not affect the vitality of THP-1 cells (Fig. 3A). In addition, the apoptosis of THP-1 cells was also not influenced by NOR at above-mentioned concentrations (Fig. 3B).
Fig. 1 Effects of norisoboldine (NOR) on TNBS-induced colitis in mice. The mice were challenged with TNBS, NOR (20 and 40 mg·kg⁻¹) and 5-aminosalicylic acid (5-ASA, 200 mg·kg⁻¹) were orally administered daily from Day 0 to 7. (A) Survival rate. (B) Body weight. (C) The colon length. (D) Clinical activity score. (E) The activity of MPO in colons. (F) The histological changes of colons. Histologic grades were evaluated according to epithelium destruction, infiltration of inflammatory cells and crypt destruction. The data are expressed as means ± S.E.M. **P < 0.01 vs Normal group; *P < 0.05 and ***P < 0.01 vs TNBS group, n = 7–12
Fig. 2  Effects of norisoboldine (NOR) on expressions of pro-inflammatory cytokines and activation of NLRP3 inflammasome in the colons of mice with TNBS-induced colitis. Mice were challenged with TNBS, NOR (20 and 40 mg·kg⁻¹) and 5-aminosalicylic acid (5-ASA, 200 mg·kg⁻¹) were orally administered daily from Day 0 to 7. Then, mice were executed by ether anesthesia, and colons were collected. (A) The mRNA expression of TNF-α, IL-1β and IL-6 were detected by using Q-PCR assay. (B) The protein expressions of NLRP3, ASC, cleaved Caspase-1 and cleaved IL-1β were detected by using western blotting assay. The data are expressed as means ± S.E.M. ##P < 0.01 vs Normal group; *P < 0.05 and **P < 0.01 vs TNBS group, n = 7–12

NOR inhibits the activation of NLRP3 inflammasome in THP-1 cells

The inflammasome is a complex composed of many proteins. It can modulate the activation of caspase-1 and promote the cleavage and maturation of pro-IL-1β. To establish the model of NLRP3 inflammasome activation, THP-1 cells were adopted as tools, LPS and ATP were used as inducers. NOR (10 and 30 μmol·L⁻¹) significantly inhibited the protein expression of NLRP3, cleaved Caspase-1, and cleaved IL-1β, but not ASC in LPS- and ATP-activated THP-1 cells (Fig. 3C). NOR activates aryl hydrocarbon receptor (AhR) in THP-1 cells

AhR, a ligand-dependent transcription factor, belongs to the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) family. In rest state, it locates at the cytosol. After binding with ligands, it translocates to the nucleus, associates with ARNT to form AhR/ARNT heterodimers, and binds to XRE in the promoter of target genes such as CYP1A1 [27]. As shown in Figs. 4A and B, NOR concentration-dependently increased the mRNA expression of CYP1A1, and the action was significantly prevented by siAhR and α-NF (1 μmol·L⁻¹). In addition, NOR (30 μmol·L⁻¹) promoted the dis-association of HSP90/AhR complexes, nuclear translocation of AhR, association of ARNT and AhR, and DNA binding activity of AhR/ARNT/ XRE (Figs. 4C–F). The activity of XRE reporter gene was also up-regulated by NOR (30 μmol·L⁻¹) treatment (Fig. 4G).

Subsequently, NOR was added in combination with si-AhR or α-NF, and the activation of NLRP3 inflammasome activation was detected. The results showed that both siAhR and α-NF (1 μmol·L⁻¹) significantly prevented NOR-induced reductions of NLRP3, cleaved Caspase-1, and cleaved IL-1β expression in THP-1 cells (Fig. 5A). Furthermore, NOR (20 and 40 mg·kg⁻¹) also up-regulated the nuclear translocation of AhR and the expression of CYP1A1 but not AhR in the colons of mice with TNBS-induced colitis (Fig. 5B–D). Taken together, NOR could activate AhR in THP-1 cells, and AhR plays an important role in the inhibitory effect of NOR on NLRP3 inflammasome activation.

Mechanisms by which NOR inhibits the activation of NLRP3 inflammasome after activating AhR in THP-1 cells

Data in mountain demonstrate that AhR activation results in a rise of Nrf2 level in cells, and subsequently decreases the level of ROS, which acts as the proximal signals for NLRP3 inflammasome activation. In the present study, after being stimulated with LPS and ATP, the levels of Nrf2 and ROS in
THP-1 cells were significantly regulated, NOR (10, 30 \(\mu\)mol·L\(^{-1}\)) showed significant up-regulation of Nrf2 level and down-regulation of ROS level, respectively (Figs. 6A and B). In addition, \(\alpha\)-NF (1 \(\mu\)mol·L\(^{-1}\)) and siAhR significantly prevented NOR-regulated levels of Nrf2 and ROS in THP-1 cells (Figs. 6C and D).

\(\alpha\)-NF almost abolishes NOR-mediated anti-colitis effect, inhibition of NLRP3 inflammasome activation, and up-regulation of Nrf2 level

Finally, to confirm the important role that AhR played in NOR-mediated protection of colitis, the model of TNBS-induced colitis was re-established in mice. NOR was administered in combination with \(\alpha\)-NF, and TCDD was taken as a positive drug. NOR (40 mg·kg\(^{-1}\), i.g.) and TCDD (25 \(\mu\)g·kg\(^{-1}\), i.p.) significantly ameliorated disease symptoms of mice with colitis, including reducing mortality, body weight loss, clinical activity score and MPO activity, alleviating the shortening of colon length and histopathological changes in colons. \(\alpha\)-NF (30 mg·kg\(^{-1}\), i.p.) itself had no significant effect, but dramatically diminished the action of NOR (Figs. 7A–F).

Fig. 3  Effects of norisoboldine (NOR) on activation of NLRP3 inflammasome in THP-1 cells. (A) The THP-1 cells were treated with NOR (0, 1, 3, 10, 30, 60, and 100 \(\mu\)mol·L\(^{-1}\)) for 24 h, the viability was detected by using MTT assay. (B) The THP-1 cells were treated with NOR (0, 1, 3, 10, 30, 60, and 100 \(\mu\)mol·L\(^{-1}\)) for 24 h, and apoptosis was detected by using Annexin V/PI assay. (C) THP-1 cells were pre-treated with PMA (50 ng·mL\(^{-1}\)) for 3 h. Then, they were incubated with NOR (3, 10, 30 \(\mu\)mol·L\(^{-1}\)) for 24 h. NLRP3 inflammasome was activated with LPS (2 \(\mu\)g·mL\(^{-1}\)) for 3 h, and followed by ATP (5 mmol·L\(^{-1}\)) for 30 min. Then, cells were collected, and protein was extracted. The protein expressions of NLRP3, ASC, cleaved Caspase-1 and cleaved IL-1\(\beta\) were detected by using western blotting assay. The data are presented as means \(\pm\) S.E.M. of three independent experiments. *\(P<0.05\), **\(P<0.01\) vs Normal group; ###\(P<0.01\) vs LPS&ATP group
Fig. 4  Effects of norisoboldine (NOR) on AhR activation in THP-1 cells. (A) The THP-1 cells were treated with NOR (3, 10, and 30 μmol·L⁻¹) and TCDD (5 nmol·L⁻¹) for 24 h, mRNA expression of CYP1A1 was detected by using Q-PCR assay. (B) THP-1 cells were treated with NOR (30 μmol·L⁻¹), siAhR, α-naphthoflavone (α-NF, 1 μmol·L⁻¹), NOR (30 μmol·L⁻¹) + α-NF (1 μmol·L⁻¹) or TCDD (5 nmol·L⁻¹) for 24 h, mRNA expression of CYP1A1 was detected by using Q-PCR assay. (C–E) THP-1 cells were treated with NOR (30 μmol·L⁻¹), α-NF (1 μmol·L⁻¹), NOR (30 μmol·L⁻¹) + α-NF (1 μmol·L⁻¹) or TCDD (5 nmol·L⁻¹) for 24 h, the association of HSP90 and AhR was detected by immunoprecipitation assay (C); the nuclear translocation of AhR was detected by western blotting assay (D); the association of ARNT and AhR was detected by immunoprecipitation assay (E). (F, G) THP-1 cells were treated with NOR (30 μmol·L⁻¹), siAhR, α-NF (1 μmol·L⁻¹), NOR (30 μmol·L⁻¹) + siAhR, NOR (30 μmol·L⁻¹) + α-NF (1 μmol·L⁻¹) or TCDD (5 nmol·L⁻¹) for 24 h, the AhR-XRE binding activity was detected by EMSA assay (F); The activity of XRE reporter gene was detected by luciferase report gene assay (G). The data are presented as means ± SEM of three independent experiments. **P < 0.01 vs LPS & ATP group; $P < 0.05$ and $$$P < 0.01$ vs NOR group.

**Fig. 5** Effects of α-naphthoflavone (α-NF) on norisoboldine (NOR)-inhibited activation of NLRP3 inflammasome in THP-1 cells. (A) The protein expressions of NLRP3, ASC, Caspase-1 and IL-1β were detected by using western blotting assay. (B–D) Mice were challenged with TNBS, NOR (20 and 40 mg·kg⁻¹) and 5-aminosalicylic acid (5-ASA, 200 mg·kg⁻¹) were orally administered daily from Day 0 to 7. Then, mice were executed by ether anesthesia, and colons were collected. The total protein in colons was isolated, and expression of AhR was detected by using western blotting assay (B); The nuclear and cytoplasm protein in colons was isolated, and nuclear translocation of AhR was detected by using western blotting assay (C); The mRNA expression of CYP1A1 in colons was detected by using Q-PCR assay (D). The data are presented as means ± SEM of three independent experiments or seven–twelve mice. ##P < 0.01 vs Normal group; *P < 0.05 and **P < 0.01 vs TNBS group

Furthermore, NOR (40 mg·kg⁻¹) and TCDD (25 μg·kg⁻¹) significantly inhibited the protein expressions of cleaved IL-1β, NLRP3 and cleaved Caspase-1 in the colons of mice with TNBS-induced colitis. When combined with α-NF (30 mg·kg⁻¹), the action of NOR almost disappeared. In addition, α-NF (30 mg·kg⁻¹) obviously reversed NOR-mediated up-regulation of Nrf2 expression in colons of mice with colitis (Fig. 7G).
Fig. 6 Effect of norisoboldine (NOR) on the levels of Nrf2 and ROS in THP-1 cells. (A) The protein expression of Nrf2 was detected by using western blotting assay. (B) The level of ROS was detected by using immunofluorescence assay. (C, D) The levels of Nrf2 and ROS were detected by using above-mentioned methods. The data are presented as means ± SEM of three independent experiments. ** \( P < 0.01 \) vs LPS&ATP group; $$$ \( P < 0.01 \) vs NOR group

**Discussion**

IBD manifests as recurring mucosal inflammation, which can spread from rectum to proximal colon, and even to ileum. The incidence of IBD rises year by year with the changes of dietary structure and environment, and 5-ASA, corticosteroids, immunosuppressive agents and biological agents are usually used in the clinic. Although the kinds of therapeutic drugs are numerous, many problems have arisen, especially in terms of cost and patients’ increased susceptibility to infection as well as occurrence of poor-responders [28-31]. Therefore, new therapeutic agents with high efficiency and few side effects are expected. NOR, the major bioactive ingredient of Radix Linderae, has anti-inflammatory and immune-regulatory effects. It could alleviate joint destruction in rats with adjuvant-induced arthritis by reducing the expression of RANKL, IL-6, PGE2, and MMP-13 at the doses of 7.5, 15, and 30 mg·kg\(^{-1}\); improve the clinical symptoms of mice with collagen-induced arthritis at the doses of 10, 20, and 40 mg·kg\(^{-1}\) [11-12]. In the present study, NOR (20 and 40 mg·kg\(^{-1}\)) was shown to attenuate TNBS-induced colitis in mice. It increased survival rate, reduced mortality, clinical activity score, body weight loss and MPO activity, prevented shortening of colon length and histological changes in colons. In the colon mucosa of IBD patients and model animals, a large population of monocytes and macrophages accumulate. They secrete multiple pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α to damage the mucosal barrier and maintain inflammation. The down-regulation of
Fig. 7  Effects of α-naphthoflavone (α-NF) on norisoboldine (NOR)-mediated inhibition of TNBS-induced colitis in mice. Mice were challenged with TNBS, NOR (40 mg·kg\(^{-1}\), i.g.), α-NF (30 mg·kg\(^{-1}\), i.p.), TCDD (25 μg·kg\(^{-1}\), i.p.) and NOR + α-NF were administered daily for consecutive 7 days. Then, mice were sacrificed, and colons were isolated. (A) Survival rate. (B) Body weight. (C) The colon length. (D) Clinical activity score. (E) The activity of MPO in colons. (F) The histological changes of colons. Histologic grades were evaluated according to epithelium destruction, infiltration of inflammatory cells and crypt destruction. (G) The expressions of Nrf2, NLRP3, cleaved Caspase-1 and cleaved IL-1β in colons were detected by using western blotting assay. The data are expressed as means ± SEM. \(n = 7\)–12. \(\#\) \(P < 0.01\) vs Normal group; \(\ast\) \(P < 0.05\) and \(\ast\ast\) \(P < 0.01\) vs TNBS group; \(\dagger\) \(P < 0.05\) and \(\dagger\dagger\) \(P < 0.01\) vs NOR group.
these cytokines will alleviate the occurrence and development of IBD [30-33]. NOR could inhibit the expressions of IL-1β, IL-6, and TNF-α in the colons of mice with TNBS-induced colitis, and the inhibitory percentage on IL-1β was highest. These findings suggested that NOR might be beneficial for the management of colitis, improving the inflammation and histological changes in colons of patients.

IL-1β is essential in the early phase of the inflammation cascade, leading to the inflamed colon. Importantly, it can modulate the function of multiple cells such as dendritic cells and neutrophils, as well as the differentiation of Th1, Th17 and Treg cells [34-35]. IL-1β is translated as an inactive 31-kDa precursor (pro-IL-1β) after the stimulation of toll like receptor (TLR), which is cleaved to its activated 17-kDa form by NLRP3 inflammasome-activated Caspase-1 [36]. The inflammasome is a multi-protein oligomer, and consists of caspase-1, PYCARD, and NALP. When activated by pathogen-associated patterns or damage-associated molecular patterns, nucleotide-binding and oligomerization domain (NOD)-like receptor (NLR) recruits pro-caspase-1 and the adaptor protein ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) to form the inflammasomes. The excessive inflammasome activation might contribute to the occurrence and development of various inflammation-related diseases including IBD [37]. There are multiple kinds of inflammasomes, and studies about NLRP3 inflammasome are most thoroughly and meticulously. Genome wide association studies (GWAS) have indicated that NLRP3 mutations are significantly and internally associated with Crohn’s disease, and six single nucleotide polymorphisms (SNPs) locate in a regulatory region of NLRP3 [38]. After oral administration of dextran sulfate sodium (DSS), NLRP3 knockout mice develop a less severe colitis than wild-type mice and produce lower levels of pro-inflammatory cytokines IL-1β, TNF-α and IFN-γ-inducible protein 10 in colon tissue [39]. In the present study, NOR reduced the expressions of NLRP3, ASC, and Caspase-1 in colons of mice with TNBS-induced colitis, which indicated that NOR could inhibit the activation of NLRP3 inflammasome. Up to now, the pharmacokinetic characteristics of NOR in mice has not been reported. However, in our previous studies, NOR (3, 10, and 30 μmol·L⁻¹) could inhibit IL-6 production in IL-1β-stimulated synovial fibroblasts and IL-1β production in LPS-stimulated RAW264.7 cells. Therefore, we adopted THP-1 as tool cells and LPS, ATP as inducers to establish model of NLRP3 inflammasome activation. The effect of NOR on NLRP3 inflammasome activation at the concentrations of 3, 10, and 30 μmol·L⁻¹ was investigated in vitro. As expected, the similar results were obtained.

AhR, a multifunctional regulator, plays a critical role in cell development, immune response and inflammatory reaction. In silent state, AhR locates in the cytosol and complexes with the chaperone proteins HSP90, XAP2, and p23. After binding with ligands, it will be released and associates with the AhR nuclear translocator (ARNT), which binds to the cognate DNA binding motifs referred to as AhR responsive elements (AhRE), dioxin response elements (DRE) or xenobiotic response elements (XRE) to regulate transcription of the target genes such as CYP1A1. Interestingly, a previous study indicated that AhR complex can interact with the region of XRE located at the sides of NF-xB binding sites, and block NF-xB DNA binding or mask the NF-xB transcription activity, and then inhibit the expression of NLRP3 [40]. The classical AhR agonist TCDD owns the ability to inhibit NLRP3 expression, caspase-1 activation, and subsequent IL-1β secretion in macrophages, while siAhR shows opposite effects [41]. Furthermore, AhR activation plays an important role in IBD. TCDD can alleviate TNBS-induced colitis in mice, recover the loss of body weight and histological changes, reduce the expressions of IL-6, IL-12, and TNF-α in colons, and increase the Foxp3+ Treg population in the gut immune tissue [42].

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Nrf2 is a nuclear transcriptional factor of Cap’n’ Collar family. In resting state, it integrates with Keap1 (a substrate adaptor, which helps Cullin 3 uniquitiname Nrf2). After being activated, it will translocate into nucleus and heterodimerize with small Maf, which can bind with anti-oxidant response elements (ARE) in the promoter of target genes such as γ-GCSc and γ-GCSm [26, 44]. The γ-GCSc and γ-GCSm work together to catalyze the rate-limiting step in GSH biosynthesis. The increased GSH will reduce level of ROS, which acts as a proximal signals for NLRP3 inflammasome activation [45-47]. In addition, Nrf2 mediates anti-inflammatory action of multiple chemical compounds. Tsai PY et al. have demonstrated that epigallocatechin-3-gallate prevents the development of lupus nephritis in NZB/WF1 mice via enhancing the activation of Nrf2 signaling pathway [48]. De S et al. have observed that allylpyrocatechol attenuates collagen-induced arthritis via attenuation of oxidative stress secondary to modulation of Nrf2/HO-1 signaling pathway [49]. Importantly, the cross talk between AhR and Nrf2 pathway is highly relevant. For examples, Nrf2 is required for TCDD-induced expression of NQO1; AhR activation can regulate the gene transcription of Nrf2; cigarette smoke condensate promotes the binding between AhR and Nrf2 in periplasmatic GC-2spd cells [50-52]. Therefore, we speculated that Nrf2/ROS signals might play key roles in NOR-inhibited NLRP3 inflammasome activation after activating AhR. As expected, NOR significantly up-regulated the protein level of Nrf2 and down-regulated the level of ROS.
in THP-1 cells.

Finally, the correlation between NOR activating AhR, up-regulating Nrf2 expression, inhibiting NLRP3 inflammasome activation, and attenuating colitis was validated in colitis mice induced by TNBS.

Conclusion

In summary, NOR could significantly alleviate TNBS-induced colitis in mice. It acted as an AhR agonist, up-regulated the expression of Nrf2, reduced the level of ROS, and ultimately inhibited the activation of NLRP3 inflammasome in colons.

References


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Prof. DAI Yue, working in China Pharmaceutical University. He focuses his research on anti-inflammatory and immunological pharmacology of traditional Chinese medicine, demonstrated that 1) Blocking synovial angiogenesis was a unique mechanism for anti-rheumatoid arthritis drug scopoletin, and evaluating the value of anti-angiogenesis therapy in RA; 2) Asiatica can not only promote the healing of the wound, but also inhibit the formation of scar. It provides a model effect for the study of dual-directional regulation of traditional Chinese medicine. Up to now, he has published 90 SCI papers, and trained more than 40 doctoral and master graduate students.