Supporting Information

Inhibitory Effect of β-Sitosterol on TNBS-Induced Colitis in Mice

In-Ah Lee, Eun-Jin Kim, Dong-Hyun Kim

Affiliation

Department of Life and Nanopharmaceutical Sciences and Department of Pharmacy, Kyung Hee University, Seoul, Korea

Correspondence

Prof Dr Dong-Hyun Kim
College of Pharmacy
Kyung-Hee University
1, Hoegi, Dongdaemun-ku
Seoul 130-701
Korea
Phone: +82/2/961/0374
Fax: +82/2/957/5030
dhkim@khu.ac.kr
Preparation of experimental colitic mice

The C57BL/6 mice were randomly divided into 5 groups: normal and TNBS-induced colitic groups treated with or without β-sitosterol or sulfasalazine. Each group consisted of 6 mice. TNBS-induced colitis was induced by the administration of 2.5% (w/v) TNBS solution (100 μL) in 50% ethanol into the colon of anesthetized mice via a thin round-tip needle equipped with a 1 mL syringe [1]. The normal group was treated with just the vehicle. The needle was inserted so that the tip was 3.5 - 4 cm proximal to the anal verge. To distribute the agents within the entire colon and cecum, mice were held in a vertical position for 30 s after the injection. Using this procedure, >95% of the mice retained the TNBS enema. If an animal quickly excreted the TNBS-ethanol solution, it was excluded from the remainder of the study. β-Sitosterol (10 and 20 mg/kg) or sulfasalazine (50 mg/kg) dissolved in 2% Tween 80 were orally administered once a day for 3 days after TNBS administration. The mice were sacrificed 8 h after the final administration of test agents. The colon was quickly removed, opened longitudinally, and gently cleared of stool by PBS. Macroscopic assessment of the disease grade was scored according to a previously reported scoring system (0, no ulcer and no inflammation; 1, ulceration and local hyperemia; 2, ulceration without hyperemia; 3, ulceration and inflammation at one site only; 4, two or more sites of ulceration and inflammation; 5, ulceration extending more than 2 cm [2]. Then, the entire colon tissue was used for immunoblotting and enzyme-linked immunosorbent assay analysis.

For the histological exam, the middle part of the colon was fixed in 10%-buffered formalin solution, cut into 7-µm sections, stained with hematoxylin–eosin, and assessed under light microscopy.

Immunostaining for neutrophils
Immunolocalization of neutrophils was analyzed using a 3-step staining procedure consisting of sequential incubation with 1st and 2nd antibodies and streptavidin-biotin complex with horseradish peroxidase. Inflammatory cell profiles in the colonic tissues were investigated using anti-neutrophil [myeloperoxidase (MPO), CD66b, and neutrophil elastase] antibodies. The serial sections were subjected to this procedure. Horseradish peroxidase activity was visualized with 3-amino-9-ethylcarbazole.

**Colon tissue preparation**

Colon tissues were excised, perfused with ice-cold perfusion solution containing 0.15 M KCl and 2 mM EDTA (pH 7.4) and homogenized in 50 mM Tris-HCl buffer (pH 7.4). The homogenates were centrifuged at 10,000 × g at 4 °C for 30 min. The supernatant was used for the estimation of the antioxidant defense system. For immunoblot analysis and ELISA, colon tissues were carefully homogenized in 1 mL of ice cold RIPA lysis buffer containing 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail.

**Assay of myeloperoxidase activity in colonic mucosa**

Colons isolated from the mice were homogenized in a solution containing 0.5% hexadecyl trimethyl ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7.0) and then centrifuged for 30 min at 20,000 × g at 4 °C. A 50 μl aliquot of the supernatant was added to the reaction mixture consisting of 1.6 mM tetramethyl benzidine and 0.1 mM H₂O₂, and incubated at 37°C. Following that, the absorbance was measured at 650 nm. The myeloperoxidase activity was defined as the quantity of enzyme degrading 1 μmol/mL of peroxide at 37°C and expressed in unit/mg protein [1]. The protein content was assayed by Bradford’s method [3].
**Immunoblotting and enzyme-linked immunosorbent assay**

Proinflammatory cytokines TNF-α, IL-1β, and IL-6 in the supernatant extracts prepared from the colon were assessed by ELISA according to the manufacturer’s instructions. For the assay of COX-2, p65, p-p65, and β-actin by immunoblotting, the supernatant extracts prepared from colon and peritoneal macrophages were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat dried-milk proteins in PBST and probed with COX-2, p65, p-p65, or β-actin antibody. After washing with phosphate buffered saline Tween 20 (PBST), proteins were detected with horseradish peroxidase-conjugated secondary antibodies for 1 h. Bands were visualized with enhanced chemiluminescence reagent.

**Isolation and culture of peritoneal macrophages**

Male C57BL/6 mice were intraperitoneally injected with 2 ml of 4% sodium thioglycolate solution. Mice were sacrificed 4 days after injection, and the peritoneal cavities were flushed with 10 mL of RPMI 1640. The peritoneal lavage fluids were centrifuged at 200 × g for 10 min, and the cells were resuspended with RPMI 1640 and plated. After incubation for 1 h at 37 °C, the cells were washed three times, and nonadherent cells were removed by aspiration. Cells were cultured in 24-well plates (0.5 × 10^6 cells/well) at 37 °C in RPMI 1640 plus 10% FBS. The attached cells were used as peritoneal macrophages. To examine the anti-inflammatory effect of β-sitosterol, peritoneal macrophages were incubated in the absence or presence of arctigenin with 50 ng/mL LPS.
Fig. 1S The effect of β-sitosterol on colonic histology in TNBS-induced colitic mice. (A) H&E staining. (B) Immunostaining for neutrophils (MPO). TNBS, except in normal control group (NOR), was intrarectally administered to mice and then treated with saline, β-sitosterol, or sulfasalazine. β-Sitosterol (S10, 10 mg/kg; S20, 20 mg/kg), sulfasalazine (SUL, 50 mg/kg), or saline (TNBS) was orally administered for 3 days after TNBS treatment. The mice were anesthetized with ether and killed 3 days after TNBS treatment.
References

