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ScienceDirect

Journal of Nutritional Biochemistry 57 (2018) 67–76

Journal of Nutritional Biochemistry

Polysaccharide of *Hericium erinaceus* attenuates colitis in C57BL/6 mice via regulation of oxidative stress, inflammation-related signaling pathways and modulating the composition of the gut microbiota

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Received 13 September 2017; received in revised form 25 January 2018; accepted 6 March 2018

Abstract

Inflammatory bowel disease (IBD) is a disease caused by a dysregulated immune with unknown etiology. *Hericium erinaceus* (*H. erinaceus*) is a Chinese medicinal fungus, with the effect of prevention and treatment of gastrointestinal disorders. In this study, we have tested the anti-inflammatory effect of polysaccharide of *H. erinaceus* (HECP, Mw: 86.67 kDa) in the model of dextran sulfate sodium (DSS)-induced colitis in C57BL/6 mice. Our data indicated that HECP could improve clinical symptoms and down-regulate key markers of oxidative stresses, including nitric oxide (NO), malondialdehyde (MDA), total superoxide dismutase (T-SOD), and myeloperoxidase (MPO). HECP also suppressed the secretion of interleukin (IL)-6, interleukin (IL)-1β, tumor necrosis factor (TNF)-α and the expression of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and decreased the expression of related mRNA. Meanwhile, HECP blocked phosphorylation of nuclear factor-κB (NF-κB) p65, NF-κB inhibitor alpha (IκB-α), mitogen-activated protein kinases (MAPK) and Protein kinase B (Akt) in DSS-treated mice. Moreover, HECP reversed DSS-induced gut dysbiosis and maintained intestinal barrier integrity. In conclusion, HECP ameliorates DSS-induced intestinal injury in mice, which suggests that HECP can serve as a protective dietary nutrient against IBD.

Keywords: Inflammatory bowel disease; Anti-inflammatory; Gut dysbiosis; *Akkermansia*; *PICRUSt*

1. Introduction

Inflammatory bowel disease (IBD) that includes ulcerative colitis (UC) and Crohn's disease (CD) is characterized by chronic inflammation of the intestinal tract [1]. The pathogenesis of IBD remains unclear, but inflammation and oxidative stress are considered as the essential mechanism underlying the pathophysiology of IBD [2].

Nuclear factor-κB (NF-κB), mitogen-activated protein kinases (MAPK) and phosphatidylinositol-3-kinase (P3IK)/protein kinase B (Akt) pathways are major inflammatory signaling pathways, which regulate the expression of inflammatory cytokines and proteins [3,4]. Cytokines, as essential mediators between activated immune and non-immune cells, are involved in intestinal immune inflammation of IBD [1]. Inflammation augments oxidative stress by stimulating reactive oxygen/nitrogen species (ROS/RNS)-generating systems, such as myeloperoxidase (MPO) and inducible nitric oxide synthase (iNOS), and oxidative stress is intimately involved in inflammatory cytokines and infiltration of inflammatory cells via stimulating NF-κB signaling pathway in IBD [5,6]. Nitric oxide (NO) production catalyzed by iNOS and expression of cyclooxygenase-2 (COX-2) catalyzed the production of prostaglandins can reflect the degree of inflammation [7]. The gut microbiota is part of a complex network responsible for maintaining normal physiological function. Recent studies indicate that gut microbiota are associated with the development of IBD [8,9]. Intestinal dysbacteriosis could cause IBD and reduce the microbial diversity in mice [10,11]. In genetically susceptible individuals, the gut mucosal integrity is impaired, and the microbial antigens escape through the

Abbreviations: Akt, Protein kinase B; CD, Crohn's disease; COX-2, cyclooxygenase-2; DAI, disease activity index; DSS, dextran sulfate sodium; ERK, extracellular signal-regulated kinase; HECP, Polysaccharide of *Hericium erinaceus* (Mw: 86.67 kDa); *H. erinaceus*, *Hericium erinaceus*; IBD, inflammatory bowel disease; IL, interleukin; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinases; MDA, malondialdehyde; MPO, myeloperoxidase; NF-κB, nuclear factor-κB; NO, nitric oxide; PICRUSt, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; PI3K, phosphatidylinositol-3-kinase; qRT-PCR, quantitative real-time polymerase chain reaction; ROS/RNS, reactive oxygen/nitrogen species; T-SOD, total superoxide dismutase; UC, ulcerative colitis; 5-ASA, 5-aminosalicylic acid; SCFAs, short chain fatty acids; TNF-α, tumor necrosis factor α.

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https://doi.org/10.1016/j.jnutbio.2018.03.005
0955-2863/

epithelial barrier more easily, thereby activating inappropriate immune response or underlying chronic inflammation [12]. Previous studies have shown that prebiotics are non-digestible, fermentable carbohydrates and fibers, such as polysaccharides, which might be useful in preventing and treating acute and chronic conditions including antibiotic associated diarrhea and IBD [11,13]. Intestinal bacteria possesses distinct polysaccharide preferences. On the other hand, polysaccharides may favor the growth of specific bacterial species and modulate the composition of the gut microbiota [11,13–15]. Also, prebiotics improve the intestinal tight junction integrity and decrease blood endotoxemia caused by LPS [13,16].

With edible values and medicinal properties, *Hericium erinaceus* (*H. erinaceus*) has a potential therapeutic effect on anticancer, immune stimulation, improving lipid metabolism and gastrointestinal diseases. Aqueous extract and polysaccharide of *H. erinaceus* can reduce ulceration, provide cytoprotection in ethanol-induced gastric ulcers to protect against gastric mucosal damage [17–22]. A protein isolated from *H. erinaceus* could improve the immune system via regulation the composition and metabolism of gut microbiota [23]. Our previous study showed that the ethanol extract from *H. erinaceus* had anti-inflammatory effects, which contributed to the effective amelioration of DSS-induced colitis symptoms in mice [24]. However, very little is known about the role of polysaccharide from *H. erinaceus* on colitis. The aim of this study was to investigate the potential protective effect of the polysaccharide of fermented *H. erinaceus* mycelium powder (HECP) against DSS-induced colitis in C57BL/6 mice.

2. Materials and methods

2.1. Materials

The dry power of fermented *H. erinaceus* mycelium was obtained from Jiangsu Shenhua Pharmaceutical Co., Ltd., China., and prepared as Fig. S1. Dextran sulfate sodium (DSS, 36–50kD) was purchased from MP Biomedical (Solon, OH, USA). 5-ASA (5-aminosalicylic acid) was purchased from Tokyo Chemical Industry Co., Ltd, Japan. Trizol, SYBR Green PCR Master Mix and BCA Protein Assay Kit were obtained from Thermo Fisher Scientific (MA. USA). MPO, NO, total superoxide dismutase (T-SOD) and malondialdehyde (MDA) assay kits were purchased from Jiancheng Bioengineering Institute (Nanjing, China). Enzyme linked immunosorbent (ELISA) assay kits for TNF-α, IL-1β and IL-6 were obtained from eBioscience (San Diego, CA, USA). Antibodies for p-p65, p65, p-IκBα, IκBα, p-Akt, Akt, p-p38, p38, p-ERK, ERK, p-JNK, JNK and GAPDH were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies for iNOS and COX-2 were purchased from Abcam (Cambridge, UK). Polyvinylidene difluoride (PVDF) membranes were purchased from GE Healthcare (England, UK). Unless otherwise specified, all chemicals were purchased from Sangon Biotech (Shanghai, China).

2.2. Chemical analysis of HECP

Total carbohydrate content was determined by the phenol sulfuric acid method. Reducing sugars were measured by the dinitrosalicylate method. Glucose was used for the standard curve. Total protein content was determined by the Lowry assay with BSA for the standard curve. The homogeneity and molecular weight of HECP were determined by high performance gel permeation chromatography (HPGPC), which was performed on a 6000 high performance liquid chromatograph (Waters, MA, USA). Ultrahydrogel™ Linear (300 mm×7.8 mmid×2) was maintained at 45 °C and the mobile phase was 0.1 M $NaNO_3$ (flow rate=0.9 ml/min) and detected by a Waters 2414 refractive index detector. The sample (2 mg) was dissolved in the mobile phase and centrifuged. A 20 μl sample was injected in each run. Pullulan polysaccharide calibration kit (Agilent technologies, CA, USA) of Mw 180, 4600, 21,400, 41,100, 133,800 and 200,000 Da was used to calibrate the average molecular weights. All samples were filtered through a 0.22 μm membrane before injection. The monosaccharide composition of HECP was determined by gas chromatography (GC) analysis. The tested sample (20 mg) dissolved in 2 ml 1 M H_2SO_4, was hydrolyzed at 100 °C for 4 h, and converted to their alditol acetates as previously described and were analyzed by GC. GC was performed on an Agilent 6280 instrument fitted with FID and equipped with an OV1701 fused-silica capillary (30 m×0.53 mm×1.00 μm). The column temperature was increased from 120 to 240 °C at a rate of 3 °C /min then hold on 10 min (Tables S1–S3).

2.3. Animals and treatment

Six-week-old male C57BL/6 mice of clean grade [SCXK(Hu)2012–002] were purchased from Shanghai SLAC Laboratories Animal Co. Ltd.. Six mice were housed per cage under a 12 h light/dark cycle at 22 °C for 7 days before the experiment. All mouse procedures and protocols were approved by the Institutional Animal Care and Use Committee of Jiangnan University, Wuxi, China [Approval No. JN. No20150103–20,150,119(2)]. Acute colitis was induced through oral administration of 2% DSS w/v in fresh tap water ad libitum for 7 days. Animals were randomly assigned to five groups: Control (CTL) group (mice received water), DSS group (mice received 2% DSS added in water from day 0), 5-ASA group (mice received 150 mg/kg/d 5-ASA by gavage and 2% DSS from day 0), 250 mg/kg HECP group (mice received 250 mg/kg/d HECP by gavage and 2% DSS from day 0), 500 mg/kg HECP group (mice received 500 mg/kg/d HECP by gavage and 2% DSS from day 0). Mice weight was recorded daily and animals were sacrificed at day 8. Colons were removed and submitted to macroscopic examination. The disease activity index (DAI), a score used to assess the severity of colitis, was evaluated as previously described [25]. Colon samples were rinsed with cold PBS, blotted dry, weighed, and their lengths were measured. Distal colon parts were fixed in 4% p-formaldehyde in PBS, embedded in paraffin and cut into sections of 4 μm. Then, hematoxylin and eosin (H&E) staining was used to observe the morphological changes under a light microscope [26].

2.4. Measurement of NO in serum

The blood samples were centrifuged (3000 rpm, 4 °C, 15 min) and the serum was collected. NO content in serum was measured using NO assay kit as the manufacturer's protocol.

2.5. Assay of MPO activity, MDA, T-SOD and inflammatory cytokines in colon tissue

The colonic sample was homogenized in ice-cold 50 mmol/L PBS solution (1:9, w/v). The activity of MPO was measured according to the manufacturer's instructions by MPO assay kit. The result was expressed as activity units per mg tissue. T-SOD and MDA were detected by assay kits as the manufacturer's protocols. The protein content was assayed by BCA protein assay kit and values were normalized to tissue protein levels. IL-1β, IL-6 and TNF-α cytokines were quantified using ELISA kits according to manufacturer's instruction. The results were expressed as per ml supernatant.

2.6. Analyses of relative mRNA expression in colon tissue

Total RNA was isolated from colonic tissues using the Trizol reagent (Thermo Fisher Scientific, MA. USA). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with a Real-time PCR Sequence Detection System (Bio-Rad Laboratories, CA, USA) using SYBR Green PCR Master Mix according to the manufacturer's protocol (Thermo Fisher Scientific, MA. USA). Primer sequences of IL-6, IL-1β, TNF-α, iNOS, COX-2 and GAPDH were shown in Table S4. The relative amount of each transcript was normalized to the amount of GAPDH in the same cDNA. All samples were run in duplicate in a single 96-well reaction plate, and data were analyzed according to the $2^{-\Delta\Delta Ct}$ method.

2.7. Western blot analysis

All colons were homogenized to extract total protein. Protein contents were quantified with the BCA protein assay kit (Thermo, USA); 30 μg protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto PVDF membranes. The membranes were blocked with 5% w/v defatted milk diluted in TBS-0.1% v/v Tween 20 for 1 h and incubated overnight at 4 °C with primary antibodies. The membranes were washed and incubated with secondary antibodies at room temperature for 2 h. Finally, the immunoreactive bands were visualized with an enhanced chemiluminiscence system (Bio-Rad Laboratories, CA, USA) and quantified using Image J software.

2.8. Bioinformatic analysis

Genomic DNA was extracted from every stool sample using the QIAamp DNA Stool Mini Kit according to manufacturer's protocols (QIAGEN, USA). Extracted DNA was diluted to 100 ng/μL, and mixed each group of samples together. The primers 338F (5′-barcode-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′- GGACTACHVGGGTWTCT AAT-3′), where barcode is an eight-base sequence unique to each sample, were used to amplify the V3-V4 region of the bacteria 16S ribosomal RNA gene. Sequencing was performed using an Illumina (La Jolla, California) MiSeq. Pair-end reads generated by 16S sequencing were merged by using the QIIME v 1.9.1 script join_paired_ends.py and the fastq-join method to obtain~460 bp V3-V4 16S sequences. Merged reads were analyzed with QIIME v1.9.1. First, reads with N bases were removed. Reads containing three or more consecutive low-quality bases (Q<20) were truncated and the length of a trimmed read should be≥75% of its original length. Reads passing the quality filter were aligned to the Greengenes Database (version Aug, 2013) for chimera check by USEARCH v6.1. Then, UCLUST4 was applied for OTU clustering at the 97% similarity level. Ribosomal Database Project (RDP) classifier v2.25 was retrained with the Greengenes Database and utilized to assign a taxonomic rank to each representative OTU. OTU relative abundances were hierarchically clustered and visualized by the R software (v3.0.1) gplots package (heatmap.2 function). The heatmap was generated for scaled, normalized titer data using a Euclidean distance function with complete linkage clustering. Phylogenetic Investigation of Communities by Reconstruction of