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Ginsenoside Rd ameliorates colitis by inducing p62-driven mitophagy-mediated NLRP3 inflammasome inactivation in mice

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ABSTRACT

Previous studies reported that Ginsenoside Rd (Rd) had anti-inflammatory and anti-cancer effects. However, the molecular mechanism underlying the inhibition effect of Rd on colitis in mice hasn't been clarified clearly. Here, in our study, we detected the effects of Rd on dextran sulfate sodium (DSS)-induced murine colitis, and found that oral administration of Rd dose-dependently alleviated DSS-induced body weight loss, colon length shortening and colonic pathological damage with lower myeloperoxidase (MPO) and inducible nitric oxide synthase (iNOS) activities and higher glutathione level. In addition, the production of pro-inflammatory cytokines (IL-1β, TNF-a and IL-6) in both serum and colonic tissues were significantly down-regulated by Rd administration. The activation of NLRP3 inflammasome was also suppressed in Rd-treated group, resulting in reduced caspase-1 production and IL-1β secretion. In vitro, Rd remarkably inhibited NLRP3 inflammasome activation which was mostly dependent on the mitochondrial translocation of p62 and mitophagy. Importantly, Rd-driven inhibition of the NLRP3 inflammasome was significantly blocked by various autophagy inhibitors. Furthermore, upregulation of AMPK/ULK1 signaling pathway accounted for Rd-induced autophagy, which was also seen in vivo. In conclusion, our results demonstrated the function of Rd on the inhibition NLRP3 inflammasome and its potential application for the treatment of NLRP3-associated diseases.

1. Introduction

Ulcerative colitis (UC) is a chronic, relapsing, immunological-caused disorder in gastrointestinal tract [1,2], which increases the risk of colon cancer of patients and has become a public health problem [3,4]. The exact etiology of UC still remains unclear. However, excessive activation of the mucosal immune system, especially innate immunity, accompanied with abundant immune cells recruitment and chronic pro-inflammatory cytokines, including interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α secretion in colon, contributes to the onset and deterioration of colitis [5,6].

It has been shown that colitis progression was associated with activation of the NOD-like receptor, pyrin domain-containing (NLRP)3 [7–9]. The inflammasome is a cytosolic multi-protein complex composed of nucleotide-binding domain and leucine-rich repeat-containing proteins (NLRs), adaptor protein ASC, and caspase-1 [10]. NLRP3

Abbreviations: Rd, Ginsenoside Rd; NLR, NOD-like receptor; DSS, dextran sulfate sodium; IL-1β, interleukin-1β; IL-18, interleukin-18; IL-6, interleukin-6; TNF-α, tumor necrosis factor α; ASC, apoptosis-associated speck-like protein which contains a CARD; NF-κB, nuclear factor-κB; ROS, reactive oxygen species; mROS, mitochondrial reactive oxygen species; 5-ASA, 5-aminosalicylic acid; THP-Ms, THP-1 cell-derived macrophages; PMA, phorbol myristate acetate; LPS, lipopolysaccharide; ATP, adenosine triphosphate; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IBD, inflammatory bowel disease; CRC, Colorectal Carcinoma

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inflammasome is best known to trigger the activation of caspase-1 and subsequent maturation of interleukin-1β (IL-1β) and IL-18 [11,12]. Accumulating evidence indicates that over-activation of the inflammasome is critical for the pathogenesis of several inflammatory disorders, such as Crohn's disease, atherosclerosis, gout, type-2 diabetes (T2D) and cancer [13–16]. Base on those facts, NLRP3 and the inflammasome pathways should be tightly controlled and the regulation of inflammasome activity is worth exploring.

Notably, autophagy has been regarded as a critical cellular process to clear damaged organelles and long-lived proteins in cytoplasm [17]. Besides, autophagy was reported to be greatly involved in degradation of bacteria and viruses [18,19]. Dysfunction of autophagy leads to many diseases, especially the imbalance of immune system. Autophagy could be induced by noxious stimuli, and then clear damaged proteins and organelles to protect cells from harmful stimulation [20,21]. It was demonstrated that genetic variants in autophagy-related molecules, including ATG16L1 (autophagy-related 16 like 1) and IRGM (immunity-related GTPase M) increased the risk to develop inflammatory bowel disease (IBD) [22,23]. Furthermore, a recent study validated that autophagy inducers could be adopted to intervene inflammation progression by inactivating NLRP3 inflammasome [24].

Ginseng, a deciduous perennial plant, belongs to the Araliaceae family, mainly growing in China and Korea. Ginsenosides are the most important components of Ginseng with various pharmacological and therapeutic functions [25]. Ginsenosides are classified into three major categories, namely protopanaxatriols (PPTs; ginsenoside Rg1, Re, Rg2, Rh1 and Rf), protopanaxadiol (PPD; ginsenoside Rb1, Rb2, Rd, Rg3 and Rh2) and oleanolic acid derivates (ginsenoside Ro) [26]. Rd was demonstrated to exhibit prominent effect on suppressing oxidative stress and inflammation in many kinds of disease, such as cerebral ischemia, chemical-induced excitotoxicity and Alzheimer's disease [27,28]. Even though the anti-inflammatory effects of Rd on colitis in rats were pinpointed [29], the regulation and mechanism of Rd on colitis in mice has not been illustrated clearly today.

In our study, we found that the natural small molecule Rd remarkably alleviated DSS-induced colitis through inactivating the NLRP3 inflammasome in macrophages. Further study showed that Rd triggered mitophagy through activating AMPK/ULK1/p62 signaling pathway, leading to a reversed mitochondrial membrane potential collapse, which inhibited the NLRP3 inflammasome. These findings obtained in this study indicated that the ameliorative effect on colitis results from Rd-driven mitophagy-mediated NLRP3 inflammasome inhibition. Accordingly, these findings may help direct clinical decisions regarding the use of Rd in patients with IBD.

2. Materials and methods

2.1. Reagents

Rd were purchased from Sigma–Aldrich (St. Louis, MO). Primary antibodies for NLRP3, β-actin, IL-1β, p62, LC3B, VDAC, caspase-1, p-ULK1, ULK and Tublin were purchased from Abcam (Cambridge, UK); Primary antibody for ASC was purchased from Santa Cruz Biotechnology (Texas, USA) and p-AMPK, AMPK from Cell Signaling Technology (Danvers, USA); Horseradish peroxidase (HRP) conjugated second antibodies were from Bioworld Technology Inc. (Minnesota, USA). p62 siRNA and control shRNA were purchased from Santa Cruz Biotechnology Inc. (Texas, USA). Phorbol myristate acetate (PMA), lipopolysaccharide (LPS), adenosine triphosphate (ATP) and carboxymethylcellulose sodium (CMC-Na) were purchased from Sigma–Aldrich (St. Louis, MO). Dextran sulfate sodium (DSS, 36–50 kDa) was from MP Biomedicals (Aurora, OH). Myeloperoxidase (MPO) and iNOS activity assay kits were from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). ELISA kit for murine IL-1β, TNF-a and IL-6 were purchased from Boster Biotech Co. Ltd. (Wuhan, China). Immunohistochemistry kit was from KeyGEN Biotech Inc. (Nanjing, China).

2.2. Cell culture

Human THP-1 cells were obtained from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China) and maintained in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY). THP-1 cells were stimulated by PMA (100 ng/ml) for 12 h to differentiate into macrophages. Cells were cultured under a humidified 5% (v/v) CO2 atmosphere at 37 °C.

2.3. Mice

6- to 8-week-old male C57BL/6 mice were purchased from 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.

2.4. Western blot assay

Cells with different treatments were washed twice with PBS, then collected and lysed in Ripa lysis for 1 h on the ice. The lysates were then subjected to centrifugation (13,000 rpm) at 4 °C for 20 min. Protein concentration in the supernatants was detected by BCA protein assay (Thermo, Waltham, MA). Then an equal amount of protein was separated with 12% or 10% SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA) using a semi-dry transfer system (Bio-rad, Hercules, CA). Proteins were detected using specific antibodies of NLRP3, caspase-1, IL-1β, β-actin, p62, LC3, VDACI, p-AMPK, AMPK, p-ULK1, ULK and Tublin overnight at 4 °C followed by HRP-conjugated secondary antibodies for 1 h at 37 °C. All of the antibodies were diluted in PBST containing 1% BSA. Enhanced chemiluminescent reagents (Beyotime, Jiangsu, China) were used to detect the HRP on the immunoblots. The signals were analyzed using the ECL chemiluminescence detection system (Tanon, Nanjing, China).

2.5. ASC oligomerization assay

THP-Ms were seeded in 6-well plates. After the treatment with indicated stimuli, cells were washed by cold PBS and resuspended in an ice-cold buffer (Buffer A: 20 mMHEPES-KOH, pH 7.5, 150 mM KCl, 1% NP-40, 0.1 mM PMSF, and protease inhibitor), and lysed by shearing 10 times through a 21-gauge needle. Nuclei and unlysed cells were removed by centrifugation at 250*g* for 5 min. The cell lysates were then centrifuged at 5000*g* for 10 min at 4 °C. After washing twice with PBS, the pellets were crosslinked with fresh Disuccinimidyl suberate (DSS) (2 mM) (Thermo Fisher Scientific, Massachusetts, USA) for 30 min at 37 °C. The crosslinked pellets were separated in 12% SDS–PAGE and immunoblotting was performed.

2.6. Measurement of production of mtROS

THP-Ms superoxide levels were measured with Mitosox Red (YEASEN, Shanghai, China). Rd-pretreated THP-Ms were exposed with different stimulations and incubated with MitoSOX Red superoxide indicator for 30 min and washed. The fluorescence intensity was measured by FACS Calibur flow cytometry (Becton Dickinson, San Jose, CA, USA).