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Original article

Enzymatically synthesized glycogen inhibits colitis through decreasing oxidative stress

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ABSTRACTS

Inflammatory bowel diseases are a group of chronic inflammation conditions of the gastrointestinal tract. Disruption of the mucosal immune response causes accumulation of oxidative stress, resulting in the induction of inflammatory bowel disease. In this study, we investigated the effect of enzymatically synthesized glycogen (ESG), which is produced from starch, on dextran sulfate sodium (DSS)- and 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis in C57BL/6 mice. Oral administration of ESG suppressed DSS- and TNBS-induced shortening of large intestine in female mice and significant decreased DSS-induced oxidative stress and TNBS-induced pro-inflammatory cytokine expression in the large intestine. ESG increase in the expression levels of heme oxygenase-1 (HO-1) and NF-E2-related factor-2 (Nrf2), a transcription factor for HO-1 expressed in the large intestine. Furthermore, ESG-induced HO-1 and Nrf2 were expressed mainly in intestinal macrophages. ESG is considered to be metabolized to resistant glycogen (RG) during digestion with α-amylase *in vivo*. In mouse macrophage RAW264.7 cells, RG, but not ESG decreased 2,2′-azobis(2-amidinopropane) dihydrochloride (A APH)-induced reactive oxygen species (ROS). Knockdown of Nrf2 inhibited RG-induced HO-1 expression and negated the decrease in AAPH-induced ROS brought about by RG. RG up-regulated the protein stability of Nrf2 to decrease the formation of Nrf2-Keap1 complexes. RG-induced phosphorylation of Nrf2 at Ser40 was suppressed by ERK1/2 and JNK inhibitors. Our data indicate that ESG, digested with α-amylase to RG, suppresses DSS- and TNBS-induced colitis by increasing the expression of HO-1 in the large intestine of mice. Furthermore, we demonstrate that RG induces HO-1 expression by promoting phosphorylation of Nrf2 at Ser40 through activation of the ERK1/2 and JNK cascade in macrophages.

1. Introduction

The inflammatory bowel diseases (IBD) are a group of inflammatory conditions of the gastrointestinal tract. Crohn's disease and ulcerative colitis are the principal types of IBD. The morbidity of IBD has been increasing worldwide, and IBD has a high recurrence rate [1]. The distinguishing feature of IBD is chronic inflammation of mucosa in the gastrointestinal tract, and genetic and environmental factors, including food, are involved in its onset. However, the precise mechanism by which the onset of IBD occurs remains poorly understood [2]. Disruption of the mucosal immune response produces excess amounts of inflammatory cytokines and matrix metalloproteinase, and also increases oxidative stress, resulting in severe damage to the intestine [3]. Antioxidative and immunosuppressive reagents are used in IBD treatment, but these therapies have been associated with severe side effects and long-term toxicity [4,5]. Therefore, it is important to investigate safe and effective regimens such as dietary interventions to complement IBD therapies.

Antioxidant enzymes play an important role in the removal of free radicals and protect against reactive oxygen species (ROS)-induced cell damage. NF 2-related factor 2 (Nrf2, encoded by the NFE2L2 gene) is a master transcription factor that regulates the expression of antioxida-

Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; DAPI, 4′,6-diamino-2-phenylindole; DCF-DA, 2′,7′-dichlorodohydrofluorescein diacetate; DSS, dextran sulfate sodium; ERK1/2, extracellular signal-regulated kinase 1/2; ESG, enzymatically synthesized glycogen; HO-1, heme oxygenase-1; IBD, inflammatory bowel diseases; IL, interleukin; JNK, c-jun N-terminal kinase; Keap1, Kelch-like ECH-associated protein 1; Nrf2, NF-E2-related factor-2; NSG, natural sources of glycogen; qPCR, quantitative real-time PCR; RG, resistant glycogen; ROS, reactive oxygen species; TBARS, 2-thiobarbituric acid-reactive substances; TNBS, 2,4,6-trinitrobenzenesulfonic acid

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tive enzymes [6]. Under normal redox conditions, Nrf2 interacts with its negative regulator, Kelch-like ECH-associated protein 1 (Keap1), in the cytoplasm and is easily degraded by the ubiquitin-proteasome pathway. Upon activation, Nrf2 is detached from Keap1 and translocates to the nucleus, where it binds to the antioxidant response elements (AREs) located in the promoter region of antioxidant enzymes, including heme oxygenase-1 (HO-1, encoded by the HMOX1 gene), and induces expression of their genes [7–9]. HO-1 expression is localized in macrophages of the intestinal mucosa [10], and HO-1 inhibitors enhance ROS accumulation and colonic damage along with a reduction in HO-1 activity [11]. Thus, HO-1 protein is a molecular target for IBD therapy.

Glycogen is a multi-branched α-D-glucan containing α−1,4 and α−1,6 linkages, and is the major form of energy storage in animals, plants, and fungi [12]. Recently, we developed an enzymatically synthesized glycogen (ESG) made from plant starch [13]. Although the physical properties of ESG are equivalent to those of natural sources of glycogen (NSGs), the two forms have a slightly different molecular structure. In an NSG molecule, α−1,6 linkages are sporadically distributed near the surface of the molecule. However, in the ESG molecule, α−1,6 linkages are buried in the center of the molecule, and its linkages form a huge cluster [14]. Thus, ESG molecules are only partially digested with α-amylase, and the remaining undigested ESG is called resistant glycogen (RG) [15]. In rats, oral administration of ESG showed that the glycemic index of ESG is about 80%, and 20% of the ESG was retained as RG [16].

Recently studies have demonstrated that oligosacchrides ameliorate intestinal inflammation in mice by increase in HO-1 expression and by decreasing the pro-inflammatory cytokines [10,17–19]. Previous studies have shown that ESG possesses anti-tumor activities in natural killer cells through Tool-like receptor, and also acts as a dietary fiber *in vivo* [20,21]. However, the effect of ESG on intestinal inflammation remains unclear. Thus, we investigated whether ESG suppresses intestinal inflammation using model mice and mouse cell lines. In the study reports that oral administration of ESG suppresses dextran sulfate sodium (DSS)- and 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis and oxidative stress in mice. Furthermore, we demonstrate that RG, the active form of ESG, decreases oxidative stress by increasing Nrf2 and HO-1 expression in macrophage cells.

2. Materials and methods

2.1. Glycogen

ESG was prepared from starch as a substrate using three enzymes as described previously [22]. The molecular weight of ESG is 8700 kDa. RG was prepared by partial digestion of ESG with α-amylase from porcine pancreas. The preparation method of RG was previously described [23].

2.2. Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee and carried out according to the Kobe University Animal Experimentation Regulation (Permission No. 26-05-12). C57BL/6 female and male mice (6-week-old) were purchased from Japan SLC (Shizuoka, Japan). The mice were inbred under temperature- and light-controlled conditions (25 ± 3 °C, 12 h light/dark cycle) and had free access to water and food for 1 week during their acclimatization.

2.3. Administration of ESG and induction of colitis

For DSS-induced colitis model, Mice were generated with minor modification of the method described [24,25]. Female and male mice (n = 24) were randomly divided into four groups of six, the DSS, ESG, DSS + ESG and control groups. ESG in water (100 mg once daily) was orally administered to mice in the ESG and DSS + ESG groups, and water was administered to the other two groups for 2 weeks. One week after start of ESG administration, colitis was induced by administration of 2% (w/v) DSS (molecular weight 36–50 kDa, MP Biomedical, OH, USA) in the drinking water of mice in the DSS and DSS + ESG groups for 1 week. The mice were anesthetized with pentobarbital sodium and sacrificed at the end of the experimental period, and the colons were collected. For TNBS-induced colitis model, female and male mice (n = 18) were randomly divided into three groups of six, the TNBS, TNBS + ESG and control groups. ESG in water (100 mg once daily) was orally administered to mice in the ESG and TNBS + ESG groups, and water was administered to the other one group for 10 days. One week after start of ESG administration, TNBS (200 mg/mouse) was administered intrarectally to anesthetized mice. Three days after TNBS administration, the mice were anesthetized with pentobarbital sodium and sacrificed at the end of the experimental period, and the colons were collected.

2.4. Cell culture

RAW264.7 macrophages were purchased from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle medium with high glucose (4.5 g/l glucose; DMEM-HG) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 5% CO_2, 95% air atmosphere at 100% humidity.

2.5. Estimation of lipid peroxidation

Lipid peroxidation was assayed with the formation of 2-thiobarbituric acid-reactive substances (TBARS) [26]. Mouse colons were homogenized in 20 mM pH 7.5 HEPES-NaOH containing 0.5% (w/v) Nonidet P-40, 1 mM EDTA, 50 μM butylated hydroxytoluene, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin. The homogenates were incubated with 20% (v/v) trichloroacetic acid and 0.8% (w/v) 2-thiobarbituric acid for 40 min at 90 °C. After cooling to room temperature, the reaction mixture was centrifuged at 4000*g* for 10 min, and the fluorescence was measured by Wallac (PerkinElmer Inc., Wellesley, MA, USA) with the excitation and emission filters set at 530 and 590 nm, respectively. The amount of TBARS was quantified using tetraethoxypropane as a reference standard. TBARS level was expressed as relative level of control groups.

2.6. Western blotting

RAW264.7 cells were cultured in DMEM-HG for 48 h, followed by incubation with various concentrations of RG for 24 h. For the cycloheximide (CHX) treatment experiment, RAW264.7 cells were cultured in DMEM-HG with 10 μg/ml CHX in the presence or absence of 400 μg/ml RG for the indicated time periods. The cells and large bowel were lysed in lysis buffer (50 mM Tris–HCl, pH 7.5, containing 150 mM NaCl, 0.5% [w/v] Nonidet P-40, 10 mM sodium pyrophosphate, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin). The lysates were subjected to SDS-PAGE and analyzed by western blotting using the following primary antibodies: rabbit polyclonal antibodies (anti-Nrf2; Cat#sc-13032 from Santa Cruz Biotechnology; anti-c-jun N-terminal kinase (JNK; Cat#9252), anti-P-JNK (Thr183/Tyr185; Cat#4671), anti-extracellular signal-regulated kinase 1/2 (ERK1/2; Cat#4695), and anti-P-ERK1/2 (Thr202/Tyr204; Cat#9101) from Cell Signaling Technology, Beverly, CA, USA; anti-HO-1; Cat#ADI-SPA-895 from Enzo Life Sciences, Farmingdale, NY, USA);