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The Preventive and Curative Effect of Cyanidin-3β-D-Glycoside and Its Metabolite Protocatechuic Acid Against TNBS-induced Colitis in Mice

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Abstract – Cyanidin-3β-D-glycoside (C3G), which is widely distributed in herbal medicines and functional foods, exhibits anti-inflammatory, anti-oxidant, and anti-scratching behavioral effects. Orally administered C3G is metabolized to protocatechuic acid (PA) by gut microbiota. Therefore, we compared the anti-colitic effect of C3G to that of PA in mice with 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis. Orally administered C3G and PA preventively and curatively ameliorated TNBS-induced colitis parameters, including macroscopic colitis score, colon shortening, and increase of myeloperoxidase activity. Treatment with C3G or PA also inhibited the expression of cyclooxygenase-2, inducible NO synthatase, IL-1β, IL-6, and TNF-α and the activation of NF-κB in the colon of mice with TNBS-induced colitis. Furthermore, these also inhibited lipopolysaccharide-induced NF-κB activation and TNF-α expression in peritoneal macrophages. The anti-colitic effect of PA was more effective than C3G. Orally administered PA more potently attenuate colitis than C3G by inhibiting NF-κB activation and the anti-colitic efficacy of C3G may be dependent on the biotransformation of C3G to PA by gut microbiota.
Keywords – Colitis, Cyanidin-3β-D-glycoside, Procatechuic acid

Introduction

Anthocyanins are widely distributed in herbal medicines and functional foods, suggesting that the considerable amounts of anthocyanins were ingested from herbal medicines and diets.[1,2] These constituents exhibit anti-diabetic, antioxidant, and anti-inflammmatory effects.[3-6] Therefore, natural products containing anthocyanins have been used frequently to prevent metabolic diseases.

Orally administered anthocyanins in humans and animals contact with gut microbiota in the gastrointestinal tracts and are transformed into their aglycones and phenolic acids.[7–10] For example, orally administered cyanidin-3β-D-glucopyranoside (C3G) is transformed to procatechuic acid via cyanidin by gut microbiota and, of these metabolites, procatechuic acid (PA) is mainly absorbed into the blood. PA inhibits inflammation in LPS-stimulated RAW264.7 cells and mouse air-pouch with carrageenan-induced inflammation.[7] Furthermore, PA did not only scavenge the radicals, but also inhibited NF-κB activation in LPS-stimulated macrophages more strongly than C3G and cyanidin. Nevertheless, their anti-colitic effects of C3G and PA have not been studied.

Here, we investigated the preventive and curative effects of C3G and PA (Fig. 1) in mice with 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis.

Experimental

Chemicals – C3G, PA, TNBS, hexadecyl trimethyl ammonium bromide, and radio-immunoprecipitation assay (RIPA) lysis buffer were purchased from Sigma (St Louis, MO, USA). A protease inhibitor cocktail was purchased from Roche Applied Science (Mannheim, Germany). Enzyme-linked immunosorbent assay (ELISA) kits were from Pierce Biotechnology (Rockford, IL, USA). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Enhanced chemiluminescence (ECL) immunoblot system was from Pierce (Rockford, IL, USA).

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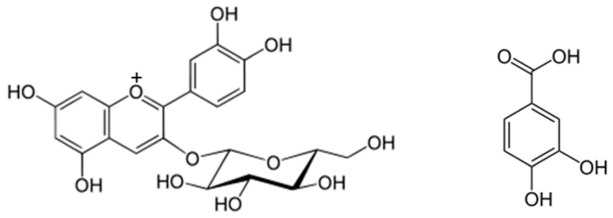

Fig. 1. The structures of C3G and PA.

Animals – Male ICR mice (23 – 25 g, 7 weeks old) were supplied from Orient Animal Breeding Center (Seoul, Korea). All animals were housed in wire cages at 20 – 22 °C and 50 ± 10% humidity, fed standard laboratory chow (Samyang, Seoul, South Korea), and allowed water ad libitum. The experiments were approved by the Committee for the Care and Use of Laboratory Animals in the College of Pharmacy, Kyung Hee University and performed in accordance with the NIH and Kyung Hee University guides for Laboratory Animals Care and Usage.

Preparation of macrophages – Mice were intraperitoneally injected with 2 mL of 4% (w/v) thioglycolate and sacrificed 4 days after the injection[7]. The fluid of peritoneal cavity was collected by 10 mL of RPMI 1640, centrifuged (300 × g, 10 min), and washed with RPMI 1640 twice. The collected cells (0.5×10^6 cells/well) were seeded in 24-well microplate, incubated in RPMI 1640 containing 1% antibiotic-antimycotic and 10% fetal bovine serum (37 °C, 20 h). Attached cells were used as macrophages. Macrophages (0.5×10^6 cells/well) were treated with LPS (100 ng/mL) in the absence or presence of test agents for 90 min (for p65 and p-p65) or 20 h (for TNF-α).

Preparation of experimental colitis mice – Colitis was prepared to the method of Lee et al.[11] Briefly, to measure the preventive effects of C3G and PA in mice with TNBS-induced colitis, mice were randomly divided into 6 groups: one normal control group and five TNBS-treated groups orally treated with vehicle alone, C3G (10 mg/kg or 20 mg/kg), protocatechuic acid (10 mg/kg), or sulfasalazine (20 mg/kg) once a day from the 3rd day before the treatment with TNBS to the 3rd day after TNBS treatment (Fig. 2A). Additionally, to evaluate the curative effects of C3G and PA in mice with TNBS-induced colitis, male ICR mice were randomly divided into 6 groups: one normal control group and five TNBS-induced colitis groups orally treated with vehicle alone, C3G (10 mg/kg or 20 mg/kg), protocatechuic acid (10 mg/kg), or sulfasalazine (20 mg/kg) once a day for 3 days after TNBS treatment (Fig. 2B). TNBS-induced colitis was induced by the intrarectal injection of 2.5% (w/v) TNBS solution (0.1 mL, dissolved in 50% ethanol) into the colon. If an animal quickly excreted the

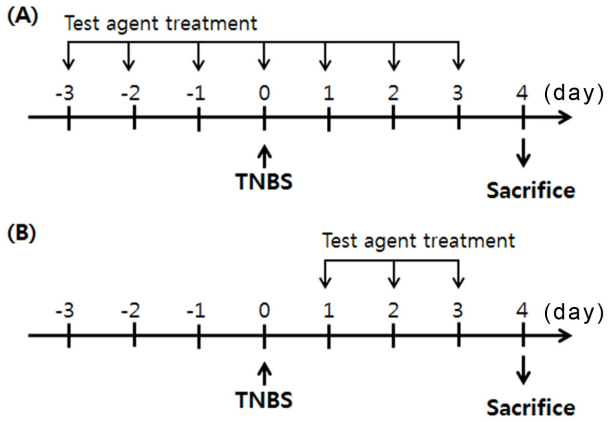

Fig. 2. Experimental protocol. (A) Preventative effect for TNBS-induced colitis in mice. (B) Curative effect for TNBS-induced colitis in mice.

TNBS-ethanol solution, it was excluded from the remainder of the study. Normal control group was treated with saline. The mice were killed 18 h after the final treatment with test agents. The colon was then quickly removed, opened longitudinally, and gently washed by ice-cold PBS. Macroscopic assessment of the colitis grade was scored (0 to 5), as previously reported.[11] The colons were stored at −80 °C until used in experiments.

Assay of myeloperoxidase activity – Colons 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 and 4 °C.[11] An aliquot (50 μL) of the supernatant was added to a reaction mixture of 1.6 mM tetramethylbenzidine and 0.1 mM H_2O_2 and incubated at 37 °C and the absorbance at 650 nm was then measured over time.

ELISA and immunoblotting – For the ELISA of TNF-α, IL-1β, and IL-6, colons were homogenized in 1 mL of ice-cold RIPA lysis buffer containing 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail.[11] The lysate was centrifuged (15,000 × g, 4 °C) for 15 min, and the supernatant was transferred to 96-well ELISA plates. TNF-α, IL-1β, and IL-6 concentrations were determined using commercial ELISA kits (Pierce Biotechnology, Rockford, IL, USA). For the immunoblot analysis, colons were homogenized in the RIPA lysis buffer (1 mL) containing 1% phosphatase inhibitor cocktail and 1% protease inhibitor cocktail at 4 °C and centrifuged at 15,000 × g and 4 °C for 15 min. Proteins of the homogenate supernatants were measured by immunoblotting according to the method of Lee et al.[11]

Statistical analysis – All data are expressed as the mean

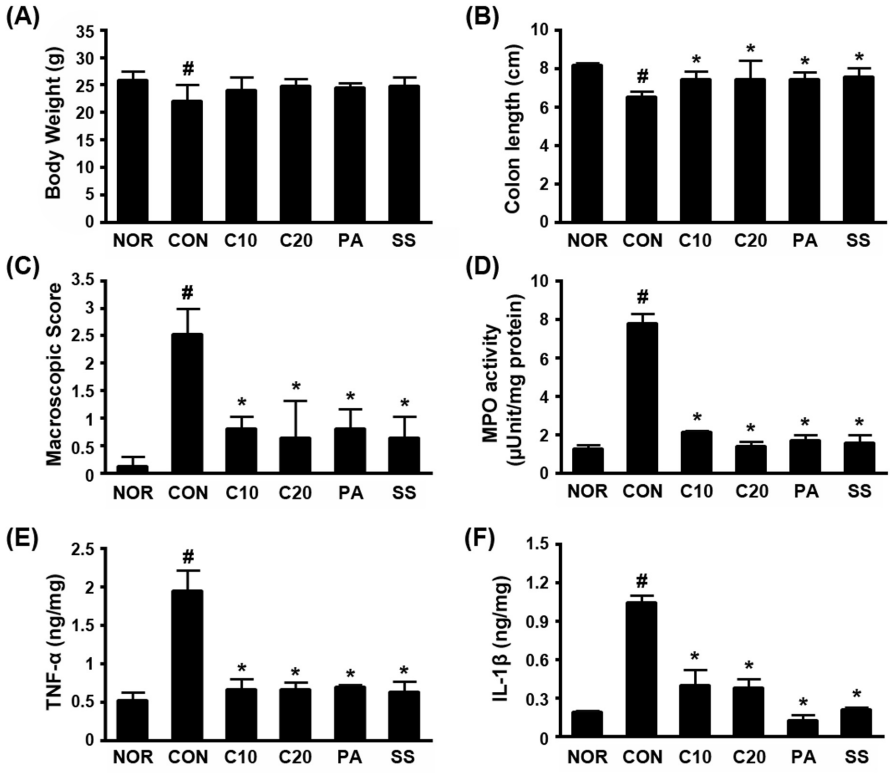

Fig. 3. The preventive effects of C3G and PA on TNBS-induced colitis in mice. (A) Effects on body weight. (B) Effects on colon length. (C) Effects on macroscopic colitis score. (D) Effects on colonic MPO activity. (E) Effects on TNF-α, IL-1β, and IL-6 expression. (F) Effects on COX2 expression. TNBS, except normal group (NOR, treated with vehicle alone), was intrarectally administered. Test agents (CON, vehicle alone; C10, 10 mg/kg; C20, 20 mg/kg; PA, 10 mg/kg; SS, 20 mg/kg sulfasalazine) were orally administered from the 3rd day before TNBS treatment to the 3rd day after TNBS treatment and sacrificed 18 h after the final administration of test agents. All values are means (n = 10). $^{\#}P < 0.05$, significantly different vs. normal group; $^{*}P < 0.05$, significantly different vs. control group.

standard deviation with statistical significance analyzed using one-way ANOVA followed by a Student-Newman-Keuls test.

Result and Discussion

To evaluate the preventive effects of C3G and PA against TNBS-induced colitis in vivo, we orally administered C3G and PA from the 3rd day before TNBS treatment to the 3rd day after TNBS treatment and measured colitic markers (Fig. 3). Treatment with TNBS caused colon shortening manifested by shortened, thickened, and erythematous, as well as the increase of colonic myeloperoxidase activity. Furthermore, TNBS treatment increased inflammatory markers such as TNF-α and IL-1β. However, oral administration of C3G or PA in mice suppressed TNBS-induced colon shortening and the increase of macroscopic score and myeloperoxidase activity, as well as the expression of TNF-α and IL-1β. The anti-colitic effect of C3G was inferior to that of PA, which was comparable to sulfasalazine.

Next, we orally administered C3G and PA for 3 day after TNBS treatment in mice and measured their colitic effects (Fig. 4). Treatment with TNBS significantly caused colitis including colon shortening, increased myeloperoxidase activity, TNF-α, IL-1β, IL-6, COX-2, and iNOS expression, and NF-κB activation. However, oral administration of C3G or PA in mice with TNBS-induced colitis inhibited shortening of colon and increase of macroscopic score and myeloperoxidase activity, as well as expression of TNF-α, IL-1β, IL-6, and COX-2. The curative anti-colitic effect of PA was superior to that of C3G. The anticolitic effect of PA was comparable to that of sulfasalazine.

To confirm whether C3G and PA could inhibit NF-κB activation, we measured their inhibitory effects in LPS-stimulated peritoneal macrophages (Fig. 5). These inhibited LPS-induced NF-κB activation and TNF-α expression. The inhibitory effect of PA against NF-κB activation was superior to that of C3G.

Acute inflammation is a beneficial immune response, whereas chronic inflammation causes the progressive damage to the body, leading to inflammatory diseases, such

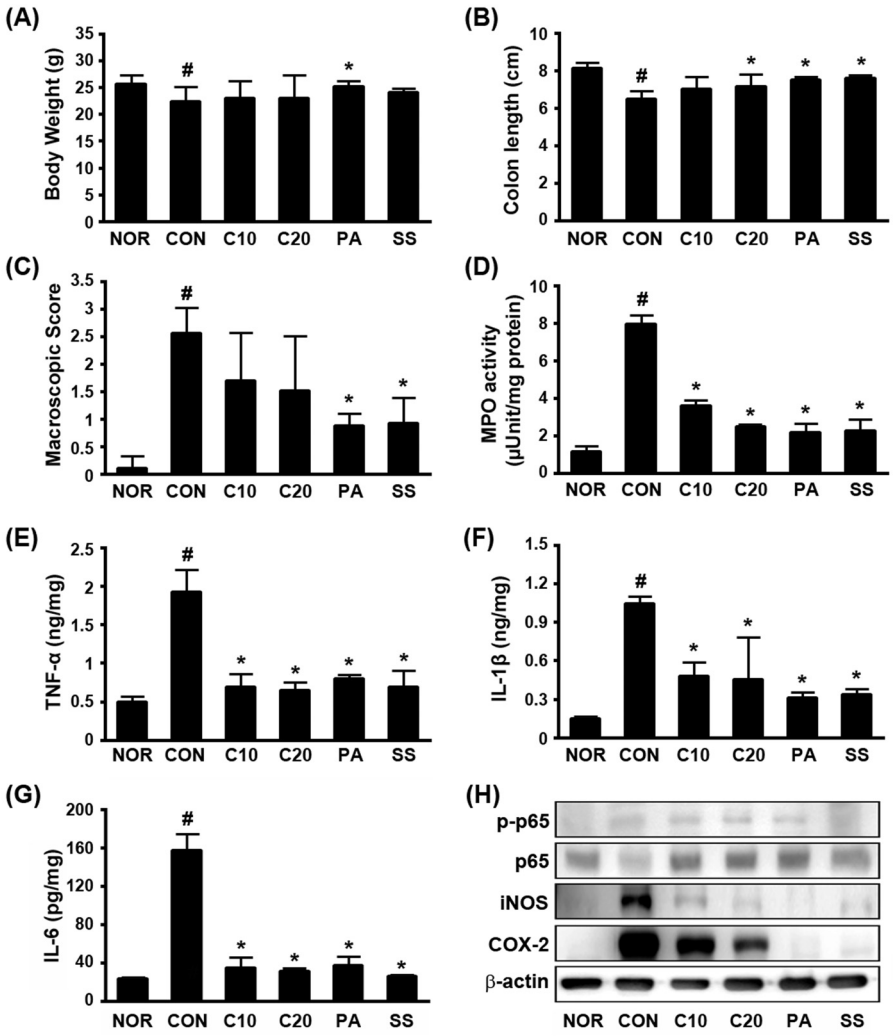

Fig. 4. The curative effects of C3G and PA on TNBS-induced colitis in mice. (A) Effects on body weight. (B) Effects on colon length. (C) Effects on macroscopic colitis score. (D) Effects on colonic MPO activity. (E) Effects on TNF-α, IL-1β, and IL-6 expression. (F) Effects on COX2 and iNOS expression and NF-κB activation. TNBS, except normal group (NOR, treated with vehicle alone), was intrarectally administered. Test agents (CON, vehicle alone; C10, 10 mg/kg; C20, 20 mg/kg; PA, 10 mg/kg; SS, 20 mg/kg sulfasalazine) were orally administered for 3 days after TNBS treatment and sacrificed 18 h after the final administration of test agents. All values are means (n = 10). $^{\#}P < 0.05$, significantly different vs. normal group; $^{*}P < 0.05$, significantly different vs. control group.

as ulcerative colitis and rheumatoid arthritis.[12,13] Ulcerative colitis is characterized by the excessive release of inflammatory mediators secreted from immune cells in all or part of your digestive tract. Of these mediators, tumor necrosis factor (TNF)-α and interleukin (IL)-1β are inducible in the presence of various stresses such as pathogens or injury.[14,15]Thus, stimulation with lipopolysaccharide (LPS) of gram-negative pathogens induces the expression of inflammatory markers such as TNF-α and COX-2 by activating NF-κB signaling pathway, leading to inflammatory diseases.[16,17] Therefore, regulating the expression of proinflammatory cytokines through NF-κB signaling pathway can be helpful in curing colitis.

In the present study, orally administered C3G and its metabolite PA showed the preventive and curative effects on TNBS-induced colitis in mice. They also inhibited the expression of TNF-α, IL-1β, IL-6, and COX-2. Min et al. also reported that C3G and PA inhibited NF-κB activation and TNF-α expression in LPS-stimulated macrophages and mice with carrageen-induced air pouch inflammation.[7] These results suggest that C3G and PA may ameliorate inflammatory diseases in vitro and in vivo by inhibiting NF-κB activation. Furthermore, PA, transformed from C3G by gut microbiota, showed the colitic effect in mice more potently than C3G. Blue berry and black rice, which contain C3G, exhibit anti-oxidant, anti-inflammatory, anti-

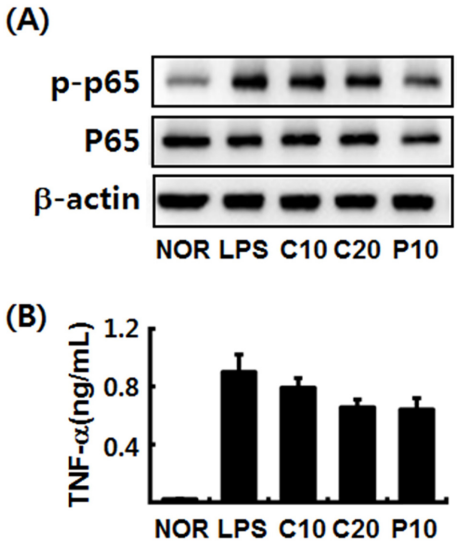

Fig. 5. Effect of C3G and PA on NF-κB activation and TNF-α expression in LPS-stimulated peritoneal macrophages. Peritoneal macrophages (0.5×10^6 cells) were incubated with LPS in the absence or presence of C3G (C10, 10 μM; C20, 20 μM) or PA (P10, 10 μM) for 90 min (for NF-κB) or 20 h (for TNF-α). All data are shown as the mean ± S.D. (n = 3). $^{\#}p < 0.05$ vs. group treated without LPS and test agents. $^{*}p < 0.05$ *vs.* group treated with LPS alone.

degranulated, anti-anaphylactic, and anti-scratching effects.[4,18,19] The bioactive constituents in their pharmacological effects are anthocyanidins, particulary C3G. Moreover, PA, a metabolite of C3G by gut microbiota, exhibits antioxidant, anti-inflammatory as well as antihyperglycemic and neuroprotective effects (20). These results suggest that orally administered C3G may be dependent on the metabolic activity of C3G to PA by gut microbiota.

Finally, orally administered PA more potently attenuated colitis than C3G, which contains in black rice and blue berry as a main constituent, by inhibiting NF-κB signaling pathway and the anti-colitic activity of C3G may be dependent on the biotransformation of C3G to PA by gut microbiota.

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