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Anti-inflammatory effect of a standardized triterpenoid-rich fraction isolated from *Rubus coreanus* on dextran sodium sulfate-induced acute colitis in mice and LPS-induced macrophages

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ABSTRACT

Ethnopharmacological relevance: *Rubus coreanus* Miquel (Rosaceae), the Korean black raspberry, has traditionally been used to treat inflammatory diseases including diarrhea, asthma, stomach ailment, and cancer. Although previous studies showed that the 19α-hydroxyursane-type triterpenoids isolated from *Rubus coreanus* exerted anti-inflammatory activities, their effects on ulcerative colitis and mode of action have not been explored. This study was designed to assess the anti-inflammatory effects and the molecular mechanisms involving19α-hydroxyursane-type triterpenoid-rich fraction from *Rubus coreanus* (TFRC) on a mice model of colitis and lipopolysaccharide (LPS)-induced RAW 264.7 macrophages.

Materials and methods: Experimental colitis was induced by DSS for 7 days in ICR mice. Disease activity indices (DAI) took into account body weight, stool consistency, and gross bleeding. Histological changes and macrophage accumulation were observed by immunohistochemical analysis. Pro-inflammatory markers were determined using immunoassays, RT-PCR, and real time PCR. Signaling pathway involving nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs) activation was determined by luciferase assay and Western blotting.

Results: In DSS-induced colitis mice, TFRC improved DAIs and pathological characteristics including colon shortening and colonic epithelium injury. TFRC suppressed tissue levels of pro-inflammatory cytokines and reduced macrophage infiltration into colonic tissues. In LPS-induced RAW 264.7 macrophages, TFRC inhibited the production of NO, PGE_2, and pro-inflammatory cytokines by down-regulating the activation of NF-κB and p38 MAPK signaling.

Conclusion: The study demonstrates that TFRC has potent anti-inflammatory effects on DSS-induced colonic injury and LPS-induced macrophage activation, and supports its possible therapeutic and preventive roles in colitis.

1. Introduction

Inflammatory bowel disease (IBD) is comprised of Crohn's disease (CD) and ulcerative colitis (UC). Both illnesses are seriously growing problems in industrialized countries and have been associated with colon cancer risks (Langholz et al., 1992; Gillen et al., 1994). The etiologies of these two diseases have not been determined but are likely to involve genetic, environmental, and immunological factors. Imbalances among pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), Interleukin (IL)-1, IL-6, and IL-12, and anti-inflammatory cytokines, such as IL-4, IL-10, IL-11, are believed to play central

Abbreviations: 5-ASA, 5-Aminosalicylic acid; CD, Crohn's disease; DAI, Disease activity index; DSS, dextran sulfate sodium; IBD, Inflammatory bowel disease; IL-1β, Interleukin-1β; IL-6, Interleukin-6; iNOS, Inducible nitric oxide synthase; MAPK, mitogen-activated protein kinase; MOMA, monocytes and macrophages; MPO, Myeloperoxidase; NF-κB, nuclear factor-κB; NO, nitric oxide; RT-PCR, Reverse-transcriptase polymerase chain reaction; TFRC, 19α-hydroxyursane-type triterpenoid-rich fraction from *Rubus coreanus*; TNF-α, tumor necrosis factor-α; UC, ulcerative colitis

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roles in the modulation of inflammation (Ardizzone and Bianchi Porro, 2005). IBD is characterized by contiguous inflammation of the colonic lamina propria with subsequent injury and disruption of the mucosal barrier (Herias et al., 2005). These changes are accompanied by the infiltration of activated cells from both the innate and adaptive immune systems and by the release of inflammatory mediators from immune cells, which mediate tissue injury (Rabbi et al., 2014).

Macrophages play a key role in host defense against bacterial pathogens when stimulated via the activation of toll-like receptors (TLRs). In IBD and experimental colitis models, total numbers of macrophages are increased and macrophages are abnormally activated in inflamed intestinal tissues (Rath et al., 2001; Stevceva et al., 2001). Furthermore, activated macrophages may contribute to intestinal damage by releasing free radicals and by secreting pro-inflammatory cytokines and other inflammatory mediators (Stevceva et al., 2001). In macrophages, TLR4 ligation with LPS induces the activations of specific intracellular pathways via receptor dimerization and the recruitments of different adapter molecules. In addition, the subsequent activations of two distinct downstream signaling pathways, the nuclear factor-κB (NF-κB) pathway and the mitogen-activated protein kinases (MAPKs) pathway, induce the expression of various inflammatory mediators (Lee et al., 2014). NF-κB is one of the pivotal regulators of pro-inflammatory gene expression, and the aberrant regulation of NF-κB activity has been implicated in the pathogenesis of immune disorders, neurodegenerative disorders, autoimmune and inflammatory diseases (Liu and Malik, 2006). Under normal conditions, NF-κB is a dimer comprised of p65 and p50, and is present in cytoplasm as a NF-κB–Inhibitory κB (IκB) complex. Activation of NF-κB–IκB involves the phosphorylation of IκB and its subsequent recognition by ubiquitinating enzymes. The resulting proteasomal degradation of IκB leads to the nuclear translocations of NF-κB, where it binds to their consensus DNA binding sites. In this manner, NF-κB activates a number of rapid response genes involved in inflammatory response (Shin et al., 2011b). MAPKs are serine/threonine protein kinases and composed of three major families; the extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 MAPK. All three can be activated independently and simultaneously in response to different microbial products, inflammatory cytokines, or cellular stress (Lagoumintzis et al., 2008). Upon activation of the MAPKs, transcription factors were phosphorylated and activated, leading to expression of pro-inflammatory mediators (El Maghraoui et al., 2009).

Rubus coreanus Miquel (Rosaceae), the Korean black raspberry, is the edible berries cultivated in the southern part of Korea and some parts of China and Japan (Seo et al., 2011). *Rubus coreanus* has traditionally been used as a remedy for prostate and liver diseases and inflammatory diseases, such as diarrhea, asthma, allergy, and stomach ailment, and also for the prevention of uterine, cervical, and colon cancer (Hummer, 2010; Kim et al., 2012b; Chae et al., 2014). Furthermore, *Rubus coreanus* are recently reported to have anti-cancer, anti-inflammatory (Lee et al., 2007), anti-bacterial (Oh et al., 2012), and anti-oxidant functions (Jeon et al., 2007). Scientists have tried to elucidate the active components in *Rubus coreanus* and found that it contains various flavonoids, anthocyanins, polyphenols (Lim et al., 2012a, 2012b) and triterpenoids (niga-ichigoside F1 and its aglycone 23-hydroxytormentic acid) (Choi et al., 2003). Previously, we found that niga-ichigoside F_1 and 23-hydroxytormentic acid isolated from *Rubus coreanus* have anti-nociceptive, anti-inflammatory (Choi et al., 2003), anti-gastropathic, anti-rheumatic (Nam et al., 2006), anti-oxidant (Kim et al., 2011), and nephroprotective (Sohn et al., 2011) effects in vivo and in vitro. Based on the anti-inflammatory effects of these 19α-hydroxyursane-type triterpenoids, we prepared a standardized 19α-hydroxyursane-type triterpenoid-rich fraction from *Rubus coreanus* (TFRC) and examined its anti-inflammatory properties and the underlying molecular mechanisms involved in a DSS-induced colitis murine model and in LPS-induced RAW 264.7 macrophages.

2. Materials and methods

2.1. Preparation and analysis of TFRC from the unripe fruits of Rubus coreanus

TFRC used for this study was prepared from the unripe fruits of *Rubus coreanus* as described previously (Choi et al., 2003). The air-dried avoiding sun-light fruits of *Rubus coreanus* (2.4 kg) were pulverized and extracted with MeOH under reflux three times, and the MeOH extract was filtered and evaporated on a rotatory evaporator under reduced pressure and then lyophilized to give a solid mass (156 g) of MeOH extract. This material (150 g) was suspended in H_2O (1 L), and then partitioned with each 0.8 L hexane three times. The residual aqueous fraction was extracted with H_2O-saturated BuOH in a separating funnel three times; then the BuOH soluble part was concentrated in vacuo. The BuOH fraction was suspended in H_2O and then filtered using a filter paper. The filtered solution was poured into Diaion HP-20 column (320 g, $5 \times 70\ cm^2$) and then eluted with 2.0 L H_2O to elute sugars or ionic substances. This column was successively eluted with 2.0 L MeOH and then the eluate was concentrated to dryness to give a TFRC fraction (18 g; yield = 0.75%). TFRC was analyzed for major 19α-hydroxyursane-type triterpenoids, niga-ichigoside F1 and 23-hydroxytormentic acid were found to be present at concentrations of 292.5 ± 55.7 mg/g and 58.4 ± 7.3 mg/g by LC-MS/MS, respectively.

2.2. Experimental animals

All animal care and experimental procedures complied with the Guidelines of the Committee for Animal Care and Use of laboratory animals, College of Pharmacy, Kyung Hee University according to an animal protocol (Approval number # KHP-2009-10-06). Male ICR mice weighing 28–30 g were purchased from the Orient Bio Inc. (Seongnam-si, Korea). All mice were housed 4/cage and fed standard laboratory chow in the animal room with 12 h dark/light cycles and constant temperature (temperature: 20 ± 5 °C, humidity: 40–60%, light/dark cycle: 12 h) for 2 weeks or more.

2.3. Induction of colitis

Experimental colitis was induced by giving mice drinking water containing 4% (w/v) DSS for 7 days. Mice of each group were monitored carefully every day to confirm that they consumed an approximately equal volume of DSS-containing water. For experiments, mice were divided into six experimental groups ($n = 8$). The first group (vehicle-treated control group) was given drinking water and administered vehicle and the second group (DSS only-treated group) was given 4% DSS and administered vehicle throughout the experimental period. Other 3 groups (TFRC + DSS-treated group) were given 4% DSS and administrated TFRC (25, 50, or 100 mg/kg/day p.o.) daily. Last group (5-ASA + DSS-treated group) was given 4% DSS and administrated 5-ASA (75 mg/kg/day p.o., a reference drug) daily. 5-ASA and TFRC were dissolved in vehicle (0.9% saline containing 0.01% tween-20 and 0.5% carboxymethyl cellulose sodium). Administration of each drug was started with the DSS treatment at the same time.

2.4. Evaluation of Disease activity indices (DAI)

Body weight, stool consistency, and gross bleeding were recorded daily. Disease activity indices (DAI) were determined by combining scores of (i) body weight loss, (ii) stool consistency, and (iii) gross bleeding. Each score was determined as follows: change in body weight loss (0: none, 1: 1–5%, 2: 5–10%, 3: 10–20%, 4: $>20\%$), stool blood (0: negative, 1: +, 2: ++, 3: +++, 4: ++++) and stool consistency (0: normal, 1 and 2: loose stool, 3 and 4: diarrhea). Body weight loss was calculated as the percent difference between the