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The Protective Effects of *Perilla frutescens* Extract against Dextran Sulfate Sodium-induced Colitis in Mice and Underlying Mechanism

Dextran Sulfate Sodium 으로 유도된 Mice 대장염에서의 *Perilla frutescens* 의 보호 효과와 그 기작

2016 년 2 월

서울대학교 대학원 분자의학 및 바이오제약학과 박 동 대
Abstract

The Protective Effects of *Perilla frutescens* Extract against Dextran Sulfate Sodium-induced Colitis in Mice and Underlying Mechanism

Deung Dae Park

Under the supervision of Professor Young-Joon Surh

Department of Molecular Medicine and Biopharmaceutical Science

Seoul National University

The inflammatory bowel disease (IBD) which could progress to colorectal cancer (CRC) is caused by inflammation of the colon and intestine. CRC is one of the most prevalent malignancies, and accounts for the high rates of cancer-associated death. *Perilla frutescens* is an aromatic and edible annual herbaceous plant used as a traditional medicine and food or spice. In addition, *Perilla frutescens* has anti-inflammatory and immune-modulatory properties. In this study, I investigated the preventive effects of *Perilla frutescens* extract (PE) against dextran sulfate sodium (DSS)-induced inflammatory damage in colonic mucosa that mimics human IBD. PE was administered orally to five-week-old ICR mice for one week before and together with 3% DSS in double distilled water for 7 days. DSS-induced colitis exhibited several symptoms, such as body weight loss, colon length shortening, diarrhea, bloody stool, etc., and these were significantly attenuated by PE treatment. PE strongly reduced expression of DSS-induced inflammatory factors including cyclooxygenase-2, inducible nitric oxide synthase (iNOS) and cyclin D1 through suppression of nuclear factor-kappa B and signal transduction and activator of transcription 3 (STAT3). PE also exerted its inhibitory effects on tumor necrosis factor-alpha-induced iNOS expression, STAT3 phosphorylation and chemokine (C-X-C motif) receptor 2 expression in CCD841CoN human normal colon epithelial cells. Moreover, in murine peritoneal
and bone marrow-derived macrophages, PE induced expression of heme oxygenase-1, the anti-inflammatory cytokine interleukin-10, and peroxisome proliferator-activated receptor-gamma. The above results indicate that PE has a therapeutic/preventive potential in the management of colitis and inflammation-associated colon carcinogenesis.

**Keywords:** *Perilla frutescens* extract, DSS, NF-κB, STAT3, Macrophage, IL-10, PPAR-γ

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Contents

Abstract.........................................................................................................I

Contents.....................................................................................................III

List of Figures............................................................................................IV

Introduction.................................................................................................1

Material and Methods..................................................................................3

Results...........................................................................................................8

Discussion...................................................................................................28

References..................................................................................................

31

Abstract in Korean....................................................................................

35
### List of Figures

**Figure 1.** Experimental design for evaluating the effects of PE on DSS-induced mouse colitis.

**Figure 2.** PE ameliorated pathological symptoms of mouse colitis.

**Figure 3.** PE ameliorated inflammatory cell infiltration and oxidative tissue damage in DSS-induced mouse colitis.

**Figure 4.** Inhibitory effects of PE on expression of COX-2, iNOS and cyclin D1.

**Figure 5.** Inhibition of NF-κB signaling in DSS-induced colitis by PE.

**Figure 6.** Suppressive effects of PE on DSS-induced activation of STAT3 signaling.

**Figure 7.** Inhibition of DNA binding of NF-κB and STAT3 by PE.

**Figure 8.** Effects of PE on the expression of COX-2 and STAT3 in mouse colon tissues.

**Figure 9.** PE reduced oxidative damage and upregulated antioxidant enzyme expression in mouse colon.

**Figure 10.** Effects of PE on TNF-α induced expression of inflammation-associated signaling molecules in the CCD841CoN human normal colon cell line.

**Figure 11.** Stimulation of anti-inflammatory cytokine and antioxidant gene expression by PE.

**Figure 12.** Effects of PE on LPS-induced overexpression of inflammatory factors.

**Figure 13.** Upregulation of PPAR-γ expression by PE in peritoneal macrophages *ex vivo*.

**Figure 14.** Enhancement of PPAR-γ accumulation in peritoneal macrophages by PE treatment.

**Figure 15.** Stimulation of efferocytic activity of macrophages by PE.

**Figure 16.** Induction of anti-inflammatory and antioxidant gene expression by PE in bone marrow-derived macrophages.

**Figure 17.** Schematic illustration of proposed mechanisms underlying protective effects of PE on DSS-induced mouse colitis.
INTRODUCTION

Colorectal cancer (CRC) is one of the most prevalent malignancies which accounts for the high rates of cancer-associated death (Torre, Bray et al. 2015). Chronic inflammation is an aberrant and prolonged response to a disturbance of tissue homeostasis and is largely responsible for development of the majority of human malignancies (West, McCuaig et al. 2015). Inflammatory bowel disease (IBD), including ulcerative colitis and Crohn’s disease, is an illness characterized by chronic inflammation of colonic mucosa which is considered to be implicated in the pathogenesis of CRC (Ananthakrishnan 2015). Although the causation of CRC progressed from ulcerative colitis which is different from sporadic CRC is not fully comprehended, an emerging phase of the diseases signifies the pathogenic association with each other (Zhiqin, Palaniappan et al. 2014).

Elevated expression and activity of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) have been implicated in inflammation-associated carcinogenesis. COX-2 catalyzes the breakdown of arachidonic acid to produce prostaglandin E\(_2\), a lipid mediator of inflammation, which promotes tumorigenesis (Wang and Dubois 2010). iNOS is responsible for production of nitric oxide (NO) derived from L-arginine which can cause cell-cycle disorders, apoptosis, inflammatory tissue insult, and DNA damage (Aktan 2004). One of the regulators of cell proliferation, cyclin D1, promotes the cell cycle through binding to cyclin-dependent kinase (CDK) 4 and CDK 6, resulting in the phosphorylation of retinoblastoma protein (pRb). Subsequently, the E2F transcriptional factor is released from pRb and promotes the cell cycle by activating gene essential for G1-S transition and progression to S-phase (Witzel, Koh et al. 2010). The transcription of genes encoding COX-2, iNOS and cyclin D1 is regulated, in common, by nuclear factor-kappa B (NF-κB) and signal transducer and activator of transcription 3 (STAT3) (Tsatsanis, Androulidaki et al. 2006, Witzel, Koh et al. 2010).

NF-κB is a key transcription factor that regulates expression of cytokines, growth factors, and pro-inflammatory enzymes in response to various stimuli. Constitutive overactivation of this transcription factor contributes to cellular transformation, proliferation, angiogenesis and metastasis (Aggarwal 2004). Activation of NF-κB signaling promotes inflammation, which often leads to cancer. STAT3 is another transcription factor that also plays an important role in inflammation, tumorigenesis and cancer metastasis by up-regulating transcription of genes involved in cell proliferation and survival and tumor invasion and angiogenesis (Aggarwal,
Kunnumakkara et al. 2009). This transcription factor contributes to inflammation and carcinogenesis by co-operating with NF-κB (Lee, Herrmann et al. 2009, Grivennikov and Karin 2010).

The intestine contains a large number of immune cells than any other tissues in the body, and it is constantly exposed to antigens and potential immune stimuli (Mowat and Agace 2014). When intestinal inflammation occurs, various immune cells including macrophages, dendritic cells, neutrophils, T cells, B cells, etc. are involved in regulating the innate and adaptive immune system. In innate immune response, monocyte-like macrophages and dendritic cells play crucial roles in chemotaxis, phagocytosis, endocytosis and secretion of factors that modulate inflammatory responses all of which are integral to homeostasis, immune defense and tissue repair (Mantovani, Sica et al. 2004). These immune responses mainly take place in the mucosa, specifically in lamina propria. Upon inflammatory stimuli, monocytes infiltrate the mucosa and differentiate into macrophages for producing pro-inflammatory mediators, cytokines and reactive oxygen species (ROS), resulting in the recruitment of other cells contributing to the development of inflammation (Lusis 2000). Macrophages are classified according to cytokines secreted from T lymphocytes. Th2 immune responses associated with interleukin-4 (IL-4) and IL-10 inhibit pro-inflammatory mediators of macrophages and stimulate them to have anti-inflammatory capability (Mills, Kincaid et al. 2000).

*Perilla frutescens* (L.) Britt. (Lamiaceae) and their varieties are annual herbs of the mint family and edible plants frequently used in Asian countries such as China, Korea, Japan and Thailand. They contain polyphenols which have various structural varieties with distinct biological activities (Asif 2012). The anti-inflammatory effects of *perilla frutescens* extract (PE) were revealed *in vivo* (Makino, Furuta et al. 2001, Ueda and Yamazaki 2001, Lim, Woo et al. 2014) and *in vitro* (Kwak and Ju 2015). Even though the anti-inflammatory effects of PE have been reported, the detailed molecular mechanism remains poorly understood. The present study demonstrates that PE inhibits dextran sulfate sodium (DSS)-induced overexpression of COX-2, iNOS and cyclin D1 in mouse colitis by targeting NF-κB and STAT3 pathways.
MATERIALS AND METHODS

Animals

Four-week-old male ICR mice and 5-week-old female C57BL/6 mice were purchased from Orient-Bio, Inc. (Republic of Korea). They were acclimated for 7 days with tap water and basal diet under the conventional housing conditions (humidity (50 ± 10%), temperature (24 ± 2°C), light (12 / 12 hour light/dark cycle)).

Induction of DSS-induced colitis

DSS (molecular weight of 36,000 – 50,000) was obtained from MP Biomedical, LLC. PE was obtained from Amino Up Chemical Co., Ltd. (Sapporo, Japan). After an acclimation for 1 week, total of 30 mice were divided into five experimental groups and treated for 2 weeks in accordance with the conditions illustrated in Fig. 1. At the end of the experimental period, mice were sacrificed by cervical dislocation. Their colon tissues were taken out and washed with phosphate-buffered saline (PBS), and separated into three parts. The distal part was fixed in 10% buffered formalin for immunohistochemical examination, and other parts were frozen in lipid nitrogen right away and kept at -70°C for Western blot analysis.

Macroscopic assessment

Since the beginning of DSS treatment, the body weight of mice was measured everyday (Fig. 2A). Rectal bleeding and stool consistency were graded from 0 to 3 (Fig. 2 B) depending on the severity of symptoms (Fukata, Michelsen et al. 2005, Lavi, Levinson et al. 2010). The disease activity index (DAI) was graded as the sum of scores of rectal bleeding and stool consistency (Fig. 2 C). The colorectal parts were photographed, and their length was measured (Fig. 2 D).

Tissue lysis and protein extraction

Mouse colon tissues were homogenized in ice-cold lysis buffer [150 mM NaCl, 0.5% triton-X 100, 50 mM Tris-HCl (pH 7.4), 20 mM ethylene glycol tetra acetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride (PMSF) and ethylenediaminetetraacetic acid (EDTA)-free cocktail tablet] followed by periodical vortex mixing for 30 min at 0°C. Lysates were centrifuged at 18,000 g for 15 min at 4°C. The supernatants were collected and stored at -70°C till use.

Western blot analysis
For Western blot analysis, the total protein concentration was determined by using the bichinconinic acid (BCA) protein assay kit (Pierce). Cell lysates (30-50 μg protein) were mixed and boiled in a sodium dodecyl sulfate (SDS) sample buffer for 5 min before 8-15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After separation by SDS-PAGE, the gels were transferred to a polyvinylidene difluoride (PVDF) membrane (Gelman Laboratory). The blots were blocked in 5% fat-free dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 hour at room temperature. The membranes were incubated for 12-24 hours at 4°C with dilutions of primary antibodies for α-tubulin, actin, lamin-B, STAT3, P-STAT3, p65, IκBα, P-IκBα, COX-2, iNOS, chemokine (C-X-C motif) receptor 2 (CXCR2), heme oxygenase-1 (HO-1), NAD(P)H: quinone oxidoreductase 1 (NQO-1), 4-hydroxynonenal (4-HNE), and nuclear factor (erythroid-derived 2)-like 2 (Nrf2). The membranes were washed, followed by incubation with 1:4000 dilution of respective horseradish peroxidase (HRP)-conjugated secondary antibodies (rabbit, mouse or goat) (Zymed Laboratories) for 2 hours, and again washed with TBST. Protein expressed was visualized with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech) and LAS-4000 image reader (Fuji film) according to the manufacturer’s instructions.

Fraction of nuclear and cytoplasmic extracts

Cytosolic extracts were obtained from lysates dissolved in hypotonic buffer A [(10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES), pH 7.8, 1.5 mM MgCl₂, 10 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, and 0.1 mM PMSF)] with 10% Nonidet P-40 (NP-40) by homogenization and vortex mixing at 10-min intervals for 3 hours. After centrifugation at 18,000 g for 15 min, the supernatant (the cytosolic extracts) was collected and stored at -70°C until use. Precipitated pellets were resuspended in buffer C [(50 mM HEPES, pH7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 20% glycerol)]. After centrifugation at 18,000 g for 15 min, the supernatants (nuclear extracts) were collected and stored at -70°C until use.

Immunohistochemical analysis

The dissected colon tissues were prepared for immunohistochemical (IHC) analysis of the expression patterns of P-STAT3 and COX-2. Four-μm sections of 10% formalin-fixed, paraffin-embedded tissues were cut on silanized glass slides and deparaffinized three times with xylene and rehydrated through graded alcohol bath. The deparaffinized sections were heated by using
microwave and boiled twice for 6 min in 10 mM citrate buffer (pH 6.0) for antigen retrieval. To diminish nonspecific staining, each section was treated with 3% hydrogen peroxide and 4% peptone casein blocking solution for 15 min. For the detection of respective protein expression, slides were incubated with P-STAT3 (Cell Signaling Technology, Inc.) and COX-2 (Cayman Chemical) antibodies at room temperature for 40 min in Tris-buffered saline containing 0.05% Tween 20, and then developed using respective HRP-conjugated secondary antibodies (rabbit) EnVision™ System (Dako). The peroxidase-binding sites were detected by staining with 3,3’-siaminobenzidinetetrahydrochloride (Dako). Finally, counterstaining was performed using Mayer’s hematoxylin.

**Cell culture**

The CCD841CoN cells were obtained from the American Type Culture Collection and maintained in MEM (Gibco) containing 10% fetal bovine serum (FBS) (GenDEPOT) and an antibiotic-antimycotic mixture (AB) (Gibco BRL) at 37°C in the atmosphere of 5% CO₂ and 95% air. The CCD841CoN cells were treated with PE (10 μg/ml) 1 hour before tumor necrosis factor (TNF-α) dissolved in 0.1% bovine serum albumin (BSA) in PBS, treatment (10 ng/ml). After TNF-α treatment, CCD841CON cells were incubated for 1 hour. The cells were harvested and centrifuged at 18,000 g for 5 min at 4°C. The cells were suspended in the cell lysis buffer (Cell Signaling Technology). After centrifugation at 18,000 g for 15 min at 4°C, the supernatant was collected and stored at -70°C until use. Isolated peritoneal and bone marrow derived macrophages were maintained in DMEM-F12 containing glutaMAX™ (Gibco), 10% FBS and AB at 37°C with 5% CO₂ and 95% air. The macrophages were incubated at least for 24 hours after isolation and treated PE (50 μg/ml) or 100 ng/ml of bacterial lipopolysaccharide (LPS), or both. In treating cells with both PE and LPS, PE was added 2 hours before LPS treatment.

**Polymerase chain reaction**

Total RNA was isolated from CCD841CoN cells by using TRIzol® (Invitrozen) according to manufacturer’s protocol. Ten μg of total RNA was reverse transcribed with MLV reverse transcriptase at 42°C for 50 min and at 72°C for 15 min. PCR was conducted according to the standard procedures. Amplified products were analyzed on 2% agarose gel electrophoresis, stained with SYBR® Green (Invitrogen) and photographed using fluorescence in LAS-4000.

**Isolation of peritoneal macrophages**
Six-week-old male C57BL/6 mice were treated intraperitonally with 3% Brewer thioglycollate medium 1 ml. After 4 days, the abdominal skin of the mice was retracted to exposure the peritoneal wall and 10 ml of cold harvest buffer (3 mM EDTA in PBS) was injected through the wall. The fluid was aspirated slowly and dispensed into a 50 ml conical tube. The peritoneal exudates cells were centrifuge at 400 g for 8 min at 4℃. After removing supernatant, the pellet was suspended in 200 μl 1X red blood cell (RBC) lysis buffer (eBioscience) and incubated in ice for 30-60 sec. Then the cells were suspended with 4 ml PBS and centrifuged at 400 g for 8 min at 4℃. Finally the cells were divided into culture dishes with DMEM-F12 (with 10% FBS, AB, glutaMAX™) after cell counting.

**Bone marrow derived macrophage**

Bones from 6-week-old C57BL/6 cervical dislocation were transferred into sterile Petri dish contacting ice-cold sterile PBS. Tibia of the bones was cut and gently smashed, and the supernatant was collected in a 15 ml conical tube. The fluid was filtered through 70 μm Nylon cell strainer (BD) to remove solid fragment and centrifuged at 400 g for 5 min at 4℃. Supernatant was gently discarded and the pellet was suspended in 200 μl 1X RBC lysis buffer (eBioscience) and incubated in ice for 30 sec. After addition of 4 ml PBS, the cells were centrifuged at 400 g for 5 min at 4℃. The pellet dissociated in DMEM (Gibco) with 10% FBS and AB. After cell counting, the cells were distributed into petri dishes in DMEM (10% FBS, AB, M-CSF) and incubated for 3 days. Three days later, the media were changed and incubated for 4 days more.

**Flow cytometry assay**

Cells were fixed with 4% formaldehyde in PBS for 1 hour at 4℃, centrifuged at 400 g for 5 min at 4℃. The cells were washed with PBS and permeabilized with a permeabilization buffer (2% BSA, 0.1% sodium azide, 0.1% Tween20 in PBS) for 30-60 min at room temperature. After washing with PBS, anti-PPAR-γ antibody, diluted 1:100 in permeabilization buffer, was applied for 30 min at 4℃. The cells were incubated with FITC conjugated anti-rabbit immunoglobulin G (IgG) secondary antibody diluted at 1:1000 in FACS buffer(2% BSA, 0.1% sodium azide in PBS) for 30 min at 4℃. Cells were analyzed using a FACS Calibur_ Flow Cytometer (BD, Franklin Lakes).

**Immunocytochemistry**
Cells were plated in a six chamber and incubated for 24 hours to be stable. After stabilization, the cells were cultured in the absence or presence of PE for 4 hours. The media were removed and the cells were fixed with 4% formaldehyde in PBS for 15 min at room temperature. After fixation, the cells were permeabilized with 0.1% Triton X-100 in PBS, incubated with PBS containing 10% BSA, washed with PBS, and incubated with a diluted (1:100) primary antibody overnight at 4°C. After washing with PBS, cells were incubated with a diluted (1:100) TRITC anti-rabbit IgG secondary antibody for 1-2 hour at room temperature and with 4', 6-diamidino-2-phenylindole for 5-10 min, and examined under a confocal microscope (Nikon).

**Electrophoresis mobility shift assay (EMSA)**

DNA binding activity of NF-κB and STAT3 was measured with EMSA. In brief, T4 polynucleotide kinase transferred $^{32}$P labeled γ-phosphate from ATP to NF-κB oligonucleotide. After purification with a G-50 micro column (GE Healthcare, UK), [γ- 32 P] labeled probes were mixed with 10 µg of nuclear extracts and incubation buffer [10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 4% glycerol and 0.1 mg/ml sonicated salmon sperm DNA]. All the samples were mixed with 2 µl of 0.1% bromophenol blue loading dye after 50 minutes incubation and separated on 6% nondenatured polyacrylamide gel in a cold room. Finally, gels were dried and exposed to X-ray films (Agfa Healthcare, Belgium).

**Statistical assay**

Except for the data on the DAI score, and colon length expressed as the mean ± standard error (SE), all other values were expressed as the mean ± standard deviation (SD) of at least three independent experiments. Statistical significance was determined by the Student’s $t$-test, and $p < 0.05$ was considered to be statistically significant. ($^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$)
RESULTS

PE ameliorated pathological symptoms in the DSS-induced colitis mouse model

The common symptoms of IBD are bloody diarrhea, fever and weight loss. The body weight of mice in the DSS group was decreased after day 3 compared to that in the control group. Oral administration of PE reduced the loss of body weight. In particular, mice given a high dose of PE (100 mg/kg) showed a similar pattern of control in terms of body weight changes (Fig. 2 A). After DSS treatment, DAI score which includes the degree of rectal bleeding and stool consistency was increased. DSS treated mice exhibited severe symptoms of IBD. The DAI score in mice treated with DSS and PE was significantly lower than that in the DSS group (Fig. 2 C). Thickening of colon and shortening of colorectal length reflecting colitis were observed in the DSS group, and these were mitigated by PE treatment (Fig. 2 D). Inhibitory effects of PE against DSS induced colitis in mice were also confirmed by immunohistochemical analysis. Histological evaluation of dysplasia in the colonic crypts was also conducted using hematoxylin and eosin (H&E) staining. While exposure to DSS completely destroyed the architecture of colonic mucosa and induced infiltration of inflammatory cells and expansion of lamina propria, mice given PE exhibited a preserved colonic structure, clearance of inflammatory cells and prevention of lamina propria expansion (Fig. 3).

PE inhibited DSS-induced activation of NF-κB and STAT3 signaling in DSS-induced mouse colitis

It has already been revealed that DSS induces activation of NF-κB and STAT3 (Youn, Lee et al. 2009). Expression of COX-2, iNOS and cyclin D1 was upregulated in the DSS group. These factors are well-known pro-inflammatory mediators that play pivotal roles in inflammatory responses (Takeuchi and Akira 2010). Their expression was inhibited by treatment of PE, particularly at a high concentration (100 mg/kg) (Fig. 4). NF-κB, a transcription factor regulating expression of COX-2 and iNOS, is overactivated in IBD (Atreya, Atreya et al. 2008), resulting in disruption of the immune system, elevation of cell survival and resistance to apoptosis (Aggarwal 2004). NF-κB activation and translocation into nucleus are dependent on phosphorylation and degradation of IκBα. DSS treatment caused IκBα phosphorylation and degradation, resulting in augmented nuclear translocation of p65 which is a major functional subunit of NF-κB. Treatment with PE (100 mg/kg) reduced the DSS-induced nuclear migration.
of p65 as well as phosphorylation and degradation of IκBα (Fig. 5). STAT3 is mainly activated by IL-6-gp30-JAK signaling through its phosphorylation at Tyr 705. After dimerization, it translocates into nucleus and binds to promoters of target genes. The phosphorylation and nuclear translocation of STAT3 were significantly elevated in the colonic mucosa from DSS-treated mice and these were inhibited by PE (100 mg/kg) administration (Fig. 6). Not only translocation of NF-κB and STAT3 into nucleus but also DNA-binding of these transcription factors was abrogated by PE in a dose-dependent manner (Fig. 7). In colorectal tissues, DSS induced collapse of epithelium, lamina propria expansion, infiltration of inflammatory immune cells and overexpression of COX-2 and P-STAT3. These detrimental effects of DSS were significantly attenuated by a high dose (100 mg/kg) of PE (Fig. 8).

**PE upregulated expression of antioxidant enzymes in mouse colon**

DSS-induced colitis has been attributed to oxidative stress caused by ROS. To determine the changes in the ROS level in DSS-induced mouse colitis, 4-HNE-modified protein was assessed as an indicator of ROS. 4-HNE-modified protein was overexpressed in mouse colon as a consequence of DSS administration, and this was suppressed by PE treatment (Fig. 9 A). Oxidative tissue damage is prevented by antioxidant enzymes and related proteins. Nrf2 is a transcription factor encoded by the *NFE2L2* gene and regulates the expression of many antioxidant enzymes, including HO-1 and NQO-1 (Moi, Chan et al. 1994). PE enhanced the expression of HO-1, NQO-1 and total Nrf2 in a dose-dependent manner (Fig. 9 B).

**PE attenuated TNF-α-induced expression of inflammatory factors in CCD841CoN human colon epithelial cells**

TNF-α is a pleiotropic inflammatory cytokine and a central regulator of inflammation. Upon stimulation by TNF-α, NF-κB and MAPK pathways are activated (Bendtzen, Ainsworth et al. 2009). Human recombinant TNF-α was treated to CCD841CoN (10 ng/ml) for stimulating inflammatory factors. At the protein level, phosphorylation of STAT3 was increased 1 hour after TNF-α treatment. Phosphorylation of total IκBα was enhanced and degradation of IκBα was decreased after 15 min. The mRNA levels of COX-2 and IL-6 related to the NF-κB and STAT3 pathways were elevated 1 hour after TNF-α treatment. In addition, IL-8 which could promote neutrophil infiltration was overexpressed at the same time (Fig. 10 A). From these results, it was confirmed that TNF-α could induce inflammatory factors in CCD841CoN cells. To investigate its anti-inflammatory potential, PE (10 ng/ml) was pre-treated to the cells 1 hour before TNF-α
treatment. The expression of iNOS, P-STAT3, P-IκBα and CXCR2 was elevated by TNF-α and inhibited by PE (Fig. 10 B).

**PE was efficacious to make macrophages manifest anti-inflammatory characteristic.**

In the IBD, a number of pro-inflammatory molecules such as iNOS, COX-2, IL-6 and TNF-α participate in mediating immune inflammatory responses (Seo and Chae 2014). Based on the results on CXCR2 suppression by PE, it is plausible that PE can modulate immune responses. For identifying PE effects on immune cells, macrophages were isolated from murine peritoneal cavity. The macrophages from 6-week-old female C57BL/6 mice were treated with PE (50 μg/ml) after 24 hours stabilization. The mRNA expression of IL-10, a cardinal anti-inflammatory cytokine, was upregulated at 1 hour and 2 hours. The expression of HO-1 was increased from 2 hours and gradually decreased, while there was no change in Nrf2 accumulation (Fig. 11). To figure out whether PE also has protective effects on the murine peritoneal macrophages, the cells were pre-treated with PE, 2 h before LPS (100 ng/ml) treatment. The mRNA expression of inflammatory cytokines, IL-6, IL-12, IL-23, and TNF-α, was increased by LPS treatment, and this was inhibited by PE except for TNF-α (Fig 12). PPAR-γ activation is known to prime human monocytes into anti-inflammatory M2 macrophages. PPAR-γ was increased in PE-treated macrophages as determined by flow cytometric and immunoblot analyses (Fig. 13) which was also verified by immunocytochemistry (Fig. 14). However, there was no change in the Nrf2 levels (Fig. 14). In parallel with the enhancement of PPAR-γ, efferocytosis was more active in PE-treated macrophages compared with control cells (Fig. 15). Because macrophages have different properties depending on their origin, bone marrow-derived macrophages from mice were also used to examine the PE effects. The mRNA levels of IL-10, PPAR-γ, HO-1 and Nrf2 were also enhanced by PE in bone marrow-derived murine macrophages (Fig. 16).
Figure 1. Experimental design for evaluating the effects of PE on DSS-induced mouse colitis.
Figure 2. PE ameliorated pathological symptoms of mouse colitis. Five-week-old male ICR mice were treated with 3% DSS in DDW for 7 days. PE (20 or 100 mg/kg) was dissolved in DDW and given orally 7 days before DSS administration or 7 days together with 3% DSS. (A) Changes of body weight. (B, C) The DAI score as the sum of stool consistency and rectal bleeding was scored as 0 to 3. (D) The comparison of colon length. Data are expressed as means ± standard deviation (n = 6, in each group). *p < 0.05, **p < 0.01 and ***p < 0.001.
Figure 3. PE ameliorated inflammatory cell infiltration and oxidative tissue damage in DSS-induced mouse colitis. In immunohistochemical detection, the collapse and expansion of epithelium caused by DSS were attenuated by treatment of PE.
**Figure 4. Inhibitory effects of PE on expression of COX-2, iNOS and cyclin D1.** The whole lysates of colonic tissues were subjected to Western blot analysis to detect COX-2, iNOS and cyclin D1. Data are expressed as means ± standard deviation. *p < 0.05, **p < 0.01 and *** p < 0.001.
Figure 5. Inhibition of NF-κB signaling in DSS-induced colitis by PE. Cytosolic and nuclear extracts were analyzed for expression of P-IκBα and p65. Data are expressed as means ± standard deviation. *p<0.05, **p < 0.01 and *** p < 0.001. W.E. : whole lysate extract, C.E. : cytosolic extract, N.E. : nuclear extract.
Figure 6. Suppressive effects of PE on DSS-induced activation of STAT3 signaling. Nuclear extracts were analyzed for the translocation of P-STAT3 into nucleus. Data are expressed as means ± standard deviation. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. 
Figure 7. Inhibition of DNA binding of NF-κB and STAT3 by PE. NF-κB and STAT3 DNA binding was determined by EMSA as described in Materials and Methods.
Figure 8. Effects of PE on the expression of COX-2 and STAT3 in mouse colon tissues. The images illustrate immunohistochemical detection of COX-2 (A) and P-STAT3 (B). The cells expressing COX-2 and P-STAT3 are shown in brown dots. The number of stained cells was increased in the DSS group, and the DSS-induced upregulation of both proteins was decreased by PE administration.
Figure 9. **PE reduced oxidative damage and upregulated antioxidant enzyme expression in mouse colon.** To measure the expression of 4-HNE and antioxidant enzymes, the whole lysates of colonic tissues were subjected to Western blot analysis. The overexpression of 4-HNE, an indicator of ROS-induced oxidative tissue damage, was reduced (A), whereas the expression of antioxidant enzymes was elevated (B) by PE. Data are expressed as means ± standard deviation. *p<0.05, **p < 0.01 and *** p < 0.001.
Figure 10. Effects of PE on TNF-α induced expression of inflammation-associated signaling molecules in the CCD841CoN human normal colon cell line. TNF-α (10 ng/ml) was treated to CCD841CoN cells. The protein and mRNA levels of inflammatory factors were increased by TNF-α time-dependently (A). The expression of TNF-α induced inflammatory factors was inhibited by PE (10 mg/ml) (B).
Figure 11. Stimulation of anti-inflammatory cytokine and antioxidant gene expression by PE. Peritoneal macrophages were treated with PE (50 μg/ml) and mRNA levels of IL-10, HO-1 and Nrf2 were determined by RT-PCR. Data are expressed as means ± standard deviation.
Figure 12. Effects of PE on LPS-induced overexpression of inflammatory factors. PE (50 μg/ml) pre-treatment (2 hours before LPS treatment) diminished LPS (100 ng/ml)-induced expression of pro-inflammatory cytokines in peritoneal macrophages. Data are expressed as means ± standard deviation.
PE (100 mg/kg) was injected into mouse peritoneal cavity with thioglycollate. After 4 days, the macrophages were isolated and subjected to flow cytometry (A) and Western blot analysis (B).
Figure 14. Enhancement of PPAR-γ accumulation in peritoneal macrophages by PE treatment. Peritoneal macrophages were cultured in the absence or presence of PE (50 μg/ml) for 4 hours. The expression of the PPAR-γ and Nrf2 was visualized under the microscope.
Figure 15. Stimulation of efferocytic activity of macrophages by PE. Peritoneal macrophages, treated with PE for 4 hours, engulfed apoptotic thymocytes (red puncta) actively.
Figure 16. Induction of anti-inflammatory and antioxidant gene expression by PE in bone marrow-derived macrophages. One hour after PE (50 μg/ml) treatment of bone marrow-derived macrophages, anti-inflammatory and antioxidant gene expression was determined by RT-PCR.
Figure 17. Schematic illustration of proposed mechanisms underlying protective effects of PE on DSS-induced mouse colitis.
DISCUSSION

This study was intended to examine the anti-inflammatory effects of PE in the DSS-induced mouse colitis model which mimics human IBD (Okayasu, Hatakeyama et al. 1990). In response to DSS administration, the expression of iNOS, 4-HNE, COX-2 and cyclinD1 was elevated. iNOS induces excessive formation of NO and causes nitrosative stress that leads to DNA damages (Aktan 2004). 4-HNE is one of the major end products of lipid peroxidation and has been widely used as a hallmark of oxidative stress (Uchida 2003) PE significantly decreased the overexpression of iNOS and 4-HNE in DSS-induced mouse colitis. PE administration also suppressed DSS-induced upregulation of COX-2 and cyclinD1. Based on these findings, it is suggested that PE inhibits DSS-induced mouse colitis by suppressing inflammation, abnormal cell proliferation and ROS production in the colonic mucosal cells. In addition, PE upregulates the expression of anti-oxidant enzymes, HO-1 and NQO-1, and their master regulator Nrf2.

NF-κB and STAT3 have been recognized as key transcriptional factors in the onset of inflammation and tumor progression (Aktan 2004, Wang and Dubois 2010). Five members of NF-κB subunits include p65 (RelA), RelB, c-Rel, p50/p1105 (NF-κB1) and p52/p100 (NF-κB2). In the canonical pathways, the p65/p50 heterodimer remains inactive by forming a complex with inhibitor of κB (IκB), but becomes activated in response to pro-inflammatory stimuli through phosphorylation and degradation of IκBα (Hayden and Ghosh 2004, Ghosh and Hayden 2008, Hayden and Ghosh 2008). Inappropriate activation of STAT3 signaling is implicated in the pathogenesis of IBD and subsequent development of CRC through enhancement of cell proliferation and tumor growth (Sugimoto 2008). Activation of STAT3 is dependent on phosphorylation of tyrosine residue 705 in the C-terminal transactivation domain, which facilitates the dimerization of this transcription factor. The activated dimer then translocates to nucleus and promotes the transcription of target genes (Aggarwal, Kunnumakkara et al. 2009, Yue and Turkson 2009). Both NF-κB and STAT3 are activated in DSS-induced mouse colitis. The DSS-induced phosphorylation of IκBα and STAT3, translocation of NF-κB and STAT3 from cytoplasm to nucleus, and their DNA binding were all inhibited by PE. These findings suggest that PE treatment attenuates DSS-induced mouse colitis by inhibiting inflammatory responses associated with NF-κB and STAT3 pathways.
Although the DSS-induced mouse colitis model mimics human IBD, it has some limitations in studying precise mechanism underlying anti-inflammatory effects of PE. Therefore, design for in vitro experiments was necessary to further assess the anti-inflammatory potential of PE and elucidate underlying mechanisms. As TNF-α provokes inflammatory signaling in the human normal colon epithelial cell line, PE was treated with TNF-α. The results showed that inflammatory responses were augmented by TNF-α treatment and inhibited by PE.

CXCR2 is a member of the G-protein-coupled receptor family and a receptor for IL-8. CXCR2 has been implicated in CRC progression (Khalili, Gong et al. 2015). IL-8, also known as neutrophil chemotactic factor, is a major ligand to CXCR2 and involved in promoting neutrophil infiltration into epithelial tissues and making epithelial tissues inflamed (Islam, Lombardini et al. 2015). TNF-α induced increase of CXCR2 was suppressed by PE, suggesting that PE could inhibit inflammation by modulating the immune responses. For this reason, investigation was extended to assessment of PE effects on immune cells, especially macrophages. Many inflammatory cytokines secreted by immune cells stimulate proliferation of intestinal epithelial cells and confer resistance to apoptosis (Grivennikov, Greten et al. 2010). IL-6 is classically regarded as a pivotal factor in CRC progression, inducing activation of STAT3 and mediating cancer cell proliferation, migration and angiogenesis (Taniguchi and Karin 2014). IL-12 and IL-23 are secreted by inflammatory myeloid cells in response to exogenous or endogenous signals associated with host defense and wound healing. These cytokines influence the development of T cells and NK cells to promote inflammation (Teng, Bowman et al. 2015). PE suppressed LPS-induced overexpression of IL-12, IL-23 and IL-6 by upregulating anti-inflammatory factors, IL-10 and HO-1, in murine peritoneal macrophages. These results indicate that PE could modulate the patterns of cytokines to reduce inflammation.

PPAR-γ is a key factor to inhibit inflammatory macrophages (Ricote, Li et al. 1998). PPAR-γ was shown to downregulate the expression of STAT, NF-κB, and AP-1 (Wang, Verna et al. 2002) and PPAR-γ target genes are associated with anti-inflammatory macrophage (Porta, Rimoldi et al. 2009). PE upregulated PPAR-γ expression, and activated the process, called efferocytosis, by which dying/dead cells are removed by phagocytic cells in peritoneal macrophages. In the present study, peritoneal macrophages were initially used for investigating PE effects on the immune system. However macrophages have different characteristics in accordance with their origin. Moreover, peritoneal macrophages are not directly related with colitis. Bone marrow-derived and colon tissue-resident macrophages are more relevant to colorectal immune
responses (Varol, Zigmund et al. 2010, Ginhoux and Jung 2014, Lavin, Winter et al. 2014). PE enhanced expression of IL-10, HO-1, PPAR-γ and Nrf2 mRNA in bone marrow-derived macrophages. For exact comprehension of regulatory capability of PE in intestinal immune environment, it will be worthwhile to determine whether PE influences macrophages originated from not only bone marrow but also colon tissue.

PE contains many polyphenols, and some of them have been reported to exert anti-inflammatory and antioxidant effects (Zhou, Yan et al. 2014). Major components of PE are luteolin (Seelinger, Merfort et al. 2008, Kritas, Saggini et al. 2013), caffeic acid (Patel, Acharya et al. 2014), apigenin (Hoensch and Oertel 2012, Gupta, Tyagi et al. 2014), and rosmarinic acid (Kamyab and Eshraghian 2013). Further studies will be necessary to identify active principle(s) of PE that can contribute to its protection against DSS-induced colitis in mice.

In summary, PE protects against DSS-induced murine colitis by inhibiting activation of NF-κB and STAT3 and expression of their target proteins. In addition, PE activates anti-inflammatory signaling while suppressing pro-inflammatory one in macrophages. PE potentiates cellular antioxidant defense, thereby attenuating oxidative stress-induced apoptosis in colon epithelial cells. These suggest that PE has a potential for ameliorating colitis and inflammation-associated colon carcinogenesis. Further clinical and mechanistical studies will be necessary.
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국문초록

염증성 장 질환은 장과 대장에서 생기는 염증에 의해 야기되는 것으로 대장암까지 진행될 수 있다. 대장암은 가장 반연하는 악성종양 중 하나로 그 사망률이 매우 높다. *Perilla frutescens*는 방향족의 다년초 식물로 전통적으로 약물, 음식, 향신료 등으로 사용되어 왔으며, *Perilla frutescens*는 항염증 기능과 면역조절 기능을 가지고 있다. 이 실험에서는 dextran sulfate sodium (DSS)에 의해 유도된 마우스 대장염에서의 *Perilla frutescens* 추출물 (PE)의 보호효과를 확인하였다. PE 를 5주령의 마우스에 7일간 경구 투여한 후, PE 경구투여를 유지하며 3% DSS 를 7일간 처리하였다. DSS 에 의해 유도된 염증으로 인해 몸무게 감소, 대장수축, 설사, 혈변 등의 증상이 나타났으며 이 증상들은 PE 처리에 의해 감소하였다. PE 는 DSS 에 의해 유도된 cyclooxygenase-2, inducible nitric oxide synthase (iNOS), cyclin D1 와 같은 염증 요인들의 발현과 이들의 전사인자인 nuclear factor-kappa B (NF-κB), signal transduction and activator of transcription 3(STAT3)의 발현도 억제하였다. PE는 CCD841CoN 세포주에서 tumor necrosis factor-alpha 에 의해 유도된 iNOS 와 chemokine (C-X-C motif) receptor 2 의 발현, 그리고 NF-κB 와 STAT 의 인산화도 억제하였다. 또한, 마우스의 복강과 골수에서 유도된 대식세포에 PE 처리 시, 항산화 효소와 항염증 사이토카인인 interleukin-10, peroxisome proliferator-activated receptor- gamma 의 발현이 증가하였다. 위의 결과들을 통해 PE 는 대장염과 염증관련 대장암을 치료하고, 이로부터 보호할 수 있는 가능성이 있음을 확인하였다.

주요어: *Perilla frutescens* 추출물, DSS, NF-κB, STAT3, 대식세포, IL-10, PPAR-γ

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