Gallic acid suppresses inflammation in dextran sodium sulfate-induced colitis in mice: Possible mechanisms

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ABSTRACT

Inflammatory bowel diseases (IBD) encompass at least two forms of intestinal inflammation: Crohn’s disease and ulcerative colitis (UC). Both conditions are chronic and inflammatory disorders in the gastrointestinal tract, with an increasing prevalence being associated with the industrialization of nations and in developing countries. Patients with these disorders are 10 to 20 times more likely to develop cancer of the colon. The aim of this study was to characterize the effects of a naturally occurring polyphenol, gallic acid (GA), in an experimental murine model of UC. A significant blunting of weight loss and clinical symptoms was observed in dextran sodium sulfate (DSS)–exposed, GA-treated mice compared with control mice. This effect was associated with a remarkable amelioration of the disruption of the colonic architecture, a significant reduction in colonic myeloperoxidase (MPO) activity, and a decrease in the expression of inflammatory mediators, such as inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)–2, and pro-inflammatory cytokines. In addition, GA reduced the activation and nuclear accumulation of p-STAT3 Y705, preventing the degradation of the inhibitory protein IκB and inhibiting of the nuclear translocation of p65-NF-κB in colonic mucosa. These findings suggest that GA exerts potentially clinically useful anti-inflammatory effects mediated through the suppression of p65-NF-κB and IL-6/p-STAT3 Y705 activation.

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1. Introduction

Inflammatory bowel diseases (IBD) encompass at least two forms of intestinal inflammation: Crohn’s disease and ulcerative colitis (UC). Both types are chronic and inflammatory disorders in the gastrointestinal tract, with an increasing prevalence being associated with the industrialization of nations and in developing countries [1]. The pathogenesis of IBD remains unclear, but imbalances between pro-inflammatory cytokines, such as tumor necrosis factor (TNF-α), interferon gamma (IFN-γ), interleukin (IL)-1β, IL-6, and IL-12, and anti-inflammatory cytokines, such as IL-4, IL-10, and IL-11, are believed to play a central role in modulating inflammation [2,3]. Patients with these diseases are 10–20 times more likely to develop cancer of the colon or bowel cancer [4,5]. Therapeutic strategies for treating inflammatory bowel disease now focus on the use of anti-inflammatory agents [6], and plant-based remedies play an important role in the therapy of many inflammatory disease conditions, including IBD [7–9].

Signal transducer and activator of transcription (STAT)-3 is a key member of the STAT protein family, which has been shown to play significant roles in cytokine signaling pathways. Under normal circumstances, STAT3 is activated in a transient manner with activation terminated by suppressors of cytokine signaling (SOCS) proteins. STAT3 is activated by phosphorylation at tyrosine 705 (Y705) and serine 727 (S727) [10]. Phosphorylation of STAT3 at Y705 is often mediated by JAK1, epidermal growth factor receptor, or Src and is required for STAT3 homo- or hetero-dimerization, nuclear translocation and DNA binding. Several target genes of STAT3 have been identified, including proteins that are involved in cell survival and proliferation, such as Bcl-2, Bcl-xl, Mcl-1, Fas, cyclin D1, cyclin E1 and p21 [4,11,12]. Constitutively activated STAT3 is associated with IBD, where it modulates gut immune cell activation [13]; the STAT3 gene is a susceptibility loci for IBD [14]. IL-6 is elevated in the serum and mucosa of patients with IBD, and the level of IL-6 in serum is an indicator of disease relapse [15]. Blocking IL-6 reduced disease severity in association with diminished STAT3 activation and lamina propria T-cell apoptosis [16]. Hence, with this evidence, IL-6/STAT3 pathway was selected as a key target to treat UC.

Gallic acid (3,4,5-trihydroxybenzoic acid, GA), is a type of phenolic acid that is found in various natural products, such as gallnuts, pineapples, sumac, oak bark, green tea, apple peels, tea leaves, grapes,
strawberries, bananas, and lemons and also in red and white wine [17]. GA possesses many beneficial effects including anti-oxidant [18], anti-inflammatory [19], anti-diabetic [20] and anti-carcinogenic [21] properties. Recently Ma et al. [22] reported that pretreatment with GA attenuated Dimethylnitrosamine-induced acute liver injury, which might be attributed to its capability of inducing nrf2 translocation and subsequent expression of hemoxygenase-1 and Glutathione-S-Transferase-α.

Previous reports have shown that GA downregulates matrix metalloproteinase-2 (MMP-2)/MMP-9 in human leukemia K562 cells [23] and inhibits gastric cancer cell metastasis and invasive growth via increased expression of RhoB, downregulation of Akt/small GTPase signals and inhibition of NF-κB activity [21]. GA shows selective cytotoxicity for cancer cells and has little toxicity against normal cells [24]. In the present study, we analyzed the anti-colitic effect of gallic acid in BALB/c mice with DSS-induced colitis. We found that GA potentially suppressed pro-inflammatory cytokines (TNF-α, IL-1β, IL-17 and IFN-γ) and inflammatory mediators, such as iNOS and COX-2. Furthermore, GA treatment inhibited the activation and translocation of p65-NF-κB and IL-6/STAT3 pathways.

2. Materials and methods

2.1. Chemicals

HistoVT antigen retrieval kit (10×, pH 7.0), propidium iodide, the BCA protein assay kit and the peroxidase stain DAB kit were purchased from Nacalai Tesque (Kyoto, Japan). Primary antibodies against iNOS, COX-2, Bcl-xL and actin were procured from Santa Cruz Biotechnology (Dallas, TX, USA); antibodies against p-STAT3Y705 and T-STAT3 were from Cell Signaling Technology (Danvers, MA, USA). QIAshredder and RNasey Kit and QuantFast SYBR Green PCR Master Mix were purchased from QIAGEN (Hilden, Germany), and Clarity western ECL substrate and Immun-Blot® PVDF membrane were from BIO-RAD (USA). All other chemicals used were analytical grade.

2.2. Animals

Male BALB/c mice weighing 25–30 g were purchased from A Sapphire Enterprises, Seri Kembangan, Selangor, Malaysia. The mice were housed individually in plastic cages at a constant temperature (21 ± 2 °C) with an alternating 12-h light/dark cycle; animal chow and water were provided ad libitum. All animal treatments adhered strictly to institutional and international ethical guidelines of the care and use of laboratory animals. The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC), Universiti Putra Malaysia (UPM), Serdang, Selangor, Malaysia for the use of animal subjects.

2.3. Experimental design

Animals were randomly divided in 3 experimental groups. Group 1 served as control received normal drinking water. In Group 2, acute...
colitis was induced by administrating 2.5% (w/v) DSS (molecular weight-36,000–50,000; MP Biomedicals, OH, USA) in the drinking water for 7 days [25]. Group 3 animals received DSS along with GA at the dose of 10 mg/kg body weight orally for 7 days. During the experimental period, the body weight of the mice was measured and recorded.

After the experimental period, the animals were sacrificed, the colon was excised from the mice, and the length and weight of the colon was measured. Colon sections were then washed with PBS; part of the colon was fixed in 10% buffered formalin, and the other part was stored in dry ice for further analyses.

2.4. Histological analysis

Paraffin-embedded samples were cut into 5-μM sections and stained with hematoxylin and eosin (HE) for light microscopic examination. The colon sections were imaged using a Nikon ECLIPSE 80i (Tokyo, Japan) photomicroscope. A histologic inflammatory score, ranging from 0–10, was assigned following the method described by [26]. The total score was assessed as the sum of the following 4 parameters: mucosal ulceration, 0–3 (0, normal; 1, surface epithelial inflammation; 2, erosions; 3, ulcerations); epithelial hyperplasia, 0–3 (0, normal; 1, mild; 2, moderate; 3, pseudopolyps); lamina propria mononuclear infiltrate, 0–2 (0, normal; 1, slightly increased; 2, markedly increased); and lamina propria neutrophil infiltrate, 0–2 (0, normal; 1, slightly increased; 2, markedly increased).

2.5. Cell culture

RAW264.7 macrophages were purchased from American Type Culture Collection (ATCC; Rockville, MD) and cultured in standard media consisting of Dulbecco’s Modified Eagle’s Medium (DMEM), 10% fetal bovine serum (FBS) and 100 U/mL penicillin–streptomycin (Gibco, New York, USA). RAW264.7 cell viability in the absence and presence of different concentrations of GA was measured. Briefly, cells were plated onto 96-well plates (5 × 10^3 cells/well) and treated with GA (0–200 μg/mL) for 24 h. MTS (Promega, WI, USA) solution was added, and the cells were further incubated for 2 h followed by viable cell detection using a Hildex Chameleon (Turku, Finland) microplate reader at 450 nm.

2.6. Scanning electron microscopy (SEM)

Colon tissue samples were washed thoroughly in PBS and cut approximately 1 cm in length then fixed overnight in 2.5% phosphate-buffered glutaraldehyde solution (0.1 M, pH 7.4). Furthermore, the samples were fixed in 1% osmium tetroxide solution and passed through an increasing alcohol and amyl acetate series. After drying the tissue
samples with a critical-point dryer, they were coated with gold. The tissue samples were examined under Jeol 5200 JSM scanning electron microscopy (Tokyo, Japan).

2.7. Measurement of Myeloperoxidase Activity

Myeloperoxidase (MPO) activity of colon homogenates was determined as previously described with some modifications [27]. Briefly, whole colon tissue was homogenized at a concentration of 0.1 g/mL in PBS containing complete protease inhibitor cocktail and centrifuged at 12,000 g for 10 min. The pellets were resuspended in 50 mM sodium phosphate, pH 6.0, contain 0.5% hexadecyltrimethylammonium bromide and crushed on ice followed by 3 freeze/thaw cycles. The lysate was centrifuged at 14,000 g for 10 min, and the supernatant was heated at 60 °C for 2 h to inhibit catalase activity. The sample was added to reagent buffer (50 mM sodium phosphate, pH 6.0, 0.8 mM 3,3′,5,5′-tetramethylbenzidine and 5 mM hydrogen peroxide). MPO-dependent oxidation of 3,3′,5,5′-tetramethylbenzidine was measured by monitoring absorbance at 650 nm, and activity was determined using an extinction coefficient of 39 mM$^{-1}$ cm$^{-1}$. Protein concentrations were determined using the BCA protein assay.

2.8. Immunohistochemical analysis

The immunohistochemical method was performed according to previously described methods [28]. Finally the sections were counterstained with hematoxylin, and the slides were imaged using a light microscope (Nikon ECLIPSE 80i, Japan).

2.9. Immunofluorescence analysis

2.9.1. Tissues

Paraffin-embedded colonic tissue sections were deparaffinized in xylene and then rehydrated in graded ethanol solutions. Then, the slides

![Fig. 3.](image-url) Gallic acid reduces the RT-PCR expression of pro-inflammatory cytokines (A) TNF-α, (B) IL-1β, (C) IL-6, (D) IL-17. The values are expressed as the mean ± S.D. Comparisons: aControl vs. DSS, bDSS vs. DSS + GA. *, significant at p < 0.05; ns, non-significant.

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were incubated with the HistoVT (10 ×, pH 7.0) antigen retrieval solution for 20 min in 90 °C followed by cooling at room temperature.

2.9.2. Cells
RAW264.7 cells were seeded in a chamber slide at the concentration of 5 × 10³ and allowed to plate overnight. Next, the cells were treated with GA at 25 or 50 μg/mL. Furthermore, the cells were treated with lipopolysaccharide (LPS; 10 ng/mL); then, the slides were washed with ice-cold PBS, and the cells were fixed with ice-cold methanol-acetone for 20 min at −20 °C (1:1).

The slides were then blocked with 5% BSA or goat serum in Tris-buffered saline (TBST) for 90 min. The sections were then immunostained with mouse monoclonal immunoglobulin G (IgG) to mouse p-STAT3(Y705) or p65-NF-κB, diluted 1:200 with 5% BSA in TBST, and incubated overnight at 4 °C. After washing the sections three times with TBS, the slides were incubated with their corresponding secondary antibodies, DyLight™ 488 or DyLight™ 550 (Thermo Scientific, MA, USA), diluted 1:500 with TBS, and incubated in dark for 60 min at room temperature. The sections were then washed with TBS and incubated with counterstain propidium iodide or Hoechst to stain the cell nuclei. The slides were cover slipped and visualized under an Olympus FS X100 fluorescent microscope (Tokyo, Japan).

2.10. RNA isolation and quantitative PCR
RNA isolation and RT-PCR were performed following previously described methods [29]. The colon tissue samples were frozen and mechanically dissociated in RNA buffer. The total RNA was then extracted using the QIAshredder and RNeasy Kit (QIAGEN, Hilden, Germany) following the manufacturer’s instructions. The quality and quantity of the total RNA was measured using a nanodrop machine. Later the Real-time PCR was performed using an Eppendorf PCR system with the QuantiFast SYBR Green PCR MasterMix (QIAGEN, Hilden, Germany), primers, and 1 μg of cDNA in a 25-mL reaction mixture. Each target and standard g of cDNA in a 25-mL reaction mixture. Each target and standard

2.11. Immunoblot analysis
Immunoblot analysis was performed as according to previously described methods [28]. The colon tissue was cut into pieces, washed and homogenized in 5 volumes of ice-cold homogenizing buffer (0.1 mM NaCl, 0.1 M Tris Cl, 0.001 M EDTA) containing 1 mM PMSF, 1 mg/mL aprotinin, and 0.1 mM leupeptin at 3000 g for 10 min at 4 °C. The supernatants were estimated for protein content using BSA as a standard. The extracts were heated in a boiling water bath for 5 min, and the protein samples, 40 μg each, were subjected to SDS-PAGE and transferred to PVDF membranes using a transfer apparatus (BIO-RAD, USA). The membranes were blocked overnight at 4 °C with blocking reagent [20 mM Tris (pH 7.4), 125 mM NaCl, 0.2% (v/v) Tween 20, 4% (w/v) nonfat dry milk and 0.1% (w/v) sodium azide]. The membranes were then incubated with the p-STAT3(Y705), T-STAT3, p65-NF-κB, Bcl-xl and β-actin primary antibodies at the appropriate dilutions recommended by the supplier overnight at 4 °C and then incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h. Protein–antibody complexes were detected by the Clarity western ECL substrate. Immunoblots were quantified using ImageJ software (NIH, Bethesda, MD).

2.12. Statistical analyses
The data are expressed as the mean ± SD. The data were processed by the statistical analysis software SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). All P values were two-tailed, and a P value of less than 0.05 was considered significant.

Fig. 4. GA reduces the expression/activity of CD68+ and MPO. (A) Confocal microscopic analysis of CD68+ in control and experimental mice. The experimental details are described in the “Materials and Methods” section. The secondary antibody used in this study was tagged with DyLight™ 550, and the slides were counterstained with Hoechst. The control animals exhibited little CD68+ expression. DSS treatment increased the expression of CD68+ (Dylight 550; red). However, GA treatment decreased CD68+ expression. (B) MPO activity in control and experimental mice. The elevated activity of MPO in DSS-induced mice was significantly (P < 0.05) reduced by GA administration. The values are expressed as the mean ± S.D. Comparisons: *Control vs. DSS, **DSS vs. DSS + GA, *significant at p < 0.05; ns, non-significant.
3. Results

3.1. GA attenuated DSS-induced experimental colitis

In the present study, a mouse model of DSS-induced experimental colitis was used to evaluate the therapeutic effect of GA. Mice were challenged with 2.5% DSS for 7 days, which led to inflammatory conditions in the colon. DSS induces severe illness in mice that is characterized by a dramatic weight loss, evident rectal bleeding and diarrhea. As shown in Fig. 1A, compared with control mice, GA administration prevented the reduction of body weight. It is generally accepted that colon length is inversely associated with the severity of DSS-induced colitis. To determine whether GA had a beneficial effect on DSS-induced colonic shortening, we measured and compared the colon lengths of control mice, mice with DSS-induced colitis, and GA (10 mg/kg body weight)-treated mice with DSS-induced UC. While a significant shortening of colon length was observed in mice with DSS-induced colitis compared with control mice, the oral administration of GA reduced this shortening of the colon length (Fig. 1B and C).

3.2. GA decreased histological changes in mice with DSS-induced colitis

Consistent with the previous findings, histological changes in the colons of mice with DSS-induced colitis showed mucosal inflammation in the rectum with reducing proximal severity [30]. Pathological examinations of colons and rectums were performed after H&E staining, and representative results are shown in Fig. 2A. Tissue sections from representative areas of the large intestine showed decreases in crypts, epithelium distortion and, in the mucosal and submucosal areas, infiltrations of acute and chronic inflammatory cells, which significantly reduced histopathological scores (Fig. 2B). Interestingly, crypt structures were rather well preserved, and inflammatory reactions were significantly reduced in tissue samples from mice with DSS-induced colitis treated with GA than control mice. Fig. 2C shows the SEM photomicrograph analysis of colon tissues in control and experimental mice. Normal mice showed an intact epithelial surface (1000×). DSS-induced colitis mice showed abnormal crypt structure in the epithelium and fibrous connective tissue (800×). Colitic mice treated with GA showed reconstruction of microvilli and reduced pathological symptoms (600×).

3.3. GA attenuated pro-inflammatory cytokines

Cytokines play a significant role in the intestinal immune system, and immune cells, such as macrophages, T cells, dendritic cells, and intestinal epithelial cells, are known to secrete various cytokines that regulate inflammatory response in UC. Fig. 3 shows the relative mRNA expression levels of TNF-α, IL-1β, IFN-γ, IL-6, and IL-17 in the mouse control and experimental groups by qRT-PCR analysis. The expression of all...
inflammatory cytokines in the mice treated with DSS was increased compared with the untreated mice. GA treatment significantly attenuated the expression of all of tested cytokines (P < 0.05).

3.4. GA reduced the expression/activity of CD68+ and MPO

Fig. 4A presents the confocal microscopic analysis of CD68+ (conjugated to DyLight™ 550) after counterstaining with Hoechst. CD68+ expression was comparatively higher in AOM/DSS-induced animals, whereas GA treatment attenuated CD68+ expression. MPO is an enzyme found in neutrophils and in substantially smaller quantities in monocytes and macrophages. Increased MPO activity indicates the degree of neutrophil infiltration, which is a marker of acute inflammation [31]. We measured MPO activity in colonic tissues of control and experimental groups of mice; the result is shown in Fig. 4B. MPO activity was significantly increased in DSS-induced colitis mice compared with control mice, and GA administration significantly suppressed this MPO activity. We conclude that GA protects the colonic tissue by reducing neutrophil infiltration.

3.5. GA inhibited expression of p65-NF-κB in DSS-induced colitis

NF-κB activation plays a central role in inflammation; hence, we examined the effect of GA on this transcription factor in the inflamed tissue. Fig. 5A shows the expression of p65-NF-κB and IκB-α in control and experimental mice via western blot. DSS-induced mice showed an increased expression of p65-NF-κB and decreased expression of IκB-α compared with control mice, and GA treatment reversed these effects. Densitometric analysis of the blots also confirmed the above findings (Fig. 5B). Immunofluorescence analysis of p65-NF-κB is portrayed in Fig. 5C. Control mice showed reduced expression of p65-NF-κB, but DSS-administered mice showed increased expression of p65-NF-κB compared with control mice. The mice treated with GA showed reduced expression of p65-NF-κB compared with DSS-induced mice. This result confirms that GA strongly inhibits p65-NF-κB expression in DSS-induced mice.

3.6. GA suppressed p65-NF-κB expression in RAW 264.7 macrophage cells

To further understand the GA-mediated amelioration of inflammation in DSS-induced colitis, we studied the in vitro effect of GA on RAW 264.7 macrophage cells stimulated with LPS. Fig. 6A shows the western blot analysis of p65-NF-κB with or without LPS treatment. We observed an increased expression of p65-NF-κB in LPS (10 ng/mL)-induced cells compared with control. GA administration to the LPS-induced cells decreased the expression of p65-NF-κB in a dose-dependent manner. We further confirmed p65-NF-κB expression by

Fig. 6. Gallic acid inhibits inflammation and p65-NF-κB expression in RAW 264.7 macrophage cells. (A) Western blot analysis of p65-NF-κB in RAW 264.7 macrophage cells with or without LPS and GA. LPS-induced cells showed increased NF-κB expression. GA administration reduced NF-κB expression in a dose-dependent manner. (B) RAW 264.7 cells were grown in chamber slides, and after treatment with LPS and GA, the cells were washed with PBS. The cells were fixed with ice-cold methanol:acetone (1:1) at −20 °C for 20 min. The cells were blocked with goat serum for 90 min to avoid non-specific expression. Then, the cells were stained with a primary antibody against p65-NF-κB and a secondary antibody conjugated to DyLight™ 488 and counter stained with Hoechst for 10 min. After washing with PBS 2–3 times, the cells were cover slipped with an anti-fading agent. Then, the cells were imaged under fluorescence microscopy. LPS administration increased p65-NF-κB expression in the nucleus. However, treatment with GA at 50 and 100 μg/ml showed complete elimination of p65-NF-κB expression.
immunofluorescence analysis (Fig. 6B). We noted a pattern of results similar to that obtained from western blot analyses.

3.7. GA inhibited the expression of iNOS and COX-2 in the colon

Fig. 7A shows the immunohistochemical expression levels of iNOS and COX-2 in control and experimental mice. We observed an increase in the expression levels of iNOS and COX-2 in DSS-induced mice compared with control. Subsequent GA treatment reduced the expression of both iNOS and COX-2. Fig. 7B shows the immunohistochemical quantifications of iNOS and COX-2 expression. We also confirmed the expression of iNOS and COX-2 by qRT-PCR, as shown in Fig. 7C. The results show that the DSS-induced mice had increased expression of iNOS (12.84 ± 1.32) and COX-2 (14.08 ± 0.62) compared with the control mice (iNOS, 1.48 ± 0.68, COX-2, 1.8 ± 1.13). The DSS-induced mice treated with GA showed a more than 50% reduction in the expression of iNOS (5.82 ± 2.06) and COX-2 (9.15 ± 1.32).

3.8. GA inhibited STAT3 activation and translocation

The western blot results for p-STAT3Y705, Bcl-xl and T-STAT3 expression are shown in Fig. 7B. The DSS-induced mice showed increased expression of p-STAT3Y705 and Bcl-xl compared with the control mice. However, GA treatment inhibited the phosphorylation of STAT3 at Tyr705 (Fig. 8A and B) and the translocation of p-STAT3Y705 into the nucleus (Fig. 8C). We did not observe any changes in the expression of T-STAT3 in any experimental group.

4. Discussion

The results of the present study have revealed the beneficial effects of GA, a polyphenol present in certain fruits, such as sumac, tea leaves, witch hazel and gallnuts, among others, in an experimental murine model of UC. GA (10 mg/kg) diminished the severity and extension of the intestinal injuries induced by DSS. The reduction in the extent of colitis was accompanied by a decrease in weight loss and an increase in the length of the colon. Likewise, an attenuation of the morphologic alterations associated with cellular injury, a good maintenance of the glandular architecture and an important decrease in inflammatory cell infiltrate were observed.

NF-κB has been recognized as a tumor promoter in inflammation-associated carcinogenesis [32]. This ubiquitous eukaryotic transcription factor has been known to regulate the expression of inflammatory proteins, such as COX-2 and iNOS [33]. One of the critical events for NF-κB activation is its dissociation from the inhibitory subunit IκB-α, which requires IκB-α phosphorylation. The phosphorylated IκB proteins are rapidly polyubiquitinated and degraded by proteasomes, releasing free NF-κB for translocation to the nucleus and subsequent regulation of the transcription of target genes. We observed an increase in p65-NF-κB expression (Fig. 5A) and a subsequent decrease in IκB-α expression (Fig. 5A) in DSS-induced mice. When the DSS-induced mice were treated with GA, p65-NF-κB expression was reduced, and IκB-α expression was increased (Fig. 5). It has been shown that natural antioxidants directly inhibit the expression of NF-κB-dependent cytokines by suppressing the activators of pro-inflammatory cytokines, such as iNOS and COX-2, and thereby reducing inflammation [34]. The antioxidant NF-κB inhibitors restrict the production of inflammatory mediators through the reduction of their gene expression to prevent inflammatory diseases [35]. GA is potent antioxidant that has been reported to decrease p65-NF-κB expression in LPS-induced RAW 264.7 cells [19] and DSS-induced mice.

NOS activity is increased in active UC and CD patients and correlates with disease activity [36,37]. NOS are induced in the inflamed human colonic epithelium and are associated with the formation of peroxynitrite and the nitration of cellular proteins [38]. A high activity of inducible NOS contributes to early-onset IBD, which may contribute to colon carcinogenesis [39,40]. On the other hand, COX-2 is an inducible enzyme, which is present at low concentrations in healthy tissues but is up-regulated in response to tissue damage during inflammatory conditions. COX-2 is known to produce prostaglandins (PGs) from arachidonic acid that is associated with the mediation of inflammation. Furthermore, it has been reported that the expression levels of COX-2...
and PGE$_2$ are elevated in the inflamed mucosal tissues of patients with UC [41,42]. We observed that the expression levels of iNOS and COX-2 (Fig. 7) were increased after exposure to DSS. We also noticed that GA prevented the degradation of the inhibitory protein I$\kappa$B-$\alpha$, which inhibited the activation of the nuclear transcription factor NF-$\kappa$B and subsequently reduced the expression of both iNOS and COX-2 (Fig. 7).

The roles of the IL-6/STAT-3 signaling pathway in the etiology of IBD have been reported previously [13,43]. It has also been demonstrated that IL-6 enhances both the initiation and progression of CAC and that STAT3 activation downstream of IL-6 has an essential role in intestinal mucosal regeneration after injury and in the development of CAC [44, 45]. Expression of TNF-$\alpha$ and IL-6 and activation of NF-$\kappa$B and STAT3 are also increased in patients with UC and in those who have progressed to colorectal cancer [4,46]. In the present study, we observed an increased expression/translocation of p-STAT3$^{Y705}$ in DSS-induced mice (Fig. 8A and C). Han et al. [47] found that STAT-3 activation promotes chronic inflammation and epithelial proliferation in murine colitis. STAT3 activation may play an important role in the inflammatory process of TNBS-induced colitis, and inhibiting STAT3 activation during the early phase of the inflammatory response may have a beneficial effect on colitis [48]. We observed a decrease in the expression of STAT3 and its downstream targets GA-treated mice (Fig. 8). Natural product-derived compounds have been shown to have protective effects by inhibiting activated STAT3 [29,49].

To summarize, it is possible that one of the underlying mechanisms in the gallic acid anti-inflammatory effect in the present mouse model of ulcerative colitis involves a reduction of the neutrophilic infiltration in the colon accompanied by a decreased expression of CD68$^+$. In addition, there was a decrease in the expression of the pro-inflammatory proteins iNOS and COX-2 by inhibiting p65-NF-$\kappa$B-mediated transcriptional activation and preventing the expressions of p-STAT3$^{Y705}$. Therefore, we propose that gallic acid may be useful in the treatment of ulcerative colitis.

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References


