The anti-inflammatory effect and potential mechanism of cardamonin in DSS-induced colitis

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CARDAMONIN AMELIORATES DSS-INDUCED COLITIS

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Abstract

Cardamonin is a naturally occurring chalcone with strong anti-inflammatory activity. However, the direct effect of cardamonin on intestinal inflammation remains elusive.

In the current study, we found that cardamonin markedly ameliorated dextran sulfate sodium (DSS)-induced mouse body weight loss, diarrhea, colon shortening, spleen swelling and histological damage, which correlated with a decline in the activity of myeloperoxidase (MPO) and the production of nitric oxide (NO), tumor necrosis factor (TNF)-α and interleukin (IL)-6 in the colon. The upregulation of toll-like receptor 4 (TLR4) after DSS treatment was associated with an increase in the activation of myeloid differentiation factor 88 (MyD88), IL-1 receptor-associated kinase (IRAK)-1, nuclear factor kappa B (NF-κB) p65, inhibitor kappa B-α (IκBα), and IκB kinase (IKK)-α/β as well as the mitogen-activated protein kinase (MAPK) molecules of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK), and this upregulation was reversed by cardamonin administration. Moreover, cardamonin blocked the nuclear translocation of NF-κB p65, inhibited NF-κB-luciferase activity, and downregulated NF-κB target genes expression. The current study clearly demonstrates a beneficial effect of cardamonin on experimental inflammatory bowel disease (IBD) via a mechanism associated with suppression of TLR4 expression and inactivation of NF-κB and MAPK pathways. This study may give insight into the further evaluation of the therapeutic potential of cardamonin or its derivatives for human IBD.

Key words: Experimental colitis; TLR4; NF-κB; MAPK; Cardamonin
**Abbreviations:**

CD, Crohn’s disease; CDN, cardamonin; cNOS, constitutive nitric oxide (NO) synthase (cNOS); COX-2, cyclooxygenase 2; DSS, dextran sodium sulfate; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; HPLC, high pressure liquid chromatography; IBD, inflammatory bowel disease; ICAM-1, intercellular adhesion molecule-1; iNOS, inducible NO synthase; IFN-γ, interferon-γ; IL-1/6/10/15, interleukin-1/6/10/15; inhibitor kappa B-α, IκBα; IKK, IκB kinase; IRAK1/4, interleukin-1 receptor-associated kinase 1/4; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemotactic protein-1; MPO, myeloperoxidase; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor kappa B; PAMPs, pathogen-associated molecular patterns; qPCR, quantitative polymerase chain reaction; siRNA, small interfering RNA; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor-α; TRAF-6, TNF receptor-associated factor-6; UC, ulcerative colitis.
INTRODUCTION

INFLAMMATORY BOWEL DISEASES (IBD), including ulcerative colitis (UC) and Crohn’s disease (CD), are chronic and inflammatory conditions of the gastrointestinal tract. Current medical treatments for IBD generally include aminosalicylates, corticosteroids, immunomodulators, antibiotics and biologic therapies (e.g., monoclonal antibodies) (7). The severity of inflammation in patients with IBD correlates with the risk of colorectal cancer (25). Despite optimal medical therapy, about one-third of patients with UC and two-thirds of those with CD eventually require surgery at certain point during the course of the treatments (11). Therefore, new agents, especially those with limited side-effects and clear molecular mechanisms have been intensively investigated in recent years (26,27).

Toll-like receptor 4 (TLR4), which is abundantly expressed in intestinal epithelial cells and recognizes pathogen-associated molecular patterns (PAMPs), is thought to play a crucial role in gut innate immunity and is implicated in human IBD pathogenesis (16). TLR4 acts through a down-stream regulator, myeloid differentiation factor 88 (MyD88), which initiates a signal cascade leading to the nuclear translocation of transcription factor kappa-B (NF-κB) and/or activation of the mitogen-activated protein kinases (MAPKs) (4). Ultimately, the TLR4 signaling pathway regulates gene expression profiles including the production of pro-inflammatory cytokines and chemokines. TLR4 is strongly upregulated in patients with IBD, as well as in mice with dextran sulfate sodium (DSS)-induced colitis, implying that changes in TLR4 expression and subsequent alterations in the innate
immune response may contribute to the pathogenesis of IBD (14,16).

Chalcones are naturally occurring compounds belonging to the flavonoid family and exhibiting a broad spectrum of biological activities including anti-cancer, anti-inflammatory, anti-oxidative, anti-viral, anti-fungal and anti-allergic activities (12). Cardamonin (2',4'-dihydroxy-6'-methoxychalcone) is a chalcone mainly existed in the seeds of *Alpinia Katsumadai Hayata*, a plant that has been widely used in oriental medicine to treat emesis and gastric disorders (20). Cardamonin has strong anti-inflammatory, anti-cancer, anti-oxidative, vasorelaxant and hypoglycemic activities (10). However, the anti-inflammatory effects of cardamonin and the mechanisms responsible for its effect on intestinal inflammation have not been investigated. In this study, for the first time, we showed that cardamonin exerted potent anti-inflammatory properties by decreasing the expression of TLR4 and blocking the activation of NF-κB and MAPKs signaling pathways.

**MATERIALS AND METHODS**

*Cell lines and reagents.* Human colon adenocarcinoma cell lines HT-29 and LS174T and mouse macrophage cell line RAW264.7 were obtained from the American Type Culture Collection (Manassas, VA). All cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and a mixture of antibiotics (100 units ml-1 penicillin and 100 μg/ml streptomycin) under 5% CO₂ at 37°C. Cardamonin (HPLC purity ≥ 98%) was kindly provided by the Shanghai R&D Center for the Standardization of Traditional Chinese Medicine.
DSS (MW, 36-50KDa) was acquired from MP Bioc hemical LLC (Solon, OH). The NF-κB reporter vector pGL4.32[luc2P/NF-κB-RE/Hygro], the Dual-Luciferase reporter assay system, and 1× Passive Lysis Buffer were form Promega (Medison, WI). Human TLR4 expression plasmid pcDNA3-TLR4-YFP was from Addgene (Cambridge, MA). PCR Master Mix (2X) was from Thermo Scientific (Waltham, MA). Antibodies for iNOS (#13120), p-IKK-α/β (#2697); NF-κB p65 (#8242), p-p65 (#3033), IκBα (#4814), p-IκBα (#2859), ERK1/2 (#4348), p-ERK1/2 (#4377), JNK (#9255), p-JNK (#9252) and β-actin (#4970) were obtained from Cell Signaling Technology (Danvers, MA). Antibodies for cNOS (AP11828a), MyD88 (AP8521C), p-IRAK1 (AP50215), IRAK1 (AP14440C), and IRAK4 (AP12875A) were obtained from Abgent (San Diego, CA). Antibody for TLR4 (#G0913) was from Santa Cruz (Santa Cruz, CA). Alexa Fluor 488-conjugated secondary antibody (A21206), Triton X-100, Trizol, DAPI and the SuperScript II Reverse Transcriptase kit were from Invitrogen (Carlsbad, CA). SYBR Premix ExTaq Mix was from Takara Biotechnology (Shiga, Japan). Protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Mouse TNF-α, IL-6 and NO ELISA kits were from R&D Systems (Minneapolis, MN). The MPO activity assay kit was from CytoStore (Calgary, AB, Canada). Donkey serum, LPS, DEPC water, formalin, paraformaldehyde, Tween-20, ethanol, DMSO, and Bicinchoninic Acid (BCA) kit were from Sigma-Aldrich (St Louis, MO). The enhanced chemiluminescence (ECL) detection kit was from Millipore (Billerica, MA).
Animals. Animal studies were conducted in accordance with the ethical guidelines approved by the Animals Ethics Committee of Shanghai University of Chinese Medicine (SHUTCM). Healthy female C57BL/6 mice at 8 weeks of age (body weight, 20 ± 2 g) were obtained from the Shanghai Laboratory Animal Center (Shanghai, China). All mice were acclimated one week after arrival and were maintained in a specific pathogen-free environment. Mice were kept five mice per cage with water and food ad libitum in a climate-controlled environment set to 25 ± 2°C and on a 12-h light/dark cycle.

Induction and assessment of colitis. DSS colitis was induced in female C57-BL/6 mice as described previously (6,27). Animals were randomly divided into the following groups (n=10 per group): vehicle group, cardamonin (50 mg/kg of body weight) group, DSS group, and DSS + cardamonin (20, 50 and 100 mg/kg of body weight) group. The vehicle group was administered 100 μl 0.5% methylcellulose by oral gavage once per day. The DSS group received 4% DSS in drinking water for seven consecutive days. For the cardamonin group and the DSS + cardamonin group, drugs were administered by oral gavage for two days prior to DSS treatment and continued to the end of the DSS treatment (Fig. 1A, upper panel). Mice were assessed daily for the development of colitis based on body weight, diarrhea, and bloody stool. After sacrifice under anesthesia, the spleen weight and the colon length were measured. The colon was opened longitudinally, flushed with ice-cold saline, fixed in 10% neutral formalin. The tissue samples were then embedded in paraffin wax,
stained with hematoxylin and eosin (H&E), and assessed under microscopy. Tissue
damage was scored as described previously (6,27).

**Immunoblotting analysis.** Total proteins extracted from colon tissues and cultured
cells were lysed and homogenized in radioimmunoprecipitation assay (RIPA) buffer
(50 mmol/L Tris-HCl, pH 7.4, 1% Nonidet P-40, 150 mmol/L NaCl, 0.5% sodium
dehydrate, 0.1% SDS, 1 mmol/L EDTA) supplemented with protease inhibitor
cocktail. Cell lysates were centrifuged at 10,000 g for 15 min at 4°C, the protein
content in the supernatants was measured using the BCA kit. 10-30 μg of protein was
separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. After
blocking with 5% skim milk, the membranes were incubated with antibodies against
TLR4, iNOS, cNOS, p-IKK-α/β, p65, p-p65, IκBα, p-IκBα, ERK1/2, p-ERK1/2, JNK,
p-JNK, MyD88, p-IRAK1, IRAK1, IRAK4 and β-actin, respectively. The membranes
were then incubated with the specific secondary antibodies conjugated to horseradish
peroxidase (HRP). The blots were developed using an ECL detection kit. The protein
bands were analyzed using a GS-700 imaging densitometer (Bio-Rad, CA) and
quantified following normalization to the expression of the housekeeping protein
β-actin.

**RNA analysis.** Total RNA were extracted from cells and colon tissues using
TRIzol reagent. Polymerase chain reaction (PCR) was performed using cDNA
generated from 1 μg total RNA with the SuperScript III Reverse Transcriptase kit.
The primer sequences used in the semi-quantitative (semi-q) PCR amplification are as follows:

5'-GGGAATCTTGGAGCGAGTTG-3'/5'-GTGAGGGCTTGGCTGAGTGA-3' for iNOS,
5'-AAGTTGACCCGTAATCTGA-3'/5'-TGAAAGGGAATACCATAACA-3' for MCP-1,
5'-GAAGTCTTTGCTGTGCT-3'/5'-GCTCCTGCTTGAGTATGTC-3' for COX-2,
5'-ACCACGGCCTCTCCTA-3'/5'-CATTTCCACGATTTCCAGA-3' for IL-6,
5'-CAGAATGGGAGGTGGTAGTGC-3'/5'-CAGAATGGGAGGTGGTAGTGC-3' for IL-15,
5'-AAGTGGCATAGATGTGGAAG-3'/5'-AAGTGGCATAGATGTGGAAG-3' for IFNγ,
5'-CGTGGAACTGGCAGAAGGG-3'/5'-AGACAGAAGAGCGTGGTAGTGC-3' for TNF-α,
5'-TGCTGTCCCTGTATGCCTCT-3'/5'-TGCTGTCCCTGTATGCCTCT-3' for β-actin. The DNA thermal cycle conditions were 94°C for 5 min (pre-denature), and 35 cycles of 94°C for 30 s, annealing at 56°C (MCP-1, IFNγ, IL-6 and β-actin), 58°C (iNOS, COX-2, and TNF-α), or 60°C (IL-15) for 30 s and extension at 72°C for 45 s, followed by a final extension of 72°C for 2 min. The PCR products were separated by electrophoresis on 2% agarose gels and stained with ethidium bromide. The amount
of the target gene was normalized to the internal control, β-actin. The primer sequences used in the qPCR amplification are as follows: 5’-GGGAATCTTTGGAGCGAGTTG-3’/5’-GTGAGGGCTTGAGCTGAGTGA-3’ for iNOS, 5’-CGCTGTGCTTTGAGAAGCTGT-3’/5’-AGGTCCCTTGCCCTACTTGCTG-3’ for ICAM-1, 5’-GAAGTCTTTGGTCTGGTGCCT-3’/5’-GCTCCTGCTTGAGATGTGCG-3’ for COX-2, 5’-CGTGGAAGCTGGCAGAAGG-3’/5’-AGACAGAAAGGCAGCTGGTGGC-3’ for TNF-α, 5’-ACCACGGCCTCCCTACTTC-3’/5’-CATTCCACGATTGCCAAGA-3’ for IL-6, and 5’-CAGCCTTCTTCTTGGTAT-3’/5’-TGCCATAGAGGTCTTTACGG-3’ for β-actin. PCR reactions were carried out using SYBR Premix ExTaQ Mix in an ABI Prism 7900 real-time PCR System (Life Technologies, Carlsbad, CA). The thermal cycle parameters were as follows: 1 cycle of 95°C for 30 s and 40 cycles of denaturation (95°C, 5 s) and combined annealing/extension (60°C, 30 s). Gene expression changes were calculated by the comparative Ct method, and the values were normalized to the internal control, β-actin.

**Immunofluorescence cytochemistry.** RAW264.7 cells were seeded on sterile coverslips with a density of 5 × 10⁴/well in a 24-well plate for 24 hours until cell attachment. The cells were pretreated with cardamonin (0, 25 μM) for 2 h following stimulation with LPS (2 μg/ml) for 12 h. The cells were fixed with 4% (w/v)
paraformaldehyde solution at 20°C for 10 min, and permeabilized with 0.3 % (w/v) Triton X-100 for 20 min. After incubation in a blocking buffer of 0.1% (w/v) Triton X-100, 1% (w/v) BSA, and 3% (w/v) donkey serum, the cells were then stained with NF-κB p65 antibody overnight at 4°C, and then incubated with Alexa Fluor 488-conjugated secondary antibody at 20°C for 1 h in the dark. To stain the nuclei, 1 µg/ml of DAPI in PBS was added. Fluorescence photographs were obtained using a fluorescence microscope (Olympus CKX41, Tokyo, Japan).

Transient transfection reporter gene assay. Using a Lonza Nucleofector II instrument (Amaxa Biosystems, Germany) as described previously (8), 1×10^6 HT-29 cells were electroporated with pGL4.32[luc2P/NF-κB-RE/Hygro] reporter vector. The cells were then seeded at a density of 5×10^4 cells in a 24-well plate. After overnight incubation, the cells were treated with cardamonin (0, 5, 10 and 25 µM) for 2 h followed by an additional treatment with or without LPS (2 µg/ml) for 12 h. Cell-free lysates in 1× Passive Lysis Buffer were obtained by centrifugation at 10,000 g for 2 min at 4°C. The effects of cardamonin on the activation of the LPS-stimulated NF-κB promoter were quantified using a luciferase assay system on a Glomax 20/20 Luminometer (Promega Corp., Madison, WI). The results were presented in relative light units (RLU), and the data were expressed as fold induction of control cells.

Cytokine bioassay. The colon tissues were homogenized using IKA T25 Digital Ultra-Turrax (Germany) in ice-cold saline and the supernatants were harvested for NO,
TNF-α and IL-6 measurement after centrifugation at 3,000 g for 10 min at 4°C. The level of each pro-inflammatory mediator in the supernatant was determined using an ELISA kit according to the manufacturer’s protocol, and the protein content was assayed by a BCA kit.

Myeloperoxidase (MPO) assay. Neutrophil infiltration into inflamed colon mucosa was quantified by MPO activity assessment as described previously (7). MPO activity in colon tissues was measured using a detection kit according to the manufacturer’s instructions. The results were expressed as activity units/mg of protein. The protein content was assayed by a BCA kit.

Statistical analysis. Data were expressed as the mean ± standard error (SE) and analyzed with Prism 5.0v software (GraphPad, La Jolla, CA). Differences among groups were compared using one-way analysis of variance (ANOVA) and multiple comparisons were performed by a post-hoc test with Bonferroni correction. A value of p < 0.05 was considered statistically significant.

RESULTS

Cardamonin alleviated DSS-induced experimental colitis. Mice administered either vehicle or cardamonin alone did not exhibit weight loss, diarrhea, colon shrinking, spleen swelling or mucosal damage in colon (Fig. 1 and Fig. 2). DSS administration dramatically decreased body weight (Fig. 1A, lower panel). Cardamonin (20-100 mg/kg) suppressed DSS-induced body weight loss. From day 4 to day 7, the mice of
the DSS group experienced bloody diarrhea (Fig. 1B). In contrast, the mice receiving both DSS and cardamonin (20-100 mg/kg) exhibited fewer diarrhea symptoms. The swelling of the spleen and the shrinking of the colon have been identified as the major signs of DSS-induced colitis (3). As expected, there was significant shortening of the colon (Fig. 1C and D) and swelling of the spleen (Fig. 2C) when assessed on day 7 of DSS treatment. Cardamonin (50 mg/kg or 100 mg/kg, but not 20 mg/kg) significantly abrogated colon length reduction and spleen swelling. In addition, DSS-exposed mice exhibited epithelial destruction, inflammatory cell infiltration with lymphoid aggregation, and submucosal edema (Fig. 2A). Cardamonin (50 mg/kg) treatment resulted in a significant reduction in these disease symptoms. However, by contrast, treatment with cardamonin (20 mg/kg or 100 mg/kg) could not reduce the inflammation score as effectively as mice dosed at 50 mg/kg (Fig. 2B). Based on these results, the dose of 50 mg/kg of cardamonin was chosen for subsequent experiments to investigate the anti-inflammatory mechanisms.

Cardamonin reduced the expression of NOS and the production of NO in the colon.

NO is produced by different NOS isoforms, constitutive NOS (cNOS) and inducible NOS (iNOS). The overproduction of NO by iNOS and the downregulation of cNOS have been observed in experimental colitis (23). Accordingly, we found that the iNOS protein expression increased, whereas the cNOS protein expression decreased in DSS-treated mice compared with the vehicle-treated group (Fig. 3A and B). The oral administration of cardamonin markedly decreased the iNOS protein expression while
increasing the cNOS protein expression. In addition, the DSS group showed a surge in NO production in the colon (Fig. 3C). Cardamonin treatment reduced NO production in DSS-treated mice.

Cardamonin decreased the activity of MPO and the production of cytokines in the colon. MPO is an enzyme produced mainly by polymorphonuclear leukocytes, and its activity in the colon is linearly related to neutrophil infiltration (27). MPO activity levels were low in the colon of vehicle-treated mice and markedly increased in mice with DSS-induced colitis (Table 1). The increased MPO activity in mice with DSS-induced colitis was significantly reduced after the administration of cardamonin. To further characterize the extent of inflammation in DSS-treated mice, the levels of cytokines in the colon were measured. The DSS group showed a significant elevation of the levels of TNF-α and IL-6 in the colon compared with the vehicle-treated group (Table 1). Cardamonin treatment remarkably decreased DSS-induced upregulation of the levels of TNF-α and IL-6 in the colon.

Cardamonin inhibited the activation of NF-κB in vivo and in vitro. NF-κB is the central transcription factor in the regulation of pro-inflammatory cytokines and chemokines. It has been clearly established that the activation of NF-κB is a crucial step in the development of experimental colitis (8). NF-κB immunoblotting was performed to assess the functional status of NF-κB activation in colon tissues. The results showed that DSS-induced phosphorylation and degradation of IκBα and
phosphorylation of NF-κB p65 were blocked by cardamonin (Fig. 4A & B). Because IKK is the kinase that causes the phosphorylation of IκBα, we next determined the effects of cardamonin on the phosphorylation (activation) of IKK. The data showed that the DSS-induced upregulation of the phosphorylation of IKK-α/β was inhibited by cardamonin (Fig. 4A & B). Consistent with the in vivo data, the nuclear translocation of NF-κB p65 in LPS-stimulated RAW264.7 cells was blocked by cardamonin treatment (Fig. 5A). On the other hand, induction of NF-κB-mediated luciferase activity in LPS-stimulated human colorectal adenocarcinoma HT-29 cells was inhibited by cardamonin treatment in a dose-dependent manner (Fig. 5B).

Cardamonin downregulated NF-κB target genes expression in vivo and in vitro. To further elucidate the impact of cardamonin on NF-κB signaling, we investigated the expression levels of representative downstream signaling genes involved in NF-κB activation. qPCR analyses of several NF-κB target genes in the colon were performed. The results showed that mRNA expression of iNOS, ICAM-1, COX-2, TNF-α and IL-6 was remarkably induced in the inflamed colons of mice with DSS administration (Fig. 6A). In contrast, the increase in these inflammatory genes following DSS treatment was significantly decreased in mice exposed to cardamonin. In concert with the in vivo data, results from semi-qPCR analysis showed that the relative increase in the mRNA expression of iNOS, COX-2, MCP-1, IFNγ, TNF-α, IL-6 and IL-15 following LPS stimulation dramatically decreased in RAW264.7 cells exposed to cardamonin (Fig. 6B).
Cardamonin decreased the activation of TLR4 signaling molecules in vivo and in vitro. The TLR4 signaling pathway, which is associated with the activation of NF-κB via MyD88, is thought to be involved in the pathogenesis of IBD (15,19). We determined the effects of cardamonin on the activation of TLR4 and MyD88 by immunoblotting analysis. The protein expression of TLR4 and MyD88 in mice after DSS treatment was upregulated compared with in normal control mice (Fig. 7). However, the relative increase in the expression of TLR4 and MyD88 after DSS treatment was significantly downregulated in mice subjected to cardamonin administration. Several previous studies have shown that the activation of IRAK-1 and IRAK-4 plays a central role in TLR-mediated signaling, and increased levels of phosphorylated IRAK-1 and IRAK-4 result in the activation of NF-κB (9). We then tested the ability of cardamonin to activate IRAK-1 and IRAK-4. The results showed that treatment with DSS increased the phosphorylation of IRAK-1 and degradation of IRAK-1 and IRAK-4 (Fig. 7). Cardamonin significantly inhibited DSS-induced phosphorylation and degradation of IRAK-1, but it did not suppress IRAK-4 degradation. The in vitro data performed in LPS-stimulated RAW264.7 cells confirmed the in vivo results (Fig. 8).

Cardamonin suppressed MAPKs phosphorylation in vivo and in vitro. Since MAPK signaling molecules play a critical role in regulating the TLR4-mediated inflammatory process, we analyzed the phosphorylation levels of MAPKs in DSS-treated mice by...
immunobloting. As depicted in Figure 9, DSS induced strong phosphorylation (activation) of ERK1/2 and JNK in the inflamed colon. Cardamonin treatment significantly attenuated the phosphorylation levels of ERK1/2 and JNK induced by DSS.

DISCUSSION

Conventional therapies for IBD are usually associated with complications or side effects. Novel therapies, including TNF-neutralizing antibodies, have demonstrated efficacy in improving mucosal healing, but their side effects and toxicity remain major clinical concerns (21). Therefore, the development of medicinal plants or their active components with minimum adverse reactions is becoming an increasingly attractive approach in the management of IBD patients (26,27). Chalcones belong to the flavonoid family, which possess a variety of biological and pharmacological properties, including antioxidant, cytotoxic, anti-cancer, anti-microbial, anti/protozoal, anti-ulcer, anti-histaminic and anti-inflammatory activities (24). Cardamonin is a chalcone mainly found in Alpinia Katsumadai. Several previous studies have shown that cardamonin has potent anti-inflammatory activities by blocking NF-κB signaling (17). However, so far, there have been no reports on the effects of cardamonin on IBD. Using a DSS-induced colitis mouse model, the clinical manifestations of which resembling human UC, we were able to demonstrate that cardamonin could ameliorate the disease hallmarks such as body weight loss, bloody diarrhea, colon shortening, spleen swelling and histological injury. Notably, none of the mice that
received cardamonin alone exhibited apparent clinical lesions or mucosal damage throughout the study, indicating the relative safety of cardamonin management.

Since its discovery by Dr. David Baltimore’s research group in 1986, NF-κB has been widely viewed as a therapeutic target for various inflammatory conditions including IBD (18). Excess or inappropriate activation of NF-κB has been observed in IBD patients and experimental IBD animal models (2). It has been demonstrated that the blockade of NF-κB activation with flavanoid agents might be an effective strategy in abrogating experimental IBD (6,26). The most abundant form of NF-κB in cells is the p50/p65 heterodimer, which is normally sequestered in the cytoplasm by binding to the inhibitory protein IκBα. Following various stimuli, IκBα is rapidly phosphorylated by IκB kinase IKK, ubiquitinated and subsequently degraded, allowing NF-κB p50/p65 to translocate to the nucleus, where it drives the expression of target genes. In our study, we found that cardamonin inhibited the phosphorylation and degradation of IκBα and the phosphorylation of NF-κB p65 and IKKa/β in the colon mucosa, blocked NF-κB p65 nuclear translocation in mouse macrophage cells, and decreased NF-κB-luciferase activity in human colorectal adenocarcinoma cells. Furthermore, the downregulation in the expression of NF-κB target genes (iNOS, ICAM-1, COX-2, TNF-α and IL-6), the activity of MPO, the accumulation of TNF-α and IL-6, the expression of iNOS and the release of NO after DSS treatment was inhibited by cardamonin administration. We hypothesize that cardamonin inhibits these cytokines and chemokines through downregulation of NF-κB activation. But further studies assessing the direct engagement of NF-κB signaling components in the
anti-inflammatory effects of cardamonin are warranted. These findings indicate that cardamonin has potential anti-inflammatory effects in DSS-induced colitis via NF-κB signaling suppression.

Among the upstream regulators of NF-κB activation, TLR4 is indicated to play a major part in regulating NF-κB activation (1). TLR4 is normally expressed at low levels in the intestinal mucosa, while it is strongly upregulated in IBD patients as well as in DSS-induced colitis mice, implying its role in the pathogenesis of IBD (16). Activation of TLR4 needs adaptor protein MyD88, which initiates a downstream signal cascade including the activation of IRAK-1, IRAK-2, IRAK-4, and TNF receptor-associated factor (TRAF)-6 (12). The activated IRAK-1 phosphorylates IKK, which leads to the translocation of NF-κB and/or the activation of MAPKs (5). Ultimately, the TLR4 signaling pathway regulates the transcription of inflammatory cytokine genes (16). In our study, cardamonin significantly reversed the upregulation of TLR4 and MyD88 in the inflamed colons as well as in LPS-stimulated RAW264.7 cells. The DSS or LPS-induced phosphorylation and degradation of IRAK-1, but not IRAK-4, were also inhibited by cardamonin treatment. Further studies revealed that DSS induced a strong phosphorylation of ERK1/2 and JNK in the inflamed colon, but cardamonin treatment markedly inhibited the increase of these phosphorylation levels. These results suggest that cardamonin appears to exert its effect in ameliorating DSS-induced colitis via decreasing the expression of TLR4 and blocking the activation of NF-κB and MAPK signaling pathways.

Interestingly, cardamonin has been shown to be a potential anti-tumor agent against
colon cancer by suppressing the proliferation of cancer cells (22). Thus, the anti-tumor properties of cardamonin increase its promising medicinal value for patients with long-standing UC because the most important clinical issue for patients with longstanding UC is an increased risk for the development of colon cancer (25). Taken together, these in vitro and in vivo results provide the first evidence that the beneficial effects of cardamonin in DSS-induced colitis might be associated with suppression of TLR4 expression and inactivation of NF-κB and MAPK pathways. These novel findings may contribute to the effective utilization of cardamonin or its derivatives in the treatment of human IBD or related complications.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.
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Figure legends.

Fig. 1. Effects of cardamonin on DSS-induced colitis in mice. (A) Experimental protocol for DSS-induced colitis model (upper panel) and effect of cardamonin on body weight changes of mice (lower panel). Data were plotted as percentage of the basal body weight. (B) The occurrence of bloody diarrhea. Data were plotted as percentage of total mice that had bloody diarrhea on different days of DSS treatment. (C) A representative view of the colon morphology at the end of DSS treatment. (D) Colon length was measured at the end of DSS treatment. Values were expressed as mean ± SD (n = 10). * p < 0.05 vs. DSS-treated group.

Fig. 2. Effects of cardamonin on DSS-induced colitis in mice. (A) Representative H&E-stained colon sections (Magnification ×200). (B) Histological score. (C) Spleen weight was measured at the end of DSS treatment. Values were expressed as mean ± SD (n = 10). * p < 0.05, ** p < 0.01 vs. DSS-treated group.

Fig. 3. Effects of cardamonin on the expression of NOS and the production of NO in the colon. (A) Mice were sacrificed after 7 days of 4% DSS exposure, and the total protein from colon tissues was subjected to SDS-PAGE. Immunoblotting was performed with antibodies against iNOS (1:1000) and cNOS (1:1000). One representative experiment from three independent experiments is shown. (B) Quantification of the protein expression was performed by densitometric analysis of the blots. (C) Colon segments were homogenized and the supernatants were assayed
for the determination of the level of NO as described in the Methods. Values were expressed as mean ± SD (n=6). * p < 0.05 vs. DSS-treated group.

**Fig. 4.** Effects of cardamonin on the activation of NF-κB *in vivo*. (A) Protein samples from colon tissues was subjected to SDS-PAGE, and immunoblotting was performed with antibodies against p-p65 (1:1000), p-ΙκBα (1:1000), ΙκBα (1:1000) and p-ΙΚΚ-α/β (1:1000). One representative experiment from three independent experiments is shown. (B) Quantification of the protein expression was performed by densitometric analysis of the blots. Data were expressed as mean ± SD (n=9). *P < 0.05, **P < 0.01 vs. DSS-treated group.

**Fig. 5.** Effects of cardamonin on the activation of NF-κB *in vitro*. (A) RAW264.7 cells were treated as described in the Methods and NF-κB p65 localization was visualized under a fluorescence microscope (magnification×200). (B) HT-29 cells were transiently transfected with pGL4.32[luc2P/NF-κB-RE/Hygro]. Cells were treated with cardamonin (0, 5, 10 and 25 µM) for 2 h followed by an additional treatment with or without LPS (2 µg/ml) for 12 hours. Cells were lysed and the lysates were analyzed using a luciferase assay system. NF-κB promoter-driven luciferase activity was expressed as fold values of control cells. Data were expressed as mean ± SD of quadruplicates of two independent experiments. *P < 0.05, **P < 0.01 vs. LPS-treated cells.
**Fig. 6.** Effect of cardamonin on the expression of NF-κB target genes. (A) mRNA expression was determined by reverse transcription qPCR in colon samples isolated from mice. Expression was normalized to β-actin (B) RAW264.7 cells were treated with cardamonin (0, 25 μM) for 2 h prior to LPS (2 µg/ml) treatment for an additional 12 h. RNA was extracted as described, and the mRNA expression of NF-κB target genes was carried out by semi-qPCR (upper panel). The expression was normalized to β-actin and quantification of the mRNA expression was performed by densitometric analysis of the bands (lower panel). Data were expressed as the mean ± SD (n=8). *P < 0.05, **P < 0.01, ***P < 0.001 vs. DSS/LPS-treated samples.

**Fig. 7.** Effects of cardamonin on the activation of TLR4 signaling molecules *in vivo*. (A) Mice were sacrificed after 7 days of 4% DSS exposure, and total protein from the colon tissues was subjected to SDS-PAGE. Immunoblotting was performed with antibodies against TLR4 (1:1000), MyD88 (1:1000), IRAK-1 (1:1000), p-IRAK-1 (1:1000), IRAK-4 (1:1000) and β-actin (1:2000). One representative experiment is shown. (B) Quantification of the protein expression was performed by densitometric analysis of the blots. Data were expressed as mean ± SD of triplicates of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 vs. DSS-treated group; ns (no significance).

**Fig. 8.** Effects of cardamonin on the activation of TLR4 signaling molecules *in vitro*. (A) RAW264.7 cells were treated with cardamonin (0, 25 μM) for 2 h followed by an
additional incubation with or without LPS (2 μg/ml) treatment for 12 h. Total protein was extracted, and immunoblotting was performed. One representative experiment is shown. (B) Quantification of the protein expression was performed by densitometric analysis of the blots. Data were expressed as mean ± SD (n=9). *P < 0.05, **P < 0.01 vs. LPS-treated group; ns (no significance)

**Fig. 9.** Effects of cardamonin on the activation of MAPK signaling molecules in the colon. (A) Mice were sacrificed after 7 days of 4% DSS exposure, and total protein from the colon tissues was subjected to SDS-PAGE. Immunoblotting was performed with antibodies against p-ERK1/2 (1:1000), ERK1/2 (1:1000), p-JNK (1:1000), JNK (1:1000), and β-actin (1:2000). One representative experiment from two independent experiments is shown. (B) The ratio of phosphorylated MAPKs to total MAPKs. Data were expressed as mean ± SD (n=9). ***P < 0.001 vs. DSS-treated group.
Figure 1

A

Dosing scheme

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<th>+DSS</th>
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Vehicle/Cardamonin

B

- DSS
- DSS 4%
- DSS+CDN 20mg/kg
- DSS+CDN 50mg/kg
- DSS+CDN 100mg/kg

Colon length (cm)

C

Vehicle
CDN (50)
CDN (0)
CDN (20)
CDN (50)
CDN (100)

D

Initial body weight %

Bloody diarrhea event %

Colon length (cm)
Figure 2

A

Vehicle                                          CDN (50 mg/kg)                                          DSS 4%

DSS + CDN (20 mg/kg)                     DSS + CDN (50 mg/kg)                      DSS + CDN (100 mg/kg)

B

C

Histological score

Spleen (mg)

Vehicle, CDN, 0, 20, 50, 100

DSS 4% + CDN (mg/kg)

Vehicle, CDN, 0, 20, 50, 100

DSS 4% + CDN (mg/kg)
Figure 3

A

Relative protein expression

B

C

NO₂ release
Figure 4

A

<table>
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<tr>
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</table>

IkBa

p-IkBa

p-p65

p-IKK α/β

Actin

B

Relative protein expression

Vehicle
CDN
DSS
DSS+CDN

lkBa
p-IkBa
p-p65
p-IKK α/β
**Figure 5**

A

![Immunofluorescence images of RAW264.7 cells showing DAPI, NF-KB p65, and Merge under different treatments: Vehicle, LPS, and LPS+CDN.](image)

B

![Graph showing luciferase activity of HT-29 cells under LPS and CDN treatments.](image)
Figure 7

A

Colon

<table>
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</table>

TLR4

MyD88

IRAK1

p-IRAK1

IRAK4

Actin

B

Relative protein expression

Vehicle  CDN  DSS  DSS+CDN

- ** ***
- * ***
- ns
A

RAW264.7

<table>
<thead>
<tr>
<th></th>
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TLR4

MyD88

IRAK1

p-IRAK1

IRAK4

Actin

B

- Vehicle
- CDN
- LPS
- LPS+CDN

Relative protein expression

- TLR4
- MYD88
- IRAK1
- p-IRAK1
- IRAK4
Figure 9

A

<table>
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</table>

- p-Erk1/2
- Erk1/2
- p-JNK
- JNK
- Actin

B

- Vehicle
- CDN
- DSS
- DSS+CDN

Relative protein expression
Table 1. Effects of cardamonin on MPO activity and the levels of TNF-α and IL-6 in DSS-induced colitis mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>TNF-α (pg/mg pr.)</th>
<th>IL-6 (pg/mg pr.)</th>
<th>MPO (U/mg pr.)</th>
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<tbody>
<tr>
<td>Vehicle</td>
<td>14.6 ± 1.7</td>
<td>28.9 ± 2.3</td>
<td>3.7 ± 0.6</td>
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<tr>
<td>Cardamonin</td>
<td>15.8 ± 0.2</td>
<td>35.7 ± 1.5</td>
<td>4.9 ± 0.3</td>
</tr>
<tr>
<td>DSS + Vehicle</td>
<td>127.4 ± 8.5</td>
<td>214.3 ± 17.9</td>
<td>26.8 ± 2.2</td>
</tr>
<tr>
<td>DSS + Cardamonin</td>
<td>87.4 ± 4.9 *</td>
<td>148.1 ± 11.3 *</td>
<td>20.7 ± 1.4 *</td>
</tr>
</tbody>
</table>

Colon segments from mice (n = 6 per group) were excised and homogenized. The supernatants were assayed for the determination of the activity of MPO and the levels of TNF-α and IL-6 as described in the Methods. Values are expressed as mean ± SD of triplicates of two independent experiments. * p < 0.05 vs. DSS-treated group.