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Peracetylated (−)-Epigallocatechin-3-gallate (AcEGCG) Potently Suppresses Dextran Sulfate Sodium-Induced Colitis and Colon Tumorigenesis in Mice

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Supporting Information

ABSTRACT: Previous studies reported that peracetylated (-)-epigallocatechin-3-gallate (AcEGCG) has antiproliferative and anti-inflammatory activities. Here, we evaluated the chemopreventive effects and underlying molecular mechanisms of dietary administration of AcEGCG and EGCG in dextran sulfate sodium (DSS)-induced colitis in mice. The mice were fed a diet supplemented with either AcEGCG or EGCG prior to DSS induction. Our results indicated that AcEGCG administration was more effective than EGCG in preventing the shortening of colon length and the formation of aberrant crypt foci (ACF) and lymphoid nodules (LN) in mouse colon stimulated by DSS. Our study observes that AcEGCG treatment inhibited histone 3 lysine 9 (H3K9) acetylation but did not affect histone acetyltransferase (HAT) activity and acetyl- CREB-binding protein (CBP)/p300 levels. In addition, pretreatment with AcEGCG decreased the proinflammatory mediator levels by down-regulating of PI3K/Akt/NFκB phosphorylation and p65 acetylation. We also found that treatment with AcEGCG increased heme oxygenase-1(HO-1) expression via activation of extracellular signal-regulated protein kinase (ERK)1/2 signaling and acetylation of NF-E2-related factor 2 (Nrf2), thereby abating DSS-induced colitis. Moreover, dietary feeding with AcEGCG markedly reduced colitis-driven colon cancer in mice. Taken together, these results demonstrated for the first time the in vivo chemopreventive efficacy and molecular mechanisms of dietary AcEGCG against inflammatory bowel disease (IBD) and potentially colon cancer associated with colitis. These findings provide insight into the biological actions of AcEGCG and might establish a molecular basis for the development of new cancer chemopreventive agents.

KEYWORDS: *DSS, colitis, Nrf-2, NFκB, IBD*

INTRODUCTION

The major inflammatory bowel diseases (IBD) forms, Crohn's disease and ulcerative colitis (UC), are chronic relapsing inflammatory conditions associated with an increased risk of colon cancer.[1] The colitis models in mice also may be induced by dextran sulfate sodium (DSS) and trinitrobenzenesulfonic acid (TNBS), increased intestinal and extraintestinal clinical manifestations of disease, including weight loss, diarrhea with blood and/or mucus, fever, shortening of the colon, and inflammatory infiltrates.[2] Experimental colitis disease is characterized by upregulated inflammatory mediators, including reactive oxygen species (ROS), prostaglandin (PG), nuclear factor kappaB (NFκB), Toll-like receptor (TLRs), and pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), interleukin-1beta (IL-1β), and IL-6, resulting in tissue damage and neoplastic transformation.[3−7] Most of the current therapeutic strategies for IBD provide limited beneficial action and possess toxic side effects. For these reasons, alternative approaches have gained increasing attention, especially those derived from natural and functional foods. Therefore, IBD experimental animals could serve as a helpful tool to establish and validate a screening assay for bioactive dietary components that may prevent and provide therapy for colitis and colon cancer associated colitis.

Various genes and proteins are involved in the tumorigenesis and progression of colorectal cancer. Epigenetic alterations (histone acetylation/deacetylation) in multiple genes are believed to play a crucial role in inflammation. NFκB also plays an important role in the pathogenesis of chronic

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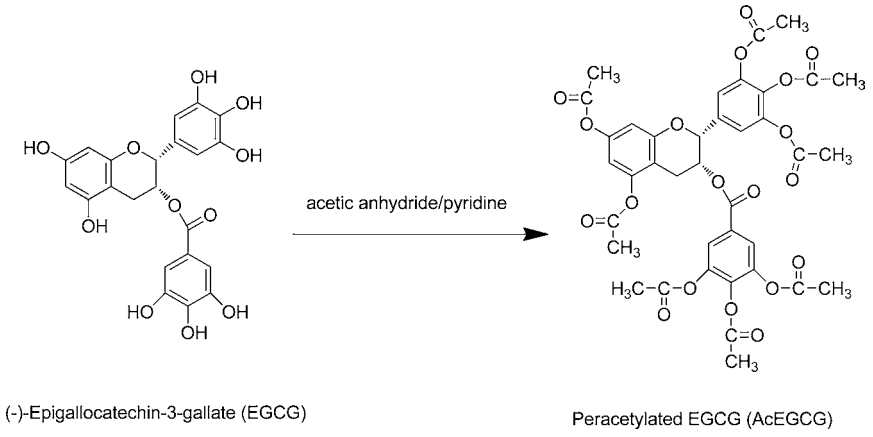

Figure 1. Synthesis of acetylated EGCG from EGCG.

inflammatory diseases and in IBD patients.[8,9] Multiple extracellular stimuli, including inflammatory cytokines can induce NFκB activation by promoting I-kappa-B-alpha (IκBα) phosphorylation and its proteasomal degradation.[10] For this purpose, more studies are needed to elucidate the mechanisms underlying epigenetic regulation of colorectal cancer.

Recently, many experimental studies with rodents have demonstrated that antioxidants reduce disease activity by preventing the activation or transcription of NFκB and inducible kinase (IKK) in the murine model of DSS-induced colitis.[11] Recent articles clearly showed that activation of NF-E2-related factor 2 (Nrf2)-antioxidant signaling could attenuate NFκB inflammatory response.[12] It has been reported that NFκB could directly repress Nrf2 signaling at the transcription level. NFκB competes against Nrf2 for transcription coactivator CREB binding protein (CBP).[13] Moreover, NFκB or Nrf2 activation and transcriptional activity are reciprocally regulated by acetylation and deacetylation, which are mediated by histone acetyltransferases (HATs) and histone deacetylases (HDACs).[14,15] However, definite evidence showing that Nrf2 can directly inhibit NF-κB signaling is still wanting. Therefore, it is important to further elucidate the cross-talk between Nrf2 and NFκB signaling pathways.

(−)-Epigallocatechin-3-gallate (EGCG) is an abundant and natural polyphenolic compound, often found in green tea. EGCG, however, is relatively unstable in alkaline or neutral solutions[16] and exhibits poor absorption and extensive biotransformation including glucuronidation, methylation, and sulfation in the colon,[17] which leads to a reduction in its bioavailability. Acylation is a very promising way to change the hydrophilicity of parent compounds and be hydrolyzed by esterases in the tissues and plasma to serve as prodrugs. To increase the bioavailability, stability, and cancer preventive activities of EGCG, peracetylated EGCG (AcEGCG) was synthesized (Figure 1).[18] EGCG (458 mg, 1 mmol) was reacted with acetic anhydride (510 μL, 50 mmol) in 20 mL of ethyl acetate catalyzed by pyridine (445 μL) overnight with stirring. This reaction was guided by high-performance liquid chromatography (HPLC) analysis. Until all the EGCG changed to peracetylated EGCG, the reaction was stopped by adding 20 mL of iced water, and the ethyl acetate layer was separated and washed by water four times, then dried using magnesium sulfate anhydrous, and evaporated to yield 730 mg of peracetylated EGCG. Previous studies have demonstrated that AcEGCG acts as a prodrug of EGCG and exhibits more potency than EGCG to inhibit proteasome activity, tumor proliferation, and inflammation, and to induce apoptosis in vitro and in vivo.[19,20] Recent research has demonstrated that EGCG and AcEGCG inhibit telomerase and induce cellular apoptosis through inhibition of DNA methyltransferases (DNMTs) and HATs activities.[21] It implied that AcEGCG may prevent carcinogenesis by modifications of epigenetic processes. However, the effects of AcEGCG on HAT activity and histone and nonhistone proteins acetylation in DSS-induced colitis have not yet been well studied. Here, we explore and compare the efficacy of EGCG and AcEGCG supplementation on the development of colonic inflammation and aberrant crypt foci (ACF) in DSS-induced experimental colitis. Our results clearly demonstrate that AcEGCG is more potent than EGCG for the improvement of colitis through blocking the pro-inflammatory mediators secretion and increasing the antioxidant enzyme expression via altering histone and nonhistone proteins acetylation/deacetylation balance. These findings support a role for AcEGCG in epigenetic regulation of intestinal inflammation.

MATERIALS AND METHODS

Reagents and Antibodies. DSS (mol wt, 36,000−50,000) was purchased from MP Biomedicals (Aurora, OH). EGCG (100% pure) was isolated from a crude green tea polyphenol extract provided by Dr. Chi-Tang Ho (Department of Food Science, Rutgers University). AcEGCG was provided by Dr. Shengmin Sang (North Carolina Agricultural and Technical State University). Briefly, AcEGCG was synthesized using a pyridine catalyzed reaction of EGCG with acetic anhydride.[18] The purity of EGCG and AcEGCG was determined by HPLC as higher than 99.2% (Figure 1). Antibodies against iNOS, Nrf2, IκBα, p-IκBα, p65, phospho-PI3K (Tyr508), and β-actin were purchased from Santa Cruz Biotechnology, CA, USA. Anti-COX-2 monoclonal antibody was purchased from BD Transduction Laboratories, Lexington, KY. p-p65 (Ser536), p-ERK1/2 (Thr202/Tyr204), ERK, and acetyl-CBP/p300 and acetyl-H3K9 were from Cell Signaling Technology, Beverly, MA. p-IκBα (Ser32/Ser36), p-Akt (Ser473), and Akt polyclonal antibodies were purchased from Upstate Biotechnology, Lake Placid, NY.

Animal Care and Experimental Design. Male ICR mice at 5 weeks of age were purchased from the BioLASCO Experimental Animal Center (Taipei, Taiwan). After 1 week of acclimation, animals were randomly distributed into control and experimental groups. All animals were housed in a controlled atmosphere (25 ± 1 °C at 50% relative humidity) under a 12 h light/12 h dark cycle. Animals had free access to the standard diet (BioLASCO, Co., Ltd., Taipei, Taiwan) and sterile water at all times. All mice were fed the experimental diets ad libitum for 2 or 20 weeks. Food cups were replenished with fresh diet every day. All animal experimental protocols used in this study was

approved by Institutional Animal Care and Use Committee of the National Kaohsiung Marine University (IAC UC, NKMU). The experimental design is summarized in Supporting Information, Figure S1A. Mice were sacrificed by euthanasia at 2 weeks, after which the colon, spleen, liver, kidney, and serum were collected.

Evaluation of DSS-Induced Colitis Severity. Colitis in mice was induced by administering 1.5% DSS orally in drinking water for 7 days in accordance with previous studies.[22] We analyzed the disease activity index (DAI), colon length, spleen weight, and histology. The DAI was evaluated by scoring changes in body weight, stool consistency, and gross bleeding, as described previously.[23] After determining the DAI, mice were sacrificed, and their entire colon was removed and fixed in 3.7% neutral formalin for colon length and histological analysis. The hematoxylin and eosin (H&E) stained colon tissues were examined and scored for inflammatory activity by an experienced pathologist in a blind fashion using a previously published scoring system.[24]

Identification of ACF and ALN. Entire colons were excised, cut longitudinally, rinsed with ice-cold phosphate- buffered saline (PBS), and fixed flat overnight between sheets of filter paper using 3.7% neutral formalin. The Bird method was used to classify ACF. The total number of ACF was determined using a microscope (40×). Multiple formalin-fixed colonic sections were stained with H&E and examined for dysplasia, and adjacent sections were used for immunostaining. H&E stain sections were examined histologically and classified as ACF and aggregates of lymphoid nodules (ALN) according to specific pathological criteria by a pathologist in accordance with previous reports.

Electrophoretic Mobility Shift Assay. Nuclear protein extracts used for EMSA. The electrophoretic mobility shift assay analysis was performed with a nonradioactive (biotin label) gel shift assay according to the manufacturer's protocol. The NFκB (5′-AGTT-GAGGGGACTTTCCCAGGC-3′) consensus oligonucleotide probe was end labeled with biotin (Pierce, Rockford, IL) with terminal deoxynucleotidyl transferase. For the binding reaction, 6 μg of nuclear extract protein was incubated in a total volume of 20 μL with binding buffer containing 50 fmol of biotin end-labeled oligonucleotide. The mixture was incubated at room temperature for 20 min. The specificity was determined by adding a 100-fold excess of unlabeled double-stranded consensus oligonucleotide to the reaction mixture to act as a competition reaction. Following the addition of 5 μL of sample buffer, the DNA−protein complexes were resolved on a 6% nondenaturing polyacrylamide gel in a 0.5× Tris-borate-EDTA buffer at 100 V for 2 h and then transferred to nylon membrane. Finally, the biotin-labeled DNA was detected by chemiluminescence using the LightShift Chemiluminescent EMSA kit (Pierce, Rockford, IL, USA) and exposed to X-ray film.

Western Blot Analysis. For protein analyses, total scraped colon mucosa was homogenized on ice for 15 s using a Polytron tissue homogenizer and then lysed in 0.5 mL of ice-cold lysis buffer [50 mM Tris-HCl, pH 7.4, 1 mM NaF, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethane-sulfonyl fluoride, 1% NP-40, and 10 mg/mL leupeptin] on ice for 30 min, followed by centrifugation at 10,000*g* for 30 min at 4 °C. The samples (50 μg of protein) were mixed with 5× sample buffer containing 0.3 M Tris-HCl (pH 6.8), 25% 2-mercapto-ethanol, 12% sodium dodecyl sulfate (SDS), 25 mM EDTA, 20% glycerol, and 0.1% bromophenol blue. The mixtures were boiled at 100 °C for 5 min and were then subjected to stacking gel, following which they were resolved by 12% SDS−polyacrylamide minigels at a constant current of 20 mA. Subsequently, electrophoresis was carried out on SDS−polyacrylamide gels. For Western blot analysis, proteins on the gel were electrotransferred onto a 45 μm immobile membrane (PVDF; Millipore Corp., Bedford, MA) with transfer buffer composed of 25 mM Tris-HCl (pH 8.9), 192 mM glycine, and 20% methanol. The membranes were blocked with a solution of 20 mM Tris-HCl, pH 7.4, 0.2% Tween 20, 1% bovine serum albumin, and 0.1% sodium azide. The membrane was further incubated with specific antibodies, at appropriate dilution (1:1000) using blocking solution with the primary antibodies overnight at 4 °C. The membranes were subsequently probed with antimouse or antirabbit IgG antibody conjugated to horseradish peroxidase (Transduction Laboratories, Lexington, KY) and visualized using enhanced chemiluminescence (ECL, Amersham). The densities of the bands were quantified using a computer densitometer (AlphaImagerTM 2200 System). All membranes were stripped and reprobed for β-actin (Sigma Chemical Co.) as the loading control. Co-immunoprecipitation was performed as described previously.[15] Briefly, 200 μg of nuclear extract was incubated with 1 μg of NFκB p65, Nrf2, or Rabbit IgG control for 4 h at 4 °C. Twenty microliters of protein A/G Plus agarose were added to each tube and then incubated for 2 h overnight at 4 °C. Immunoprecipitates were washed 4 times each with 1 mL of wash buffer using a magnetic stand, after which the pellets were resuspended with 5× reducing loading buffer. Western blot analysis was performed using antiacetyl-lysine antibody (Cell Signaling Technology, Beverly, MA).

HAT Activity Assay. HAT activity and coimmunoprecipitation were prepared as described previously. Fifty micrograms of nuclear extracts were taken for the determination of HAT activity. HAT activity was measured using a HAT activity colorimetric assay kit from BioVision (Mountain View, CA). The experiment was done according to the manufacturer's instructions. Absorbance was taken at 440 nm.

Reverse Transcription−Polymerase Chain Reaction. Total RNA were extracted from scraped colonic mucosa using TRIZOL reagent according to the supplier's protocol. The template used was 4 μg of total cellular RNA in a 20 μL reaction solution with Super Script II RNase H-reverse transcriptase (Invitrogen, Renfrewshire, UK). The cDNA (2 μL) was amplified by polymerase chain reaction (PCR) with the following primers: IL-6, forward primer 5′-GAGGATAC-CACTCCCAACAGACC-3′ (sense), 5′-AAGTGCATCATCGTTGT TCATACA-3′ (antisense); IL-1β 5′-AC-CTGCTGGTGTGTGACGTT-3′ (sense), 5′-TCGTTGCTTGGTTCTCCTTG-3′ (antisense); TNF-α 5′-AGCA-CAGAAAG CATGATCCG-3′ (sense), 5′-CTGATGAGAGG-GAGGCCATT-3′ (antisense); HO-1 5′-GAGCAGAACCAGCCT-GAACTA-3′ (sense), 5′-GGTACAAGGAAGCCATC ACCA-3′ (antisense); β-actin (286 bp) 5′-AAGAG AGGCATCCTCACCCT-3′ (sense); and 5′-TACATGGCTGGGGTGTTGAA-3′ (antisense). PCR (GeneAmp PCR System 9700, Applied Biosystems, CA, USA) and amplification was performed under the following conditions: 30 cycles at 94 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min, and followed by a final incubation at 72 °C for 10 min. PCR products were analyzed on 1% agarose gel and visualized by ethidium bromide staining.

Immunohistochemical Analysis. Three micrometer sections of colonic mucosa in ACF and inflammatory segments were deparaffinized, rehydrated, and treated with 0.3% hydrogen peroxide (H_2O_2) for 15 min to block endogenous peroxidase. Sections were pressure cooked for (4 × 7 min) in 10 mM citrate buffer, pH 6.0 (Immuno DNA retriever with citrate, BIO SB, Inc., Santa Barbara, CA), to unmask epitopes. Sections were incubated with primary antibody to AR and Nrf2 (1:100 dilutions in PBS) for 1 h. Immunoreactivity was determined using biotin-labeled secondary antibody and streptavidin−biotinperoxidase for 30 min each. 3,3′-Diaminobenzidine tetrahydrochloride (0.05%, DAB) was used as the substrate, and a positive signal was detected as a brown color under a light microscope. The detailed procedures for the stained tissue analysis method were reported previously.[25] For Nrf2, the criterion for positive expression was nuclear staining. For the immunoreactive score (IRS), the scores for the percentage of positive cells and the staining intensity was multiplied.

Azoxymethane/DSS-Induced Colon Cancer Model. The experimental design is summarized in Supporting Information, Figure S1B. Mice were sacrificed by euthanasia at 20 weeks, after which the colon, spleen, liver, kidney, and serum were collected. Colons were cut longitudinally and fixed in 10% buffered formalin overnight. The colons were stained with methylene blue and macroscopically scored for the number of colonic tumors.

Statistical Analysis. Relative expression values are given as the mean ± SE for the indicated fold of expression in the colon mucosa of mice. SigmaPlot 10.0 software was used for illustration and analysis. Statistical analyses were performed using 2-way analysis of variance (ANOVA) with subsequent Bonferroni posthoc test for pairwise

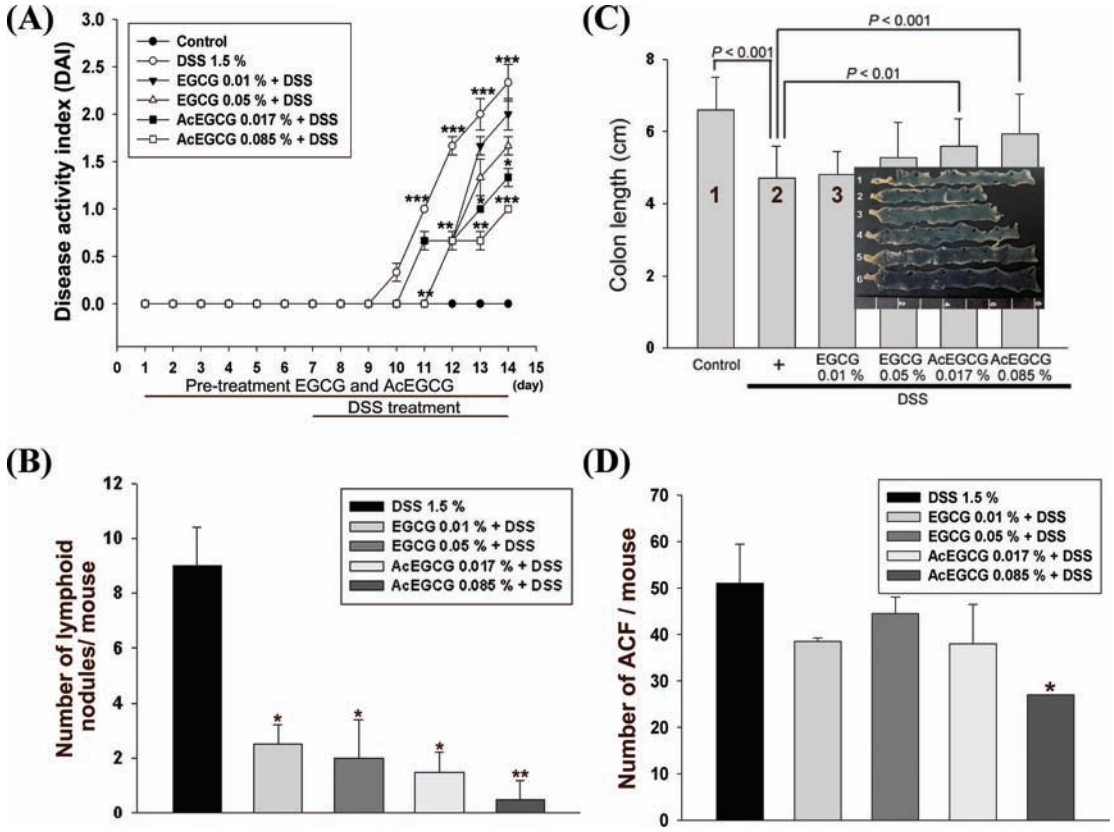

Figure 2. EGCG and AcEGCG inhibited DSS-induced colonic shortening, LN, and ACF. All mice were killed after 14 days of feeding, and the colons were fixed with 10% 3.7% buffered formalin for 24 h and (A) DAI values, (B) colon lengths, (C) lymphoid nodules (LN), and (D) aberrant crypt foci (ACF) were measured. Each bar represents the mean ± SE of the averages of 6 mice scored. Statistical significance, $*p < 0.05$; $**p < 0.01$ compared with the DSS-treated group.

comparisons between two particular groups. A *P*-value of <0.05 was considered statistically significant.

RESULTS

AcEGCG Improved DSS-Induced Body Weight Loss, Clinical Symptoms, and Spleen Enlargement Better than EGCG. We first monitored symptomatic colitis parameters including body weight and the disease activity index (DAI) caused by colitis 7 days after starting 1.5% DSS oral administration (Figure 2A). Pretreatment of mice with EGCG or AcEGCG significantly attenuated the weight loss (Supporting Information, Figure S1B) induced by DSS. There was no difference in the kidney and liver weights among the different experimental groups (Supporting Information, Figure S2A and B). The gain of spleen weight observed, however, after exposure to DSS was reduced remarkably in mice fed with 0.017 ($P < 0.05$) and 0.085% ($P < 0.01$) of AcEGCG.

AcEGCG Treatment Prevented DSS-Induced Colitis Better than EGCG. On the basis of these results, we evaluated the protective efficacy of EGCG or AcEGCG in the treatment of DSS-induced colitis. Colon lengths were measured due to the observation that a short colon can be used as a morphological parameter of colon inflammation in the DSS-induced colitis model. As shown in Figure 2B, the colon length of the mice given DSS was significantly shortened ($P < 0.001$) compared to the nontreated control group. The dietary administration of 0.017 ($P < 0.01$) and 0.085% ($P < 0.001$) AcEGCG was markedly longer than that in the DSS alone group. EGCG, however, did have a mild effect in increasing colon length. Next, we observed that DSS-induced colonic ACF, lymphoid nodules (LN), and inflammatory status could be quantified by a histopathological study. As shown in Figure 2C, the number of colonic LN was significantly decreased by pretreatment with EGCG or AcEGCG ($P < 0.05$). In contrast, the number of ACF caused by DSS was only significantly reduced following pretreatment with 0.085% in the AcEGCG group ($P < 0.05$) (Figure 2D). Histopathological analysis confirmed the DSS-induced inflammation (Figure 3). In the DSS-treated group, but not nontreated group, colonic sections presented typical inflammatory characteristics such as infiltration of inflammatory cells, distortion of crypts structure, reduction of goblet cells, and ulcerations. In general, pretreatment with EGCG or AcEGCG revealed dramatically attenuated histological scores of inflammation in the colon induced by DSS ($P < 0.05$). More noteworthy was the fact that the total inflammation scores were significantly lower in the AcEGCG treated group compared with the EGCG treated group (Figure 3A–D). These results demonstrate that the dietary consumption of AcEGCG may be more effective than EGCG in preventing DSS-induced colitis and colonic preneoplastic lesions such as ACF and aggregates of lymphoid nodule (ALN) formation.

AcEGCG Suppresses DSS-Induced Inflammatory Mediator Production by Inhibiting the NFκB-Activating Signaling Pathway. The phosphatidylinositol 3-kinase (PI3K)/Akt signal transduction pathway is involved in the regulation and release of pro-inflammatory mediators and plays an important role in the development and progression of

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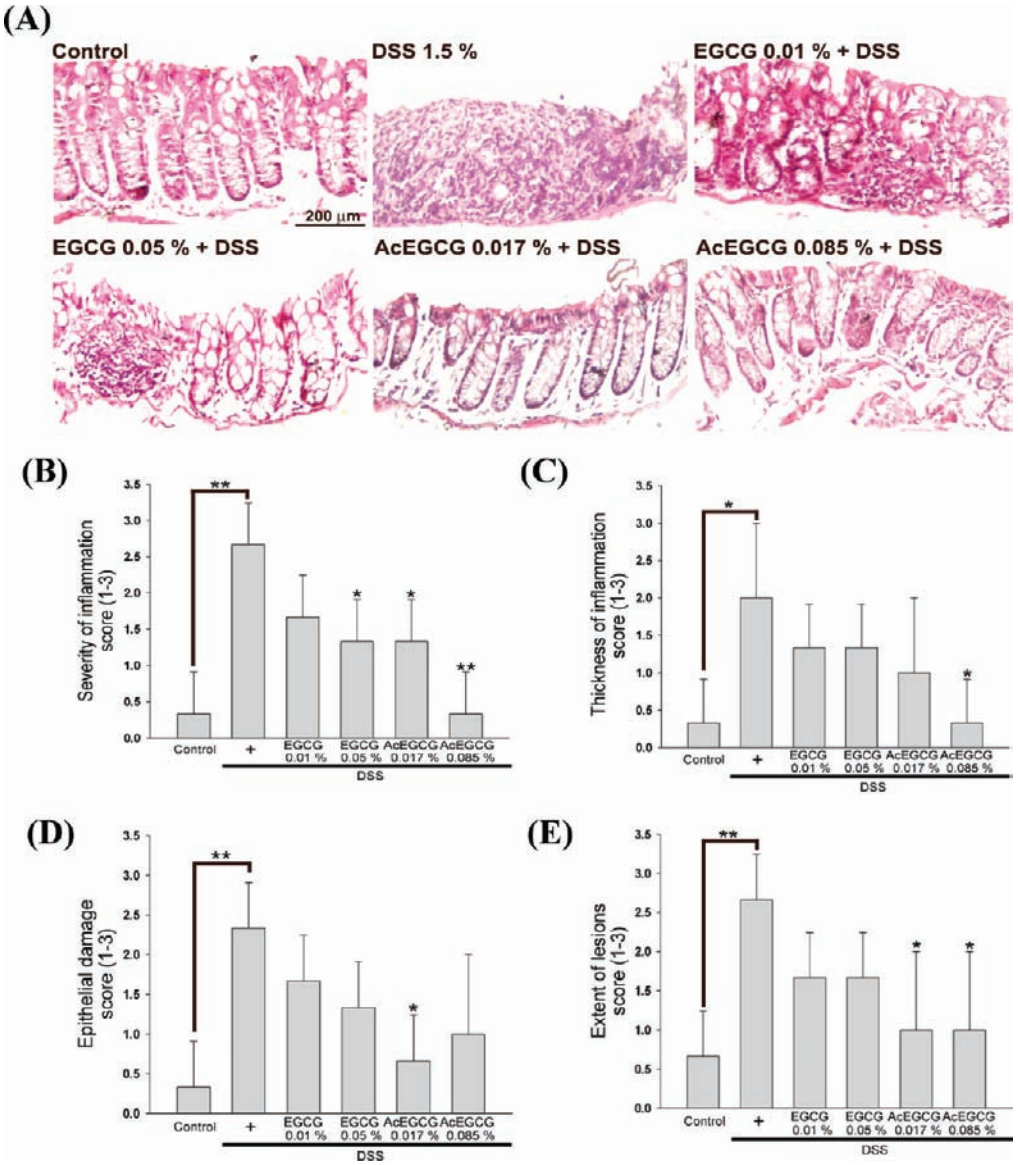

Figure 3. EGCG and AcEGCG attenuated DSS-induced inflammation grading. Colons were obtained after DSS administration, then sectioned, stained with H&E, and scored histopathologically (200× magnification). (A) Histological features of colonic tissue from the six groups of mice were presented (as shown in Figure 1A). (B–E) The histological scores of inflammation in the colons of mice were evaluated as described in Materials and Methods. Each bar represents the mean ± SE of the averages of 6 mice scored. Statistical significance, $*p < 0.05$; $**p < 0.01$ compared with the DSS-treated group.

ulcerative colitis (UC).[26] Activation of PI3K/Akt could mediate phosphorylation of p65 at Ser536, IKKα/β, IκBα, and CBP/300, resulting in the activations of NFκB.[27] Therefore, we investigated the effects of AcEGCG on the DSS-induced phosphorylation of PI3K/Akt and expression of pro-inflammatory mediators in mouse colitic colon. As depicted in the immunoblot (Figure 4A and B), the levels of PI3K/Akt, iNOS and COX-2 expression were significantly elevated compared with those of the control-treated group ($P < 0.001$). Importantly, administration of AcEGCG markedly decreased PI3K/Akt phosphorylation and iNOS and COX-2 expression after DSS challenge. Next, we asked whether mice fed with AcEGCG may show a reduced capacity for DSS-induced production of inflammatory cytokines. As shown in figure Figure 4C, TNF-α, IL-1β, and IL-6 were all upregulated in the DSS group compared with the control group. Preadministration with AcEGCG at 0.085% significantly decreased the DSS-induced IL-6 ($P < 0.05$) and TNF-α expression ($P < 0.01$). There was, however, no significant suppression of levels of IL-1β pretreatment with AcEGCG. Because of the essential role of the NFκB signaling pathways in the regulation of pro-inflammatory mediator expression in DSS-induced colitis,[28] we determined the effect of AcEGCG on the activation of NFκB. NFκB activation was analyzed by NFκB-DNA-binding activity using immunoblot and electrophoretic mobility shift (EMSA) assays of nuclear protein. We found that DSS-induced NFκB-DNA-binding activity was inhibited by pretreatment with AcEGCG in a dose-dependent manner (Figure 5A). The phosphorylation of p65/RelA was also inhibited by the AcEGCG pretreated (Figure 5B).

In more recent studies, ameliorate inflammatory bowel disease was related to the inhibition of histone deacetylase (HDAC) and acetylation of histone 3 and 4.[29−31] Histone and non-histone protein substrate acetylation by HATs is crucial for

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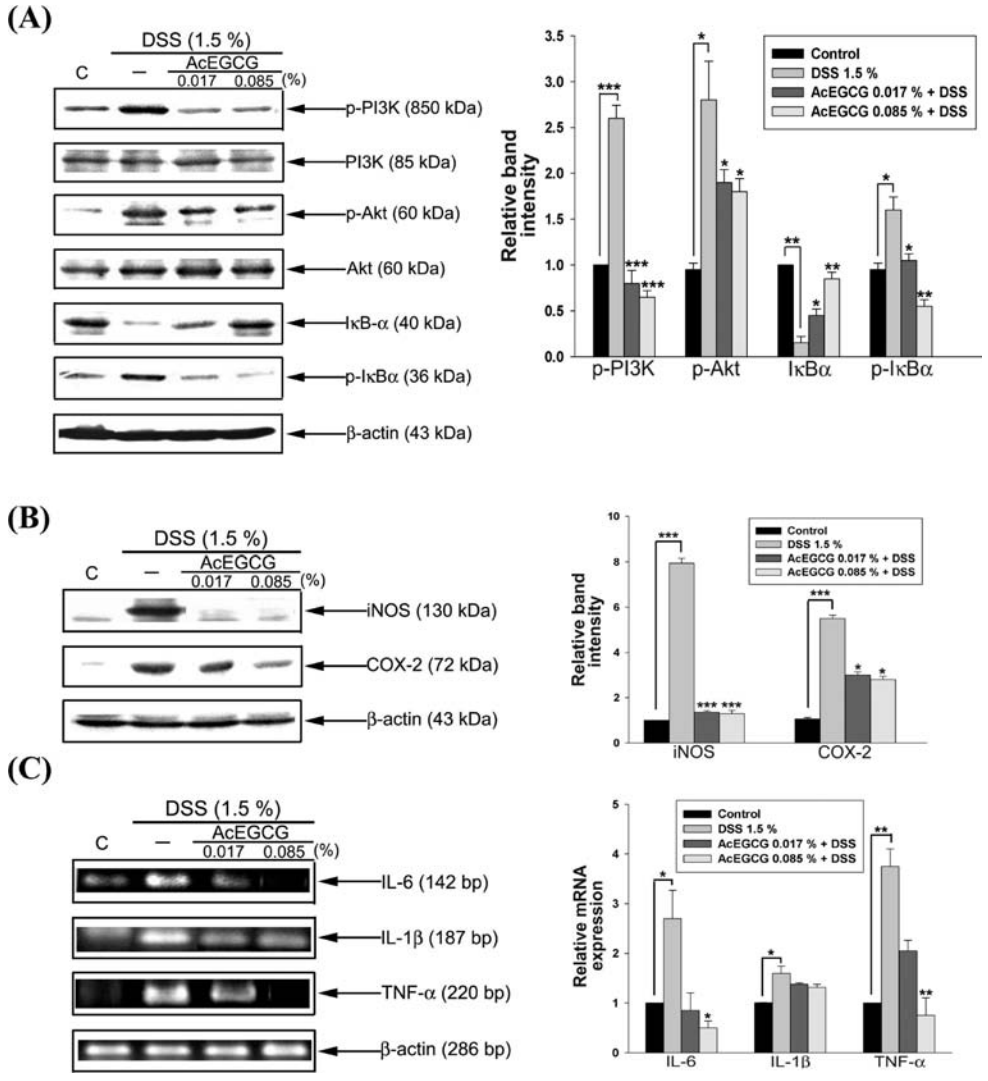

Figure 4. AcEGCG decreased DSS-induced inflammatory signaling. ICR mice were treated as described in Figure S2A (Supporting Information), and after 14 days, the colonic mucosas were scraped and homogenized. (A and B) Equal amounts of cell lysates (50 μg of protein) were measured by Western blot assay. (C) RNA was subjected to RT-PCR analysis using appropriate primers for IL-6, IL-1β, and TNF-α. Each sample was normalized to β-actin. Quantification of protein and gene expression was normalized to β-actin using a densitometer. Each bar represents the mean ± SE (n = 3) of the averages of three independent experiments. Statistical significance, *p < 0.05; **p < 0.01; ***p < 0.001compared with the DSS-treated group.

chromatin-compaction status and gene transcription.[32] We observed that AcEGCG resulted in markedly decreased DSS-induced acetylation of histone 3 lysine 9 (H3K9) and p65/RelA (Figure 5B and C). Thus, to confirm the epigenetic regulation of AcEGCG on inflammation, we next examined whether AcEGCG pretreatment could inhibit HAT activity. DSS could increase acetyl-CBP/p300 level and HATs activity, but pretreatment with AcEGCG does not affect it (Figure 5D and E). However, inhibition of acetyl-H3K9 leading to downregulation of NF-κB transcriptional activity may be one mechanism behind the action of the AcEGCG extract against DSS-induced colitis.

AcEGCG Blocks DSS-Induced Inflammation by Activating the Nrf2 Signaling Pathway and Enhancing Antioxidant Enzyme Expression. Several studies have demonstrated that activation of Nrf2 signaling and the induction of its target genes could exert anti-inflammation and antioxidative effects.[33] A previous study showed that ablation of Nrf2 resulted in increased expression of pro-inflammatory mediators, and it will be interesting to know if overexpression of Nrf2 can suppress NFκB signaling pathway. The question as to whether modulation of these pro-inflammatory mediators by Nrf2 is a direct transcriptional regulation or is indirect through its transactivated target genes such as HO-1 requires further investigation.

To clearly interpret the anti-inflammatory effects of AcEGCG, the Nrf2 mediated signaling pathway was examined. A widely accepted model for the induction of ARE-mediated antioxidant gene expression involves phosphorylation of serine/threonine residues of Nrf2 by ERK (p44/42) MAPK kinases, leading to enhanced nuclear accumulation of Nrf2 and subsequent ARE binding.[34] Besides, previous studies indicated that the acetylation condition modulates nuclear localization and the transcriptional activity of Nrf2.[15] Therefore, we investigated the effects of AcEGCG on the phosphorylation of ERK MAPK and acetylation of Nrf2.

The results displayed that administration of AcEGCG caused a significant (P < 0.05) enhancement in the activation and nuclear translocation of Nrf2 (Figure 6A) and induction of Nrf2-mediated downstream antioxidant enzyme HO-1(Figure

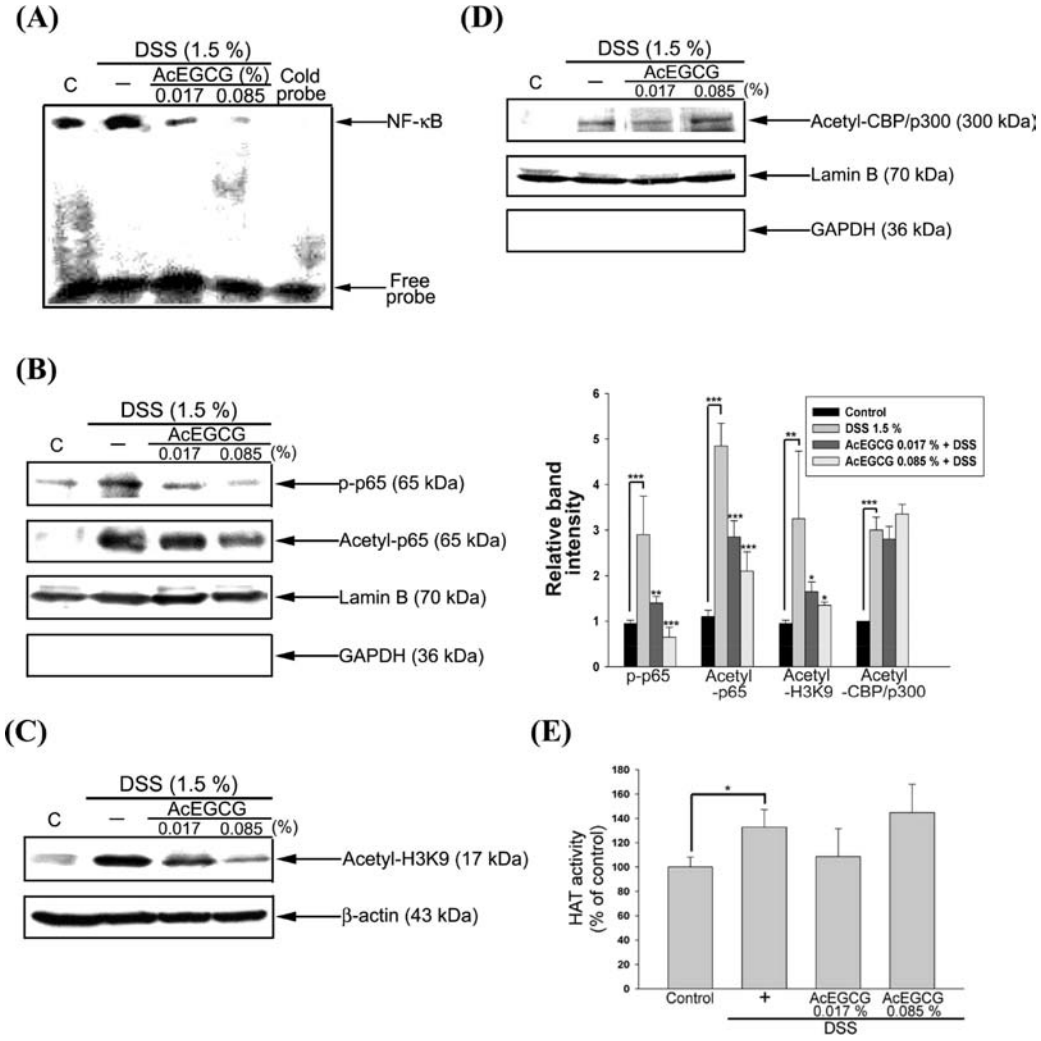

Figure 5. AcEGCG blocked activation of NFκB signaling and acetylation of histone 3 lysine 9 (acetyl-H3K9). Electrophoretic mobility shift assay analysis was performed by equal 6 μg of nuclear extracts from colonic mucosa with a biotin-labeled (nonradioactive) (A) NFκB probe. The expressions of (B) p-p65 (Ser536) and acetyl-p65, (C) acetyl-H3K9, and (D) acetyl-CBP/p300 were measured using the Western blot assay. (E) Histone acetyltransferase (HAT) activity was assessed with proteins of nuclear extracts using the HAT activity assay kit following the manufacturer's instructions. Quantification of protein expression was normalized to β-actin and lamin B using a densitometer. Each bar represents the mean ± SE (n = 3) of the averages of three independent experiments. Statistical significance,*p < 0.05; **p < 0.01; ***p < 0.001compared with the DSS-treated group.

6C) in the colonic tissue. We also found that AcEGCG pretreatment could increase ERK (p44/42) phosphorylation (Figure 6B) and Nrf2 acetylation (Figure 6D), consequently reinforcing Nrf2 signaling. Thus, AcEGCG appears to suppress the pro-inflammatory signaling pathway via activating Nrf2 signaling through increasing Nrf2 acetylation and inducing Nrf2 transcriptional activity, which results in inactivation of NFκB. Taken together, this study also supported the idea that acetyl-Nrf2 expression in the intestinal epithelium during IBD is a potential epigenetic therapeutic strategy to prevent inflammation and mucosa disruption by increased oxidants. Furthermore, the present preliminary results aim to provide further understanding of the role that Nrf2 and NFκB acetylation plays in the regulation of colitis.

AcEGCG Prevents Colitis-Related Colon Tumorigenesis Induced by AOM Plus DDS Better than EGCG. We have shown that AcEGCG suppresses colitis. Because both mice and humans with chronic colitis are at a high risk for colon cancer, here we assessed the preventive potential of AcEGCG in a mouse model of colitis-driven colon cancer. As shown in Figure 7A, the number of macroscopic colon tumors in the AOM + DSS group was 26 ± 9, and the number of macroscopic colon tumors in the AOM + DSS + EGCG group was 22 ± 8. However, this difference between the AOM + DSS and AOM + DSS + AcEGCG (4.7 ± 2 tumors per animal) was statistically significant (P < 0.001). Additionally, the multiplicities of colon tumors in AcEGCG treated mice were significantly lower than that in EGCG treated mice (P < 0.001). In the present study, this is the first report showing that AcEGCG more effectively exerts cancer chemopreventive ability in colitis-related colon carcinogenesis than EGCG.

DISCUSSION

Epidemiological studies have suggested that green tea consumption has cancer preventive effects. Laboratory studies have identified EGCG as a chemopreventive agent for prevention of colon carcinogenesis and presented evidence for its efficacy and safety.[35] Additionally, EGCG has also been

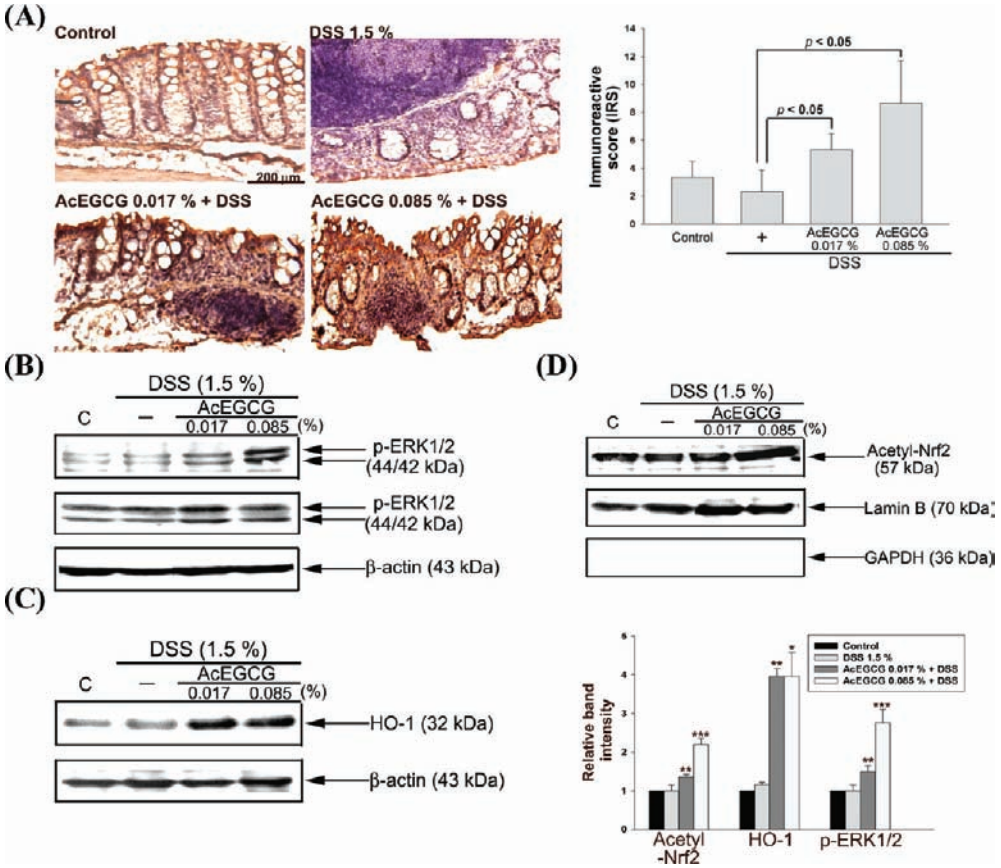

Figure 6. AcEGCG enhanced expression of Nrf2-regulated antioxidant enzyme. (A) Staining of Nrf2 expression was measured 14 days after the DSS induction of colitis by IHC methods. (200× magnification). Positive expressing cells are stained brown. (B and C) The expressions of (B) p-ERK1/2 and (C) HO-1 were measured using the Western blot assay. (D) Acetylation was measured in nuclear cell lysates by a coimmuno-precipitation assay. The nuclear cell lysates (200 μg of protein) were first precipitated with anti-Nrf2 antibody as described under Materials and Methods, followed by Western blotting of the IP samples, using an anti-acetyl-lysine antibody. Quantification of gene expression was normalized to β-actin and lamin B using a densitometer. Each bar represents the mean ± SE of the averages of 3−6 mice scored. Statistical significance, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$compared with the DSS-treated group.

suggested as a potential therapeutic agent in colitis and colon cancer associated with colitis because of its anti-inflammatory effects.[36,37] EGCG, however, is rapidly degraded, metabolized, and excreted in the colon, which limits its bioavailability.[38] Therefore, the AcEGCG, was synthesized and was quite successful in improving the biological activity and bioavailability of EGCG. Joshua et al. have reported that intragastric administration of AcEGCG to CF-1 mice resulted in higher bioavailability and cancer preventive activity compared with the administration of equimolar doses of EGCG. On the contrary, we also believe that consuming more cups of green tea daily may mitigate the poor bioavailability of EGCG compared to a single administration. More importantly, the concentration of EGCG in colonic tissues was 6.0-fold increased following the administration of AcEGCG compared with equimolar doses of EGCG. However, the molecular mechanism of how AcEGCG suppresses DSS-induced colitis and colon cancer associated with colitis in mice is unclear. In this study, for the first time, we compare the chemopreventive effect of AcEGCG and EGCG in DSS- and AOM/DSS-induced colon carcinogenesis in mice. We further elucidate their molecular mechanisms of anti-inflammation and epigenetics in DSS-induced colitis. For the first time, we found that AcEGCG ameliorates DSS-induced inflammation and tissue injury in mouse colon in a dose-dependent manner.

The intestinal inflammatory model involves supplementing the drinking water of mice with DSS, which is characterized by inflammatory cell infiltration, epithelial crypt loss, and injury, and results in acute clinical symptoms (weight loss, bloody stools, and diarrhea). Multiple lines of evidence suggest that inflammatory enzymes and cytokines play a pathophysiologic role in IBD. Previous reports have also demonstrated that the formation of ACF and ALN are associated with colonic preneoplastic stage and inflammatory cell infiltration. Our results indicated that preadministration of AcEGCG at a significantly lower dose compared to EGCG was able to significantly reduce the ACF and ALN number in DSS-induced mice (Figure 2) and improved inflammation grading (Figure 3) and DAI scores (Figure 2). Moreover, dietary administration of AcEGCG also markedly diminished the tumor numbers compared with those in AOM/DSS-induced mice (Figure 7A). These results of our study clearly indicate that AcEGCG is more effective than EGCG in alleviating the symptoms of colitis and preventing the progression of colonic tumors. This cancer chemopreventive ability is considered to be associated with the suppression of inflammation (Figure 4) and the induction of antioxidant mechanisms (Figure 6).

Several previous studies have indicated that DSS could induce colitis and possible colorectal carcinogenesis by increasing lipid peroxidation, inflammation, and up-regulating

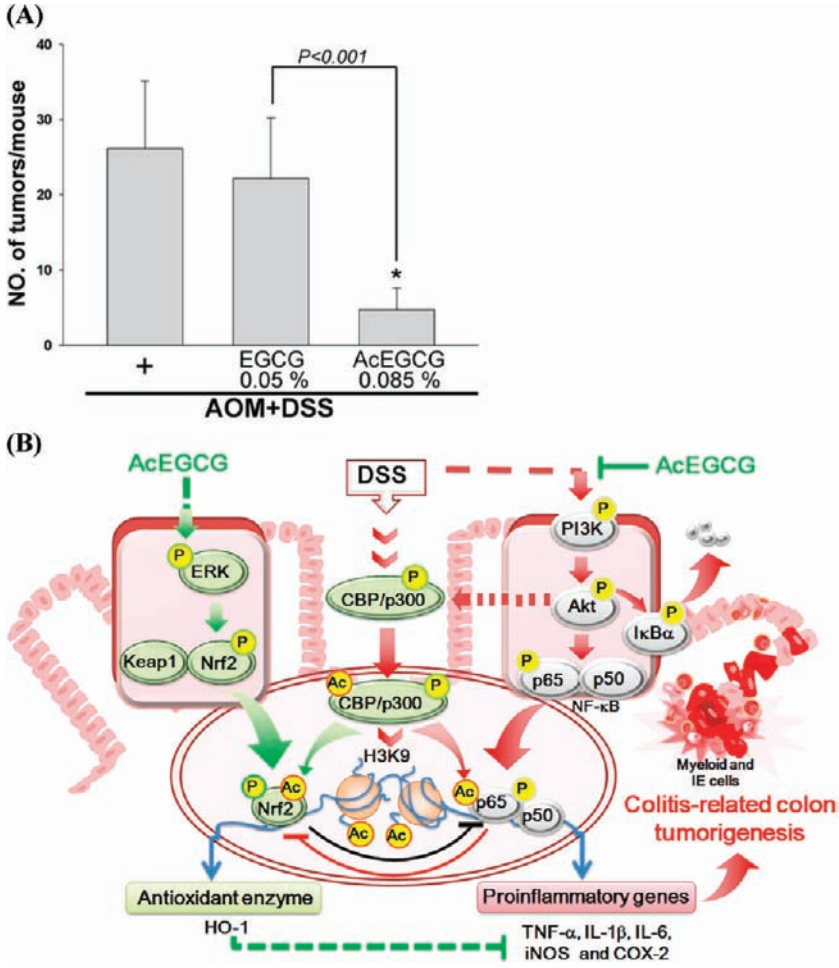

Figure 7. AcEGCG prevents DSS-induced colitis and AOM/DSS-induced colon tumorigenesis in ICR mice. (A) Number of colonic tumors per mouse. Each bar represents the mean ± SE of the averages of 6−8 mice scored. Statistical significance,*$p < 0.001$ compared with the DSS-treated group. (B) Possible molecular targets for chemopreventive effects of AcEGCG in DSS-induced colitis. DSS activated HAT activity and the PI3K/Akt-mediated NFκB signaling pathway leading to pro-inflammatory gene expression. Administration of AcEGCG activated ERK1/2 mediated Nrf2 transcriptional activity through enhanced acetylation-dependent transactivation, leads to deacetylation of p65, subsequently protecting against inflammation. IE, intestinal epithelial.

of oxidative stress signaling.[39] In addition, orally administered DSS also reduced the glutathione (GSH) content and superoxide dismutase and catalase activities in the intestinal mucosa.[40] Findings from our laboratory and those of others have demonstrated that activation of Nrf2 signaling could inhibit oxidative stress and inflammatory response by up-regulating of HO-1 expression. Numerous in vivo studies have revealed that Nrf2-deficient mice exhibited increased DSS or AOM/DSS-mediated colonic inflammatory injury and formation of ACF and tumor compared to Nrf2 wild type mice.[41] In fact, HO-1 is reported to inhibit inflammation by the induction of CO and bilirubin.[42] Interestingly, intraperitoneal injections of hemin in mice ameliorated DSS-induced colitis, suggesting that up-regulation of HO-1 plays a protective role in IBD.[43] Thus, targeting antioxidation could be more effective as a potential therapeutic or prevention strategy in IBD.

Inflammatory signaling is a manifestation of epigenetic modifications by histone acetylation/deacetylation in the inhibition of colitis. Histone acetylation modifies the chromatin structure that allows access to the various transcriptional activators at gene promoters, and they therefore play an important role in gene regulation and tumorigenesis.[44] Previously, studies have reported that acetylation of histone H4 and H3 was significantly elevated in the inflamed mucosa. Furthermore, several HAT inhibitors were also used in the inhibition of growth in HCT116 and HT-29 human colon tumor cells. Thus, inhibiting of histone acetylation and understanding of its mechanisms are also potential candidates for the development of anti-inflammation and anticancer agents.

It is now well established that lysine acetylation of NFκB plays a crucial part in regulating the NFκB-dependent gene transcription through the recruitment of HATs (e.g., CBP/p300 and PCAF) to gene promoters. CBP and its homologue p300 are common transcriptional coactivators that possess acetyl-transferase activity. In addition, recent studies have demonstrated that CBP/p300 could promote transcriptional potential and acetyl-transferase activity by p300 autoacetylation and that Akt binds to p300 and phosphorylates its Ser-1834.[45] More specifically, Liu et al. have found that NFκB p65 could antagonize this pathway through the deprivation of the coactivator, CBP, from Nrf2.[13] A previous study also indicated that Nrf2-dependent transcription and nuclear localization were shown to require CBP/p300 augments, which resulted in the induction of Nrf2-modulating antioxidant response.[15] Thus,

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acetylation of Nrf2 will be a novel epigenetic regulatory mechanism.

However, its specific regulation mechanisms at the level of target proteins (histone or nonhistone) are not known in colitis conditions. The goal of this study was to determine whether AcEGCG can be used as a specific and selective epigenetic therapeutic agent for IBD. In this study, we demonstrated that AcEGCG markedly inhibited H3K9 acetylation (Figure 5C) but did not affect HAT activity (Figure 5D). It is clear that AcEGCG protects against DSS-induced inflammatory signaling by reducing p65 phosphorylation and NFκB-DNA-binding activity and selectively abating p65 acetylation (Figure 5A and B). In contrast, the protein level of Nrf2 was apparently stronger in the AcEGCG-treated mucosa than in DSS-treated (Figure 6A). The activation of Nrf2 would be dependent on HAT activity and ERK (p44/42) MAPK phosphorylation, which may raise the antioxidant stress proteins HO-1 expression (Figure 6B–D). Our findings support the Nrf2 as a novel epigenetic therapeutic intervention to control various forms of inflammatory disorders.

In conclusion, we clearly demonstrated that the EGCG derivative, AcEGCG, showed much higher potency than natural EGCG to prevent DSS-induced colitis. We first reported that DSS phosphorylates PI3K/Akt and activates HAT (CBP/p300), which in turn acetylates H3K9 and p65 resulting in the induction NFκB activation and increased transcription of inflammatory genes (Figure 7B). Another important finding is that, for the first time, we showed that AcEGCG acts at different levels and induces epigenetic changes by specifically increasing acetyl-Nrf2, activating ERK signaling, thereby resulting in enhancing antioxidant enzyme expression (HO-1) and suppressing DSS-induced NFκB activation and inflammation (Figure 7B). Our findings propose that AcEGCG may be developed into a novel epigenetic therapeutic agent by selective regulation of epigenetic mechanisms. In summary, this is the first investigation with evidence that dietary AcEGCG has great potential as a novel chemopreventive agent than EGCG to be used in the treatment of inflammation associated with tumorigenesis, especially in the prevention and treatment of colorectal cancer. These results provide insight into the biological actions of AcEGCG and might establish a molecular basis for the development of new cancer chemopreventive agents.

■ ASSOCIATED CONTENT

Supporting Information

EGCG and AcEGCG suppression of DSS-induced clinical symptoms and effects of EGCG and AcEGCG on changes in organ weight during DSS-induced colitis. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

EGCG, epigallocatechin-3-gallate; AcEGCG, peracetylated EGCG; DSS, dextran sulfate sodium; ACF, aberrant crypt foci; iNOS, inducible nitric oxide synthase; COX-2, cycoloxygenase-2; PCR, polymerase chain reaction; Nrf2, NF-E2-related factor 2; HO-1, heme oxygenase-1; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; IL-1β, interleukin-1β; AOM, azoxymethane; ICR, Institute of Cancer Research

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