Anti-inflammatory effects of betaine on AOM/DSS-induced colon tumorigenesis in ICR male mice

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Abstract. Betaine is an important human nutrient obtained from various foods and studies in animals and humans have provided results suggesting their pathogenesis of various chronic diseases and points to a role in risk assessment and disease prevention. However, the molecular mechanisms of its activity remain poorly understood and warrant further investigation. This study was performed to investigate the anti-inflammation and tumor preventing capacity of betaine on colitis-associated cancer in mice. In *in vivo* experiments, we induced colon tumors in mice by azoxymethane (AOM) and dextran sulfate sodium (DSS) and evaluated the effects of betaine on tumor growth. Administration with betaine significantly decreased the incidence of tumor formation with downregulation of inflammation. Treatment with betaine inhibited ROS generation and GSSG concentration in colonic mucosa. Based on the qPCR data, administration of betaine inhibited inflammatory cytokines such TNF-α, IL-6, iNOS and COX-2. In *in vitro* experiments, LPS-induced NF-κB and inflammatory-related cytokines were inhibited by betaine treatment in RAW 264.7 murine macrophage cells. Our findings suggest that betaine is one of the candidates for the prevention of inflammation-associated colon carcinogenesis.

Introduction

Cancer of colon and rectum (colorectal cancer, CRC) is a malignant neoplasm arising from the lining of the large intestine. Patients with inflammatory bowel disease (IBD), including both ulcerative colitis (UC) and Crohn's disease (CD), are at increased risk of developing CRC (1). CRC is a worldwide health-care problem with a continually increasing incidence. In Asia, IBD was considered rare until two decades ago (2), but recent population-based and referral center cohorts have shown a rising incidence and prevalence of IBD in Asia (3). Importantly, the incidence and prevalence of CD and UC in Korea are still lower than those in Western countries, but are rapidly increasing (4). IBD can occur in combination of defected immune response, luminal, environment and genetic factors including tumor necrosis factor (TNF)-α. The evaluated level of TNF-α was especially found in the blood, intestinal mucosa and stools of patients with IBD. In addition to TNF-α, the increases of other pro-inflammatory mediators have been observed in stools and rectal dialysates from patients with IBD as well (5). The existing data revealed that TNF-α inhibitors are increasingly being used in IBD-related studies and patients. The most commonly used biologics in IBD are TNF-α antibodies, such as infliximab, a chimeric IgG1 monoclonal antibody; adalimumab, a human monoclonal IgG1 antibody; and certolizumab pegol, a pegylated Fab fragment of a humanized IgG4 isotype monoclonal antibody (6). Even though these TNF inhibitors may increase the risk of tuberculosis, varicella and other opportunistic infections, there is little evidence suggesting that anti-TNF agents specifically raise the overall risk of serious infections. Similarly, there is little evidence that TNF antagonists raise the risk of developing malignancy over and above the risks from concomitant therapies and the underlying disease process (7). Therefore, more studies are needed on the use of TNF inhibitors in patients with IBD.

Betaine is an essential biochemical molecule of the methionine/homeocysteine cycle and is synthesized by conversion of choline. It was first discovered in the juice of sugar beets (*Beta vulgaris*) in the 19th century (8), and since then has been found in various microorganisms, plants and animals (9,10). It plays central roles in choline-mediated one-carbon metabolism, structural integrity and signaling functions of cell membranes, and neurotransmitter synthesis (11). Previous studies showed dietary choline and betaine intakes and associations with inflammatory markers in healthy free-eating adults enrolled

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in the ATTICA study. Moreover, betaine and choline may be involved in reducing inflammation, including their important role as a source of one-carbon units for the metabolism of homocysteine. In highest tertile for dietary intake of choline and betaine had significantly lower plasma C-reactive protein, interleukin (IL)-6, and TNF-α concentration than did persons in the lowest tertile of intake (12).

Previous studies have shown that betaine has anti-inflammatory activity through inhibition of reactive species (RS) and modulation of reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio in aging process both *in vitro* and *in vivo* studies (13-15). However, the protective role of betaine on the expression and regulation of inflammatory mediators associated with colon cancer has not explored yet. Hence, in the present study we aimed to evaluate the anti-inflammatory effects of betaine on AOM/DSS-induced colitis-associated colon cancer in mice. We demonstrate that betaine is a potent anti-inflammatory agent that may act through the inactivation of inflammatory cytokines.

Materials and methods

Chemicals. Betaine was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Betaine was freshly prepared before each experiment and was solubilized with phosphate buffered saline (PBS).

Animal study. The animal protocol used in this study has been reviewed by the Pusan National University-Institutional Animal Care and Use Committee (PNU-IACUC, Busan, Korea) on their ethical procedures and scientific care, and it has been approved (PNU-2013-0318) as previously described (16). Five-week-old male ICR mice were purchased from Samtako Co., Ltd. (Osan, Korea). All animals were housed in plastic cages (4 mice/cage) and had free access to drinking water and a basal diet (Formula M07; Feed Lab, Guri, Korea) *ad libitum*, under controlled conditions of humidity (50±10%), light (12/12 h light/dark cycle), and temperature (23±1˚C). After arrival, the animals were quarantined for 7 days, and then randomized by body weights into experimental and control groups. A colonic carcinogen AOM was purchased from Sigma-Aldrich. DSS with a molecular weight of 36,000-50,000 (cat. no. 160110) was purchased from MP Biomedicals, LLC (Aurora, OH, USA). The experimental protocol is shown in Fig. 1A. Animals were divided into four experimental groups and control group (n=15 per group). Group 1 was the control. Animals in group 2 through 5 were given a single intraperitoneal injection of AOM (10 mg/kg body weight). Seven days after the AOM injection, animals received 2% DSS (w/v) in the drinking water for 7 days. Seven days after the DSS treatment, betaine-containing diets were started. Subsequently, groups 3 through 5 received the diets containing 1, 5 and 10 mg/kg betaine for 16 weeks, respectively. All animals were sacrificed at week 16 after administration of betaine. At sacrifice, a complete necropsy was performed on all mice. Histopathological examination was performed on paraffin-embedded sections after hematoxylin and eosin (H&E) staining.

GSH assay. To analyze the total glutathione level, using OxiSelect™ Total Glutathione (GSSG/GSH) Assay kit (Cell Biolabs, Inc., San Diego, CA, USA) according to company protocol. GSH reductase solution (25 µl), was added to each well in a 96-well plate and 25 µl of the NADPH was added onto it; 100 µl of the prepared GSH standards or samples was added to each well and mixed thoroughly. Then 50 µl of the chromogen was added and the absorbance was read at 405 nm by using a multi-well reader (Thermo Fisher Scientific, Vantaa, Finland).

Assessment of reactive species (RS) generation. RS generation was measured as previously described using a fluorescence probe (17). Briefly, 2',7'-dichlorofluorescin diacetate (DCF-DA; final concentration 2.5 µM) was added to homogenates and the changes in fluorescence intensity were measured every 5 min for 30 min using a fluorescence plate reader (GENios, Tecan Instruments, Salzburg, Austria) at excitation and emission wavelengths of 485 and 530 nm, respectively.

Total RNA extraction and quantitative real-time PCR. Total RNA was extracted from colonic mucosa using the TRIzol (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. The cDNA was then synthesized from total RNA using TOPscript™ RT DryMix (Enzynomics, Daejeon, Korea). A quantitative real-time PCR (qPCR) analysis of individual cDNA was performed with Takara TP800 instrument (Takara Bio Inc., Shiga, Japan) using SYBR-Green gene expression assay (Enzynomics). The primers used for each reverse transcription-polymerase chain reaction reactions are as follows: GAPDH, 5'-aactttggcattgtggaagg-3' and 5'-acacattgggggtaggaaca-3'; TNF-α, 5'-cgtcagccgatttgctatct-3' and 5'-cggactccgcaaagtctaag-3'; inducible nitric oxide synthase (iNOS), 5'-ctcactgggacagcacagaa-3' and 5'-gcttgtctctgggtcct ctg-3'; cyclooxygenase (COX)-2; 5'-gctgtacaagcagtggcaaa-3' and 5'-ccccaaagatagcatctgga-3'; IL-6, 5'-agttgccttcttgggactga-3' and 5'-ttctgcaagtgcatcatcgt-3' (forward and reverse, respectively). The PCR cycling conditions were 95˚C for 15 min, followed by 40 cycles of 95˚C for 15 sec and 54˚C for 10 sec and 72˚C for 20 sec. The expression level of each gene was normalized to the GAPDH expression level. Each assay was performed in triplicate and the average was calculated.

Cell culture and cell viability assay. The murine macrophage RAW 264.7 cells were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, HyClone), 100 U/ml penicillin and 100 µg/ml streptomycin (HyClone) at 37˚C in a humidified 5% CO_2. Cell viability was determined by MTT assay. For the MTT assay, RAW 264.7 cells were seeded in a 24-well culture plate at a density of $4x10^4$ cells/well, cultured for 24 h in the growth media and then treated with or without betaine for the indicated concentrations. The cells were incubated with 0.5 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma-Aldrich) at 37˚C for 2 h. The formazan granules generated by the live cells were dissolved in DMSO and the absorbance at 540 nm was monitored by using a multi-well reader (Thermo Fisher Scientific).

Western blot analysis. The cells were treated under the appropriate conditions, harvested and washed with cold PBS and

were lysed in lysis buffer [40 mM Tris (pH 8.0), 120 mM NaCl, 0.5% NP-40, 0.1 mM sodium orthovanadate, 2 μg/ml aprotinin, 2 μg/ml leupeptin and 100 μg/ml phenymethylsulfonyl fluoride (PMSF)]. The supernatant was collected and protein concentrations were measured (Bio-Rad, Hercules, CA, USA). Protein extracts were denatured by boiling at 100˚C for 5 min in sample buffer [0.5 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.1% bromophenol blue, 10% β-mercaptoethanol]. Equal amount of the total proteins were subjected to 6-15% SDS-PAGE and transferred to PVDF. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline with Tween-20 buffer (TBS-T) (20 mM Tris, 100 mM NaCl, pH 7.5 and 0.1% Tween-20) for 1 h at room temperature. Then, the membranes were incubated overnight at 4˚C with the primary antibodies. The membranes were washed 4 times for 10 min with TBS-T buffer and then incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The membranes were washed again for 10 min with TBS-T buffer. Antigen-antibody complexes were detected by the enhanced chemiluminescence (ECL) detection system (GE Healthcare Biosciences, Pittsburgh, PA, USA).

Statistical analysis. Measurements of cell viability of RAW 264.7 cells and multiplicity of colonic lesions were analyzed using Student's t-test. The statistical analysis of mRNA expression was performed by the Kruskal-Wallis test. P-value of <0.05 was considered to be statistically significant.

Results

Betaine inhibits AOM/DSS-induced colitis-associated tumorigenesis. The AOM/DSS model is a widely used inflammation-associated colon cancer model in rodents (18-22). In the present study, antitumor effect of dietary administration of betaine was evaluated in AOM/DSS-induced tumorigenesis model. The study protocol is summarized in Fig. 1A. During the experiments, feeding the mice with the different doses of betaine did not produce any observable clinical toxicity or significant changes in body weight compared to control (Fig. 1B). There were no relative changes in colon length between AOM/DSS-induced tumorigenesis groups and betaine-containing diets groups (Fig. 1C and D). Although slight changes were found in colon length, other positive effects such as tumor incidence and inflammatory cytokines were observed on betaine-treated groups with AOM/DSS-induced tumorigenesis.

Macroscopically, the AOM/DSS model resulted in 100% incidence of colonic tumors, which were most frequently observed in the middle and distal colon (Fig. 1C, group 2, arrow). The colonic tumors developed in the mice of groups 2 through 5 with different incidence rate and multiplicity (Fig. 2A). AOM/DSS group (group 2) had mainly adenocarcinoma (ADC) with a multiplicity of 9.67±5.03. The incidence of ADC in betaine fed (groups 3-5) was less than that of group 2 and the multiplicity of ADC in groups 3, 4 and 5 is 7.03±2.69, 5.25±2.24 and 3.48±1.41, respectively. The multiplicity of colonic ADC in groups 3, 4 and 5 was significantly smaller than group 2 ($p<0.01$) (Fig. 2A). In H&E staining, group 2 (AOM/DSS group) animals showed increased high grade dysplasia, colonic adenoma and tissue inflammation, but administration of betaine resulted in reduction of all of these phenomena in groups 3-5 compared to group 2 dose-dependently (Fig. 2B).

Betaine suppresses inflammatory mediators. Expression of inflammation-associated genes was further confirmed in colonic mucosa by using real-time PCR. AOM/DSS upregulated the mRNA expression of pro-inflammatory genes such as TNF-α, IL-6, iNOS and COX-2, compared with group 1 (control group). In contrast, when betaine was given to mice, mRNA expression of TNF-α, IL-6, iNOS and COX-2 was decreased when compared with the group 2 (AOM/DSS group) (Fig. 3). The expression level of TNF-α and iNOS was decreased 2.5- and 5.25-fold, respectively, compared to group 2. These results suggest that administration of betaine could reduce inflammation, which may contribute, at least in part, the anticancer effect in AOM/DSS-induced colon tumorigenesis.

Betaine inhibits RS generation and GSSG concentration. Previous studies have shown that betaine suppressed aging-related inflammatory status through inhibition of RS generation and modulation of glutathione (13-15). Thus, we determined the effect of betaine on AOM/DSS-induced RS generation in colonic mucosa using DCF-DA, which is oxidized by RS to fluorescence DCF. As shown in Fig. 4A, the RS formation increased due to AOM/DSS group (group 2). In contrast, betaine-treated groups (groups 3-5) showed decreased RS generation in colonic mucosa. Glutathione is a key intracellular thiol composed of glutamic acid, cysteine, and glycine. Glutathione protects cells from free radical damage by acting as an antioxidant. Within cells, glutathione exists in reduced (GSH) and oxidized (GSSG) states. In healthy cell and tissue, more than 90% of the total glutathione pool is in the reduced form (GSH) while less than 10% exists in the disulfide form (GSSG). Thus, GSH depletion can cause redox imbalance through increased oxidative stress (23). To confirm whether betaine can modulate the glutathione levels, we measured GSSG concentration in the homogenate from colonic mucosa. AOM/DSS (group 2) was increased in GSSG concentration compared to group 1 (control group). However, betaine-treated groups (groups 3-5) showed decreased GSSG concentration compared to group 2 (Fig. 4B). Taken together, in part, betaine can overcome oxidative stress including AOM/DSS-induced tumorigenesis through modulation of RS and glutathione levels.

Betaine inhibits LPS-induced pro-inflammatory gene expression in RAW 264.7 cells. After evaluation of anti-inflammatory activity of betaine in the AOM/DSS-induced mouse colon cancer model, we examined its effect on expression of pro-inflammatory genes in macrophages upon stimulation with lipopolysaccharide (LPS), which is one of the most potent pro-inflammatory agonists for monocytes and macrophages. Betaine pretreatment down-regulated LPS-induced expression of p50, p65, iNOS and COX-2 protein levels in murine macrophage RAW 264.7 cells (Fig. 5). No cytotoxicity was observed under the experimental condition (data not shown). These data suggested that betaine may regulate the macrophage functions in AOM/DSS-induced mouse colon cancer, thereby decreasing pro-inflammatory cytokine productions.

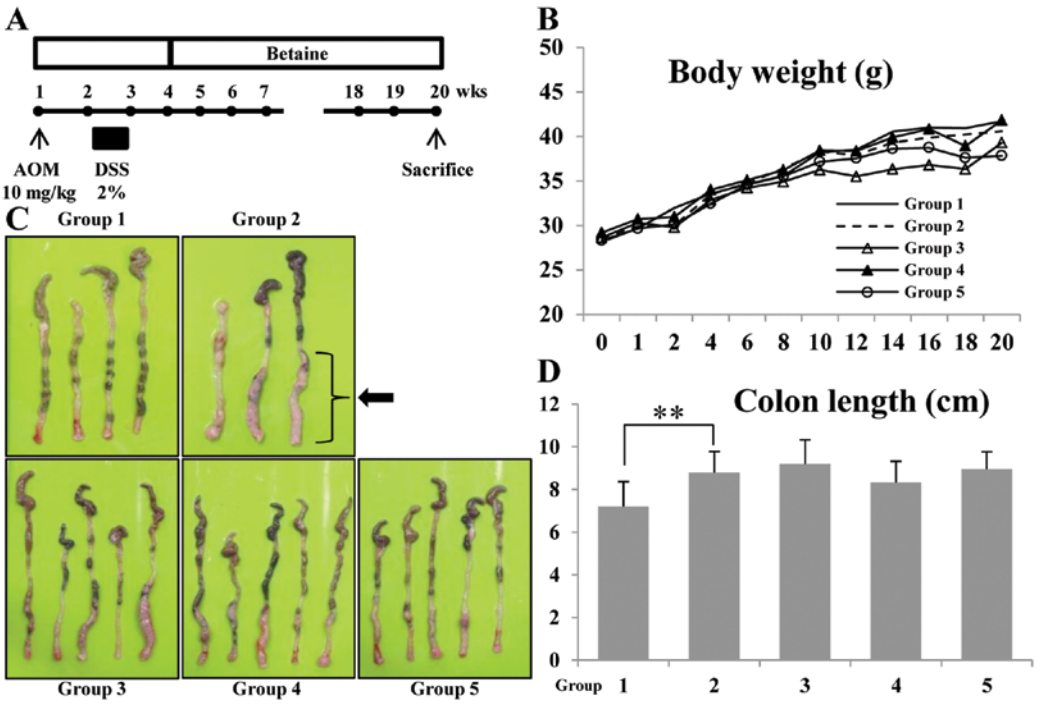

Figure 1. Administration of betaine suppressed AOM/DSS-induced colon carcinogenesis. (A) Experimental protocols. (B) Body weight changes in mice. (C) Representative macroscopic views of the colon of mice. (D) Comparison of the colon length at 16 week in control colon (group 1, control), AOM/DSS-treated mice (group 2, the arrow indicates colonic tumors), and AOM/DSS with different doses (1, 5 and 10 mg/kg in diet) of betaine (groups 3-5). $^{**}p<0.01$ compared with the AOM/DSS treatment.

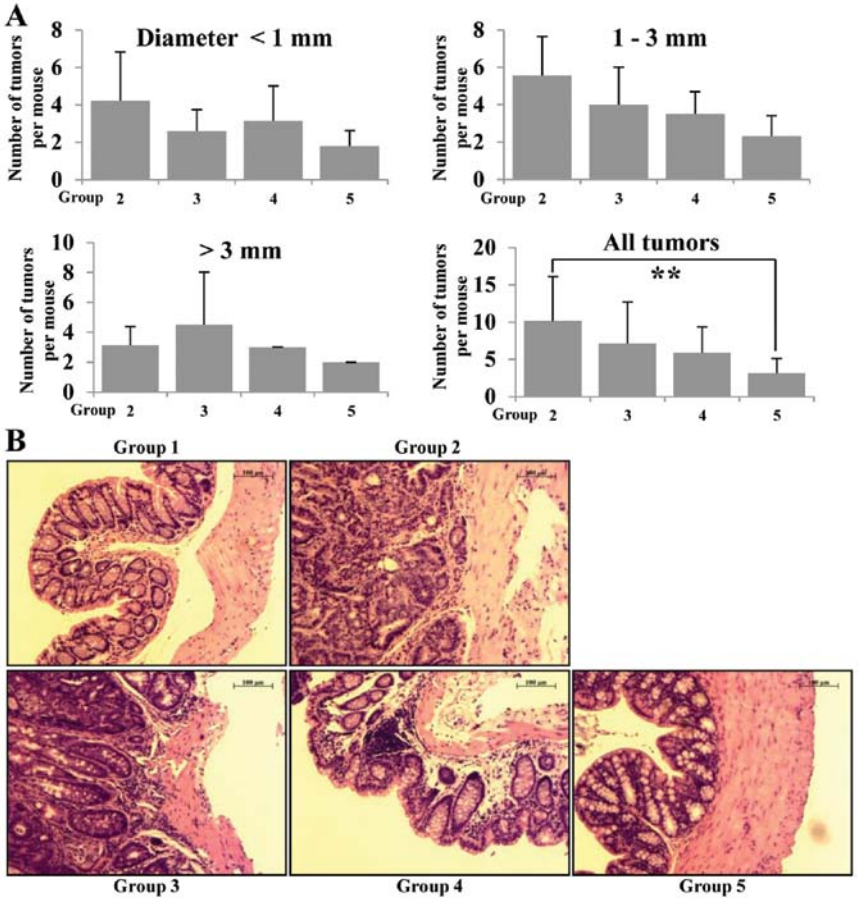

Figure 2. Betaine-containing diets decreased AOM/DSS-induced tumor incidence with inflammation. (A) Incidence and size of colitis-associated colon tumors. $^{**}p<0.01$ compared with the AOM/DSS treatment (group 2). (B) Representative histopathological images of control colon (group 1), colonic proliferation lesions developed in mice that received AOM/DSS (group 2) and AOM/DSS with different doses of betaine (groups 3-5). H&E stain. Bar, 100 μm.

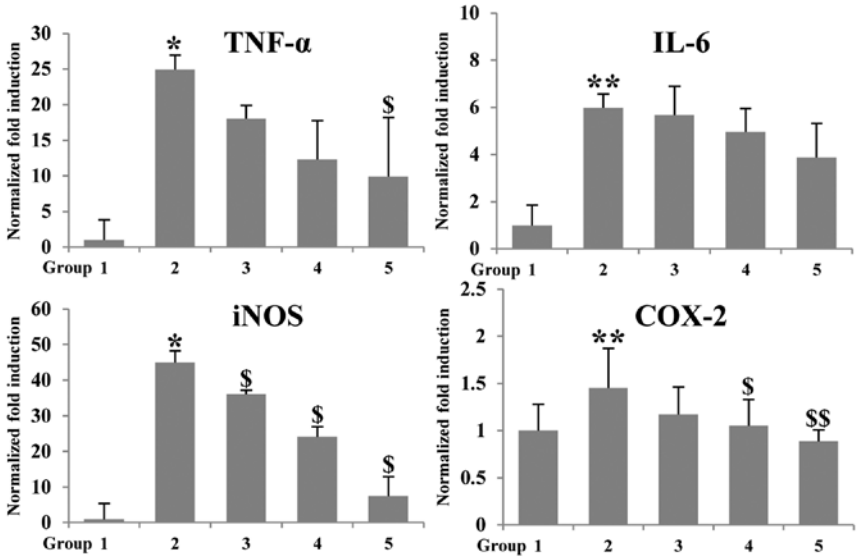

Figure 3. Treatment with betaine inhibited AOM/DSS-induced inflammatory-related cytokines in colonic mucosa. Total RNA was extracted from colonic mucosa from control (group 1), AOM/DSS (group 2) and AOM/DSS followed by various doses of betaine fed mice (groups 3-5). Real-time PCR analyses were carried out by using specific gene primers. $^{*}p<0.05$, $^{**}p<0.01$ versus the control group. $^{\$}p<0.05$, $^{\$\$}p<0.01$ versus the AOM/DSS group.

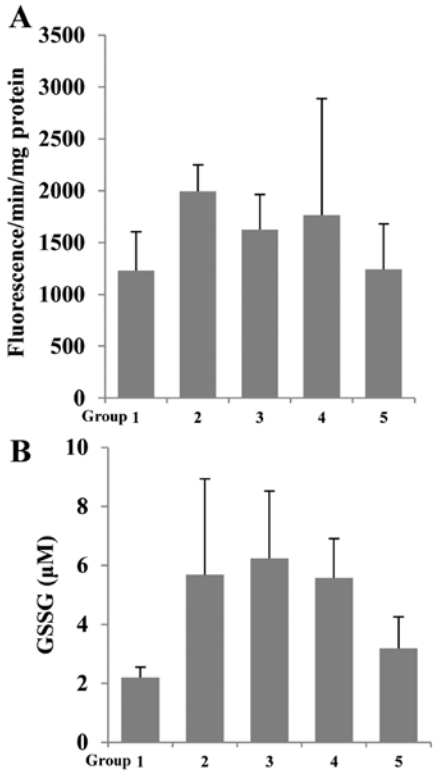

Figure 4. Treatment with betaine inhibits AOM/DSS-induced RS generation and GSSG levels. (A) The DCF-DA method was used to determine the effect of betaine on AOM/DSS-induced RS generation in colonic mucosa homogenates. Each value is the mean ± SD of five mice (n=5). (B) The GSSG levels in colonic mucosa homogenates were determined as described in Materials and methods. Each value is the mean ± SD of five mice (n=5).

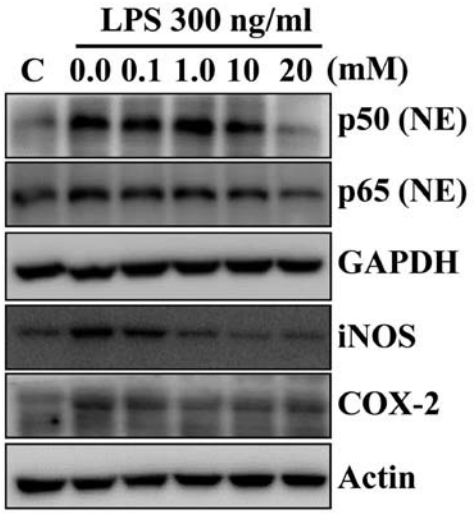

Figure 5. Treatment with betaine inhibited LPS-induced proinflammatory gene expression in murine macrophage RAW 264.7 cells. The cells were pretreated with betaine for 2 h, and then treated LPS for 24 h. Western blot analysis was performed to detect NF-κB p50, NF-κB p65, COX-2 and iNOS in RAW 264.7 cells. Representative results from three independent experiments are shown. GAPDH and actin were used as a loading control. C, control.

Discussion

Inflammation is an important tumor promoter, and several cytokines including TNF-α, IL-6, induced by inflammation can promote tumor growth (18,24). Many proto-oncogenes and carcinogens cause activation of NF-κB, whereas chemicals with known chemopreventive properties can suppress NF-κB activation (25). Previous studies showed treatment with betaine inhibited NF-κB activation through modulation of ROS and thiol homeostasis during aging process *in vitro* and *in vivo* (13-15). In this study, we demonstrated that three doses of betaine administration (1, 5, and 10 mg/kg in diet) inhibited colitis-associated colon tumorigenesis in ICR male mice.

Choline and its oxidation product betaine are nutrients involved in one-carbon metabolism (26). Betaine is a nutrient

abundant in animal foods, especially seafoods, and plant foods including wheat bran and spinach (27). Betaine can donate a methyl group to homocysteine to methionine. It serves as an osmolyte that regulates cell volume and protect cells and protein from environmental stresses including ionic stress (28). Methylation of homocyteine by betaine is confined to the liver and the kidney, but the pathway involving folate exists in all body cells (29). Betaine has been reported as a nutrient preventing inflammatory processes by blocking the expression of pro-inflammatory genes as a consequence of suppressing the NF-κB activation in aging process (13). It also suppresses the production of RS and modulation of GSH levels (14). So far, there are limited studies on intake of choline and betaine and cancer risk in humans, because food composition data were not available until recently (30). Several epidemiologic studies have examined the association between dietary intake of choline and betaine and cancer risk. Especially, higher betaine intake, may be protective against lung cancer through mitigating the adverse effect of smoking (31). In the current study, we found that administration of betaine inhibited tumor incidence with inflammation in AOM/DSS-induced colon tumorigenesis in ICR male mice (Fig. 2A). During the experiments, betaine-containing diets did not show any cytotoxicity regarding body weight or food intake. Unfortunately, we did not observe difference of colon length compared to AOM/DSS group (group 2) and betaine-treated groups (groups 3-5). However, other factors including gene expression of pro-inflammatory mediators, oxidative stress status (e.g., RS generation and GSSG concentration), and H&E staining results, were affected by betaine treatment. The qPCR data showed that AOM/DSS-induced inflammatory cytokines including TNF-α, IL-6, COX-2 and iNOS were inhibited by betaine treatment in colonic mucosa (Fig. 3). Furthermore, H&E staining data showed administration of betaine (groups 3-5) decreased AOM/DSS-induced inflammatory-related damage in colonic mucosa compared to group 2 (Fig. 2B).

Redox homeostasis plays a critical role in the protection of cells from both internal and external oxidative and other forms of stress, and it maintains the regulatory role of redox-sensitive transcription factors including NF-κB (32,33). A previous study showed ROS to play an important role in cancer development, both in the initiation and promotion stages of carcinogenesis (34). In the multi-step process of colon carcinogenesis, ROS were also found to enhance colon carcinogenesis at all stages (35). It has been reported that carcinogenic metals, such as As(III) and/or Cr(VI), in drinking water promoted tumorigenesis in murine AOM/DSS colitis-associated colorectal cancer model through modulation of the redox status. Importantly, ROS-mediated β-catenin activation by carcinogenic metals, As(III) and/or Cr(VI), may play an important role in this promotion effect (36). Betaine has been reported to prevent lysophosphatidylcholine-triggered RS generation and NF-κB activation in endothelial cells (15). In addition, a previous study reported that dietary betaine supplementation was capable of restoring the redox balance by maintaining thiol homeostasis, thereby suppressing pro-inflammatory NF-κB activation during aging (14). Betaine-treated groups showed inhibition of AOM/DSS-induced RS generation and GSSG levels in colonic mucosa (Fig. 4). Therefore, in part, betaine can reduce oxidative stress by modulation of GSSG levels in various types of stress.

LPS is an endotoxin released by gram-negative bacteria that can be transferred to cluster of differentiation 14 by LPS-binding protein and recognized by Toll-like receptor 4 on the cellular surface of macrophages (37). LPS triggers the translocation of NF-κB lead to the expression of NF-κB-regulated genes including TNF-α, IL-6, COX-2 and iNOS in murine macrophage RAW 264.7 cells (38). Our results demonstrated that betaine treatment inhibited the LPS-induced TNF-α, IL-6, COX-2 and iNOS in RAW 264.7 cells (Fig. 5).

In conclusion, administration with betaine effectively suppressed AOM/DSS-induced mouse colon tumor incidence with inflammation by suppressing the expression of cytokines, such as TNF-α, IL-6, COX-2 and iNOS. In addition, treatment with betaine decreased RS generation and modulation of total glutathione concentration. Collectively, betaine is a candidate cancer chemopreventive agent against cancer development in inflammation-associated colon tumorigenesis.

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