Protection from inflammatory bowel disease and colitis-associated carcinogenesis with 4-vinyl-2,6-dimethoxyphenol (canolol) involves suppression of oxidative stress and inflammatory cytokines

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Oxidative stress is associated with various pathological processes including inflammatory bowel disease, which is a major cause of colon cancer. Here, we examined the antioxidative and anti-inflammatory effects of 4-vinyl-2,6-dimethoxyphenol (canolol), a potent antioxidant compound obtained from crude canola oil. Oral administration of 2% dextran sulfate sodium (DSS) resulted in the progression of colitis with shortening of the large bowel length. Administering a diet containing canolol significantly suppressed pathogenesis; diarrhea markedly improved and the length of large bowel returned to almost normal. Pathological examination clearly revealed improvement of colonic ulcers. Production of inflammatory cytokines, i.e. interleukin-12 and tumor necrosis factor-α, was significantly increased during this pathological process; their production was markedly inhibited by canolol. In the azoxymethane/DSS-induced colon cancer model, mice receiving canolol had a reduced occurrence of cancer, to 60%, compared with control mice, 100% of which had colon cancer. The numbers of tumors in each mouse were also significantly reduced in mice receiving the canolol-containing diet (5.6+2.0) compared with azoxymethane/DSS control mice (10.8±4.2). No apparent toxicity of canolol was observed. Moreover, inflammatory cytokines (i.e. cyclooxygenase-2, inducible nitric oxide synthase and tumor necrosis factor-α) and oxidative responding molecules, i.e. heme oxygenase-1, in colon were suppressed during this treatment. In a mouse colon 26 solid tumor model, canolol significantly suppressed cyclooxygenase-2 expression; however, no significant tumor growth inhibition was observed, suggesting that canolol preferably shows chemopreventive effects during the stages of initiation/promotion. Canolol may, thus, be considered a potential cancer preventive agent or supplement.

Abbreviations: 8-OHdG, 8-hydroxydeoxyguanosine; AOM, azoxymethane; BHT, butylated hydroxytoluene; COX-2, cyclooxygenase-2; DI, disease activity index; DSS, dextran sulfate sodium; ELISA, enzyme-linked immuno-sorbent assay; HO-1, heme oxygenase-1; IB, inflammatory bowel disease; IL-12, interleukin-12; INOS; inducible NO synthase; LPS, lipopolysaccharide; NO, nitric oxide; ROS, reactive oxygen species; SIN-1, 3-(4-morpholino)hydroxynonimine hydrochloride; TNF-α, tumor necrosis factor-α.

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Introduction

Inflammatory bowel disease (IBD) comprises a group of common diseases that manifest chronic inflammation of the colon and small intestine (1–3). The major types of IBD are Crohn’s disease and ulcerative colitis. Although IBD itself is rarely fatal, it can greatly diminish the quality of life because of pain, vomiting, diarrhea and other socially unacceptable symptoms. More important, patients with IBD commonly have an increased risk of colorectal cancer, i.e. the risk of colon cancer in patients with ulcerative colitis begins to rise significantly above that of the general population approximately 8–10 years after diagnosis (1–4).

At present, a common therapeutic modality for IBD is use of anti-inflammatory agents, including sulfasalazine (Salazopyrin) and acetylsalicylic acid, steroid hormone and other immunosuppressive agents. Most of these treatments are symptomatic and palliative because the etiology of the disease is not yet established. As a result, the disease persists for a long time. Therefore, a therapeutic/preventive strategy that is based on the mechanism of IBD is an urgent necessity. Although the exact cause of IBD must be determined, dysfunctional immunoregulation is thought to be the primary reason (1–4). Genetic, infectious, immunological, and psychological factors have also been implicated as influencing the development of IBD. Recently it was also reported that, similar to Helicobacter pylori-induced gastritis, bacterial infection may be involved in pathogenesis of IBD, and combination therapy with antibiotics produced a significant therapeutic effect (5–8).

Another possibility concerns reactive oxygen species (ROS): high levels were produced in IBD, which suggests that ROS may be implicated in the molecular etiology of IBD (9,10). The destructive effects of ROS on DNA, proteins and lipids, because of the highly reactive nature of ROS, may contribute to initiation and propagation of the disease (6,7). The investigation of antioxidant agents may, thus, help elucidate the etiology, treatment and prevention of IBD. Indeed, many researchers proved antioxidant treatment of IBD to be effective, not only in animal experiments but also in clinical settings (9,11).

In our laboratory, we identified a potent antioxidant phenolic compound in crude canola (rapeseed) oil, 4-vinyl-2,6-dimethoxyphenol (canolol), which exhibits a more potent alkylperoxyl (ROO•) radical scavenging activity than many well-known antioxidants, such as α-tocopherol, vitamin C, β-carotene, rutin and quercetin (12). Recently canolol was also found in mustard seed oil (13). We previously reported a strong inhibitory capacity of canolol against the endogenous mutagen peroxynitrite (ONOO•), which is a potent oxidizing and nitrating agent, and suppression by canolol of bacterial mutation, via protection from DNA damage (14,15). In related studies, we demonstrated a protective effect of canolol against gastritis and gastric ulcers and a preventive effect on gastric carcinogenesis in the H.pylori-infected, carcinogen-treated Mongolian gerbil, which is an excellent animal model of H.pylori-induced, chronic active gastritis similar to IBD and involving ROS (16). Addition of dextran sulfate sodium (DSS) to the drinking water of mice induced acute colitis characterized by bloody diarrhea, ulceration and inflammatory infiltration of leukocytes in the colon, as a result of toxicity to gut epithelial cells and distortion of the integrity of the mucosal barrier (17). The DSS-induced colitis model, which we used in this study, is commonly utilized as a model of inflammatory colitis (5,6). Application of azoxymethane (AOM) together with DSS produces a model of chronic colitis and colitis-associated colon carcinogenesis (18). The purpose of our present study was to evaluate the effectiveness of canolol for inhibition of IBD and
coliitis-associated carcinogenesis using a DSS-induced mouse colitis model and AOM/DSS-induced colin carcinogenesis in mice, respectively. We also investigated the effect of canolol on oxidative stress and inflammatory cytokines during development of colitis and colon carcinogenesis. The toxicity of canolol and its effect on a mouse colon 26 solid tumor model were also examined.

Material and Methods

Chemicals

Canolol (molecular weight, 180 Da), with >95% purity, was synthesized by Junsei Chemical Co., Ltd. (Tokyo, Japan). Antioxidant 2,6-di-t-butyl-4-methylphenol (butylated hydroxytoluene (BHT), Sigma, St Louis, MO) was added to canolol solution (in ethanol) at the concentration of 300 ppm. BHT at this concentration had no significant therapeutic effect on colitits and colon cancer prevention (16). The preparation in solid form or solution was sealed under helium or nitrogen, and stock solution in ethanol was kept at –80°C. DSS was purchased from Wako Pure Chemical (Osaka, Japan), and AOM was from Sigma. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide was purchased from Dojindo Chemical Laboratory (Kumamoto, Japan).

Diets

The AIN93G diet containing canolol was used in this study with some modifications. Components of the modified AIN93G diet are as follows (g/kg): corn starch, 397; casein, 200; α-corn starch, 132; sucrose, 100; soybean oil, 70; cellulose, 50; AIN93G mineral mixture, 35; AIN93G vitamin mixture, 10; L-cystine, 3.0; choline bitartrate, 2.5; and BHT, 0.014. α-Cystine and BHT were purchased from Sigma; other components were from Oriental Yeast Co., Ltd (Tokyo, Japan). Canolol was first dissolved in soybean oil and then mixed into the diet to the concentration of 0.1 or 0.3%. The control diet contained the same components but no canolol. The diets were sealed under vacuum and were stored at –30°C; they were given daily after being thawed. Each day, leftovers from the previous day’s feeding were measured, and new food was provided to replace the amount eaten.

Cell culture

Human embryonic kidney cells HEK293 and human colon cancer cells Caco-2 were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA), and mouse colon cancer cells colon 26 were cultured in Roswell Park Memorial Institute 1640 medium (Invitrogen), at 37°C in an atmosphere of 5% CO2/95% air.

Animals and experimental protocol

Female ICR mice, 6 weeks old and weighing 20 to 25 g, and female BALB/c mice, 8 weeks old, were obtained from Kyudo (Tosu city, Saga, Japan). All animals were maintained under standard conditions and were fed water and murine chow ad libitum. All experiments were carried out according to the Guidelines of the Laboratory Protocol of Animal Handling, Sojo University, and were approved by the Animal Care Committee of Sojo University.

As to the experimental protocol for the DSS-induced colitis model, ICR mice of canolol treatment groups were fed with diet containing different concentrations of canolol during the entire experimental period (7 days). Control ICR mice were fed with the same diets but without canolol. Two hours after feeding canolol-containing diet, water containing 2% DSS was supplied to all groups except the healthy normal ICR mouse group, for entire 7 days (Supplementary Figure 1A, available at Carcinogenesis Online). Fresh diet was supplied daily, and the body weights of mice and amounts of consumed diet were determined each day. According to this protocol, symptoms indicating the severity of colitis obtained by macroscopic observation, such as characteristics of fecal pellets, diarrhea and hematochezia, were recorded. On day 7, the mice were killed, and specimens of blood, colon and liver were collected for biochemical and pathological examinations. After the length of each colon was measured, the colon specimen was fixed with 20% formalin solution and embedded in paraffin. Paraffin-embedded sections (6 μm thick) were prepared as usual for histological examination after hematoxylin and eosin staining, as well as for immunohistochemical staining as described below. Serum obtained from the blood collected was used to determine levels of tumor necrosis factor-α (TNF-α) and interleukin-12 (IL-12), as described below.

As to the experimental protocol for colon carcinogenesis in ICR mice induced by AOM/DSS, on day 1, AOM (at 10 mg/kg) dissolved in saline was administered intraperitoneally, and after 1 week, 2% DSS was given orally in the drinking water for 1 week. The diet was changed to the canolol-containing diet from 2h before AOM administration and was continued for the entire experimental period of 6 weeks (Supplementary Figure 1B, available at Carcinogenesis Online). The amount of food consumed was calculated daily. Six weeks after the AOM injection, mice were killed, and colon and liver specimens were collected. The numbers of tumors in the colon of each mouse were measured.

Evaluation of colitis severity

We evaluated the colitis severity by measuring disease activity index (DAI) semiquantitatively, by measuring colon length as an indirect marker of inflammation, and by using histology after hematoxylin and eosin staining. The DAI was determined by scoring changes in animal weight, presence of occult blood, gross bleeding and stool consistency, as described in the literature (19). We used five grades of weight loss (0: either a gain of weight or no weight loss; 1: 1% to 5% loss; 2: 5% to 10% loss; 3: 10% to 20% loss; 4: more than 20% loss), three grades of stool consistency (0: normal; 2: loose; and 4: diarrhea) and three grades of occult blood (0: negative; 2: occult blood-positive; and 4: gross bleeding). Individual mice were graded, and the mean value for each experimental group was obtained.

Further, histological evaluation of ulcer was carried out to quantitate the degree of colitis. The numbers of ulcer regions were counted in whole-colon mucosa and divided by the total length of the evaluated colon specimens. The numbers of ulcers are expressed in unit length (mm).

Effect of canolol on colon 26 transplanted tumor

The effect of canolol on tumor was further investigated in a mouse colon cancer model. Cultured colon 26 cells (2 × 10⁶) were implanted subcutaneously in the dorsal skin of Balb/c mice. Ten days after tumor inoculation, when tumor reached a diameter of 5–6 mm, canolol (dissolved in corn oil) was orally administered at the dose of 100 mg/kg (0.1 ml), and corn oil without canolol was used for control mice. Administration was carried out every second day, totally for three times. Growth of the tumors was monitored every 2–3 days by measuring tumor volume with a digital caliper, which was estimated by measuring longitudinal cross-section (L) and transverse section (W) according to the formula V = (L × W²)/2. On day 15 after the first canolol administration when tumor reached a diameter ~12–13 mm, mice were killed and tumor tissues were excised for histological examination and immunohistochemical analysis as described below.

Immunohistochemical analyses of cyclooxygenase-2

Expressions of cyclooxygenase-2 (COX-2) in colon mucosa of mice with DSS-induced colitis and in mice with AOM/DSS-induced colon carcinogenesis, and also in colon 26-implanted syngeneic solid tumor, were detected immunohistochemically as described previously (16), using a rabbit anti-mouse COX-2 polyclonal antibody (diluted 1:500, Cayman Chemical, Ann Arbor, MI) with 3,3′-diaminobenzidine (Wako Pure Chemical) for visualization. Images were analyzed with ImageJ software (National Institutes of Health, Bethesda, MD) for brown deposition of 3,3′-diaminobenzidine as COX-2 positive. One pathologist (T.T.) who was not informed about the samples examined the immunostained slides.

To quantitate the degree of staining, numbers of COX-2-positive cells were counted in whole-colon mucosa in DSS-induced colitis experiment, or counted in a distal quarter of colon mucosa, which is the target region of AOM/DSS in colon carcinogenesis experiment, and divided by the total length of the evaluated colon specimens to compare each sample equally. The numbers of COX-2-positive cells are illustrated in unit length (mm).

In the experiments using colon 26 solid tumor, three representative photographs were taken from each tumor using an AxioCam HRc digital camera and AxioVision v.4.8.2.0 software (Carl Zeiss, Oberkochen, Germany), and average positive areas in the each frame were compared between control and canolol groups.

Enzyme-linked immunosorbent assay for 8-hydroxydeoxyguanosine in the plasma of DSS-induced colitis mice with/without canolol treatment

Oxidative stress in the DSS-induced colitis mice with or without canolol treatment was examined by detecting 8-hydroxydeoxyguanosine (8-OHdG) in plasma, using an enzyme-linked immunosorbent assay (ELISA) kit (8-OHdG Check, JalCA, Fukuroi, Shizuoka, Japan). In brief, blood was drawn from the inferior vena cava after mice were killed, plasma samples were obtained by centrifugation (4°C, 5000g for 20 min) and DNA in each sample was then extracted using QuickGene DNA tissue kit (DT-S, Wako Pure Chemical). The ELISA was then performed to detect 8-OHdG according to the manufacturer’s instructions.

Effects of canolol on production of IL-12 and TNF-α in DSS-induced colitis

Serum samples from mice with DSS-induced colitis were obtained as described above, and levels of TNF-α and IL-12 were quantified by using an

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Effect of canolol on IBD and colon carcinogenesis

Inhibitory effect of canolol on activation of macrophages from the BALB/c mouse

Macrophages were obtained from the peritoneal fluid of mice stimulated with casein. In brief, 1 ml of 5% casein sodium (Wako Pure Chemical) in phosphate-buffered saline was injected intraperitoneally into BALB/c mice. After 3 days, mice were killed, and 5 ml of cold phosphate-buffered saline was injected into the peritoneal cavity, after which peritoneal lavage fluid (~5 ml) was collected, followed by centrifugation of the fluid (1000 r.p.m., 5 min) at 4°C. The macrophages were washed with phosphate-buffered saline three times by centrifugation, and then 15 ml of Roswell Park Memorial Institute 1640 medium (Invitrogen) with 10% FBS was added and macrophages were cultured in a plastic petri dish (100 x 26 mm; Nunc A/S, Roskilde, Denmark).

To activate the macrophages in culture, lipopolysaccharide (LPS) (1.0 μg/ml) and interferon-γ (0.1 μg/ml) (Sigma) were added to the cells for 24 h. Culture medium was then collected for measurement of the concentration of nitrite, which is formed from nitric oxide (NO). A significantly high amount of NO was generated by activated macrophages, which was attributable to the action of inducible NO synthase (iNOS). The nitrite concentration was quantified by using a Griess reagent kit (NO₂⁻/NO₃⁻, Assay Kit-CII; Dojindo Laboratories), according to the manufacturer’s instructions. The production of inflammatory cytokines, i.e. TNF-α and IL-12, was also measured in culture media by using ELISA as mentioned above.

Protective effect of canolol against ONOO⁻-induced cytotoxicity

HEK293 cells were plated at 3000 cells/well in a 96-well plate (Nunc A/S). After overnight preincubation, 1 mM or 2 mM 3-(4-morpholinyl)sydnonimine hydrochloride [SIN-1 (Dojindo Laboratories)], from which ONOO⁻ was produced, was added to the cells. Canolol at various concentrations was then added. After an additional 48 h of incubation, cell viability was determined by using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay.

Expression of COX-2, TNF-α, iNOS and heme oxygenase-1 in colon tissues of AOM/DSS-induced carcinogenesis mice with/without feeding canolol

To examine the antioxidant, anti-inflammatory mechanisms of canolol in chemoprevention against AOM/DSS-induced colon carcinogenesis, mRNA expressions of representative oxidative inflammatory molecules [i.e. COX-2, TNF-α, heme oxygenase-1 (HO-1) and iNOS] were detected by reverse transcription–polymerase chain reaction. Briefly, after the protocol of AOM/DSS-induced carcinogenesis, total RNA from colon tissues of distal quarter in which most tumors were observed was extracted by using Sepasol-RNA I Super reagent (NACALAI TESQUE, Kyoto, Japan), according to the manufacturer’s instruction. The nucleotide sequences of the oligonucleotide primers and cycle conditions of PCR are as follows: COX-2: forward, 5′-ACA CCT ACT ATC ACT GGC ACC-3′; reverse, 5′-TTC AGG AAG GAG CGT TTG C-3′; 35 cycles of 15 s at 94°C, 15 s at 55°C and 1 min at 72°C to obtain a 274-bp cDNA; TNF-α: forward, 5′-CTA TGT AGC CGT TCT TTC TC-3′; reverse, 5′-CAG CCT TGT CCC TTG AAG AG-3′; 40 cycles of 15 s at 94°C, 30 s at 56°C and 30 s at 68°C to obtain a 353-bp cDNA; HO-1: forward, 5′-GCC CCT GGA AGA GAT AG-3′; reverse, 5′-GCT GGA TGT TTT GGT G-3′; 30 cycles of 30 s at 94°C, 30 s at 56°C and 30 s at 72°C to obtain a 888-bp cDNA; iNOS: forward, 5′-CCC TTC CGA AGT TTC TGG CAG CAG C-3′; reverse, 5′-GCG TGT CAG AGC TCG GTG CCT TTG G-3′; 35 cycles of 1 min at 95°C, 1 min at 65°C and 2 min at 72°C to obtain a 406-bp cDNA; GAPDH (inner control): forward, 5′-CAT GTG GCC CAT GAC GTG CAC CAC-3′; reverse, 5′-TGA AGG TCG GAG TCA AGG GAT GT-3′; 30 cycles of 1 min at 94°C, 1 min at 56°C and 1 min at 72°C to obtain a 983-bp cDNA. PCR products then underwent electrophoresis on ethidium bromide-stained 1.2% agarose gels.

Safety of canolol

Female ICR mice, beginning 6 weeks of age, were fed normal diet (untreated control) or 0.3% canolol for 6 weeks. Then, mice were killed, and blood samples were obtained. Red blood cell count, white blood cell count and hemoglobin levels were determined using an automated blood counter (F-800 Microcell Counter, Toa Medical Electronics, Kobe, Japan). Plasma obtained by centrifugation was used for measurement of the liver and the kidney functions including alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, blood urea nitrogen and total creatine using a sequential multiple Auto Analyzer system (Hitachi Ltd., Tokyo, Japan).

Statistical analyses

Data were analyzed by one-way analysis of variance followed by the Bonferroni t-test. Some studies with two experiments were analyzed by Mann–Whitney U-test, and a Fisher’s exact test was used to analyze the data of tumor incidence. A difference was considered statistically significant when P < 0.05.

Results

Protective effect of canolol against DSS-induced colitis

Severe diarrhea accompanied by hematochezia, characterized by significant increase in DAI, was observed on day 7 in the DSS-induced colitis group without canolol; these DSS-treated mice showed a decrease in body weight though no statistical significance was found (Table I). These symptoms were markedly improved with significantly decreased DAI when canolol was added to the diet in a dose-dependent manner, and these mice showed no apparent loss of body weight (Table I). Moreover, mice with DSS-induced colitis demonstrated shortening of the large bowel, which is one of the indexes of colitis, and this pathological change was significantly improved by canolol dose-dependently (Table I). No significant differences in DAI and in length of large bowel were observed in mice receiving 1% of canolol compared with normal mice (Table I), suggesting an almost complete cure of colitis. However, 1% of canolol becomes impracticable as a chemopreventive agent or supplement especially for long-term application, i.e. canolol

Table I. Protective effect of canolol against DSS-induced colitis (on day 7) and AOM/DSS-induced colon carcinogenesis (at 6 weeks)

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>Liver weight (g)</th>
<th>Length of large bowel (cm)</th>
<th>DAIa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>29.7 ± 2.0</td>
<td>3.1 ± 0.7</td>
<td>15.0 ± 1.5**</td>
<td>0</td>
</tr>
<tr>
<td>DSS</td>
<td>26.9 ± 4.4</td>
<td>2.4 ± 0.6</td>
<td>9.6 ± 2.1</td>
<td>9.6 ± 0.3</td>
</tr>
<tr>
<td>DSS + 0.1% canolol</td>
<td>27.6 ± 2.2</td>
<td>2.8 ± 0.5</td>
<td>12.8 ± 1.2*</td>
<td>5.2 ± 0.7**</td>
</tr>
<tr>
<td>DSS + 0.3% canolol</td>
<td>28.3 ± 3.3</td>
<td>2.7 ± 0.5</td>
<td>13.3 ± 2.1**</td>
<td>3.7 ± 0.6**</td>
</tr>
<tr>
<td>DSS + 1% canolol</td>
<td>29.3 ± 1.5</td>
<td>2.9 ± 0.1</td>
<td>13.7 ± 1.7***</td>
<td>1.2 ± 1.8***</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>Liver weight (g)</th>
<th>Length of large bowel (cm)b</th>
<th>Incidence and multiplicity of tumors</th>
<th>Tumor incidence (%)b</th>
<th>Tumor multiplicity (no. of tumors/animal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>48.0 ± 4.7</td>
<td>2.5 ± 0.4</td>
<td>13.0 ± 0.9**</td>
<td>0**</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>AOM/DSS</td>
<td>44.0 ± 3.3</td>
<td>2.0 ± 0.2</td>
<td>8.7 ± 0.6</td>
<td>100</td>
<td>10.8 ± 4.2</td>
<td></td>
</tr>
<tr>
<td>AOM/DSS + 0.1% canolol</td>
<td>44.6 ± 6.1</td>
<td>2.1 ± 0.1</td>
<td>10.9 ± 0.9*</td>
<td>60**</td>
<td>5.3 ± 2.7*</td>
<td></td>
</tr>
<tr>
<td>AOM/DSS + 0.3% canolol</td>
<td>44.7 ± 1.9</td>
<td>2.0 ± 0.1</td>
<td>10.7 ± 0.3</td>
<td>57**</td>
<td>5.6 ± 2.7*</td>
<td></td>
</tr>
</tbody>
</table>

Data are means ± SD, n = 5–14 for DSS-induced colitis experiments, and n = 10–20 for AOM/DSS-induced colon carcinogenesis experiments.

*See text for details.

Statistical significance was analyzed by Fisher’s exact test.

*P < 0.05, **P < 0.01, versus the DSS control group, or versus the AOM/DSS control group.

No significant difference (P > 0.05), versus normal group.
needs to be given more than 10 g a day in humans. Canolol at higher than 0.3% was not pursued in the carcinogenesis study and was not subjected for further investigations, i.e. pathological studies and examination of inflammatory cytokines.

Regarding the histopathology of the colon, as Figure 1 shows, the canolol-treated groups (Figure 1C and D) had much less tissue damage compared with the DSS control (Figure 1B) showing severe inflammation and erosion. Inflammation looks more alleviated in the higher dose (0.3%) group (Figure 1D) resembling normal mucosa (Figure 1A) than in the lower dose (0.1%) group (Figure 1C). After 7 days of consumption of the canolol diet, mice in both canolol groups showed significantly suppressed formation of ulcers in the colonic mucosa compared with the DSS-induced colitis group without canolol (Figure 1E).

Canolol exhibits anti-inflammatory and antioxidative activity in DSS-induced colitis

COX-2-specific immunostaining confirmed that DSS-induced colitis clearly associated with colon inflammation (Figure 2A). The COX-2 expressions in DSS control mice were significantly higher than that in normal ICR mice. However, we found the scores of COX-2 in the canolol-treated groups were reduced compared with the DSS control, though no significance was observed ($P = 0.063$).

Consistent with these findings, amount of free 8-OHdG in the plasma, that is a common index for oxidative injury of DNA, was significantly increased after DSS treatment, whereas it was suppressed dose dependently by canolol; a significant difference was observed between DSS group and DSS + 0.3% canolol group (Figure 2B).

Suppression of inflammatory cytokine production in vivo by canolol treatment in the DSS-induced colitis model

The anti-inflammatory tissue protective effect of canolol was further confirmed by measuring the IL-12 and TNF-α levels, which are major cytokines involved in cell killing, in the serum of mice with DSS-induced colitis. As seen in Figure 2C and D, DSS-treated mice had significantly elevated levels of both cytokines, whereas these levels decreased after treatment with canolol in a dose-dependent manner, though no significance was observed for 0.1% canolol group compared with DSS control group. This finding is consistent with the improved symptoms and pathology of colitis as noted in Table I.

Suppression of macrophage activation and cytokine production by canolol in vitro

The effect of canolol on the progression of inflammation as manifested by macrophage activation was investigated in vitro with macrophages from BALB/c mice. Canolol, at concentrations up to 200 μM, showed no apparent cytotoxicity in macrophages and human colon cancer Caco-2 cells (Supplementary Figure 2A and B, available at Carcinogenesis Online). Activation of macrophages was induced by simultaneously adding LPS and interferon-γ, and activation was assessed by measuring the generation of NO as nitrite (Figure 3A). Under the same conditions, when canolol was added to the cells, macrophage activation was significantly inhibited in a dose-dependent manner (Figure 3A). Moreover, canolol treatment significantly suppressed generation of inflammatory cytokines (i.e. IL-12 and TNF-α) by the macrophages (Figure 3B and C). These data clearly indicate the anti-inflammatory effect of canolol.

Protective effect of canolol against ONOO−-induced cytotoxicity

Canolol is known as a compound with potent antioxidative activity, which is thought to contribute to its anti-inflammatory and cancer-preventive effects. To evaluate this, we investigated the cytoprotective effect of canolol against ONOO−, which is highly cytotoxic to many cells including bacteria (14,15,20,21). ONOO− is an endogenous product of NO plus superoxide anion radical (O2−) in inflammatory reactions (22), and it can damage DNA, RNA, proteins and other critical molecules by means of oxidation, nitration and hydroxylation (23,24). To investigate the cytotoxicity of ONOO− and the antioxidative cytoprotection of canolol, we selected a normal cell line, human embryonic kidney cells HEK293. In this in vitro system, we found that ~50–60% of cells died after treatment with ONOO−, which was supplied by means of a donor, SIN-1, at 1 mM (Figure 3D). Because of the short half-lives of SIN-1 (1–2 h in plasma) and ONOO− (2–3 s at physiological pH), the cytotoxicity of ONOO− in this in vitro culture study may be underestimated. However, the important finding is the significant inhibition of ONOO−-induced cytotoxicity by canolol. In addition, a dose-dependent effect of canolol observed in HEK293 cells and the cytotoxicity of 1 mM SIN-1 was completely inhibited by 50 μM canolol (Figure 3D). In addition, canolol itself had no apparent cytotoxicity for this cell line, at least up to 100 μM (Supplementary Figure 2C, available at Carcinogenesis Online), which suggests that canolol is safe.

Preventive effect of canolol on AOM/DSS-induced colon carcinogenesis

Inflammatory colitis is believed to be closely associated with the occurrence of colon cancer (1–4). We, thus, investigated the preventive effect of canolol in the AOM/DSS-induced colon carcinogenesis model. The results, as shown in Table I, clearly indicated the suppressive effect of canolol on the occurrence of colon cancer. Compared with AOM/DSS control mice, 100% of which had colon tumors, ~40% of canolol-treated mice did not have these tumors. In addition, the multiplicity was significantly reduced by ~50% in the canolol-treated group compared with the untreated control group (Table I). This effect showed no clear dose dependence, as 0.1 and 0.3% canolol produced similar effect.

Suppression of COX-2, TNF-α, iNOS and HO-1 expression by canolol in AOM/DSS-induced colon carcinogenesis

To investigate the chemopreventive mechanisms of canolol, we measured mRNA expression of proinflammatory cytokines, i.e. COX-2,
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Similar to the findings in DSS colitis experiments (Figure 2A and D), significant decreases of TNF-α and iNOS expression were observed (Figure 4B and C). As to COX-2, though no significance was observed (P = 0.054), apparent lowered expression was found after feeding 0.3% of canolol (Figure 4A), and further immunohistochemical staining of COX-2 in colon mucosa also showed that average number of COX-2-positive cells in unit length tended to be lower at 0.46 ± 0.31/mm in canolol group compared with 0.89 ± 0.39/mm in AOM/DSS control group, although without statistical significance (data are mean ± SD; P = 0.082, Mann–Whitney U-test) (Figure 4E).

Moreover, when we examined the expression of HO-1, a major antioxidative antiapoptotic molecule in various tumors reflecting oxidative and other cellular stresses (25), a significantly decreased expression was observed in canolol group compared with AOM/DSS control group (Figure 4D), which in part supported the antioxidative effect of canolol, i.e. higher oxidative levels in AOM/DSS group inducing higher expression of HO-1, whereas suppressed oxidative stress by canolol resulted in lower expression of HO-1.

**Effect of canolol on colon 26 solid tumor model**

To further examine the effect of canolol on tumor growth, a syngeneic mouse colon tumor model (colon 26) was used. After oral administration of canolol (100 mg/kg) for three times, COX-2 expressions in tumors were significantly lowered (Figure 4F): average area of COX-2 was 1.42 ± 0.47% in the control (no canolol) group, whereas 0.215 ± 0.072% in the group fed with 0.3% canolol (data are mean ± SD; P < 0.002, Mann–Whitney U-test). However, only a slight but no significant suppression of tumor growth was found (Supplementary Figure 3, available at Carcinogenesis Online).

**Safety of canolol**

As summarized in Table II, no significant adverse effects such as decreases in red blood cell and white blood cell counts and hemoglobin levels were found in ICR mice after feeding 0.3% canolol for 6 weeks, which is the same dose for preventing AOM/DSS-induced colon carcinogenesis. Also, no significant changes in the liver enzymes and kidney functions were found under the same conditions.

**Discussion**

In this study, we demonstrated the protective effect of canolol, a potent antioxidant that was recently isolated from canola (rapeseed) oil (12), on IBD in a DSS-induced mouse model. Oral administration of a diet containing canolol to the mice significantly reduced the symptoms and suppressed the progression of this disease, as supported by the lengthening of the large bowel (Table I), as well as by reduced severity and numbers of ulcers in the colonic mucosa (Figure 1) and lower levels of COX-2 expression and inflammatory cytokines (Figure 2A, C and D). These findings were associated with a decreased occurrence of colon carcinogenesis induced by AOM/DSS (Table I). However, though suppression of COX-2 expression by canolol was also found in colon 26 solid tumor (Figure 4F), no significant delay of tumor growth was observed (Supplementary Figure 3, available at Carcinogenesis Online).
Figure 4D, 32–34. This finding may indicate Figure 4D and Supplementary A). We described similar results < 0.01. α2+D C6–8). *< 0.05, **< 0.01. -30. However, in this study we found the 20–30), and moreover it also significantly 25–28). Moreover, these findings partly agreed 24–31 α). This notion is supported by results of the present study, in 35–36. During the process of inflammation, O 2- was 23–25). Furthermore, removal of NO by reaction with O 2- on the vascular endothelial surface causes vasoconstriction and triggers neutrophil adherence and accumulation, which will promote the pathological process of inflammation (20,21). This notion is supported by results of the present study, in which canolol treatment significantly protected cells against the toxicity of ONOO− (Figure 4D and E), and moreover it also significantly decreased the levels of 8-OHdG, one of the major indicators of oxidative stress, in DSS-induced colitis model (Figure 2B).

Moreover, it was also reported that the antioxidant and cytoprotective effect of canolol is probably partly through upregulating antioxidative molecules such as NF-E2-related factor, HO-1, catalase and glutathione S-transferase-pi via an extracellular signal-regulated kinase-mediated pathway (35). However, in this study we found the decrease of HO-1 expression in the distal quarter of the colon where tumors occurred most frequently (Figure 4D). This finding may indicate the different expression profile of HO-1 in normal tissues and tumor tissues. In normal tissues, upregulated HO-1 protects against oxidative stress and other damages, whereas many tumors highly express HO-1 to support their rapid growth and protect against various oxidative stresses (25). Moreover, these findings partly agreed with a recent report showing that HO-1 may protect healthy tissues against carcinogen-induced injury, but in already growing tumors, it seems to favor their progression toward more malignant forms (36).

available at Carcinogenesis Online). Partly consistent with these findings, canolol does not show apparent cytotoxicity against cultured cells including colon cancer cells Caco-2 (Supplementary Figure 2, available at Carcinogenesis Online). These data suggested that canolol might not exhibit chemotherapeutic/cytotoxic effect against the growing tumors, whereas it exhibited significant chemopreventive effect probably during the stages of initiation and/or promotion via its antioxidative and anti-inflammatory activities.

Part of this anti-inflammatory effect of canolol may be attributable to its antioxidative or scavenging activity against the excess ROS that are produced during inflammation. ROS are known to be involved in many diseases including inflammation, infections, ischemia/reperfusion injury, neurological disorders, Parkinson’s disease, hypertension and cancer (26–28). During the process of inflammation, O 2- is extensively produced in infiltrated neutrophils and activated macrophages by means of reduced nicotinamide adenine dinucleotide phosphate oxidase and probably even more by xanthine oxidase, which is highly expressed in inflamed tissues (27–30). We described similar results in our previous study with an influenza virus infection model (29,31) and in our more recent study with a xanthine oxidase inhibitor in a rat liver ischemia/reperfusion model (30). Excess generation of ROS was also observed in DSS-induced colitis model and could be suppressed by xanthine oxidase inhibitor (Fang,J., Yin,H.Z., Liao,L., Qin,H.B., Ueda,F., Uemura,K., Eguchi,K., Bharate,G.Y., Nakamura,H., and Maeda,H., unpublished data). O 2- is then converted to hydrogen peroxide by superoxide dismutase and/or glutathione peroxide, after which the hydrogen peroxide is converted to hydroxyl radicals in the presence of transition metals (e.g. Fe2+). A massive amount of NO is also generated by iNOS that is upregulated in activated macrophages (22,28), and NO can react rapidly with O 2- to form the more toxic species ONOO−. All of these highly reactive biological radicals readily cross cell membranes and react with proteins, DNA and lipids (23,24,32–34), which results in cell damage. Furthermore, removal of NO by reaction with O 2- on the vascular endothelial surface causes vasoconstriction and triggers neutrophil adherence and accumulation, which will promote the pathological process of inflammation (20,21). This notion is supported by results of the present study, in which canolol treatment significantly protected cells against the toxicity of ONOO− (Figure 4D and E), and moreover it also significantly decreased the levels of 8-OHdG, one of the major indicators of oxidative stress, in DSS-induced colitis model (Figure 2B).
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Taken together, the association of canolol with HO-1 in AOM/DSS colon carcinogenesis seems to be different from that in normal tissues during stresses and damages as described earlier (35); further investigations are thus warranted to make clear the mechanisms involved in the effect of canolol in different conditions.

COX-2 is the enzyme that catalyzes the conversion of arachidonic acid to prostaglandins, it is unexpressed under normal conditions in most cells, but elevated levels are found under inflammatory condition and is thus largely responsible for causing inflammation (37). Many studies revealed that the products of COX-2 prostaglandins are highly involved in the carcinogenesis of many tumors including colorectal cancer metastasis and tongue and esophageal cancers (38,39). In this study, we found a decrease in COX-2 expression (both in mRNA and protein levels) after canolol treatment, though not statistically significant, in DSS-induced colitis (Figure 2A) and in AOM/DSS-induced colon carcinogenesis (Figure 4A). These findings at least partly suggested that suppression of COX-2 expression is probably involved in the effect of canolol on DSS-induced colitis and colon carcinogenesis.

Both the present study and our previous study (16) of canolol in vivo showed suppression of a number of inflammatory mediators

Table II. Change in RBC, WBC, hemoglobin and plasma liver enzyme levels and kidney function after feeding canolol (0.3% for 6 weeks) in ICR mice

<table>
<thead>
<tr>
<th></th>
<th>RBC (10⁴/µl)</th>
<th>WBC (10²/µl)</th>
<th>Hb (g/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>992.2 ± 35.9</td>
<td>30.4 ± 4.2</td>
<td>16.0 ± 0.6</td>
</tr>
<tr>
<td>Canolol⁹</td>
<td>953.0 ± 20.2</td>
<td>28.8 ± 1.9</td>
<td>15.1 ± 0.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>BUN (mg/dl)</th>
<th>Cr (mg/dl)</th>
<th>AST (IU/l)</th>
<th>ALT (IU/l)</th>
<th>LDH (IU/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>20.7 ± 0.8</td>
<td>0.15 ± 0.02</td>
<td>67.3 ± 2.8</td>
<td>28.4 ± 2.2</td>
<td>1383.0 ± 54.7</td>
</tr>
<tr>
<td>Canolol⁹</td>
<td>21.5 ± 1.3</td>
<td>0.13 ± 0.01</td>
<td>65.1 ± 4.5</td>
<td>37.6 ± 4.2</td>
<td>1036.4 ± 149.3</td>
</tr>
</tbody>
</table>

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; Cr, creatinine; Hb, hemoglobin; LDH, lactate dehydrogenase; RBC, red blood cells; WBC, white blood cells.

⁹No significant difference was found between canolol feeding mice and normal mice in all selected indices. Values are presented as means ± SE, n = 5–8.

Canolol was administered at 0.3% (w/w) in diet. Assays were carried out at 6 weeks after feeding canolol.

Fig. 4. Suppression of COX-2 (A), TNF-α (B) and iNOS (C) and upregulation of HO-1 by canolol as evaluated by reverse transcription–polymerase chain reaction, and the decreased COX-2 expression in colon tissue of AOM/DSS-treated ICR mice (E) and colon 26 (C26) mouse tumor (F) in vivo after canolol treatment. In (A–D), two representative DNA bands of each group (control and canolol group) are shown, and the results were semiquantitatively as relative intensity compared with the intrinsic DNA expression of GAPDH as control. Immunohistochemical staining of COX-2 in colon tissue of AOM/DSS control mice (E-a) and canolol-treated mice (E-b) is shown in (E), and that in C26 tumor tissues without and with canolol treatment is shown in (F-a) and (F-b), respectively; in both cases, the COX-2-positive (brown colored) area was quantitated (E-c and F-c). See text for details. Data are means ± SD (n = 5–12). *P < 0.05, **P < 0.01 by Mann–Whitney U-test.
such as TNF-α, IL-12, IL-1β, iNOS and COX-2 (Figure 2A, C, and D, Figure 4A–C and ref. 16), which confirms that this suppression will contribute to the anti-inflammatory activity and the antioxidative effect of canolol against IBD and the subsequent colon carcinogenesis. Infiltrated neutrophils and activated macrophages are major producers of these inflammatory cytokines during inflammatory diseases including IBD (40, 41). ROS play an important initial role in both the activation of macrophages and the induction of inflammatory cytokines. Bulua et al. (42) recently reported that ROS are crucial in LPS-stimulated macrophages for inducing production of several proinflammatory cytokines through an mitogen-activated protein kinase signaling pathway, as an essential feature of innate immunity. Apoptosis signal-regulating kinase 1 is also involved in this immune response (43). Consistent with this result, we found in this study that the ROS scavenger canolol suppressed activation of macrophages stimulated by LPS and interferon-γ, as evidenced by reduced NO generation (Figure 3A) and lower levels of IL-12 and TNF-α (Figure 3B and C). Also, certain cytokines, i.e. TNF-α and IL-12, secreted by activated phagocytic cells, can enhance ROS generation (44, 45), which explains the important role of cytokines in the pathogenic process of inflammation. These findings suggested that the chemopreventive effect of canolol is mostly via its antioxidative and anti-inflammatory activity to inhibit the oxidative stress and inflammation, thus inhibiting the carcinogenesis cascades.

Canolol is extracted from crude canola oil after roasting the rape seeds and is a naturally occurring compound in this edible oil whose concentration is estimated to be ~220–1200 ppm (12), which could provide doses similar to that used in our study. The amounts of canolol administered orally in the diet in this study were 0.1% and 0.3%, or equivalent to 1–3 g/kg (dry weight) of feed for humans, which is a reasonable range for supplement diet. In our previous study, we also applied the 0.1% concentration in an *H. pylori*-induced gastric carcinogenesis model and showed a significant cancer-preventive effect (15). Because the present colon carcinogenesis prevention study revealed no dose dependency (Table I), the 0.1% canolol concentration may be the level of saturation. That is, 0.1% canolol may be sufficient to prevent colon carcinogenesis.

Furthermore, canolol showed very little cytotoxicity to cells in culture: it had no apparent toxicity in human HEK293 cells at least up to 100 μM, in human Caco-2 cells up to 200 μM (Supplementary Figure 2B and C, available at Carcinogenesis Online) or in macrophages up to 300 μM (0.054 mg/ml), as described previously (16) and in present study (Supplementary Figure 2A, available at Carcinogenesis Online), which is a far higher concentration than the concentration for effective scavenging of ROS (i.e. 1–20 μM) (12, 46). Similar results were obtained in our in vivo study. Mice receiving a diet containing canolol up to 0.3% for 6 weeks showed no apparent change in body weight (Table II) and no apparent toxicity as reflected by blood cell count and biochemistry of liver and kidney functions (Table II). This safety profile suggests that canolol has the potential to be not only a drug but also a food supplement for disease prevention.

Colon cancer is the most common type of cancer in developed countries, with the highest incidence and mortality rates (47). With regard to the mechanisms of colon carcinogenesis, genetic factors seem to play an important role, as in familial adenomatous polyposis (48). However, ROS were recently found to be one of the critical factors in colon carcinogenesis and in familial adenomatous polyposis (49). Dietary habits are known to be highly associated with the occurrence of colon cancer (50, 51). For example, oxidized oils in high-fat diets, which are a risk factor for colon cancer, generate lipid peroxyl radicals in the presence of heme or iron, damage DNA and consequently induce colon cancer (50). Also, ROS contribute to many conditions other than inflammation, such as virus infections and ischemia/reperfusion injury, as described above.

Moreover, an unhealthy diet, with a low consumption of green vegetables and thus less antioxidants, may lead to the adverse consequences of these ROS-related diseases. It should be noted that purified canola oil that is available in large supermarkets does not contain canolol because the refining process removes it (12). It should be also noted that the content of canolol increases dramatically by roasting process as used in traditional oil refining process (12). Thus, the refining process should be modified so that canolol is retained. The canolol used in this study was synthesized, so synthetic canolol may be used as a preventive agent for these diseases.

For IBD treatment, drugs commonly used in clinical settings provide symptomatic or palliative relief. Canolol treatment, however, aims more at the cause of the disease, i.e. ROS. All these data therefore suggest that canolol may be effective not only for IBD-associated colon cancer but also for ROS-dependent carcinogenesis, as described for gastric cancer involving *H. pylori* infection (16). Canolol thus holds promise as a preventive agent or supplement for both IBD and colon cancer.

**Supplementary material**

Supplementary Figures 1–3 can be found at http://carcin.oxfordjournals.org/

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**References**

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