TC-3', reverse, 5'- CAG CCT TGT CCC TTG AAG AG -3', 40 cycles of 15 sec at 94°C, 30 sec at 56°C and 30 sec at 68°C to obtain a 353 bp cDNA; HO-1, forward 5'- GGC CCT GGA AGA GGA GAT AG -3', reverse, 5'- GCT GGA TGT GCT TTT GGT G -3', 30 cycles of 30 sec at 94°C, 30 sec at 56°C and 30 sec at 72°C to obtain a 888 bp cDNA; iNOS, forward 5'- CCC TTC CGA AGT TTC TGG CAG CAG C -3', reverse, 5'- GGC TGT CAG AGC CTC GTG GCT TTG G -3', 35 cycles of I min at 95°C, 1 min at 65°C and 2 min at 72°C to obtain a 496 bp cDNA; GAPDH (inner control), forward 5'- CAT GTG GGC CAT GAG GTC CAC CAC -3', reverse, 5'- TGA AGG TCG GAG TCA ACG GAT TTG 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 of 6 weeks old was fed with 0.3% canolol for 6 weeks. Then, mice were killed and blood samples were obtained. RBC, WBC counts, and hemoglobin levels were determined by 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 values by using a sequential multiple Auto Analyzer system (Hitachi Ltd., Tokyo, Japan).

Statistical analyses

All data are expressed as means ± SEM. Student *t* test or Mann-Whitney U test was used to determine the significance between two experimental groups, and a one-way ANOVA was used

to analyze the significance among all experimental groups. A difference was considered statistically significant when $P < 0.05$.

Results

Protective effect of canolol against DSS-induced colitis

The experimental protocol described in Figure 1A resulted in severe diarrhea accompanied by hematochezia observed on day 7 in the DSS-induced colitis group without canolol; these DSS-treated mice showed a decrease in body weight (Table 1). Diarrhea was markedly improved when canolol was added to the diet, and these mice evidenced no apparent loss of body weight (Table 1).

Moreover, mice with DSS-induced colitis demonstrated shortening of the large bowel, which is an index of colitis, and this symptom significantly improved after canolol treatment (Table 1). As Figure 2 shows, the canolol-treated groups (Figure 2, C and D) had much less tissue damage compared with the no-canolol group (Figure 2B) showing severe inflammation and erosion. Inflammation looks more alleviated in the higher dose (0.3%) group (Figure 2D) resembling normal mucosa (Figure 2A) than the lower dose (0.1%) group (Figure 2C). After 7 days of consumption of the canolol diet, mice in both canolol groups evidenced significantly suppressed formation of ulcers in the colonic mucosa compared with the DSS-induced colitis group without canolol (Figure 2E).

Immunohistochemistry of COX-2

Immunoreactivity against COX-2 (Figure 3A) was evident in all DSS induced colitis mice. However, we found the scores in the canolol-treated groups were reduced compared to the

canolol-untreated groups, though no significance was observed (P = 0.063); however, a significant difference (P = 0.043) was found when one-way ANOVA was carried out (Figure 3A). Consistent with these findings, amount of free 8-OHdG in the plasma of mice that is a common index for oxidative injury of DNA, was remarkably increased after DSS treatment, whereas it was significantly suppressed when canolol was administered (Figure 3B)

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 3C, D, DSS-treated mice had significantly elevated levels of both cytokines, whereas these levels decreased markedly after treatment with canolol in a dose-dependent manner. This finding is consistent with the improved symptoms and pathology of colitis as noted in Table 1.

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 as well as human colon cancer Caco-2 cells (Supplemental data Figure S1A, B). Activation of macrophages was induced by simultaneously adding LPS and IFN-γ, and activation was assessed by measuring the generation of NO as nitrite (Figure 4A). Under the same conditions, when canolol was added to the cells, macrophage activation was significantly inhibited in a

dose-dependent manner (Figure 4A). Moreover, canolol treatment similarly suppressed generation of inflammatory cytokines (i.e., IL-12 and TNF-α) by the macrophages to a significant degree (Figures 4B 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 idea, 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 (O_2^-) 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 vivo system, we found that about 50% to 60% of cells died after treatment with $ONOO^-$, which was supplied by means of a donor, SIN-1, at 1 mM (Figure 4D). Because of the short half-lives of SIN-1 (1–2 hours in plasma) and $ONOO^-$ (2–3 seconds 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 4D). In addition, canolol itself had no apparent cytotoxicity for this cell line, at least up to 100 μM (Supplemental data Figure S1C), 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, as described in Figure 1B. The results, as shown in Table 2, clearly indicated the suppressive effect of canolol on the occurrence of colon cancer. Compared with mice that had no canolol treatment, 100% of which had colon tumors, about 60% of canolol-treated mice had these tumors. In addition, the number of tumors was significantly reduced by about 50% in the canolol-treated group compared with the untreated control group (Table 2). This effect showed no clear dose dependence, however: 0.1% and 0.3% canolol produced similar effects.

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 pro-inflammatory cytokines, i.e., COX-2, TNF-α and iNOS in AOM/DSS induced colon carcinogenesis. Similar to the findings in DSS colitis experiments (Figure 3A, D), significant decreases of TNF-α and iNOS expression were observed (Figure 5B, C). As to COX-2, though no significance was obtained ($P = 0.054$), apparent lowered expression was found after feeding 0.3% of canolol (Figure 5A), 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 0.56 ± 0.15/mm in canolol group ($P = 0.202$, Mann-Whitney U test) compared with 0.89 ± 0.17/mm in control group, although without statistical significance (supplemental data, Figure

S2).

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 control group (Figure 5D), 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 oxystress by canolol resulted in lower expression of HO-1.

Effect of canolol on colon 26 solid tumor model

To further study the chemopreventive effect of canolol, a mouse solid colon tumor model (colon 26) was used. After oral administration of canolol (100 mg/kg) for 3 times, a slight but not significant suppression of tumor growth was found (Supplemental data, Figure S3). More important, COX-2 expressions in tumors were significantly lowered by canolol treatment (Figure 5E): average area of COX-2 were 1.42 ± 0.47 % in the control group, whereas 0.215 ± 0.072 % in the 0.3% canolol fed group ($P < 0.002$, Mann-Whitney U test).

Safety of canolol

As summarized in Table 3, no significant adverse effects such as decreases in RBC and WBC counts, and hemoglobin levels were found after feeding 0.3% canolol for 6 weeks, which is the same dose for preventing AOM/DSS induced colon carcinogenesis. Also, no significant changes of 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), against IBD in a DSS-induced mouse model. Oral administration of a diet containing 0.1% or 0.3% 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 1), as well as reduced severity and numbers of ulcers in the colonic mucosa (Figure 2) and lower levels of COX-2 expression and inflammatory cytokines (Figure 3A, C, D). These findings were associated with a decreased occurrence of colon carcinogenesis induced by AOM/DSS (Table 2).

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 (I/R) 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 NADPH 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 our more recent study with a xanthine oxidase inhibitor in a rat liver I/R model (30). Excess generation of ROS was also observed in DSS-induced colitis model and could be suppressed by xanthine oxidase inhibitor (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., Fe^{2+}). 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 idea was supported by results in the present study, in which canolol treatment significantly protected cells against the toxicity of $ONOO^-$ (Figure 4D, E), moreover it also significantly decreased the levels of 8-OHdG, one of the major indicators of oxidative stress, in DSS induced colitis model (Figure 3B).

Moreover, it was also reported that the antioxidative and cytoportective 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 ERK mediated pathway (35). In this study, however, we found the decrease of HO-1 expression in AOM/DSS induced colon tumors (Figure 5D). This finding may indicate the different functions of HO-1 in normal tissues and tumor tissues. Namely, in normal tissues upregualted HO-1 serves as protection against oxystress and other damages; whereas many tumors highly express HO-1 to support their rapid growth and protect against various oxidative stresses (25). The colon tissues used in this study is distal quarter in which most tumors occurred, this difference of HO-1 expression thus in other side confirmed the difference of tumor occurrence between canolol treated group and untreated group (Table 2). Moreover, these findings partly coincided 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). Taken together, the association of canolol with HO-1 in AOM/DSS colon carcinogenesis seems to be different to 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 under different circumstances.

COX-2 is the enzyme that catalyze the conversion of arachidonic acid to prostaglandins, it is unexpressed under normal conditions in most cells, but elevated levels are found under inflammatory situation, 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, tongue and esophageal cancers (38, 39). In this study, we found a tendency of decreased COX-2 expression (both in mRNA and protein levels) after canolol treatment, though not statistically significantly, in DSS induced colitis (Figure 3A) as well as AOM/DSS induced colon carcinogenesis (Figure 5A). Moreover, treatment of colon 26 tumors with canolol significantly decreased the COX-2 expression in tumors (Figure 5E), which possibly associated with the slightly delayed tumor growth (Supplemental data, Figure S3). These findings at least partly suggested that suppression of COX-2 expression is probably be involved in the effect of canolol against 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 such as TNF-α, IL-12, IL-1β, iNOS, and COX-2 (Figure 3A, C, D, Figure 5A-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. recently reported that ROS are crucial in

LPS-stimulated macrophages for inducing production of several proinflammatory cytokines through an MAPK signaling pathway, as an essential feature of innate immunity (42). 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 IFN-γ, as evidenced by reduced NO generation (Figure 4A) and lower levels of IL-12 and TNF-α (Figure 4B and C). Also, certain cytokines, i.e., TNF-α and IL-12, secreted by activated phagocytic cells, can enhance ROS generation (44, 45), which is evidence of the important role of cytokines in the pathogenic process of inflammation.

In addition, when canolol was administered into colon 26 soild tumor bearing mice orally, no significant suppression of tumor growth was observed (supplemental data Figure S3). All these findings suggested that the chemopreventive effect of canolol is not directly via killing or suppressing already existed tumor growth, but mostly via its antioxidative, aninflammatory effect to inhibit the oxystress and inflammation, thus inhibiting the carcinogenesis cascades.

Canolol is extracted from crude canola oil after roasting the rape seed, 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 amount of canolol administered orally in the diet in this study were 0.1% and 0.3%, or equivalent to 1 to 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 dependence (Table 2), 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, or human Caco-2 cells up to 200 μM (supplemental data Figure S1B, C), or in macrophages up to 300 μM (0.054 mg/mL), as described previously (16) as well as in present study (supplemental data Figure S1A), 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 2), as well as no any apparent toxicity as reflected by blood cells count and biochemistry of liver and kidney functions (Table 3). This safety profile suggests that canolol has the potential to be not only a drug but also a food supplement for disease prevention.

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. Canolol thus holds promise as a drug for IBD treatment and a preventive agent or supplement for colon cancer.

Colon cancer is the most common type of cancer in developed countries, with incidence and mortality rates among the highest of all cancers (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 as well as 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 I/R

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 increase 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.

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Figure legends

Fig. 1. Experimental design of DSS-induced colitis (A) and AOM/DSS-induced colon carcinogenesis (B).

Fig. 2. Histological examination of the large bowel in DSS-induced colitis, with and without canolol treatment. A–D, H&E staining of colon tissue from each experimental group. The arrow indicates the ulcer (necrosis) in the colonic mucosa. E, Quantification and summary of the numbers of ulcers in each experimental group. See text for details. Data are presented as means ± SEM; $n = 6$–10. *, $P < 0.05$, **, $P < 0.01$ by Mann Whitney U test. One-way ANOVA showed a significant difference among the experimental groups ($P < 0.05$).

Fig. 3. Immunohistochemistry of COX-2 (A) in colorectal lesions, and plasma levels of 8-OHdG (B), as well as production of IL-12 (C) and TNF-α (D) in DSS-induced colitis and the protective effect of canolol. The protocols of DSS-induced colitis and canolol treatment are presented in Figure 1. At 7 days after the start of DSS administration, mice were killed and serum samples were collected for measuring IL-12 and TNF-α by means of ELISA. See text for details. Data are means ± SEM ($n = 6$–10). *, $P < 0.05$, **, $P < 0.01$ by Mann Whitney U test. One-way ANOVA showed a significant difference among the experimental groups in each figure ($P < 0.05$).

Fig. 4. Suppression of macrophage activation (A) and subsequent generation of IL-12 (B) and TNF-α (C), and cytoprotective effect against the toxicity of peroxynitrite (ONOO-) in HEK293 (D) by canolol. Mouse macrophages were obtained from BALB/c mice. Macrophage

activation, as generation of NO, was evaluated by using a Griess Reagent kit. IL-12 and TNF-α were measured via ELISA. See text for details. For cytotoxicity study, 3,000 cells/well were plated in a 96-well plate. After overnight preincubation, 1 mM SIN-1 (ONOO- donor) or 2 mM SIN-1 was added to the cells. Different concentrations of canolol were administered. After an additional 48 hours of incubation, cell viability was determined by using the MTT assay. Data are means ± SEM (n = 6–8). *, $P < 0.05$, **, $P < 0.01$ by t test. One-way ANOVA showed a significant difference among the experimental groups in each figure ($P < 0.01$).

Fig. 5. Suppression of COX-2 (A), TNF-α (B), iNOS (C) and upregulation of HO-1 by canolol as evaluated by RT-PCR, and decreased COX-2 expression in colon 26 (C26) mouse tumor in vivo after canolol treatment (E). In A-E, two representative DNA bands of each group (control and canolol group) were showed, and the results were semiquantitated as a relative intensity referred to intrinsic control GAPDH. In E, immunohistochemical staining of COX-2 in tumor tissues was showed in upper panel, and the COX-2 positive area was quantitated. See text for details. Data are means ± SEM (n = 8–12). *, $P < 0.05$, **, $P < 0.01$ by Mann Whitney U test. One-way ANOVA showed a significant difference among the experimental groups in A-D ($P <$ 0.01).