Pterostilbene, an Active Constituent of Blueberries, Suppresses Aberrant Crypt Foci Formation in the Azoxymethane-Induced Colon Carcinogenesis Model in Rats

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Abstract

Purpose: Epidemiologic studies have linked the consumption of fruits and vegetables to reduced risk of several types of cancer. Laboratory animal model studies have provided evidence that stilbenes, phenolic compounds present in grapes and blueberries, play a role in inhibiting the risk of certain cancers. Pterostilbene, a naturally occurring stilbene from blueberries, was tested for its preventive activity against colon carcinogenesis.

Experimental Design: Experiments were designed to study the inhibitory effect of pterostilbene against the formation of azoxymethane-induced colonic aberrant crypt foci (ACF) preneoplastic lesions in male F344 rats. Beginning at 7 weeks of age, rats were treated with azoxymethane (15 mg/kg body weight s.c., once weekly for 2 weeks). One day after the second azoxymethane treatment, rats were fed experimental diets containing 0 or 40 ppm of pterostilbene. At 8 weeks after the second azoxymethane treatment, all rats were sacrificed, and colons were evaluated for ACF formation and for inhibition of inducible nitric oxide synthase (iNOS) and proliferating cell nuclear antigen. Effects on mucin MUC2 were also determined.

Results: Administration of pterostilbene for 8 weeks significantly suppressed azoxymethane-induced formation of ACF (57% inhibition, $P < 0.001$) and multiple clusters of aberrant crypts (29% inhibition, $P < 0.01$). Importantly, dietary pterostilbene also suppressed azoxymethane-induced colonic cell proliferation and iNOS expression. Inhibition of iNOS expression by pterostilbene was confirmed in cultured human colon cancer cells.

Conclusions: The results of the present study suggest that pterostilbene, a compound present in blueberries, is of great interest for the prevention of colon cancer.

Stilbenes, such as resveratrol and pterostilbene, are a subset of naturally occurring phenolic compounds known to have diverse pharmacologic activities including cancer chemopreventive activity (1–4). Stilbenes have been found in some berries (e.g., blueberries, cranberries, sparkleberries, lingonberries, and grapes; ref. 5), thus, consumption of these small fruits may help improve health. It is interesting that dietary black raspberries significantly suppressed *N*-nitrosomethylbenzylamine–induced rat esophageal carcinogenesis (6). The discovery of resveratrol as a cancer-preventive agent has fostered interest in testing the cancer-preventive activity of other naturally occurring stilbenes in many laboratories. Notably, pterostilbene, a dimethylether analogue of resveratrol, was found to be as effective as resveratrol in preventing carcinogen-induced preneoplastic lesions in a mouse mammary organ culture model (2). Additionally, i.v. administration of pterostilbene to mice inhibited metastatic growth of B16M-F10 melanoma cells in the liver, a common site for metastasis development (7).

Pterostilbene and resveratrol have very similar pharmacologic properties (2, 8). In addition to the aforementioned activity in the mouse mammary organ culture model, both compounds are strong antioxidants and are also hypolipidemic (2, 8–10). Pterostilbene is used as a chemical marker for extracts of *Pterocarpus marsupium*, from which it has been previously isolated and shown to lower serum glucose in rats (11). Resveratrol has been reported to reduce the growth of colorectal aberrant crypt foci (ACF) in rats (12, 13). However, lack of data on the colon cancer–preventive activity of pterostilbene, together with its seemingly similar biological properties with that of resveratrol, prompted our investigation on the efficacy of this agent against colon carcinogenesis.

Experiments to determine the chemopreventive efficacy of pterostilbene on colon carcinogenesis were planned using ACF

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induced by azoxymethane, a colon-specific carcinogen, in a relevant laboratory animal model. ACF are recognized as early preneoplastic lesions consistently observed in experimentally induced colon carcinogenesis in laboratory animals (14). Aberrant crypts are precursor lesions from which adenoma and carcinoma develop in the colon, and these lesions have been shown to occur in the colonic mucosa of patients with colon cancer (15). There is also evidence that several inhibitors of ACF development reduce colon tumorigenesis in laboratory animals (14, 16).

Interestingly, increased aberrant expression of inflammatory genes, such as inducible nitric oxide synthase (iNOS), has been shown in the azoxymethane-induced colon cancer model from the early stage of hyperplastic ACF to late stage adenocarcinoma (17–21). Selective iNOS inhibitors have been tested in several animal models of colon cancer because increased expressions of iNOS have been shown in colon cancer models (6, 22). Many studies report that selective iNOS inhibitors exerted suppressive effects in colon cancer (17, 22–26). The effect of pterostilbene on the inhibition of iNOS has not been investigated. Because iNOS has been observed in neoplastic lesions of the colon (20), there is a rationale for testing the ability of pterostilbene to inhibit iNOS in a colon cancer model in which inflammatory genes play a key role in carcinogenesis.

The present study was designed to examine the chemopreventive efficacy of pterostilbene against colon carcinogenesis using colonic ACF as an end point. Whether pterostilbene could suppress colon carcinogenesis by exerting anti-inflammatory activity, such as an inhibitory effect on iNOS, was also investigated.

Materials and Methods

Synthesis of pterostilbene. Pterostilbene was synthesized following a published procedure with minor modifications, and its structure confirmed by UV, mass spectrometry, and nuclear magnetic resonance spectra (ref. 27; Fig. 1).

Animals and diets. Weanling male F344 rats were obtained from Charles River Breeding Laboratories (Kingston, NY). All experimental diets were purchased from Research Diets (New Brunswick, NJ) and stored at 4°C. All animals were randomly distributed by weight into control and experimental groups and housed in plastic cages with filter tops (three per cage) under controlled conditions of a 12-h light and dark cycle, 50% humidity, and 21°C temperature. Animals had access to food and water at all times. Food cups were replenished with fresh diet twice weekly.

Experimental procedure. Beginning at 5 weeks of age, all rats were fed the modified American Institute of Nutrition-76A (AIN-76A) diet. At 7 weeks of age, the animals were given s.c. injections of azoxymethane (CAS no. 25843-45-2; Ash Stevens, Detroit, MI) once weekly for 2 weeks at a dose rate of 15 mg/kg body weight. One day after the second azoxymethane injection, groups of animals (n = 9 per group) were maintained on AIN-76A diet alone and AIN-76A diet containing 40 ppm of pterostilbene. Dose selection of pterostilbene was based on our earlier study that a 25 mg pterostilbene/kg diet lowered plasma cholesterol and lipoproteins in hypercholesterolemic hamsters (9). On the average, the animal consumed ~0.6 mg pterostilbene per day. All rats were weighed once weekly until termination of the study at 8 weeks after the second azoxymethane treatment. The animals were sacrificed by CO_2 asphyxiation. After laparotomy, the entire stomach, small intestine, and large intestine were resected. The organs were opened longitudinally, and the contents were flushed with normal saline.

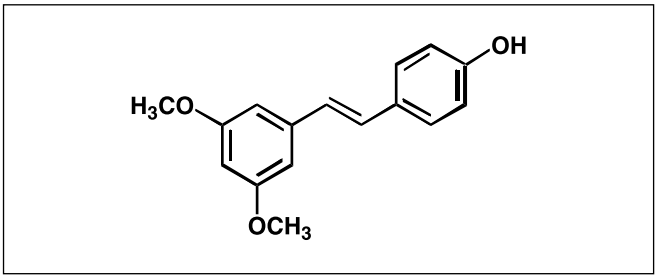

Fig. 1. Structure of pterostilbene.

ACF analysis. For the ACF analysis, the colons were fixed flat between two pieces of filter paper in 10% buffered formalin for a minimum of 24 h. The colons were then cut into 2 cm segments, starting at the anus. They were stained with 0.2% methylene blue in Krebs-Ringer solution for 5 to 10 min, and were then placed mucosal side up on a microscope slide and observed through a light microscope. ACF were counted and recorded according to standard procedures that are used routinely in our laboratory (18). Aberrant crypts were distinguished from the surrounding normal crypts by their increased size, the significantly increased distance from lamina to basal surface of cells, and the easily discernible pericryptal zone. The variables used to assess the aberrant crypts were their occurrence and multiplicity. Crypt multiplicity was determined as the number of crypts in each focus. Multicrypts were categorized as containing up to four or more aberrant crypts/focus.

Immunohistochemistry. Colon samples from each group were harvested at autopsy and fixed in 10% formalin for 24 h. They were sectioned into 8 to 10 segments, paraffin embedded, and microtomed into 4-μm-thick tissue sections. The slides were incubated overnight at room temperature with antibody to proliferating cell nuclear antigen (PCNA; 1:1,000 diluted, BD PharMingen, San Diego, CA), iNOS (1:100 diluted, Santa Cruz Biotechnology, Santa Cruz, CA), or mucin MUC2 (1:250 diluted, Santa Cruz Biotechnology). The slides were incubated with biotinylated secondary antibody, and then with avidin/biotinylated peroxidase complex for 30 min at room temperature (Vector Labs, Burlingame, CA), and were then incubated with 3′-diaminobenzamine substrate. The sections were then counterstained with modified Harris hematoxylin. The images were taken randomly at 400× using Zeiss AxioCam HRc camera fitted to a Zeiss Axioskope 2 Plus microscope. A positive reaction is noted by a reddish brown precipitate in the nucleus for PCNA, in the cytoplasm for iNOS or in the colon crypts for mucin MUC2.

Cell culture, reagents, and Western blot analysis of iNOS. Recombinant human IFN-γ and tumor necrosis factor-α were purchased from R&D Systems, Inc. (Minneapolis, MN), lipopolysaccharide (from *Escherichia coli* 0111:B4 γ-irradiated) and all other chemicals were purchased from Sigma (St. Louis, MO). HT-29 human colon carcinoma cells (American Type Culture Collection, Manassas, VA) were grown in complete medium (DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin) at 37°C, 5% CO_2. At day 0, HT-29 cells were plated in a 100 mm dish (2×10^6 cells per dish). Cells were then treated with pterostilbene together with cytokine mixtures (IFN-γ, tumor necrosis factor-α, and lipopolysaccharide, each 10 ng/mL) and cell lysates were harvested and subjected to Western blot analysis. These procedures have been described previously (28). The primary antibodies against iNOS (Santa Cruz Biotechnology), actin (Sigma), and secondary antibodies (Santa Cruz Biotechnology) were used.

Statistical analysis. The total number of ACF/colon and multiple aberrant crypts/focus were counted and the data were analyzed by Student's t test. The PCNA labeling index (PI) was calculated as the [(number of positive cells) / (total number of cells)] × 100 for each field, which is averaged to get the PI for each section. The significance of treatment between the groups was analyzed by Student's t test.

Table 1. Inhibitory effects of dietary pterostilbene on the formation of azoxymethane-induced ACF in male F344 rats

Experimental diets	ACF/colon*	Multicrypt foci*
Control diet (AIN-76A)	273 ± 17	35.6 ± 8.3
40 ppm pterostilbene	117 ± 12†	25.1 ± 5.6‡

*Mean ± SD (n = 9).
†Significantly different from the control diet group, $P < 0.001$ by Student's t test.
‡Significantly different from the control diet group, $P < 0.01$ by Student's t test.

Results

General observations. Body weights of animals fed the experimental diet containing pterostilbene were comparable to those fed the control diet throughout the study, indicating that the dose of pterostilbene used did not cause any overt toxicity (data not shown).

Efficacy of pterostilbene on ACF formation. ACF were predominantly observed in the distal colon. The end points used in this study were the occurrence of total ACF as well as multicrypt clusters (more than four crypts/focus) of aberrant crypts (Table 1). Rats treated with azoxymethane and fed with the pterostilbene diet showed a significantly lower number of total ACF/colon compared with azoxymethane-treated rats fed the control diet (57% inhibition, $P < 0.001$). The incidence of multiple aberrant crypts/focus was also significantly inhibited in rats fed the pterostilbene diet as compared with those fed the control diet (29% inhibition, $P < 0.01$).

PCNA staining of colons and cell counting. The PCNA was evaluated as a marker for cell proliferation in the colon specimens. Sections of colon samples from the control group or pterostilbene-fed group are shown (Fig. 2). The PCNA staining of the normal-appearing mucosa of the colon tissue was much stronger in azoxymethane-treated rats fed the control diet (A) than in the pterostilbene-fed group (B). The PCNA labeling index is also shown in Fig. 2. The colon sections from the azoxymethane + control group showed a higher number of positive cells than those from the azoxymethane + pterostilbene diet group. The PCNA-positive cells (%) of the colon tissue in the control group were 56.6 ± 2.8%, whereas PCNA-positive cells (%) from the pterostilbene-fed group were 44.2 ± 2.9%. The two groups were significantly different ($P < 0.01$).

iNOS staining of colons. Because the inhibition of inflammatory genes such as iNOS may contribute to the suppression of ACF formation in colon carcinogenesis, it was important to determine whether pterostilbene might inhibit azoxymethane-induced iNOS in the colon. The iNOS expression was evaluated as a marker for inflammatory response in the colon specimens. Two independent sections of colon samples from the control group or the pterostilbene-fed group are shown (Fig. 3A). The iNOS staining of the colon tissue was stronger in the control group than in the pterostilbene-fed group. The colon sections from the control group showed higher staining of iNOS in the cytoplasm than those from pterostilbene-treated rat colons. We found that ACF with moderate dysplasia from the control group displayed strong cytoplasmic staining, whereas ACF with moderate dysplasia from the pterostilbene-fed group showed weaker cytoplasmic staining.

Increased staining of mucin MUC2 in the colons by pterostilbene. We determined changes in the secretion of mucin MUC2 in the colonic crypts. MUC2 is the structural component of the colonic mucus layer which is critical for colonic protection. The colon mucosa from the azoxymethane-treated control diet group showed little expression of mucin MUC2. However, there was abundant secretion of mucin MUC2 from goblet cells lining the colonic crypts in the azoxymethane + pterostilbene-fed group. The staining of cross-sections are also shown in the bottom of Fig. 3B.

Inhibition of iNOS protein expression in a colon cancer cell line. When HT-29 human colon adenocarcinoma cells were treated with a cytokine mixture (IFN-γ, tumor necrosis factor-α, and lipopolysaccharide, each at 10 ng/mL) for 15 h, there was a great induction of the synthesis of iNOS protein. As shown in Fig. 4, pterostilbene inhibited the induction of iNOS protein

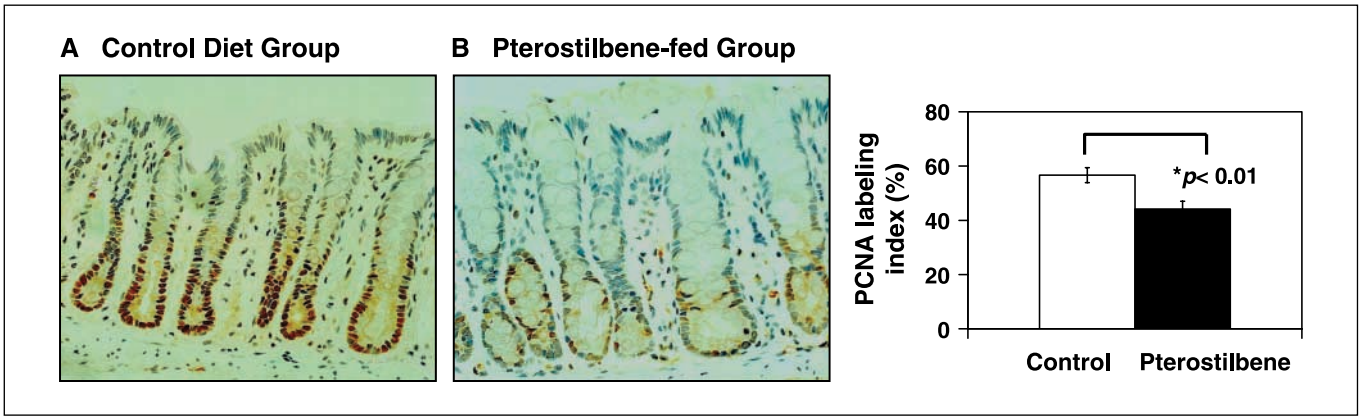

Fig. 2. PCNA staining of colon mucosa and cell counting. A representative section of colon samples from the control group (*A*) or pterostilbene-fed group (*B*). PCNA-positive cells in the nucleus (*brown*) and PCNA-negative cells (*blue*), were stained with hematoxylin. Four independent sections of the colon per animal were stained, and ~1,500 cells were counted from each section in total. The PCNA labeling index (PI) was calculated as the [(number of positive cells) / (total number of epithelial cells)] × 100 for each field. These PI values for all the different colon sections from the animals belonging to same group were then averaged. Statistical significance of treatment between the groups was analyzed by Student's t test (*, $P < 0.01$).

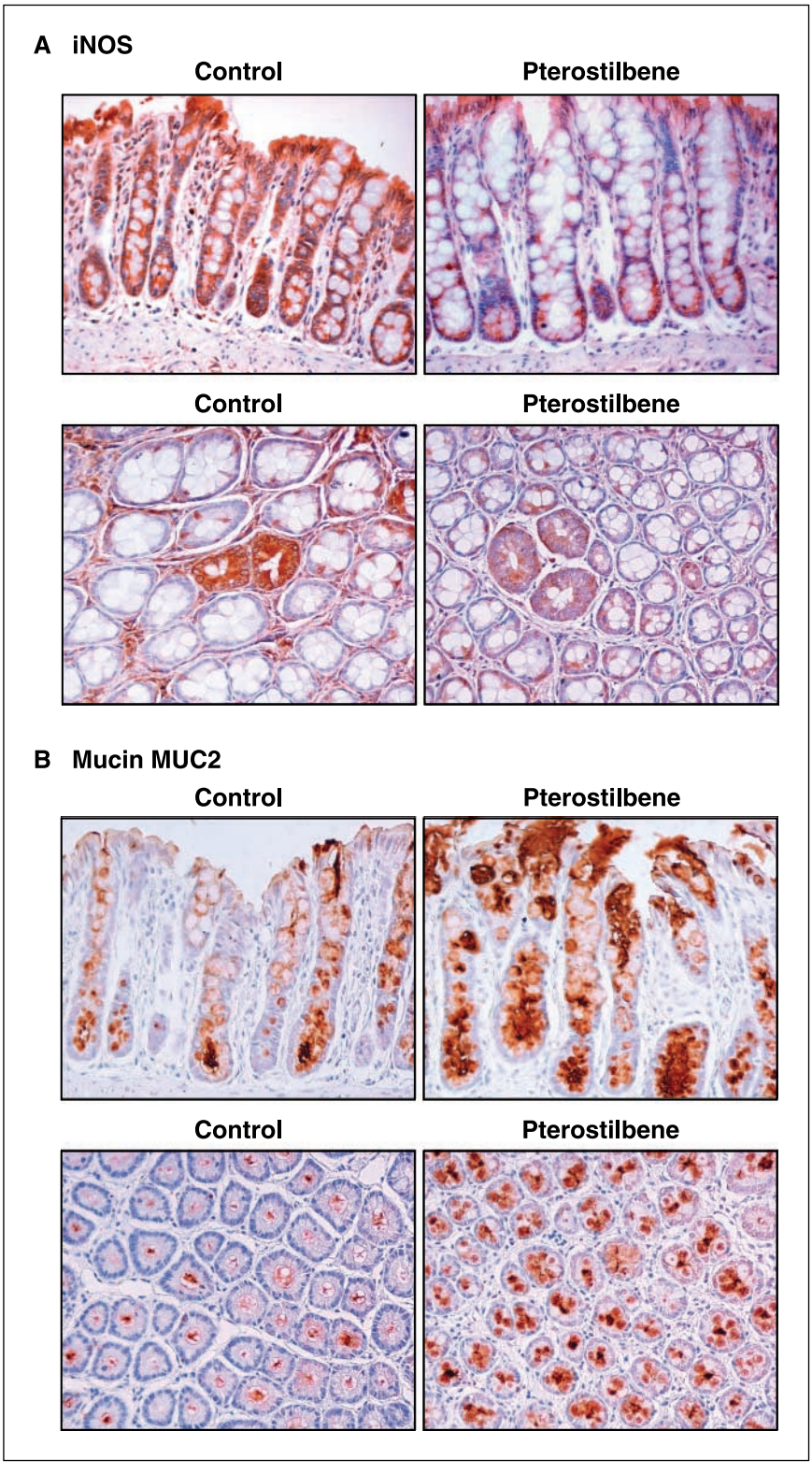

Fig. 3. *A,* inhibition of iNOS expression in the colons by pterostilbene. Eight different sections of the colon per animal were stained, and a representative section for each group is shown. The positive cells for iNOS show cytoplasmic staining. The effect of pterostilbene on histologically normal colon sections (*top*), and the effect of pterostilbene on ACF (*bottom*). *B,* increased mucin MUC2 expression in the colons by pterostilbene. Eight different sections of the colon per animal were stained, and a representative section is shown for each group. Positive staining for mucin MUC2 (*brown-stained crypts*). Colonic crypt sections (*top*), and cross-sections of the colon (*bottom*).

expression in the colon cancer cell line *in vitro*. When pterostilbene was given together at 1, 10, or 30 μmol/L concentrations, pterostilbene inhibited the induction of iNOS protein expression in a dose-dependent manner (14%, 61%, and 77% inhibition, respectively, of iNOS expression) in HT-29 cells.

Discussion

The present study is part of an ongoing preclinical investigation of the effects of naturally occurring agents against colon carcinogenesis. Polyphenolic compounds from red wine and black tea have been reported to modulate iNOS in

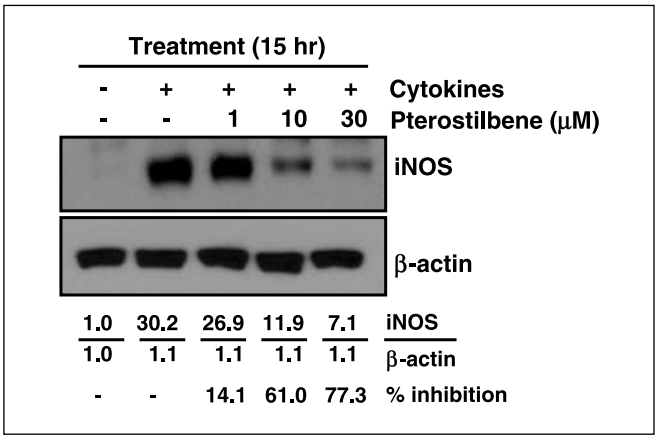

Fig. 4. Inhibition of iNOS protein by pterostilbene in HT-29 colon carcinoma cells. HT-29 human colon carcinoma cells were grown in complete medium (DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin) at 37°C, 5% CO_2. At day 0, HT-29 cells were plated in 100 mm dish (2×10^6 cells per dish). Cells were then treated with pterostilbene (1, 10, or 30 μmol/L) together with a cytokine mixture (IFN-γ, tumor necrosis factor-α, and lipopolysaccharide, each at 10 ng/mL) for 15 h and cell lysates were harvested and subjected to Western blot analysis.

azoxymethane-induced tumors in F344 rats (26); however, to our knowledge, this is the first study to show that administration of pterostilbene, a constituent present in blueberries and in the heartwood of *P. marsupium*, inhibits the development of colonic ACF, early preneoplastic lesions in the colon.

iNOS is overexpressed in colonic tumors of humans as well as in rats treated with a colon carcinogen azoxymethane (21, 22, 25, 29). Using the same animal model of azoxymethane-induced tumors in F344 rats, the selective iNOS inhibitor L-N^6-(1-iminoethyl)lysine tetrazole-amide was shown to significantly suppress azoxymethane-induced colonic ACF at 100 ppm (24). Another report of a selective iNOS-specific inhibitor, *S*,*S*′-1,4-phenylene-bis(1,2-ethanediyl)bis-isothiourea, showed an inhibitory effect against the formation of azoxymethane-induced colonic ACF. Phenylene-bis(1,2-ethanediyl)-bis-isothiourea at 50 ppm suppressed azoxymethane-induced colonic ACF formation and crypt multiplicity containing four or more crypts (22). The inhibitory effect of an iNOS inhibitor, ONO-1714 ([1S,5S,6R,7R]-7-chloro-3-imino-5-methyl-2-azabicyclo[4.1.0]heptane hydrochloride), on azoxymethane-induced rat colon carcinogenesis has also been reported with 100 ppm (17). In our study, pterostilbene showed the inhibition of ACF (57% inhibition) at 40 ppm. Our present study also shows that the suppression of ACF formation is mediated through the inhibition of colonic cell proliferation and iNOS expression. The exact mechanism by which pterostilbene reduces cell proliferation and iNOS expression is yet to be established.

The precise pathologic functions of iNOS in colorectal cancer are more difficult to specify. Recent reports suggest that iNOS may contribute to tumor development or acceleration of the progression stage (16, 21, 25). The expression of iNOS is markedly elevated in rat colon cancers induced by azoxymethane (20). In addition, iNOS can be detected in most adenomas and dysplastic ACF, suggesting that iNOS plays an important role in colon carcinogenesis (17, 20). The inhibition of carcinogenesis by pterostilbene underscores the fact that iNOS plays a role in tumorigenesis. These results suggest that the suppression of iNOS activity by pterostilbene might lead to the down-regulation of the formation of proinflammatory cytokines.

Mucins are secreted gastrointestinal proteins that protect underlying intestinal epithelium, and mucin MUC2 is critical for colonic protection (30). The expression of mucin MUC2 is lowered in inflammatory bowel disease, whereas mucin MUC2 has been implicated in the suppression of colorectal cancer (30, 31). We found that mucin MUC2 expression is higher in the pterostilbene-fed group than in the control group, suggesting that pterostilbene may maintain the normal function of the colon and protect the colonic mucus layer.

Pterostilbene and other related analogues, such as resveratrol, are commonly found in berries and grapes (4). The level of pterostilbene or resveratrol depends on the type of berries. Some varieties of blueberries contain as much as 15 μg of pterostilbene per 100 g of blueberry (5). Dietary black raspberries significantly suppress the *N*-nitrosomethylbenzylamine–induced rat esophageal carcinogenesis, and inhibition of esophageal carcinogenesis is associated with down-regulation of iNOS, inducible cyclooxygenase-2, and c-Jun in papillomatous lesions of the esophagus. Additional potential molecular targets of dietary constituents from grapes and berries include nuclear factor κB, activator protein 1, signal transducers and activators of transcription 3, Akt, Bcl-2, caspases, mitogen-activated protein kinase, and 5-lipoxygenase (3, 32, 33).

There is currently an intense effort to develop natural products containing iNOS inhibitors as chemopreventive agents. It is clear that iNOS plays a role in both early and late stages of colon carcinogenesis (17, 20–22, 24–26, 29, 34, 35). On the basis of the data presented here, we believe that naturally occurring iNOS inhibitors may be potential chemopreventive agents. The results of the present study suggest that natural products present in fruits, as exemplified by pterostilbene, are of great interest and offer alternatives for the prevention of colon cancer.

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