Chemoprevention of Colon Carcinogenesis by Sulindac, a Nonsteroidal Anti-inflammatory Agent

Chinthalapally V. Rao, Abraham Rivenson, Barbara Simi, Edith Zang, Gary Kelloff, Vernon Steele, and Bandaru S. Reddy

Divisions of Nutritional Carcinogenesis [C. V. R., B. S., B. S. RI, Pathology and Toxicology [A. RI, and Epidemiology [E. ii, American Health Foundation, Valhalla, New York 10595, and Chemoprevention Branch, Division of Cancer Control and Prevention, National Cancer Institute, Bethesda, Maryland 20892 [G. K. V. S.]

ABSTRACT

Epidemiological and laboratory animal model studies have suggested that nonsteroidal anti-inflammatory drugs reduce the risk of development of colon cancer. The present study was designed to investigate the chemopreventive action of 160 and 320 ppm (equivalent to 40 and 80% maximum tolerated doses) sulindac, a nonsteroidal anti-inflammatory drug, fed during initiation and postinitiation stages and 320 ppm sulindac fed during promotion/progression stages of azoxymethane-induced colon carcinogenesis in male F344 rats. Also investigated was the modulating effect of this agent on the colonic mucosal and tumor phospholipase A₂, phosphatidyl ethanolamine-specific phospholipase C, lipooxygenase, and cyclooxygenase activities. At 5 weeks of age, groups of male F344 rats were fed control diet or diets containing 160 and 320 ppm of sulindac. At 7 weeks of age, all animals except those in the vehicle-treated groups were given two weekly s.c. injections of azoxymethane at a dose rate of 15 mg/kg body weight/week. Animals intended for tumor promotion/progression study were administered 320 ppm of sulindac in diet starting at 14 weeks after a second azoxymethane treatment. All animals continued on their respective dietary regimen until the termination of the experiment at 52 weeks after the carcinogen treatment. Colonic tumors were evaluated histopathologically. Colonic mucosa and tumors were analyzed for phospholipase A₂, phosphatidyl ethanolamine-specific phospholipase C, prostaglandin E₂, cyclooxygenase, and lipooxygenase activities. The levels of sulindac and its metabolites in stomach, cecal, and fecal contents and in serum were analyzed. The results indicate that dietary sulindac at 160 and 320 ppm levels inhibited the incidence of invasive and noninvasive adenocarcinomas of the colon (P < 0.01-0.001) as well as their multiplicity (P < 0.01-0.0001) in a dose-dependent manner. Also, feeding sulindac during promotion/progression stages significantly suppressed the incidence (P < 0.01) and multiplicity (P < 0.001) of colonic adenocarcinomas. Dietary sulindac also suppressed the colon tumor volume by 52-62% compared to the control diet. Dietary sulindac significantly decreased the activities of phosphatidyl ethanolamine-specific phospholipase C (32-51%) and levels of prostaglandin E₂ (>40%) in the colonic mucosa and tumors, but it had no significant (P > 0.05) effect on phospholipase A₂ activity. The formation of cyclooxygenase metabolites, particularly prostaglandin F₂α, prostaglandin D₂, 6-ketoprostaglandin F₁α, and thromboxane B₂, and lipooxygenase metabolites such as 8(S)- and 12(S)-hydroxyeicosatetraenoic acids were significantly reduced in colonic mucosa and tumors of animals fed 320 ppm sulindac. Also, animals fed 320 ppm sulindac showed increased levels of microbial metabolites of sulindac in cecal and fecal contents and in serum as compared to those fed 160 ppm sulindac. Although the exact mechanism by which sulindac inhibits colon tumorigenesis remains to be elucidated, it is likely that its chemopreventive action, at least in part, may be related to the modulation of arachidonic acid metabolism.

INTRODUCTION

Large bowel cancer is one of the leading causes of cancer deaths in Western countries including North America (1). Marked variations in dietary habits among populations of different cultures and life-styles have been associated with a risk for the development of cancer of the colon (2, 3). Although several epidemiological and laboratory animal model studies suggest a relationship between the risk of development of colon cancer and dietary factors, the etiology of colon cancer is multifactorial and complex (4, 5). There is also increasing evidence that minor compounds present in the diet or their synthetic analogues reduce the risk of colon cancer development in laboratory rodent models (3-5). An attempt to identify naturally occurring as well as synthetic anticarcinogens should lead to new strategies for secondary prevention of cancer. It is noteworthy that several agents including ω-3-fatty acids, calcium salts, inorganic and organic selenium compounds, substituted dithiolethiones, polyamine synthetic inhibitors, and AA³ cascade inhibitors have been shown to possess chemopreventive properties in colon carcinogenesis (6, 7).

Several experimental animal and human colon tumors contain high levels of PGs, particularly those of type-2 series which have been shown to affect cell proliferation and tumor growth and suppress immune responsiveness (8, 9). It is therefore reasonable to predict that inhibition of colonic mucosal or tumor PGs by specific agents may suppress the tumorigenicity of colon. We and others have previously shown that several PG synthesis inhibitors, namely indomethacin, piroxicam, aspirin, ibuprofen, and sulindac (cis-5-fluoro-2-methyl-1-[(methylsulfonyl)benzyldiene]indene-3-acetic acid) suppress colon carcinogenesis in laboratory animals (10-15). It was reported that several NSAIDs were able to control growth of desmoid tumors in patients affected by FAP or Gardner’s syndrome (16-19). Labaye et al. (20) reported that, in a randomized, placebo-controlled, double-blind crossover study in patients with FAP, administration of sulindac at dose of 300 mg/day for 6-12 months caused disappearance of all polyps. In several recent studies, a reduction of the number and size of adenomas was reported in FAP patients after long-term therapy with sulindac (18-23).

Laboratory animal model studies showed that sulindac inhibits DMH-induced colon tumor incidence and multiplicity in mice when administered in the diet throughout the period of carcinogen administration (15, 24). However, when sulindac was administered starting 17 weeks after DMH administration, no reduction in tumor growth or development was observed. In another study, DMH-induced primary colon tumor development and growth in rats was inhibited by sulindac when administered by gavage at a dose level of 10 mg/kg body weight, twice daily. In a recent study, Ahnen et al. (25) showed that dietary administration of sulindac and its metabolite sulindac sulfone significantly inhibited AOM-induced colon carcinogenesis in F344 rats. Because no dose-related study on the inhibition of colon carcinogenesis by sulindac has been reported in any of the previous studies and the dose selection of sulindac in earlier investigation was not

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2 To whom requests for reprints should be addressed.

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The abbreviations used are: AA, arachidonic acid; AOM, azoxymethane; COX, cyclooxygenase; DMH, 1,2-dimethylhydrazine; FAP, familial adenomatous polyposis; HETEs, hydroxyeicosatetraenoic acids; LOX, lipooxygenase; MTD, maximum tolerated dose; NSAIDs, nonsteroidal anti-inflammatory drugs; PLA₂, phospholipase A₂; PI-PLC, phosphatidyl ethanolamine-specific phospholipase C; PIP₁, 1,2-diacylglycerol; HPLC, high-performance liquid chromatography; PGs, prostaglandins; TX, thromboxane.
Based on subchronic toxicity testing, it is necessary to evaluate the efficacy of this agent using different levels based on toxicity data. Also, the length of time that sulindac treatment can be delayed after carcinogen administration in experimental carcinogenesis is very important in chemoprevention programs for possible clinical use of sulindac in the secondary prevention of colon cancer. Since the reduced form of sulindac is a COX inhibitor, its ability to induce polyp regression may provide additional support for the hypothesis that COX inhibition is probably one of the modes of action of NSAIDs to reduce colon cancer mortality (8). In addition, Rigau et al. (26) have demonstrated a reduced PG-biosynthetic capacity of colon mucosal samples taken from the patients on long-term sulindac therapy.

Although there has been a great deal of interest in the involvement of AA metabolites in cancer, there is also uncertainty as to which metabolites are critical and how they contribute to specific steps in cell transformation, tumor growth, and metastasis (27-30). In general AA mobilization is linked to signal transduction pathways that result in the activation of PLA2 and PLC (8, 31, 32). Once AA is released from phospholipids, it is metabolized to PGs through the COX system and to hydroxy fatty acids via the LOX pathway. Among the several forms of PLA2 and PLC, cytosolic PLA2 and membrane bound PI-PLC are implicated in the regulation of eicosanoid biosynthesis and cell proliferation (33, 34). It is noteworthy that increased levels of PLA2 and PI-PLC and AA metabolite activities were observed in human colon and breast tumors and melanomas compared to normal tissues (35-37). It is therefore reasonable to predict that the changes in the activities of PLA2, PI-PLC, and AA metabolite formation through COX and LOX activities produced by exogenous agents may alter the colon tumorigenesis.

The present study was designed to investigate the chemopreventive efficacy and modulating role of sulindac on colon carcinogenesis. Dose selection of sulindac was based on MTD values determined in the current study. In the present study, we report the efficacy of 160 and 320 ppm of sulindac during initiation and postinitiation stages and 320 ppm of sulindac on promotion/progression stage of AOM-induced colon carcinogenesis. In addition, the effect of dietary sulindac on colon mucosal and tumor PLA2, PI-PLC, COX, and LOX biosynthetic activities was studied to understand the possible modulating role of this agent in colon tumorigenesis. Further, we analyzed the levels of sulindac and its active metabolites, sulindac sulfone and sulindac sulfide (Fig. 1) in gastrointestinal contents and in serum to understand the biotransformation of sulindac in the intestine and levels of sulindac metabolites in the serum.

**MATERIALS AND METHODS**

**Materials**

AOM (CAS 25843-45-2) was purchased from Ash Stevens (Detroit, MI). [14C]AA and 1-α-1-palmitoyl-2-[14C]arachidonoylphosphatidylcholine were purchased from NEN-Dupont (Boston, MA). PIP2 and a 125I-PGE2 RIA kit were obtained from Amersham (Arlington Heights, IL). AA; 5(S), 8(S), 9(S), 11(S), 12(S), and 15(S)-HETEs; PGE2; PGF2a; 6-keto-PGF1α; PGG2; TXB2, were purchased from Cayman Chemical Company (Ann Arbor, MI); and sulindac was kindly provided by National Cancer Institute’s Division of Cancer Control and Prevention. Sulindac sulfone and sulindac sulfide were kindly provided by Cell Pathways, Inc. (Denver, CO). The normal phase HPLC (4.6 x 250-mm) silica columns were obtained from Altitech Associates, Inc. (Deerfield, IL), and the reverse phase HPLC (3.9 x 300 mm) μBondapak C18 column was purchased from Waters Associates (Milford, MA). Precoated Silica G plastic TLC plates were purchased from Fisher Scientific Co. (Springfield, NJ).

**Animals and Diets**

Weanling male F344 rats were purchased from Charles River Breeding Laboratories (Kingston, NY). All ingredients of the semipurified diet were obtained from Dyets, Inc. (Bethlehem, PA), and stored at 4°C prior to preparation of diets. Male F344 rats received at weaning were quarantined for 10 days and had access to modified AIN-76A control diet (38). Following quarantine, all animals were randomly distributed by weight into various groups and transferred to an animal holding room. They were housed in plastic cages with filter tops (3/cage) under controlled conditions of a 12-h light, 12-h dark cycle, 50% humidity, and 21°C temperature. Experimental diets were prepared by adding sulindac to the control diet. The incorporation of sulindac into control diet was done with a V-blender after sulindac was premixed with a small quantity of diet in a food mixer to ensure its uniform distribution. All control and experimental diets were prepared weekly in our laboratory and stored in a cold room. Animals had access to food and water at all times and food cups were replenished with fresh diet three times weekly.

The stability of sulindac in the diet was analyzed by HPLC. Sulindac was extracted from the diet with 2 volumes of acetone:acetic acid (95:5). The organic layer was dried and redissolved in a mobile phase containing 0.1 M sodium phosphate (pH 4) and acetone and injected into HPLC. Sulindac was separated on a Waters C18 column with gradient elution and was monitored at 335 nm wavelength in a Waters 990 Photodiode Array Detector. The results indicate that >96% of sulindac could be accounted for in feed samples stored in a cold room for 14 days.

![Fig. 1. Structure of sulindac and its metabolites.](cancerres.aacrjournals.org)
CHEMOPREVENTION OF COLON CANCER BY SULINDAC

Experimental Procedure

The experiment was designed to determine the efficacy of 160 (40% MTD) and 320 ppm (80% MTD) of sulindac administered before, during, and after carcinogen treatment (during initiation and postinitiation stages) and 320 ppm of sulindac during the promotion/progression stage (starting 14 weeks after carcinogen administration) of AOM-induced colon carcinogenesis. As indicated in Fig. 2, beginning at 5 weeks of age, groups of animals intended for initiation and postinitiation study were fed their respective control diet and experimental diets containing 160 and 320 ppm sulindac whereas those intended for promotion/progression study were fed the control diet. At 7 weeks of age, groups of animals intended for carcinogen treatment received AOM s.c. once weekly for 2 weeks at a dose rate of 15 mg/kg body weight. All animals were killed after 6 weeks and the organs were examined grossly for any abnormalities. The body weights of control diet and diets containing 125 and 250 ppm sulindac were comparable throughout the study (data not shown). The animals fed 500 ppm sulindac diet showed more than 10% body weight loss by the end of 3 weeks, but later on the body weight loss was less than 10%. On the other hand, the animals fed 750 and 1000 ppm sulindac exhibited significant toxicity and most of the animals in 750-ppm sulindac group and all of the animals in 1000-ppm sulindac diet died due to the toxicity. The results of this study indicate that the MTD of sulindac is less than 500 ppm in the diet but more than 250 ppm. In the current study, we considered 400 ppm as MTD of sulindac.

Experimental Procedure

The major purpose of this MTD study was to determine the tolerable dose of sulindac in F344 rats. MTD is defined as the highest dose that causes no more than 10% weight decrement compared to the appropriate control diet group and does not produce mortality or any clinical signs of toxicity that would be predicted to shorten the natural life span of the animal. At 5 weeks of age, groups of male F344 rats (9 in each group) were fed the AIN-76A diet containing 0 and 125, 250, 500, 750, and 1000 ppm sulindac. Body weights were recorded twice weekly for 6 weeks. All animals were killed after 6 weeks and the organs were examined grossly for any abnormalities. The body weights of control diet and diets containing 125 and 250 ppm sulindac were comparable throughout the study (data not shown). The animals fed 500 ppm sulindac diet showed more than 10% body weight loss by the end of 3 weeks, but later on the body weight loss was less than 10%. On the other hand, the animals fed 750 and 1000 ppm sulindac exhibited significant toxicity and most of the animals in 750-ppm sulindac group and all of the animals in 1000-ppm sulindac diet died due to the toxicity. The results of this study indicate that the MTD of sulindac is less than 500 ppm in the diet but more than 250 ppm. In the current study, we considered 400 ppm as MTD of sulindac.

Experimental Procedure

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\[
V = \frac{L \cdot W \cdot D \cdot \pi}{6}
\]

(39). All other organs including kidney and liver were also grossly examined under the dissection microscope for any abnormalities. Colon tumors with diameters of more than 0.4 cm were cut into two halves; one portion of the tumor was used for analyses of PLAA, PI-PLC, and COX and LOX metabolites; the other half was used for histopathological examination of tumor types. Colon mucosa free of tumors from AOM-treated animals and from saline-treated animals was scraped from each animal for biochemical analysis according to our previously described method (39). Colonic mucosa and portions of tumors intended for biochemical determinations were quickly frozen in liquid nitrogen and stored at −80°C.

For histopathological evaluation, tumors were fixed in 10% buffered formalin, embedded in paraffin blocks, and processed by routine procedures with hematoxylin and eosin staining. The stained sections were examined histologically for tumor types according to the classification of Poharisskii (40) with minor modifications. Most of the colon tumors in this experiment were adenocarcinomas, either invasive or noninvasive. The invasive adenocarcinomas were mostly the signet-ring mucinous type invading the muscularis mucosa deep into the intestinal wall and beyond. The noninvasive adenocarcinomas were those growing outward towards the intestinal lumen and not invading the muscularis mucosa. They were usually well differentiated adenocarcinomas.

Biochemical Analysis

Samples of colonic mucosa and tumors for PLAA and PI-PLC assays were homogenized in 1:3 (w/v) volumes of homogenizing buffer containing 30 mM Tris-HCl (pH 7.4), 140 mM NaCl, 5 mM KCl, 20 μM EDTA, 10 μg/ml leupeptin, 50 μg/ml trypsin inhibitor, and 1 μM phenylmethylsulfonyl fluoride and the homogenates were centrifuged at 100,000 × g at 4°C for 1 h. The resulting supernatant fraction was used for cytosolic PLAA activity and the pellet fraction was resuspended in 30 mM HEPES-NaOH buffer (pH 7.2) containing 0.2% Triton X-100 and used for the analysis of membrane bound PI-PLC activity.

PLAA Activity. Cytosolic PLAA activity was measured by the method of Leslie (41) with some modifications using [14C]l-α-1-palmitoyl-2-arachidonoylphosphatidylcholine (40–60 μCi/mmol) as substrate. PLAA activity of cytosolic proteins was carried out in a total volume of 100 μl reaction mixture containing 30 mM sodium HEPES (pH 7.3), 0.8 mM CaCl2, 0.02% Triton X-100, and 20–30 μg of cytosolic protein. The reaction was initiated by adding 40 μM l-α-1-palmitoyl-2-arachidonoylphosphatidylcholine [10 μCi/μmol, adjusted with cold substrate. The reaction mixture was incubated at
37°C in a shaking water bath for 30 min. Reaction was terminated by the addition of 300 μl chloroform:methanol (3:2, v/v). An additional 200 μl of chloroform were added to each sample and mixed thoroughly. The samples were then centrifuged and the chloroform layer was separated and evaporated to dryness under N₂. Five μg of AA were added to dried extract and redisolved in chloroform. An aliquot of chloroform extract was then subjected to TLC on precoated plastic TLC plates (Silica G). The TLC plates were developed with a solvent system containing chloroform:methanol:acetic acid:water (90:12:2:1, v/v/v/v) and exposed in an iodide chamber for 5 min for visualization of AA. The area of each [¹⁴C]AA metabolite was determined with Bioscan system 200 image-scanning counter (Bioscan, Inc., Washington, DC) equipped with a β-detector. Protein content was determined by the Bio-Rad method. Results are expressed as pmol [¹⁴C]AA released/mg protein/min.

PI-PLC Activity. Membrane bound PI-PLC activity was measured by the method of Bleasdale et al. (42) with some modifications using PI₂₅ (1–5 Ci/mmol) as a substrate. The PI-PLC activity of membrane proteins (100–200 μg) was determined in a total volume of 250 μl reaction mixture containing 30 mM HEPES-NaOH buffer (pH 7.2), 5 mM DTT, 4 mM CaC₂₂, 2 mM [ethylphenidox(oxyethylenenitrilo)tetraacetic acid, 0.9 mM MgSO₄, and 50 μM PIP₂ (50 μCi/mmol). The reaction was initiated by adding substrate to the mixture and incubating at 37°C for 20 min in a shaking water bath. The reaction was terminated by addition of 0.2 ml of chloroform:methanol (1:2, v/v), followed by 0.3 ml of 1 M HCl. The incubation mixture was mixed vigorously and then centrifuged to yield two phases. An aliquot of 0.3 ml of aqueous layer containing [³H]inositol 1,4,5-triphosphate was transferred to a scintillation vial containing 10 ml of scintillation cocktail and radioactivity was counted in a Beckman model LD6800 scintillation counter. The activity is expressed as pmol of [³H]inositol 1,4,5-triphosphate formed from PIP₂/mg protein/15 min.

Prostaglandin E₂ Production

Colonie mucosa and tumors intended for PGE₂ analysis were homogenized using a Polytron homogenizer in 0.1 M Tris-HCl buffer (pH 7.4), containing 20 mM EDTA. Ex vivo production of PGE₂ was determined in colonic mucosal and tumor homogenates (0.3 ml) by incubating them at 37°C for 0 (basal level) and 15 min (ex vivo PGE₂ production). All the incubations were terminated by adding 5.6 μM indomethacin. Then 0.5 ml of ice-cold methanol was added to each incubation mixture and mixed vigorously. After acidifying the samples to pH 3.5 with 1% formic acid, the aqueous layer was extracted with an equal volume of chloroform. Following centrifugation at 9000 X g at 4°C for 10 min, the organic phase was removed and evaporated to dryness under N₂. The dried extract was resuspended in the assay buffer for RIA of PGE₂. Extraction of samples typically results in <100% recovery of PGE₂. Consequently, recovery estimates were made by adding 3000 dpm of tritiated PGE₂ to the sample prior to processing. Corrections for the proportion of analytically recoverable PGE₂ were made by measuring the tracer in an aliquot after extraction. RIA was done under equilibrium conditions according to standard methodology (43). The results are expressed as ng PGE₂/mg protein.

Cyclooxygenase and Lipoygenase Activities

Colonie mucosa and tumors from individual animals were homogenized with a Polytron tissue homogenizer. The samples were then centrifuged at 9000 x g at 4°C for 10 min. The supernatant was centrifuged at 100,000 x g for 1 h. The resulting supernatant was used for LOX activity. The micromolar pellet was resuspended in 50 mM potassium phosphate buffer (pH 7.4) for assay of COX activity.

Cyclooxygenase Activity. The COX activity of colonie mucosa and tumors was measured by previously published methods (44, 45). Briefly, 150 μl reaction mixture containing 12 μM [¹⁴C]AA (400,000 dpm), 1 mM epinephrine, 1 mM glutathione in 50 mM phosphate buffer, and 25–35 μg of mucosal or tumor microsomal protein were incubated at 37°C for 15 min. The reaction was then terminated by the addition of 40 μl of 0.2 M HCl. The COX metabolites of AA were extracted three times with 0.5 ml of ethyl acetate. The combined extracts were evaporated to dryness under N₂ and redissolved in chloroform and subjected to TLC using Silica G plates. The TLC plates were developed in a solvent system containing chloroform:methanol:acetic acid:water (100:15:1.25:1, v/v/v/v) and were exposed in an iodide chamber for 5 min for visualization of the standards. The metabolites of [¹⁴C]AA corresponding to PGE₂, PGF₂α, PGD₂, 5-keto-PGF₁α, and TXB₂ were detected by their comigration (Rᵢ) with authentic standards. The area of each metabolite was determined with a Bioscan System 200 image-scanning counter equipped with a β-detector.

Lipoxygenase Activity. Colonic mucosal and tumor LOX activity was determined by the modification method of Rao et al. (45) and Huang et al. (46). In brief, this method involved HPLC measurement of [¹⁴C]-labeled 5(S), 8(S), 9(S), 11(S), 12(S), and 15(S)-HETEs that were formed from the [¹⁴C]AA. The reaction mixture (200 μl) containing 100 mM Tris-HCl (pH 7.2), 2 mM CaC₂₂ [¹⁴C]AA (6 nmol, 480,000 dpm), and cytosol fraction (30–500 μg protein) was incubated for 15 min at 37°C. The reaction was terminated by the addition of 12 μl of 0.2 M HCl, and the metabolites of [¹⁴C]AA were extracted with 0.6 ml of ethyl acetate 3 times. The HETEs were analyzed by normal phase HPLC as described previously (45, 46).

Estimation of Sulindac and its Metabolites. Sulindac and its metabolites were analyzed in stomach, cecal, and fecal contents and in serum. Stomach, cecal, and blood samples were collected from individual animals during the termination of the experiment. Fecal samples were collected from individual animals 1 week before sacrifice using metabolic cages. All samples were stored at −70°C until the analysis. Samples were extracted with 2 volumes of acetonitrile:acetate acid (95:5). The organic layer was dried under a stream of nitrogen and redissolved in acetonitrile. Aliquots of each extract was injected into a Waters HPLC system. Sulindac and its metabolites were separated on a Waters μBondapak C₁₈ column (4.6 x 300 mm) with 0.1 mM phosphate buffer and acetonitrile gradient (60:40 to 10:90) solvent elution system. Elution of sulindac and sulindac sulfone were monitored at 335 nm, and sulindac sulfide was monitored at 350 nm wavelength in a Waters 990 Photodiode Array Detector. Sulindac and its metabolites were identified by comparing to authentic standards analyzed under the same conditions and by the comparison of retention times.

Statistical Analysis

Body weights, tumor incidence, tumor multiplicity, tumor volume, and biochemical parameters were compared between the animals fed the control and those fed the sulindac diets. Tumor incidence, which is expressed as percentage of animal with tumors, was analyzed statistically by the χ² test. Tumor multiplicity, expressed as mean number of tumors/animal, was analyzed by the unpaired t test accounting for unequal variance. Differences in body weights, tumor volume, and biochemical parameters between the groups were analyzed statistically by Student t test and ANOVA. Differences were considered statistically significant at P < 0.05.

RESULTS

General Observations. The body weights of animals treated with vehicle or AOM and fed the control and experimental diets were comparable throughout the study (P > 0.05) (data not shown). In vehicle-treated animals, 320 ppm of sulindac did not produce any gross or histopathological changes in liver, kidney, stomach, intestine, or lungs attributable to toxicity.

Tumor Incidence. Effect of dietary sulindac on AOM-induced colon tumor incidence and multiplicity is summarized in Table 1. There was no evidence of tumors in vehicle-treated animals. The results indicate that administration of AOM induced colon adenocarcinomas in about 81% of animals fed the control diet, of which 39% of animals had invasive adenocarcinomas and 67% of animals had noninvasive adenocarcinomas. Feeding of 160 ppm sulindac during the initiation and postinitiation stage significantly inhibited the incidence of colon invasive (P < 0.02), noninvasive (P < 0.0001), and total (invasive and noninvasive) adenocarcinomas (P < 0.01). Administration of 320 ppm sulindac diet significantly inhibited the incidences of invasive (P < 0.05), noninvasive, and total adenocarcinomas (P < 0.0001). Interestingly, the feeding of 320 ppm sulindac diet 14 weeks after AOM treatment (promotion/progression period) produced a better inhibitory effect on the incidence of colon adenocarcinomas (P < 0.01–0.0001) than the administration of sulindac during initation and postinitiation period. With regard to tumor multiplicity, the
feeding of 160 ppm sulindac significantly inhibited invasive (P < 0.01), noninvasive (P < 0.02), and total adenocarcinomas (P < 0.001) of the colon. Also, the feeding of 320 ppm sulindac significantly inhibited invasive (P < 0.01), noninvasive (P < 0.02), and total adenocarcinomas (P < 0.001) of the colon. Administration of sulindac during the promotion/progression stage significantly suppressed the multiplicity of invasive (P < 0.02), noninvasive, and total adenocarcinomas of invasive (P < 0.02), noninvasive, and total adenocarcinomas more than 1 cm in diameter were greatly inhibited in animals fed the sulindac diet. It is noteworthy that colon tumors more than 1 cm in diameter were greatly inhibited in animals given sulindac.

Biochemical Studies. The activities of PLA2 and PI-PLC in colonic mucosa and tumors are summarized in Table 3. Carcinogen administration significantly elevated the activities of colonic mucosal PLA2 and PI-PLC in all dietary groups. Also, there was a 3- to 4-fold increase in the activities of PLA2 and PI-PLC in colon tumors when compared to the surrounding colonic mucosa. Sulindac decreased the PI-PLC activity in the colonic mucosa of saline- and AOM-treated animals and in the colonic tumors of AOM-treated animals. However, administration of sulindac had no effect on colon mucosal and tumor PLA2 activity.

Table 4 summarizes the effect of dietary sulindac on basal levels and ex vivo synthetic activity of PGE2 in the colonic mucosa and tumors. Carcinogen treatment significantly increased the basal and ex vivo production of PGE2 in the mucosa of the animals as compared to their vehicle-treated counterparts. As expected, ex vivo production of PGE2 was higher than their respective basal levels. Also, PGE2 levels were markedly higher in colon tumors than in mucosa. Administration of sulindac significantly suppressed both basal levels as well as ex vivo production of PGE2 in the colonic mucosa and tumors of animals treated with AOM and in the colonic mucosa of vehicle-treated animals. The results also indicate that the degree of inhibition is more pronounced in animals fed 320 ppm sulindac than in those given 160 ppm sulindac.

As shown in Table 5, AOM treatment produced minimal effect on the levels of colonic mucosal PGs and TXB2 compared to saline treatment. However, a markedly increased levels (3- to 5-fold) of PGs and TXB2 were observed in colonic tumors compared to surrounding colonic mucosa of animals fed the control diet. Animals fed sulindac diet showed significantly lower levels of PGs (37- to 66%) and TXB2 (32- to 39%) in the colon mucosa and in tumors compared to those fed the control diet.

The effect of dietary sulindac on colonic mucosal and tumor LOX metabolites is shown in Table 6. The levels of 5(S)-, 12(S)-, and 15(S)-HETEs were significantly (P < 0.001) higher in colonic tumors compared to colonic mucosa. Administration of sulindac significantly suppressed 8(S)- and 12(S)-HETEs formation in colonic mucosa and tumors; however, 5(S)- and 15(S)-HETEs formation was not significantly (P > 0.05) inhibited by sulindac administration.

Table 7 summarizes the levels of sulindac and its metabolites, sulindac sulfone and sulindac sulfide, in the gastrointestinal contents, feces, and serum of animals fed 160 and 320 ppm sulindac. Increasing levels of dietary sulindac produced a dose-dependent increase in the concentration of sulindac in its microbial metabolites in gastrointestinal contents, feces, and serum. As expected, there was no microbial modification of sulindac in the gastric contents since only sulindac was identified in the contents. Microbially modified metabolites of sulindac were not detected in the small and large intestinal contents.
CHEMOPREVENTION OF COLON CANCER BY SULINDAC

The major purpose of this investigation, which is a part of a large scale investigation on the evaluation of naturally occurring and their synthetic analogues as chemopreventive agents, is to study the efficacy of several NSAIDs for their potential chemopreventive properties in colon carcinogenesis. NSAIDs such as piroxicam, indomethacin, ibuprofen, aspirin, and/or sulindac have been shown to have anticarcinogenic properties against chemically induced cancers of the colon (12–15), esophagus (47), tongue (48), pancreas (49), bladder (50), liver (51), and mammary gland (52). A recent study of colon polyp reduction and regression by sulindac in FAP patients provided a rationale to investigate the effect of sulindac in a well designed colon cancer model (23).

The results of the present study are of great interest because long-term feeding of sulindac at levels of 160 and 320 ppm (approximately 40 and 80% MTD) did not induce any histopathological changes in liver, kidney, and intestine attributable to its toxicity. Administration of 160 and 320 ppm sulindac before, during, and after carcinogen treatment significantly suppressed the colon adenocarcinoma formation in a dose-dependent manner. These results are in agreement with previous studies from other laboratories, which showed that administration of sulindac inhibited DMH- and AOM-induced intestinal tumor formation in mice and rats, respectively (15, 24, 25). The current study is the first investigation to demonstrate colon tumor inhibition by sulindac administered during the promotion/progression period similar to a study of FAP patients that administration of sulindac causes regression of colon polyps (22, 23). The present results further support our earlier observations that administration of piroxicam, a NSAID, during the promotion/progression period protected against colon tumorigenesis in F344 rats (14).

Several other NSAIDs have been studied for their chemopreventive efficacy in colon carcinogenesis in our laboratory and elsewhere (10–16). When compared to indomethacin, ketoprofen, ibuprofen, and aspirin, sulindac induced a better protective effect in colon carcinogenesis; however, the chemopreventive efficacy of sulindac in

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Basal levels</th>
<th>Ex vivo&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Basal levels</th>
<th>Ex vivo&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control diet</td>
<td>0.24 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.93 ± 0.14&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.63 ± 0.09&lt;sup&gt;f&lt;/sup&gt;</td>
<td>2.10 ± 0.32&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>160 ppm sulindac</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.18 ± 0.03&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1.21 ± 0.12&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>320 ppm sulindac</td>
<td>0.12 ± 0.03&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.32 ± 0.06&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.12 ± 0.02&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.75 ± 0.13&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>320 ppm sulindac&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.13 ± 0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* Samples were incubated at 37°C for 15 min for PGE<sub>2</sub>-synthetic activity.
* Mean ± SEM (<i>n</i> = 5).
* ND, not determined.
* Significantly different from control diet fed animals, by Student <i>t</i> test, at <i>P</i> < 0.01—< 0.0001.
* Animals were fed sulindac 14 weeks after carcinogen treatment.
* Significantly different from colon mucosal values within the control diet fed animals by Student <i>t</i> test, at <i>P</i> < 0.0001.

Table 4 Effect of dietary sulindac on AOM-induced colonic mucosal and tumor PGE<sub>2</sub> levels in male F344 rats

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Basal levels</th>
<th>Ex vivo&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Basal levels</th>
<th>Ex vivo&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control diet</td>
<td>4.74 ± 0.52&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11.47 ± 1.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.80 ± 0.33&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.38 ± 0.83&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>160 ppm sulindac</td>
<td>2.14 ± 0.32&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.76 ± 0.50&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* Significantly different from control diet fed animals, by Student <i>t</i> test, at <i>P</i> < 0.01—< 0.0001.
* Significantly different from AOM-treated control group (colonic mucosal values) by Student <i>t</i> test, at <i>P</i> < 0.0001.

Table 5 Effect of dietary sulindac on colonic mucosal and tumor cyclooxygenase activity in male F344 rats

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>PGE&lt;sub&gt;2&lt;/sub&gt; pmol</th>
<th>PGF&lt;sub&gt;2α&lt;/sub&gt; pmol</th>
<th>PGD&lt;sub&gt;2&lt;/sub&gt; pmol</th>
<th>6-keto-PGF&lt;sub&gt;1α&lt;/sub&gt; pmol</th>
<th>TXB&lt;sub&gt;2&lt;/sub&gt; pmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control diet</td>
<td>245 ± 12.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>280 ± 11.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>157 ± 11.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>325 ± 12.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>278 ± 13.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>160 ppm sulindac</td>
<td>127 ± 8.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>17.5 ± 7.5&lt;sup&gt;c&lt;/sup&gt; (37)</td>
<td>99 ± 4.3&lt;sup&gt;c&lt;/sup&gt; (37)</td>
<td>188 ± 10.2&lt;sup&gt;c&lt;/sup&gt; (42)</td>
<td>169 ± 11.2&lt;sup&gt;c&lt;/sup&gt; (39)</td>
</tr>
</tbody>
</table>

* Mean ± SEM (<i>n</i> = 6).
* Significantly different from their respective control groups by Student <i>t</i> test, at <i>P</i> < 0.05—< 0.0001.

Table 6 Effect of dietary sulindac on colonic mucosal and tumor lipoxxygenase activity in male F344 rats

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>5(S)-HETE pmol</th>
<th>8(S)-HETE pmol</th>
<th>12(S)-HETE pmol</th>
<th>15(S)-HETE pmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control diet</td>
<td>178 ± 10.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>272 ± 17.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>242 ± 14.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>237 ± 18&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>160 ppm sulindac</td>
<td>139 ± 8.3&lt;sup&gt;c&lt;/sup&gt; (23)</td>
<td>198 ± 13.7&lt;sup&gt;c&lt;/sup&gt; (27)</td>
<td>165 ± 9.3&lt;sup&gt;c&lt;/sup&gt; (32)</td>
<td>198 ± 9.4&lt;sup&gt;c&lt;/sup&gt; (16)</td>
</tr>
</tbody>
</table>

* Mean ± SEM (<i>n</i> = 4—6).
* Numbers in parentheses, percentage of inhibition compared to their respective controls.
* Significantly different from their respective controls by Student <i>t</i> test, at <i>P</i> < 0.05—< 0.0001.

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CHEMOPREVENTION OF COLON CANCER BY SULINDAC

Table 7 Levels of sulindac or its metabolites in male F344 rat gastrointestinal tract and blood serum

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>µg/g content or ml serum</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Sulindac</td>
</tr>
<tr>
<td>Stomach</td>
<td></td>
</tr>
<tr>
<td>160 ppm sulindac</td>
<td>210 ± 18* (100)*</td>
</tr>
<tr>
<td>320 ppm sulindac</td>
<td>349 ± 23 (100)</td>
</tr>
<tr>
<td>Cecum</td>
<td></td>
</tr>
<tr>
<td>160 ppm sulindac</td>
<td>16.4 ± 2.8 (8.6)</td>
</tr>
<tr>
<td>320 ppm sulindac</td>
<td>28.5 ± 3.5 (7.3)</td>
</tr>
<tr>
<td>Fecal</td>
<td></td>
</tr>
<tr>
<td>160 ppm sulindac</td>
<td>8.2 ± 1.2 (4.1)</td>
</tr>
<tr>
<td>320 ppm sulindac</td>
<td>18.0 ± 1.8 (4.9)</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
</tr>
<tr>
<td>160 ppm sulindac</td>
<td>0.51 ± 0.09 (7.6)</td>
</tr>
<tr>
<td>320 ppm sulindac</td>
<td>1.2 ± 0.07 (7.1)</td>
</tr>
</tbody>
</table>

* Mean ± SEM (n = 4–6).

Colon cancer is similar to that of piroxicam (14). It is noteworthy that sulindac suppressed both the invasive as well as noninvasive adenocarcinomas of the colon. Further, most of the NSAIDs, which have been shown to inhibit colon carcinogenesis, induce significant effect on renal PG synthesis (53). The fact that sulindac had little or no effect on the renal prostanooid synthesis (54) provides additional advantage for sulindac use in human clinical trials.

Although the precise mechanism by which sulindac inhibits colon tumorigenesis is not clearly known, the available data support the possibility involving the anti-inflammatory activity (55, 56). Previously, several studies demonstrated the anti-inflammatory activity of sulindac and its metabolites in vivo and in vitro (for a review, see Ref. 57). In addition, several studies also demonstrated that sulindac not only inhibits the prostanooid synthesis by acting on the COX enzyme activity but also modulates the activities of PLC, LOX, AA uptake, NADPH oxidase, oxidative phosphorylation, and rheumatoid factor production, which are known to play a role in inflammation and cell proliferation (8, 56).

The results of our present study demonstrated that the levels and production of PGs in colon tumors were higher than in colonic mucosa suggesting an enhanced synthesis of COX metabolites in tumors. In addition, the role of COX metabolites, particularly PGE₂, in colon tumor promotion has been well established (8, 11, 12). Previous studies from our laboratory and elsewhere have shown that PG inhibitors such as piroxicam, indomethacin, aspirin, and sulindac decrease colon tumorigenesis in rodents (12–16). LOX metabolites such as 12(S)-HETE promote tumor cell adhesion, stimulate tumor cell spreading, and augment the tumor cell metastatic potential (58–59). Further, a positive correlation was observed between the levels of 8(S)-HETE and degree of inflammation, hyperproliferation, clastogenicity, and tumor development induced by TPA (60). Also, the activities of 5(S)- and 15(S)-HETEs, which are potent modulators of inflammation, were suppressed by lipoxygenase inhibitors, indicating mediating role of HETEs in tumor promotion (61). Thus, the inhibition of colon adenocarcinomas by sulindac in the present study was consistent with the reduction in most of COX and LOX metabolites in colonic mucosa and tumors suggesting that the colon cancer-inhibitory activity of sulindac might be mediated through the inhibition of COX and LOX metabolite production.

In the present study, we analyzed the colon mucosal and tumors PLAn and PI-PLC activities, which are dominant pathways for AA release. One of the pathways leading to generation of AA involves a direct action of PLA₄ on a phospholipid that could include diacyl- or alkylacylphosphatidylinositol, phosphatidylethanolamine, or phosphatidylcholine. The second pathway mediated by PI-PLC involves the degradation of phosphatidylinositol 4,5-biphosphate via a sequence of reactions beginning with PI-PLC, followed by diglyceride and monoglyceride lipases (33). Further, PI-PLC activity is responsible for diacylglycerol formation, PKC-dependent signal transduction, and cell proliferation (34, 62). Our results demonstrated that dietary sulindac significantly inhibited the PI-PLC activity in colonic mucosa and tumors but had little or no effect on the PLA₂ activity. The exact mechanism by which sulindac or its metabolites inhibits PI-PLC activity is not clear. It may be possible that sulindac or its metabolites may exert inhibitory activity by directly acting on PI-PLC or alternatively by acting on the regulators of PI-PLC, resulting in decreased levels of AA and its metabolite formation. Also, like most NSAIDs, sulindac may also influence the membrane-integrated enzyme activities including the PI-PLC. With regard to PLA₂, we are not aware of any previous studies in which sulindac or its metabolites were tested on the cytosolic PLA₂ activity. Based on these results, it is reasonable to state that sulindac modulates not only the PI-PLC to alter endogenous AA availability but also COX and LOX activities.

The results of the present study indicate that the feeding of 320 ppm sulindac 14 weeks after carcinogen treatment (promotional/progression) stage produced a better inhibitory effect on colon invasive adenocarcinomas than the continuous feeding. The mechanism of such a phenomenon is not clear, although it may be possible that the antitumorigenic activity of sulindac is not effective until a critical tumor type/mass or size is present. Understanding the exact mechanisms of the above possibility may provide clear insights in application of sulindac in human clinical trials. Studies carried by Tsukada et al. (63) on patients with FAP having desmoid tumors had shown a delayed response to sulindac administration in some patients. The results from the promotion/progression study provided further evidence that administration of sulindac provides a beneficial effect not only in FAP patients but also for colon cancer patients and people with high risk of the colon cancer.

The results of the present study demonstrate the microbial modification of sulindac involving oxidation of the sulfanyl group of sulindac to sulfone and reduction to the corresponding sulfide in large intestine. In the cecum and colonic contents, more than 90% of sulindac was transformed to sulindac sulfone (60–84%) and sulindac sulfide (9–36%) indicating that sulindac sulfone is a major form in the colonic contents. The presence of sulindac sulfone and sulindac sulfide in the blood indicates that the microbially modified sulindac is absorbed in the colon. These results support the previous studies that sulindac is transformed to its metabolites mainly by intestinal bacteria and the sulindac sulfone is the predominant form in the serum of the rats and humans (64).

In conclusion, the present study demonstrated that dietary sulindac significantly inhibited AOM-induced colon tumorigenesis in a dose-dependent manner in male F344 rats. In addition, sulindac administered during the postinitiation/progression stage suppressed colon adenocarcinoma formation effectively. Also, sulindac significantly suppressed the colonic mucosal and tumor PI-PLC, COX, and LOX activities, which are relevant to colon carcinogenesis. Although the exact mechanisms of colon cancer inhibition by sulindac remain to be elucidated, it would appear that modulation of AA metabolism by sulindac may play a role in its chemopreventive activity. The results of current and earlier studies from other laboratories in animal models and intervention studies with FAP patients clearly suggest that further human clinical trials are warranted to test the chemopreventive action of sulindac in colon cancer patients and patients who are at high risk for colon cancer.
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REFERENCES


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