Published in final edited form as:

Nutr Cancer. 2006 ; 56(2): 172–181. doi:10.1207/s15327914nc5602_8.

Dietary Putrescine Reduces the Anticarcinogenic Intestinal Activity of Sulindac in a Murine Model of Familial Adenomatous Polyposis

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Abstract

The nonsteroidal antiinflammatory drug sulindac displays chemopreventive activity in patients with familial adenomatous polyposis (*FAP*). Sulindac metabolites induce apoptosis in colon tumor cells, in part, by a polyamine-dependent mechanism that can be suppressed with exogenous putrescine. To determine the relevance of this mechanism in animals, we treated *Apc*^{Min/+} mice, a model of human *FAP*, with sulindac alone or in combination with dietary putrescine. Sulindac increased steady-state RNA levels and enzymatic activity of the polyamine catabolic enzyme spermidine/spermine N¹-acetyltransferase and intestinal levels of monoacetylspermidine, spermidine, and spermine in the small intestine of mice. Sulindac also decreased the activity of the biosynthetic enzyme ornithine decarboxylase but not adenosylmethionine decarboxylase (*AMD*).

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Dietary putrescine increased intestinal putrescine contents, whereas the combination of dietary putrescine and sulindac yielded the highest levels of intestinal putrescine and correlated with a statistically significant reduction in *AMD* enzyme activity. Dietary putrescine did not statistically significantly increase tumorigenesis, although it significantly increased the grade of adenoma dysplasia ($P < 0.05$). The effectiveness of sulindac to suppress intestinal carcinogenesis was partially abrogated by dietary putrescine. These data suggest that sulindac exerts at least some of its anticarcinogenic effects in mice via a polyamine-dependent mechanism. Because high concentrations of putrescine can be found in certain dietary components, it may be advantageous to restrict dietary putrescine consumption in patients undergoing treatment with sulindac.

Introduction

Familial adenomatous polyposis (FAP) is an inherited cancer syndrome, where afflicted individuals develop colon cancer with a high frequency (1). FAP is caused by a mutation or deletion of the adenomatous polyposis coli (APC) tumor suppressor gene (2). Although FAP syndrome accounts for less than 1% of colorectal cancers in the United States, somatic mutations of the APC tumor suppressor gene are found in more than 90% of sporadic colorectal cancers (3). In both inherited and sporadic colonic tumors, the loss of function of the APC gene product appears to be an early step in tumorigenesis.

The nonsteroidal antiinflammatory drug (NSAID) sulindac has been shown in randomized clinical trials to induce polyp shrinkage in FAP patients (4–6). A number of studies suggest that the antitumor properties of NSAIDs result from their ability to induce apoptosis and to inhibit prostaglandin synthesis (7–10). In recent years, it has become evident that the chemopreventive action of NSAIDs also includes cyclooxygenase (COX)-independent mechanisms. Studies using colorectal cell lines show that piroxicam, aspirin, and indomethacin influence polyamine metabolism (11–14).

Polyamines (putrescine, spermidine, and spermine), which are important for cell proliferation and differentiation, are downstream mediators of genetic risk factors in human intestinal cancers (15). Cellular polyamine pools are derived from intercellular biosynthesis and uptake of amines from other host cell microorganisms in the intestinal tract and/or dietary contributions (16,17).

Recently, we have reported that sulindac decreases intracellular polyamine levels and induces apoptosis in colon cancer cell lines via the transcriptional activation of the key polyamine catabolic enzyme spermidine/spermine N^1 -acetyltransferase (SSAT) (18). Sulindac is a prodrug metabolized by the liver and intestinal flora to a sulfide, which is the active antiinflammatory metabolite, primarily responsible for blockage of prostaglandin synthesis, and to a sulfone, which has no COX-inhibitory activity (19,20). The apoptosis induced by sulindac sulfone can be rescued by exogenous polyamines in colon cancer cell lines (18,21).

Apc^{Min/+} or Min (multiple intestinal neoplasia) mice are phenotypically similar to humans with FAP, in that they develop macroscopically visible pan-intestinal and desmoid intestinal tumors and, infrequently, colonic tumors (22,23). Reduction of COX enzyme activity by

NSAIDs suppresses intestinal carcinogenesis in *Apc^{Min/+}* mice (24–26) as well as other models of intestinal cancer (7,27–30). Expression of polyamine biosynthetic enzyme, ornithine decarboxylase (ODC), is elevated in the intestinal tissues of *Apc^{Min/+}* mice and in apparently normal colonic mucosal biopsies from FAP patients (31,32). Inhibition of ODC activity, using the enzyme-activated irreversible inhibitor α -difluoromethylornithine, suppresses intestinal carcinogenesis in this model (32). Exogenous polyamines from the diet and intestinal bacteria, through intestinal transport, can reverse the growth-inhibiting effect of the ODC inhibitor on transplanted tumors and promote the growth of azoxymethane-induced aberrant crypt foci in rat colon (16,17,33–35).

Our goal in this study was to evaluate the polyamine-dependent mechanisms of antitumor activity of sulindac in vivo using the *Apc^{Min/+}* mouse model of FAP.

Materials and Methods

Rodent Model

C57BL/6J-*Apc^{Min/+}* mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and bred in the University of Arizona's Animal Care Facility in accordance with The University of Arizona Institutional Animal Care and Use Committee guidelines. These mice have a dominant mutation at codon 850 of the murine *Apc* gene that converts a leucine into a stop codon and causes the truncation of an APC protein. Affected *Apc^{Min/+}* animals were obtained by crossing *Apc^{Min/+}* males with B6 females. The presence of the mutant APC allele was detected in the DNA extracted from tail tissue using an allele-specific polymerase chain reaction (PCR) assay (23). Animals were housed in groups of one to four in microisolator cages under fluorescent lighting on a 12-h cycle. Mice were fed the defined synthetic diet AIN93G (Harlan Teklad, Indianapolis, IN). Irradiated distilled water was available ad libitum for the duration of the experiment.

Study Design

Experimental protocols were approved by the Institutional Animal Care and Use Committee. *Apc^{Min/+}* mice were treated with the nonselective COX-2 inhibitor sulindac at a dose of 167 ppm administered in the custom-prepared AIN93G diet (Harlan Teklad). The dietary preparations of sulindac have been successfully used in murine cancer models in many prior chemoprevention studies and are based on the recommended human doses (36–38). Putrescine (Sigma-Aldrich, St. Louis, MO) was administered continuously as a 1% solution in the drinking water. The treatments started at 5 wk of age. For the SSAT expression study, mice were treated for 1, 4, or 10 wk. For biochemical studies, animals were treated for 1 wk. For the tumorigenesis study, animals received treatments until 110–115 days old. Animals were housed in groups of one to four in microisolator cages under fluorescent lighting on a 12-h cycle. Throughout the experiment, animals had access to drinking water and food ad libitum. Food and water were replaced weekly or as needed.

Tissue Collection

Mice were consistently sacrificed (at approximately 10:00 am) by CO₂ inhalation after being without food for 1 h. The intestinal tract was removed, opened, and flushed with ice-cold

saline. The proximal and distal parts of the small intestine were used for measuring polyamine contents. For enzyme activity assays, the distal portion of the small intestine was used. The middle part of the distal portion of the small intestine was used for RNA isolation. Only the apparently normal intestinal tissue was used for these analyses.

RNA Isolation and Analysis

Intestinal tissue samples were homogenized in TRIzol[®] reagent (Gibco, BLR) using a Polytron homogenizer and were processed for the isolation of total tissue RNA (39). Twentymicrograms of total RNA from each sample was separated on a 1% agarose/formaldehyde gel and transferred to a nylon membrane (Hybond-N, Amersham, Arlington Heights, IL). Membranes were subsequently hybridized with ³²P-labeled cDNA encoding for the human SSAT gene (0.67-kb EcoRI-EcoRI fragment) by random priming using a kit from Boehringer Mannheim (Indianapolis, IN). ³²P-labeled cDNA for the glyceraldehyde-3'-phosphate dehydrogenase (GAPDH) gene (0.75-kb PstI-XbaI fragment) was used to normalize for the variation in loading. Northern blot autoradiographs were quantitated by densitometric analysis (Imagequant Densitometer, Molecular Dynamics, Sunnyvale, CA). Amounts of SSAT RNA were expressed as relative arbitrary units of integrated density bands normalized to GAPDH bands.

Real-Time PCR Analysis of SSAT RNA

Total RNA was isolated from control and sulindac-treated animals using the Qiagen RNeasyKit according to the manufacturer's protocol. Intestinal tissue samples were homogenized in RLT buffer using a Polytron homogenizer. Reverse transcription was completed using the TaqMan Reverse Transcription Reagents Kit (Applied Biosystems, Foster City, CA). One microgram of total RNA was transcribed into cDNA in a 100-μl reaction using random hexamers under thermal conditions recommended by the protocol. Real-time PCR amplification was performed with the ABI PRISM 7700 SDS instrument (Applied Biosystems) under the universal thermal cycling conditions recommended by the Assay-on-Demand products protocol. Each 50-μl reaction included 25 μl TaqMan Universal PCR mastermix, 10 μl of the resulting cDNA from the reverse transcription step, and 15 μl diluted SSAT primer and probe mixes ordered from Assay-on-Demand products (Applied Biosystems). No-template controls were included in each plate to monitor the potential PCR contamination. SSAT gene expression was analyzed in animals treated with sulindac for 1 or 4 wk. Each reaction was run in duplicate. To determine the relative expression level of the SSAT gene, the comparative C_T method was used. The C_T values of the SSAT gene in control and treated animals were normalized by the endogenous reference ($C_T = C_{T(\text{target})} - C_{T(18S)}$). Normalized C_T values from treated animals were compared with ones from control animals ($C_T = C_{T(\text{treatment})} - C_{T(\text{control})}$). The relative expression of the SSAT gene was calculated via the equation 2^{-C_T} . Data were presented as a ratio of the relative expression of SSAT in sulindac-treated animals to the relative expression SSAT in control animals.

SSAT Enzyme Activity

Measurement of SSAT enzyme activity was performed according to the protocol previously described (40). Briefly, distal portions of the small intestine (100–120 mg of wet weight)

were opened longitudinally, and their luminal contents were removed by washing the tissues in phosphate-buffered saline (140 mM NaCl, 2 mM KCl, 8.1 mM Na₂HPO₄, and 0.9 mM K₂HPO₄). Tissues were homogenized in lysis buffer [50 mM Tris-HCl, 2.5 mM DTT, and 0.1 mM EDTA (pH 7.5)] and frozen at -80°C until the SSAT enzyme analysis was performed. After thawing, samples were spun for 10 min at 13,200 rpm at 4°C, and supernatants were used for SSAT activity assay. The radiochemical assay of SSAT activity was performed by estimation of labeled acetylspermidine synthesized from [¹⁴C]-acetylcoenzyme A and unlabeled spermidine. One unit of enzyme activity is defined as the amount that catalyzes the formation of 1 pmol acetylspermidine per minute per milligram of protein at 30°C.

ODC Enzyme Activity

Tissue samples were collected and homogenized as described in **SSAT Enzyme Activity**. ODC activity was determined by measuring the liberation of ¹⁴CO₂ from L-[¹⁴C]ornithine (Du Pont-New England Nuclear), as described by Sertich et al. (41). The volume of 150 µl of ODC buffer (0.5 M sodium phosphate buffer containing 0.1 mM EDTA, 1 mM dithiothreitol, 0.05 mg/ml pyridoxal-5-phosphate, and 0.1 mM phenylmethylsulfonyl fluoride) was added to 100 µl of tissue lysate. The samples were mixed on ice with 0.5 µCi labeled ornithine. The tubes were capped and incubated at 37°C for 1 h. The reaction was terminated by adding 500 µl of 1 M citric acid on ice. Tubes were left overnight, and the released ¹⁴CO₂ was captured on Whatman no. 3 (2.5-cm filters) treated with 75 µl of NCS-II Tissue Solubilizer (Amersham Biosciences) suspended from the cup. The filters were then counted in 10 ml of UniverSol (ICN, Costa Mesa, CA) on a liquid scintillation counter, and ODC activity was expressed as picomoles ¹⁴CO₂ per milligram of protein per minute.

AMD Enzyme Activity

Tissue samples were collected and homogenized as described in **SSAT Enzyme Activity**. Adenosylmethionine decarboxylase (AMD) activity was determined by measuring the liberation of ¹⁴CO₂ from S-adenosyl-L-[carboxy-¹⁴C] methionine (Amersham Biosciences), as described by Shantz and Pegg (42). The volume of 150 µl of AMD buffer (0.5 M phosphate buffer containing 25 mM dithiothreitol, 15 mM putrescine, 4 mM S-adenosylmethionine, and 0.2 µCi S-adenosyl-L-[carboxy-¹⁴C] methionine) was added to 100 µl of tissue lysates. Tubes were incubated at 37°C for 2 h. The reaction was terminated by adding 300 µl of 5 M sulfuric acid on ice. Tubes were incubated for an additional 30 min at 37°C, and the released ¹⁴CO₂ was captured on Whatman no. 3 (2.5-cm filters) treated with 75 µl of NCS-II Tissue Solubilizer (Amersham Biosciences) suspended from the cup. The filters were then counted in 10 ml of UniverSol (ICN) on a liquid scintillation counter, and AMD activity was expressed as picomoles ¹⁴CO₂ per milligram of protein per minute.

Polyamine Analysis

After collection, tissue samples were stored frozen at -80°C. Samples were processed and assayed for polyamine (putrescine, spermidine, and spermine) content by reverse-phase high-performance liquid chromatography (HPLC) with 1,7-diaminoheptane as an internal standard (39). Protein content in each sample was determined by the BCA protein assay kit

(Pierce, Rockford, IL). Data are expressed as nanomoles of polyamine permilligram of protein.

Tumor Scoring

Mice were sacrificed at the age of 110–115 days by CO₂ inhalation, and whole small intestine and colon segments were removed, flushed with buffered saline, opened longitudinally, and spread out, mucosal surface up. Tissues were fixed in 10% neutral buffered formalin for 24 h and then placed in 70% ethanol. Tumor number per mouse was assessed using an LSGA epiiluminator dissecting microscope (Olympus Optical, Tokyo) at ×20 magnification. Tumors larger than 0.5 mm were counted, and their maximal diameter was measured. All assessments were done by the same observer, who was blinded to the mice genotypes and treatments. Afterward, a Swiss gut roll of small intestine and all grossly evident colon tumors were fixed in 10% buffered formalin, processed, and paraffin embedded, and 5-μm sections were stained with hematoxylin and eosin and evaluated by a veterinary pathologist. The grade of dysplasia for each adenoma examined histologically was determined on the basis of criteria outlined in a recent review on the pathology of mouse models of intestinal cancer (43).

Statistics

All of the statistical analyses were carried out with Statistical Analysis System software (version 9.1 for Windows, SAS Institute, Cary, NC), and *P* values of <0.05 were considered significant. A nonparametric test (Wilcoxon rank test) was performed for the analysis of SSAT gene expression measured using Northern blotting due to the small sample sizes. For multiple comparison, the Tukey Student range test was used. For the difference in the number of tumors between control and treated mice, Poisson regression analysis was used. For the tumor-grade analysis, Dunnett's test and Bonferroni multiple comparison test were used. The analysis of variance (ANOVA) test was used for the statistical analysis of enzyme activities.

Results

Sulindac Induces SSAT Expression in Intestinal Tissues of Min Mice

Mice carrying the wild-type (*Apc*^{+/+}) or mutant *Apc* (*Apc*^{Min/+}) allele were given sulindac mixed with the AIN93G diet at a concentration of 167 ppm starting at 5 wk of age. Mice were maintained on the sulindac-containing diet for 10 wk and sacrificed at the age of 110–115 days. Total RNA was isolated from distal portions of the small intestine, which were free of visible tumors, and analyzed for SSAT gene expression by Northern blot analysis. Figure 1A shows that steady-state SSAT RNA levels were elevated in the small intestine of both the *Apc* wild-type (*P* = 0.02) and *Apc*^{Min/+} animals (*P* = 0.004) after administration of sulindac.

Subsequently, *Apc*^{Min/+} mice were treated with the same dose of sulindac for shorter terms, 1 or 4 wk, and the SSAT expression was assessed in the small intestine by real-time PCR analysis. SSAT transcript levels were induced by sulindac in the small intestine of *Apc*^{Min/+}

mice in a statistically significant manner as early as after 1 wk of treatment (Fig. 1B, $P < 0.001$).

SSAT Enzyme Activity Is Induced in the Intestinal Tissue of *Apc^{Min/+}* Mice

We measured the SSAT enzyme activity in *Apc^{Min/+}* mice treated with 167 ppm of sulindac for 1 wk. Animals ($n = 6$) were put on study when 5 wk old. The SSAT enzyme activity was measured in the distal portion of the small intestine. The statistically significant increase in the SSAT enzyme activity ($P < 0.003$) was observed in the small intestine of treated *Apc^{Min/+}* animals compared with untreated littermates (Fig. 2). We also evaluated the effect of sulindac on enzymatic activity of two polyamine biosynthetic enzymes, ODC and AMD. The ODC enzyme activity was decreased ($P < 0.0001$) in *Apc^{Min/+}* mice treated with sulindac for 1 wk; the AMD enzyme activity was not changed in a statistically significant manner.

Sulindac Induces Spermidine Acetylation and Putrescine Supplementation Reverses It

We determined the sulindac effect on the intestinal polyamine pool in *Apc^{Min/+}* mice with and without putrescine supplementation. Mice were given the AIN93G diet, which is deficient in polyamines, or the same diet supplemented with a 1% putrescine and/or 167 ppm sulindac for 1 wk. Adding either sulindac or 1% putrescine or both to mice did not affect their weight. All the mice weighed 19–20 g, that is, they absorbed similar amounts of food and water in the different treatments. Spermidine, spermine, and N¹-acetylspermidine contents were increased with sulindac treatment (Table 1; $P < 0.001$ for spermidine and spermine; $P = 0.002$ for N¹-acetylspermidine). *Apc^{Min/+}* mice treated with 1% putrescine showed a significant increase in the intestinal putrescine and spermidine levels with no changes in N¹-acetylspermidine compared with untreated animals. Putrescine and spermidine levels were further increased in the small intestine of mice treated with both sulindac and putrescine compared with untreated mice (Table 1; $P < 0.05$ for putrescine; $P < 0.001$ for spermidine). When compared with sulindac-only-treated mice, mice that consumed the diet supplemented with both sulindac and putrescine had significantly increased putrescine levels and decreased spermine levels (Table 1; $P < 0.001$ for both). The level of N¹-acetylspermidine was significantly lower in animals maintained on the combined sulindac and putrescine diet compared with the sulindac-only diet (Table 1; $P = 0.008$).

Sulindac and Putrescine Combination Suppresses Polyamine Biosynthesis Enzyme Activities in *Apc^{Min/+}* Mice

We assessed the activities of ODC, AMD, and SSAT in *Apc^{Min/+}* mice treated for 1 wk with putrescine alone or with the combination of sulindac and putrescine starting at 5 wk of age (Fig. 2). Mice supplemented with putrescine had lower ODC enzyme activity compared with untreated mice ($P < 0.0001$). Both ODC and AMD enzyme activities were significantly suppressed in the small intestine of mice on the combined sulindac and putrescine diet compared with untreated littermates ($P < 0.0001$). SSAT enzyme activity was elevated in the small intestine of mice treated with the combination of sulindac and putrescine in a similar manner as in the sulindac-treated animals ($P < 0.05$ compared with untreated mice).

Effect of Sulindac on Tumorigenesis in Min Mice Is Polyamine Dependent

We assessed the effect of sulindac on intestinal tumorigenesis in *Apc^{Min/+}* animals fed the polyamine-deficient AIN93G diet. Sulindac significantly suppressed small intestinal tumorigenesis in these mice, as shown in Fig. 3. Untreated mice developed 32.5 ± 4.74 (mean $\pm$ SE) intestinal tumors per mouse, whereas the sulindac-containing diet reduced this number to 2.5 ± 0.9 tumors per mouse ($P < 0.0001$). In contrast to the small intestine, sulindac had no effect on colonic tumorigenesis in this model (data not shown). No sulindac-related toxicity was observed in treated mice as measured by body weight changes.

To determine the effect of dietary polyamine modifications on the antitumor effect of sulindac, we established two groups of *Apc^{Min/+}* mice that were put on the AIN93G diet either with a 1% putrescine solution in the drinking water or else on the AIN93G diet supplemented with sulindac. Feeding with these diets commenced at 5 wk of age and was terminated at 110–115 days. As summarized in Fig. 3, putrescine-fed *Apc^{Min/+}* mice exhibited an increase in tumor number per animal compared with control mice, but this increase was not statistically significant. We also analyzed the effect of sulindac and/or putrescine supplementation on adenoma grade in the small intestine of *Apc^{Min/+}* animals. Sulindac treatment decreased the number of low-grade adenomas in a statistically significant manner ($P < 0.05$). Putrescine-fed animals had a threefold increase in the number of high-grade adenomas compared with control animals ($P < 0.05$) (Fig. 3B). Mice, which were maintained on both sulindac and putrescine diet, had a decreased number of both low- and high-grade adenomas, but this decrease was not statistically significant. The histopathological analysis of intestinal and colon tumors in Min mice treated with putrescine revealed the presence of neutrophilic infiltrates in lamina propria of three of the total four colon tumors developed in this treatment group ($n = 6$ mice per group). The average intestinal tumor number per mouse in the group treated with the combination of sulindac and putrescine (19 ± 4.79 tumors per animal) was substantially higher compared with the sulindac-only-treated group (2.5 ± 0.9 ; $P < 0.0001$; or 7.6-fold increase). No sign of inflammation was observed in the colon of combined sulindac and putrescine-treated mice by histopathological analysis.

Discussion

Epidemiological studies have demonstrated that NSAIDs' use in the general population is associated with a reduced risk of colon cancer death (4,44). The understanding of the mechanisms through which NSAIDs exert their protective action is important for further progress in the area of prevention of colon cancer. Established mechanisms by which NSAIDs alter intestinal and colonic tumorigenesis include the inhibition of COXs and the synthesis of prostaglandins (24,27,45). However, NSAIDs also work by COX-independent mechanisms to induce apoptosis (9,18), and one of these latter mechanisms involves the activation of the polyamine pathway (15,46).

Endogenous polyamine synthesis is controlled by activities of three key enzymes in polyamine metabolism. These include the biosynthetic enzymes ODC and AMD, which catalyze the production of putrescine, and decarboxylated *S*-adenosylmethionine (dcSAM), respectively. dcSAM serves as the donor of aminopropyl groups in spermidine and spermine

synthesis. The catabolic enzyme SSAT acetylates spermidine and spermine and targets these molecules for export in some cases (47).

We reported recently that the sulfone metabolite of sulindac induces the transcription of SSAT via activation of peroxisome proliferator-activated receptor gamma (18). In Caco-2 colon cancer cells, induction of the SSAT gene transcription resulted in a decrease in intracellular polyamine levels. Sulindac sulfone also induced apoptosis in this cell line, partially via a polyamine-dependent mechanism because exogenous putrescine could partially rescue cells from apoptosis.

In this study, we evaluated the effects of the nonselective COX-2 inhibitor sulindac on polyamine metabolism in vivo using the *Apc^{Min/+}* mouse model of FAP. Northern blot analysis showed induction of steady-state levels of SSAT RNA in the intestinal tissue of *Apc^{Min/+}* mice treated with sulindac for 10 wk. The sulindac effect on SSAT was independent of the mutation in the APC gene. The 1-wk treatment with sulindac was sufficient to induce SSAT RNA levels, SSAT enzyme activity, and spermidine acetylation in the small intestine of *Apc^{Min/+}* mice. In sulindac-treated animals, spermidine and spermine levels were increased in a statistically significant manner. In our cell culture models, we provided evidence that these steps lead to polyamine export and decreased levels of the longer-chain amines spermidine and spermine. Because we found that sulindac suppresses the activity of polyamine biosynthetic enzymes, the observed increase in spermidine and spermine is likely not due to drug-induced biosynthesis. An alternative explanation is that intestinal spermidine and spermine pools increase due to increased uptake, possibly associated with sulindac-induced polyamine export into other tissues.

Our animals were fed the restricted AIN93G diet, which contains a very low amount of polyamines, as assessed by HPLC analysis (1.5 pmol/mg diet of putrescine, 1.2 pmol/mg diet of spermidine, and 0.85 pmol/mg diet of spermine). The *Apc^{Min/+}* mice express a functional intestinal diamine oxidase, which is capable of metabolizing intestinal luminal putrescine (data not shown). Addition of 1% putrescine to the drinking water of mice increased intestinal putrescine and spermidine contents ($P < 0.05$ for putrescine and $P < 0.001$ for spermidine compared with control mice). Dietary putrescine did not statistically significantly increase tumorigenesis, although it significantly increased the number of high-grade adenomas ($P < 0.05$). However, the consumption of 1% putrescine by the sulindac-treated mice resulted in the significant increase in the intestinal tumor number ($P < 0.0001$ compared with sulindac-only-treated mice) and suppression of N¹-acetylspermidine levels. Sulindac suppressed polyamine biosynthetic ODC enzyme activity and induced the catabolic SSAT activity while reducing intestinal tumor formation. When *Apc^{Min/+}* mice were provided dietary putrescine in combination with sulindac, the antitumor effects of sulindac were at least partially reversed. This combination caused dysregulation of polyamine metabolic flux and resulted in more than 10-fold elevation in the intestinal putrescine concentrations in treated *Apc^{Min/+}* mice compared with controls. Suppression of AMD by the combined action of putrescine and sulindac likely contributed to the high tissue levels of putrescine via reduction of dcSAM pools.

Gastrointestinal polyamines can be synthesized by intestinal epithelial cells and bacteria or can come from the diet. Analysis of luminal polyamines in the intestine of germ-free and conventional rats showed that the polyamine content in the gut of conventional rats is highly influenced by the intestinal bacteria (48). Noack et al. (48) estimated that the total amount of polyamines that might be synthesized by the intestinal flora in human colon would contain 155 μmol spermidine and 670 μmol putrescine. Polyamines synthesized by intestinal bacteria may contribute significantly to the intestinal polyamine pool if the dietary source of polyamines is low and polyamine synthesis is pharmacologically inhibited (16,48). Dietary polyamines can easily be absorbed by the gut and could play an important role in maintaining the optimal levels of polyamines in various organs (49,50). The importance of dietary polyamines in tumorigenesis was originally suggested by animal studies when the antitumor effect of polyamine biosynthetic inhibitors was enhanced with the use of a polyamine-deficient diet (51).

Our data support the conclusion that the polyamine pathway influences the antitumor activity of the nonselective COX-2 inhibitor sulindac. Our results also emphasize the potential impact of a diet on the efficacy of anticancer treatment regimes. Estimation of the dietary contribution of polyamines may be important for evaluating the effects of NSAIDs such as sulindac on the treatment of FAP patients. Polyamines are components of a variety of foods (17,49), and they may negate the anticancer effects of these drugs. For example, putrescine is found in high concentrations in grapefruit and orange juices (0.2–0.3 mmol/cup) (Dr. Cheryl Rock, personal communication, and manuscript submitted). Mice supplemented with 1% putrescine in the drinking water consume between 1 and 2 mmol/day of this amine. Even without considering the difference in metabolic rates between mice and humans, our results suggest that certain sources of dietary putrescine could reach levels capable of modifying sulindac responses in humans. Our data add to the growing recognition of the potential importance of dietary polyamines for their effects on cancer progression (52,53).

Acknowledgments

This research was supported by NIH Grant CA 72008 and Arizona Disease Control Research Commission (ADCRC) contract #8004.

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Figure 1.

(A) Effect of sulindac on steady-state levels of spermidine/spermine N¹-acetyltransferase (SSAT) RNA in the small intestine of wild-type *Apc* (*Apc*^{+/+}) and *Apc*^{Min/+} mice. Wild-type *Apc* ($n = 9$ per group) and *Apc*^{Min/+} mice ($n = 6$ per group) 110 days old were fed AIN93G diet (control) or AIN93G diet supplemented with 167 ppm of sulindac. Intestinal tissues were collected and processed for total RNA isolation and Northern blotting as described in **Materials and Methods**. SSAT expression in each animal was normalized using glyceraldehyde-3'-phosphate dehydrogenase (GAPDH) and presented as SSAT/GAPDH ratios of relative arbitrary units. *P* values were calculated between control and treatment

groups using the Wilcoxon rank nonparametric test (* $P = 0.02$ for *Apc* wild-type mice; ** $P < 0.004$ for *Apc*^{Min/+} mice). (B) Real-time polymerase chain reaction (PCR) analysis of SSAT gene expression in *Apc*^{Min/+} control animals and animals treated with 167 ppm of sulindac for 1 ($n = 4$ per group) or 4 ($n = 6$ per treatment group) wk. Intestinal and colonic tissues were harvested, and total RNA was extracted according to a protocol supplied with the Qiagen RNeasy Kit. RT and real-time PCR were performed as described in **Materials and Methods**. Individual bars represent the relative expression of the SSAT gene in the intestinal tissue of sulindac-treated animals to the SSAT expression in untreated animals. P values were calculated with the analysis of variance test. * $P = 0.001$.

Figure 2.

Sulindac and putrescine combination suppresses polyamine biosynthesis enzyme activities. Ornithine decarboxylase (ODC), spermidine/spermine N¹-acetyltransferase (SSAT), and adenosylmethionine decarboxylase (AMD) enzyme activities were measured in 6-wk-old control mice ($n = 4$ per group) and mice receiving 167 ppm sulindac, 1% putrescine, or both for 1 wk ($n = 6$ per group). The intestinal tissues were harvested and processed for enzyme activities as described in **Materials and Methods**. P values between control and treatment groups were calculated with the analysis of variance test (* $P < 0.05$; ** $P < 0.003$; *** $P < 0.0001$).

A

B

Figure 3.

Suppression of intestinal tumorigenesis by sulindac can be reversed by exogenous putrescine. (A) Tumor counts in small intestine of *Apc^{Min/+}* control mice ($n = 11$ mice per group) or mice treated with either sulindac (167 ppm in AIN93G diet, $n = 10$ mice per group), putrescine (1% in the drinking water, $n = 8$ mice per group), or a combination of sulindac and putrescine ($n = 10$ mice per group). Tumor number was determined as described in **Materials and Methods**. Poisson regression analysis was used to calculate P values between control and sulindac- or sulindac plus putrescine-treated groups as well as between sulindac- and sulindac plus putrescine-treated groups ($*P < 0.0001$ for all comparisons). (B) The effect of putrescine on high-grade adenoma number in the small intestine of *Apc^{Min/+}* mice. Mice were fed AIN93G diet with or without supplementation of 1% putrescine in the drinking water. Tumor grade was evaluated as described in **Materials and Methods**. The Bonferroni multiple comparison test was used to calculate the P value between control and putrescine-treated groups. $*P < 0.05$.

Table 1

Polyamine Contents (mean $\pm$ SD) in the Small Intestine of *Apc*^{Min/+} Mice Treated With Sulindac and/or Putrescine for 1 Week^a

Group	Polyamine Content (nmol/mg protein)			
	Putrescine	Spermidine	Spermine	N ¹ -Acetylspermidine
Control	0.27 $\pm$ 0.29	6.14 $\pm$ 1.31	4.76 $\pm$ 0.73	0.12 $\pm$ 0.1
Sulindac	0.96 $\pm$ 0.17	8.42 $\pm$ 1.15 *	6.32 $\pm$ 0.86 *	0.32 $\pm$ 0.21 **
Putrescine	1.61 $\pm$ 1.06 ***	8.47 $\pm$ 0.90 *	4.09 $\pm$ 0.31 ***	0.15 $\pm$ 0.12
Sulindac + putrescine	3.83 $\pm$ 1.84 ***	8.20 $\pm$ 1.01 *	4.80 $\pm$ 0.90	0.13 $\pm$ 0.14

^a $P < 0.001$ for putrescine and spermine; $P = 0.008$ for N¹-acetylspermidine between sulindac and sulindac + putrescine groups.

* $P < 0.001$ compared with control;

** $P = 0.002$ compared with control;

*** $P < 0.05$ compared with control.