[CANCER RESEARCH 62, 403–408, January 15, 2002]

Prostaglandin E_2 Protects Intestinal Tumors from Nonsteroidal Anti-inflammatory Drug-induced Regression in $Apc^{Min/+}$ Mice[1]

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ABSTRACT

Nonsteroidal anti-inflammatory drugs (NSAIDs) are antitumorigenic in humans as well as in animal models of intestinal neoplasia, such as the *adenomatous polyposis coli* $^{Min/+}$ ($Apc^{Min/+}$) mouse. NSAIDs inhibit cyclooxygenase (COX) isozymes, which are responsible for the committed step in prostaglandin biosynthesis, and this has been considered the primary mechanism by which NSAIDs exert their antitumorigenic effects. However, mounting evidence suggests the existence of COX-independent mechanisms. In the present study, we attempted to clarify this issue by treating $Apc^{Min/+}$ mice bearing established tumors with NSAIDs (piroxicam and sulindac, 0.5 and 0.6 mg/mouse/day, respectively) for 6 days and concomitantly bypassing COX inhibition by treatment with the E prostaglandin (EP) receptor agonists 16,16-dimethyl-prostaglandin E_2 (PGE_2) and 17-phenyl-trinor-PGE_2 (10 μg each, three times daily) administered via gavage and/or i.p. routes. Treatment with piroxicam and sulindac resulted in 95% and 52% fewer tumors, respectively, and a higher ratio of apoptosis:mitosis in tumors from sulindac-treated mice as compared with controls. These effects were attenuated by concomitant EP receptor agonist treatment, suggesting PGE_2 is important in the maintenance of tumor integrity. Immunological sequestration of PGE_2 with an anti-PGE_2 monoclonal antibody likewise resulted in 33% fewer tumors in $Apc^{Min/+}$ mice relative to untreated controls, additionally substantiating a role for PGE_2 in tumorigenesis. The EP receptor subtype EP1 mediates the effects of PGE_2 by increasing intracellular calcium levels ($[Ca^{2+}]_i$), whereas antagonism of EP1 has been shown to attenuate tumorigenesis in $Apc^{Min/+}$ mice. We demonstrate that $[Ca^{2+}]_i$ is significantly elevated in tumors of $Apc^{Min/+}$ mice relative to the adjacent normal-appearing mucosa. Furthermore, treatment with piroxicam results in significantly lower $[Ca^{2+}]_i$ in tumors, and this effect is attenuated by concomitant treatment with the EP1/EP3 receptor agonist 17-phenyl-trinor-PGE_2. Overall, our results suggest that NSAIDs exert their antitumorigenic effects, in part, via interference with PGE_2 biosynthesis, and these effects may be mediated through changes in intracellular calcium levels.

INTRODUCTION

Colorectal cancer is the second leading cause of cancer-related mortality in the United States with 56,785 deaths reported in 1998 (1). Over the last several years, the $Apc^{Min/+}$ mouse has been extensively used to evaluate the effects of pharmacological and nutritional intervention on intestinal tumorigenesis because of its recognized value in modeling human colorectal carcinogenesis (2). Development of colorectal cancer in humans from dysplastic crypts to metastatic carcinoma involves a series of genetic mutations, the earliest often involving *APC*[3] (3). Individuals with familial adenomatous polyposis, like $Apc^{Min/+}$ mice, possess a germ-line mutation in *APC*, and mutational damage or loss of the wild-type allele initiates intestinal tumor formation (4). Although familial adenomatous polyposis accounts for $<$1% of all of the human colorectal cancer cases, somatic mutations resulting in loss of full-length *APC* protein also occur early in the majority of spontaneous forms of the disease (5, 6), indicating the central importance of *APC* in colorectal carcinogenesis (5, 7, 8).

Multiple studies using the $Apc^{Min/+}$ mouse model have clearly established the antitumorigenic efficacy of NSAIDs, inhibitors of COX-1 and COX-2, isozymes responsible for the committed step in prostaglandin biosynthesis. The COX-inhibitory effects of NSAIDs have been considered key to their antitumorigenic efficacy, and this hypothesis is supported by several lines of evidence. COX-2, the inducible isoform, is overexpressed in intestinal tumor tissue but not normal intestinal tissue of both humans and $Apc^{Min/+}$ mice (9–11). Corresponding to the overexpression of COX-2, PGE_2, the COX product of arachidonic acid (20:4 n-6) metabolism, is elevated in human colonic tumors (12, 13) and in intestinal tumors from $Apc^{Min/+}$ mice compared with normal intestinal tissue (14). Furthermore, inhibition of both COX-1 and COX-2 by n-3 polyunsaturated fatty acids and nonselective inhibitors, including the NSAIDs sulindac, indomethacin, piroxicam, and aspirin, reduces tumor number in $Apc^{Min/+}$ mice by 44–96% (14–21), and selective inhibition of COX-2 reduces tumor number by 52–71% (22, 23). Likewise, crossing COX-2 knockout mice with $Apc^{\Delta 716}$ mice or $Apc^{Min/+}$ mice reduced tumors by ~85% (24, 25), and crossing $Apc^{Min/+}$ mice with COX-1 knockouts similarly reduced tumor multiplicity by 77% (24).

Despite these supportive data, mounting evidence suggests that NSAIDs may also work via COX-independent mechanisms. For example, *S*-flurbiprofen, a nonselective COX inhibitor, and its inactive enantiomer (*R*-flurbiprofen) reportedly act via COX-independent mechanisms (26), but were equally effective in reducing tumor number in $Apc^{Min/+}$ mice (27). Additionally, NSAIDs have been shown to modulate cell proliferation and cell death in cultured colon cancer cells lacking COX, suggesting that not all of the NSAID effects are based on COX inhibition (28–30). Multiple COX-independent mechanisms have been investigated to date including those involving 15-lipoxygenase-1 (31), ceramide (32, 33), p21 (34), β-catenin (29, 35, 36), peroxisome proliferator-activated receptors (37), inhibitor of nuclear factor-κB kinase (38), and cyclic GMP phosphodiesterase (39). Whereas many of these alternative mechanisms may involve signaling pathways related to COX inhibition and prostaglandin biosynthesis, these links have yet to be definitively established.

Thus, to more clearly establish the role of NSAIDs and prostaglandins in the maintenance of tumor integrity, we endeavored to attenuate NSAID-induced regression of intestinal tumors through a series of "add-back" experiments involving EPR-A and by systemically sequestering PGE_2 using an anti-PGE_2 antibody. To do this, we capitalized on previous results demonstrating that NSAIDs (*i.e.*, piroxicam and sulindac) could eliminate up to 95% of preexisting tumors within 6 days (14, 21) and that i.p. administration of the EPR-A 16,16-dimethyl-PGE_2 had direct effects on the stem cell population in small intestinal crypts of mice (40). The results of these *in vivo* experiments will demonstrate the following: (*a*) PGE_2 is important in maintaining tumor integrity; (*b*) PGE_2-mediated modulation of $[Ca^{2+}]_i$ may be involved in tumorigenesis; and (*c*) NSAIDs may reduce tumor burden by modulating $[Ca^{2+}]_i$.

Received 6/19/01; accepted 11/14/01.

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[1] Supported in part by American Institute for Cancer Research Grant 99A095-REN (to J. W.) and the Tennessee Agricultural Experiment Station, Current Research Information System Accession no. 0173340 (to J. W.).

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[3] The abbreviations used are: *APC*, adenomatous polyposis coli; $[Ca^{2+}]_i$, intracellular calcium concentration; CCE, capacitative calcium entry; COX, cyclooxygenase; EP, E prostaglandin; EPR-A, E prostaglandin receptor agonist; NSAID, nonsteroidal anti-inflammatory drug; PGE_2, prostaglandin E_2; CCE, calcium capacitative entry.

MATERIALS AND METHODS

Animals

Male C57BL/6J $Apc^{Min/+}$ mice (Jackson Laboratories, Bar Harbor, ME), were obtained at 38–45 days of age. They were housed in a temperature-controlled room with 14-h periods of light and 10-h periods of darkness, and given free access to food and water. The health of the animals was checked daily. Food was withheld overnight before sacrifice. All of the animal procedures were approved by the University of Tennessee Animal Care and Use Committee and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Diets

Diets for Experiments 1–3 were composed of purified AIN-93G powder diet (Dyets, Inc., Bethlehem, PA). Experimental diets containing NSAIDs were prepared daily by thoroughly mixing piroxicam (Sigma Chemical Co., St. Louis, MO) or sulindac (Sigma Chemical Co.) with the control diet. Diets for experiment 4 were AIN-93G powdered diet (calcium 0.4–0.5 g/100 g diet) with or without piroxicam. Diets were stored at −20°C, and all of the mice were provided fresh food daily. Food consumption was monitored daily, and body weights were recorded weekly.

Experimental Design

Experiment 1. Mice ($n = 23$) were maintained on the AIN-93G diet until 78–79 days of age at which time they were randomly assigned to one of four groups (control, EPR-A, piroxicam, or piroxicam + EPR-A). Groups receiving piroxicam (0.5 mg/mouse/day) were pair-fed to ensure equivalent dosing. The EPR-A 16,16-dimethyl-PGE_2 and 17-phenyl-trinor-PGE_2 (Cayman Chemical, Ann Arbor, MI), 10 μg each in sterile PBS, or vehicle were administered in two daily i.p. injections (8 a.m. and 4 p.m.) and once daily via gavage feeding (12 p.m.) to maximize exposure to the gastrointestinal tract over a 6-day period. They were sacrificed at 85–86 days of age, and tumor number, size, and location were determined as described previously (14).

Experiment 2. Mice ($n = 30$) were maintained on the AIN-93G diet until 80–81 days of age at which time they were randomly assigned to one of four groups (control, EPR-A, sulindac, or sulindac + EPR-A). Groups receiving sulindac (0.6 mg/mouse/day) were pair-fed to ensure equivalent dosing. The EPR-A 16,16-dimethyl-PGE_2 and 17-phenyl-trinor-PGE_2 (Cayman Chemical), 10 μg each in sterile PBS or vehicle were administered every 8 h via i.p. injection over a 6-day period. Mice were sacrificed at 86–87 days of age and treated as in Experiment 1.

Experiment 3. Mice ($n = 17$) were maintained on the AIN-93G diet until 82 days of age at which time they were randomly assigned to one of two groups (control or PGE_2 antibody). Control mice received the MOPC21 mouse IgG1-purified immunoglobulin (Sigma Chemical Co.; 280 μg in 280 μl) that was filtered (0.45 μm filter) and administered daily via i.p. injection on days 82–85. The anti-PGE_2 monoclonal antibody 2B5 (Monsanto Co., St. Louis, MO; Ref. 41; 283 μg/day in 250 μl sterile PBS) was administered daily via i.p. injection on days 82–85. All of the mice were sacrificed on day 87 and treated as in experiments 1 and 2.

Experiment 4. In experiment 4*A*, mice ($n = 3$) were maintained on the control diet until 80 days of age at which time mice were sacrificed, and normal-appearing intestinal tissue and tumors were collected for $[Ca^{2+}]_i$ analysis. In experiment 4*B*, mice ($n = 12$) were maintained on the control diet until 85 days of age at which time they were randomized into two groups (control or piroxicam at a dose of 200 mg/kg diet) for 2 days. Mice were sacrificed at 87 days of age, and tumors were collected for $[Ca^{2+}]_i$ analysis. In experiment 4*C*, mice ($n = 20$) were maintained on the control diet until 72 days of age and then randomized into one of four groups (control, EPR-A, piroxicam, or piroxicam + EPR-A). The EPR-A 17-phenyl-trinor-PGE_2, 10 μg in sterile PBS or vehicle, was administered in two daily i.p. injections (8 a.m. and 4 p.m.) and once daily via gavage feeding (12 p.m.) on days 72–74. Piroxicam (200 mg/kg diet) was provided in the diet for 2 days before sacrifice at 76 days of age, at which time tumors were collected for $[Ca^{2+}]_i$ analysis.

Measurement of Apoptosis and Mitosis

Tumors were immediately placed in 10% neutral buffered formalin. After 8–10 h of fixation, tissues were routinely processed into paraffin and 4-μm H&E-stained sections prepared for histological examination. Neoplastic epithelial cells undergoing apoptosis or mitosis were identified under ×400 magnification in H&E stained sections of small intestinal tumors according to well-characterized morphological criteria and without previous knowledge of the study group. The number of apoptotic or mitotic events were simultaneously enumerated per 1000 cells for each tumor and recorded as:

[number of apoptotic or mitotic cells ÷ total number of cells counted] × 1000.

Intracellular Calcium Measurement

$[Ca^{2+}]_i$ in normal-appearing small intestine and tumors was measured using a fura-2 dual-wavelength fluorescence imaging system and methodology described previously (42). Normal intestinal or tumor cells were isolated as described by Evans *et al.* (43). These cells were then loaded with fura-2 acetoxymethyl ester (10 μM) in HEPES balanced salt solution containing the following components (in mM): 138 NaCl, 1.8 $CaCl_2$, 0.8 $MgSO_4$, 0.9 NaH_2PO_4, 4 $NaHCO_3$, 5 glucose, 6 glutamine, 20 HEPES, and 10 mg/ml BSA for 2 h at 37°C in a dark incubator with 5% CO_2. To remove extracellular dye, cells were rinsed with HEPES balanced salt solution three times and then plated in 35-mm dishes (P35G-0–14-C; MatTek, Ashland, MA). Cells were postincubated at room temperature for an additional 1 h for complete hydrolysis of cytoplasmic fura-2 acetoxymethyl ester. The dishes with dye-loaded cells were mounted on the stage of a Nikon TMS-F fluorescence-inverted microscope with a Cohu model 4915 charge-coupled device camera. Fluorescent images were captured alternatively at excitation wavelength of 340 and 380 nm with an emission wavelength of 520 nm. $[Ca^{2+}]_i$ was calculated using a ratio equation as described previously (44). Each analysis evaluated responses of 8–10 representative whole cells. Images were analyzed with InCyt Im2 version 4.62 imaging software (Intracellular Imaging, Cincinnati, OH). Images were calibrated using a fura-2 calcium imaging calibration kit (Molecular Probes, Eugene, OR) to create a calibration curve in solution, and cellular calibration was accomplished using digitonin (25 μM) and pH 8.7 Tris-EGTA (100 mM) to measure maximal and minimal $[Ca^{2+}]_i$ levels (44).

Statistical Analyses

Values are expressed as means ± SE. With the exception of tumor number in experiment 1, data for experiments 1, 2, and 4*C* (differences in tumor number, tumor size, mitotic index, and $[Ca^{2+}]_i$) were analyzed statistically by one-way ANOVA followed by Fisher's least significant difference multiple comparison method to determine differences among groups. Mitotic index data were transformed [log(Y+1)] to normalize sample distributions before analysis. Tumor number in experiment 1, apoptotic index, and apoptosis:mitosis ratio were analyzed by the Kruskal-Wallis (rank sums) test with post hoc Bonferroni adjustment to control the experimentwise error rate. Student's *t* test was used to analyze data in experiments 3, 4*A*, and 4*B*. The Statistical Analysis System (SAS Version 6.12; SAS Institute, Inc., Cary, NC) was used to evaluate the data. Differences were considered significant at $P < 0.05$.

RESULTS

Effects of Piroxicam, Sulindac, EPR-A, and PGE_2 Antibody Treatment on Tumor Burden. Mice treated with piroxicam and sulindac had 95% and 52% fewer intestinal tumors, respectively, as compared with control mice, and this effect was significantly attenuated by concomitant EPR-A treatment (Tables 1 and 2). Antagonism of PGE_2 with 2B5 anti-PGE_2 antibody treatment also resulted in 33% fewer

Table 1 *Intestinal tumor load in $Apc^{Min/+}$ mice treated with and without piroxicam +/− EPR-A*

Groups are control (C), EPR-A treatment (E), piroxicam (P), or piroxicam plus EPR-A treatment (P+E). Values shown are means ± SE. Tumor size was calculated as a weighted average.

	C ($n = 6$)	E ($n = 5$)	P ($n = 5$)	P+E ($n = 6$)
Tumors/mouse	$48.8^a \pm 6.2$	$30.0^a \pm 6.5$	$2.4^b \pm 0.8$	$19.3^a \pm 4.8$
Tumor size (mm)	$1.10^a \pm 0.06$	$0.99^{a,b} \pm 0.04$	$1.03^{a,b} \pm 0.12$	$0.88^b \pm 0.05$

^{a,b} Different superscripts within each row indicate significant differences at $P < 0.05$.

Table 2 *Intestinal tumor load in Apc$^{Min/+}$ mice treated with and without sulindac +/− EPR-A*

Groups are control (C), EPR-A treatment (E), sulindac (S), or sulindac plus EPR-A treatment (S+E). Values shown are means ± SE. Tumor size was calculated as a weighted average.

	C (n = 7)	E (n = 7)	S (n = 8)	S+E (n = 8)
Tumors/mouse	$46.0^{a} \pm 6.7$	$38.0^{a,b} \pm 2.6$	$22.1^{b} \pm 5.8$	$38.4^{a} \pm 6.2$
Tumor size (mm)	$1.30^{a} \pm 0.05$	$1.07^{b} \pm 0.06$	$0.97^{b} \pm 0.04$	$1.10^{b} \pm 0.04$

^{a,b} Different superscripts within each row indicate significant differences at $P < 0.05$. Tumors/mouse in S *vs.* E, $P = 0.059$.

tumors compared with controls ($P < 0.05$; Table 3). Mucosal erosions and/or ulcers were commonly identified in the small intestine of piroxicam- and, to a lesser extent, sulindac-treated mice. In contrast, there was no evidence of such damage in the other treatment groups, particularly those treated with a NSAID plus EPR-A. Select small intestinal tumors in the sulindac and piroxicam treatment groups contained histological evidence of regression, as described previously (14).

Comparison of Apoptosis and Mitosis in Tumors. In comparison to controls, the mean apoptotic index was significantly higher in tumors from sulindac-treated mice, but the addition of EPR-A (sulindac + EPR-A) attenuated this effect (Fig. 1*A*). The EPR-A treatment alone had no impact on the apoptotic index. In contrast, the mitotic index was significantly lower in tumors from sulindac-treated mice as compared with controls, and EPR-A treatment (sulindac + EPR-A) attenuated this response (Fig. 1*B*). Overall, tumors from the control group had an apoptosis:mitosis ratio of 0.56 (a ratio of <1 is consistent with tumor growth), and this ratio increased to 2.52 with sulindac treatment (Fig. 1*C*). The addition of EPR-A (sulindac + EPR-A) attenuated the impact of sulindac on this ratio (to 1.13).

Comparison of $[Ca^{2+}]_i$ in Tumors *versus* Normal-appearing Small Intestine and the Effects of Piroxicam with or without EPR-A Treatment. $[Ca^{2+}]_i$ was 54% higher in tumor tissue than in adjacent normal-appearing small-intestinal mucosa of *Apc$^{Min/+}$* mice (Fig. 2, *Experiment A*). Furthermore, 2-day piroxicam treatment resulted in $[Ca^{2+}]_i$ in tumors that was significantly lower compared with tumors from control mice (Fig. 2, *Experiments B* and *C*), and this effect was attenuated by concomitant treatment with the EPR-A 17-phenyl-trinor-PGE$_2$ (Fig. 2, *Experiment C*).

DISCUSSION

Many NSAIDs are clearly antitumorigenic in the *Apc$^{Min/+}$* mouse model. These antitumorigenic effects have largely been ascribed to inhibition of prostaglandin biosynthesis, although recent evidence suggests multiple mechanisms may be involved. In our previous study, we reported that the antitumorigenic effect of sulindac seemed to be independent of prostaglandin biosynthesis (14). Similar results were reported by others, wherein sulindac treatment exhibited antitumorigenic properties but had variable effects on prostaglandins (9, 14, 19, 20, 45–48). Tissue preparation, methodological differences, and pharmacokinetics of the drug could account for the variability. However, the data presented in this report clearly suggest that sulindac, like other NSAIDs, is antitumorigenic, and the mechanism involves PGE$_2$.

To more clearly establish the role of NSAIDs and prostaglandins (particularly PGE$_2$) in maintaining tumor integrity, we circumvented NSAID-induced COX inhibition with concomitant EPR-A administration in *Apc$^{Min/+}$* mice. In the first experiment, mice treated simultaneously with EPR-A and piroxicam had an 8-fold greater tumor number than those treated with piroxicam alone, indicating that PGE$_2$ is important in maintaining intestinal tumor integrity and that its reduction accounts, at least in part, for the antitumorigenic effects of NSAIDs. Treatment with a dose of sulindac designed to yield incomplete tumor regression verified results observed with piroxicam and also allowed for subsequent tumor tissue analysis. Histological evaluation of tumors from sulindac-treated mice indicated that PGE$_2$ modulates apoptosis and proliferation of neoplastic cells in intestinal tumors *in vivo* (Fig. 1). Whether this is a direct effect on the epithelium, disruption of paracrine signaling, or secondary to stromal changes remains to be determined.

The antitumorigenic effects of NSAIDs have been linked to inhibition of COX-2. Expression of COX-2 was reported to be localized within stromal cells of tumors in *Apc$^{Min/+}$* (9, 49) and *Apc$^{\Delta 716}$* mice (25), but others have reported that COX-2 is also expressed in the epithelial cells (10). These results mimic localization of COX-2 expression in human colorectal adenomas, wherein COX-2 was preferentially localized within interstitial macrophages and to a lesser extent within dysplastic epithelial cells (50). If COX-2 is not expressed by epithelial cells, any direct effect by NSAIDs on the epithelial cells would have to be independent of COX-2 inhibition. Nevertheless, there is sufficient evidence in the literature suggesting much of these effects on the tumors are likely mediated by COX-2 and its inhibition. Our data cannot rule out the contribution of PGE$_2$ from COX-1 in the epithelial or stromal cells, because the NSAIDs used in these studies inhibit both COX-1 and -2; however, because aspirin can inhibit

Table 3 *Intestinal tumor load in Apc$^{Min/+}$ mice treated with the anti-PGE$_2$ MAb 2B5*

Apc$^{Min/+}$ mice were treated with MOPC21 control antibody (C) or an anti-PGE$_2$ monoclonal antibody (2B5). Values shown are means ± SE. Tumor size was calculated as a weighted average.

	C (n = 9)	2B5 (n = 8)
Tumors/mouse	$58.6^{a} \pm 6.0$	$39.0^{b} \pm 6.3$
Tumor size (mm)	$1.14^{a} \pm 0.04$	$1.17^{a} \pm 0.04$

^{a,b} Different superscripts within each row indicate significant differences at $P < 0.05$.

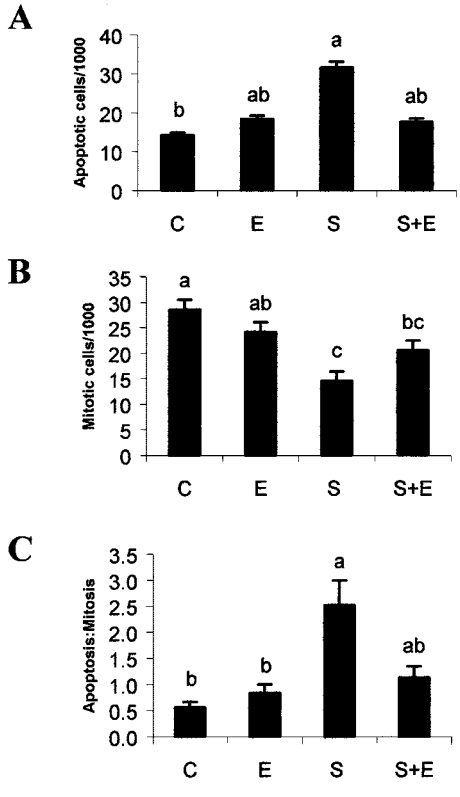

Fig. 1. Effect of sulindac (*S*), EPR-A (*E*), and sulindac + EPR-A (*S+E*) compared with control (*C*) on (*A*) apoptosis, (*B*) mitosis, and (*C*) ratio of apoptosis:mitosis in tumors of *Apc$^{Min/+}$* mice as determined histologically after H&E staining. Graphs show mean values in each group (*bars*, ± SE). Different superscripts indicate differences among groups at $P < 0.05$.

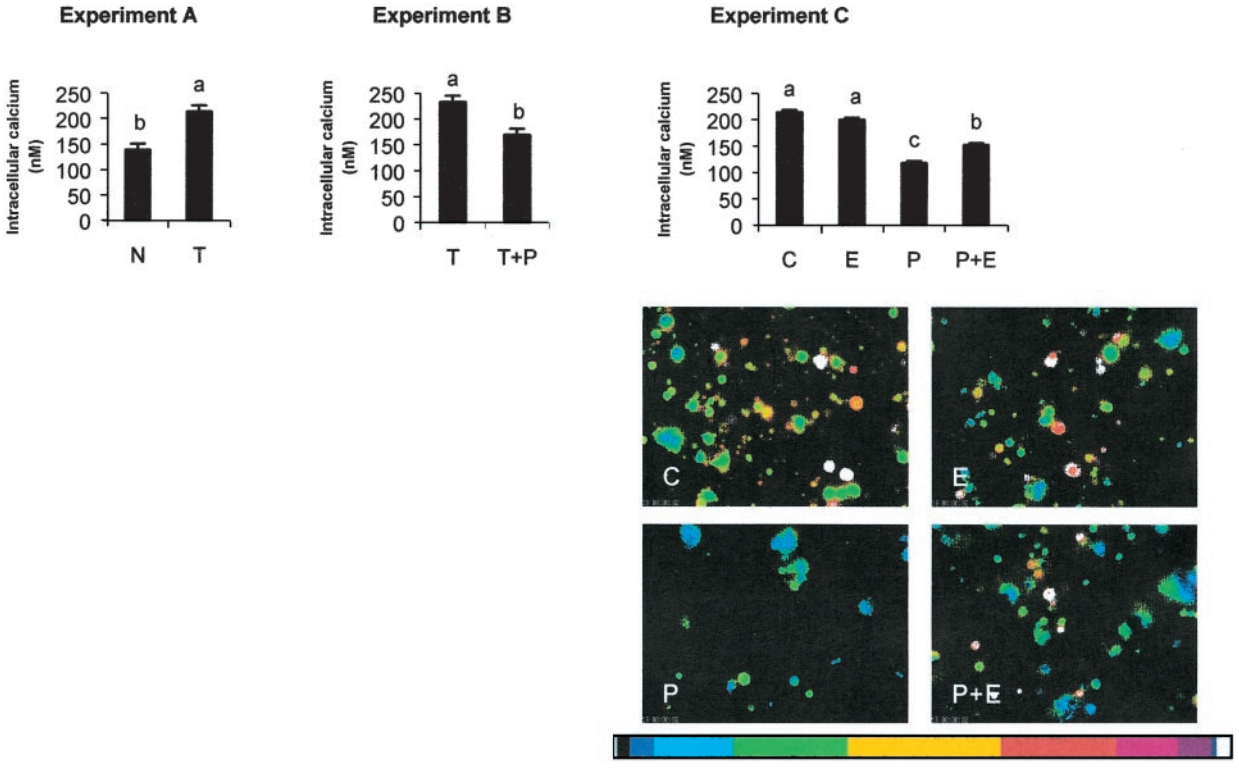

Fig. 2. The $[Ca^{2+}]_i$ in tumor cells (*T*) was compared with $[Ca^{2+}]_i$ in normal-appearing intestinal mucosal cells (*N*) from *Apc$^{Min/+}$* mice in *Experiment A* ($n = 3$/group). In *Experiment B*, $[Ca^{2+}]_i$ in tumors from untreated mice was compared with $[Ca^{2+}]_i$ in tumors from piroxicam-treated mice (*T+P*; $n =$ six/group). In *Experiment C*, the effects of the EP receptor agonist 17-phenyl-trinor-PGE$_2$ (*E*), piroxicam (*P*), and P+E on $[Ca^{2+}]_i$ in tumors were compared with tumors from untreated control mice (*C*; $n =$ five/group). Graphs show mean $[Ca^{2+}]_i$ in each group within each experiment (*error bars*, ± SE). A fura-2 dual-wavelength fluorescence imaging system was used to determine $[Ca^{2+}]_i$. The pseudocolor images are representative of variations of $[Ca^{2+}]_i$ within cells. Changes in pseudocolor, as illustrated by the *color bar*, from *blue* to *green* to *yellow* to *red* to *white* indicate increasing $[Ca^{2+}]_i$. Different superscripts indicate differences among groups at $P < 0.05$.

COX-1 but does not necessarily reduce tumor number in *Apc$^{Min/+}$* mice (17), this suggests that COX-1 involvement may be minimal.

In a follow-up experiment, we confirmed the importance of PGE$_2$ in tumorigenesis by administering an antibody (2B5) that neutralizes PGE$_2$ *in vivo* (41). If NSAIDs induce tumor regression by reducing PGE$_2$ formation, then immunological sequestration of PGE$_2$ should have a similar effect. Accordingly, administration of 2B5 to *Apc$^{Min/+}$* mice with preexisting tumors resulted in significantly fewer tumors relative to controls after 4 days of treatment. Similarly, Stolina *et al.* (51) observed attenuated tumor growth in mice bearing Lewis lung carcinoma xenografts after treatment with 2B5 along with a concomitant decrease in tissue PGE$_2$ levels. We failed to see differences in PGE$_2$ in our tissue samples (data not shown), most likely because of the length of time between administration of the final dose of 2B5 and time of sacrifice (48 h). These experiments suggest that PGE$_2$ mediates intestinal tumorigenesis and may be required for the maintenance of tumor integrity.

PGE$_2$ evokes its cellular responses via one or more of the four EP receptors (EP1-EP4), and the EPR-A used in this study were ligands for all of the receptor subtypes. Watanabe *et al.* (52) have shown that selective antagonism of EP1 results in 44% fewer tumors in *Apc$^{Min/+}$* mice, suggesting EP1 may be an important receptor in mediating the effects of PGE$_2$ in intestinal tumorigenesis. Involvement of the other EP receptors is unclear, but it seems less likely that EP3 is involved. Although EP3 is strongly expressed in myenteric ganglia and weakly expressed in longitudinal smooth muscle throughout the murine intestinal tract (53), development of aberrant crypt foci was not different in C57BL/6J EP3−/− mice compared with controls after azoxymethane treatment, whereas targeted mutations of EP1 attenuated development of azoxymethane-induced lesions in EP1−/− mice (52). EP2 was not shown to be expressed in murine small intestine (53). However, EP4 and EP1 are expressed in the intestines of mice, with EP4 being highly expressed in epithelial cells and EP1 in the muscularis mucosa (53) and, thus, are potential candidate receptors for the effects observed with the EPR-A. We used an admixture of two stable analogues of PGE$_2$ capable of mimicking the actions of endogenous PGE$_2$. Because of the putative involvement of EP1, we used the EP1/EP3 receptor agonist 17-phenyl-trinor-PGE$_2$. We also used 16,16-dimethyl-PGE$_2$ (EP2/EP3/EP4 agonist; Ref. 54), because i.p. administration has been shown previously to partially reverse indomethacin-induced reduction in survival of small intestinal crypt stem cells, the primary site for intestinal tumorigenesis in this model (40). There have been no previous reports on the effects of 17-phenyl-trinor-PGE$_2$ on the intestinal tract *in vivo* when administered either i.p. or p.o.

It has been reported that activation of EP1 augments intracellular calcium mobilization through a phospholipase C/inositol triphosphate (IP_3)-mediated signaling pathway and also effects CCE in some cell types (55–57). Our data suggest that NSAIDs may be reducing tumorigenesis in part by attenuating $[Ca^{2+}]_i$ and that these effects may involve prostaglandin-mediated pathways. When nonexcitable cells are triggered to mobilize intracellular calcium, this is followed by an influx of extracellular calcium to replenish stores (CCE). NSAIDs and n-3 polyunsaturated fatty acids, both of which inhibit PGE$_2$ biosynthesis and tumorigenesis in *Apc$^{Min/+}$* mice, also prevent CCE in cancer cells *in vitro* and *in vivo*, and add-back experiments designed to increase PGE$_2$ biosynthesis result in augmentation of tumor growth (17–19, 58–61). The link between $[Ca^{2+}]_i$ and tumorigenesis is strengthened by the fact that voltage-gated L-type calcium channel expression is elevated in colon cancer (62) and colonic tumor cell lines, and blockade of these calcium channels can trigger apoptosis via a caspase-3-dependent mechanism (63). Furthermore, calcium mobi-

lization, CCE, and mitogenesis are increased by epidermal growth factor via a phospholipase C/IP_3-mediated pathway (64), effects inhibited by NSAIDs (59, 60). Interruption of this pathway reduced tumor frequency by 52% in $Apc^{Min/+}$ mice, and this effect was augmented by concomitant treatment with sulindac (65), additionally implicating calcium involvement in tumorigenesis.

We report for the first time that intestinal tumors from $Apc^{Min/+}$ mice have elevated $[Ca^{2+}]_i$ compared with adjacent normal epithelium. Moreover, we have shown that NSAIDs exert their antitumorigenic effects, in part, by interfering with PGE_2 and possibly intracellular calcium signaling. Treatment with the NSAID piroxicam appears to normalize $[Ca^{2+}]_i$ in actively regressing tumors, and this effect is attenuated by concomitant treatment with 17-phenyl-trinor-PGE_2. These results suggest that NSAIDs may affect tumor integrity, at least in part, via reductions in $[Ca^{2+}]_i$, and this effect on $[Ca^{2+}]_i$ may be attributable to reductions in PGE_2 and its subsequent signaling through EP1. Unlike EP1, EP2–4 act primarily through cAMP-mediated signaling pathways, either by inhibiting or stimulating adenylate cyclase (reviewed in Ref. 66). Treatment of $Apc^{Min/+}$ mice with an EP2–4 agonist has been shown to reduce tumor number by as much as 50%, suggesting the various EP receptors may be associated with dichotomous outcomes (67).

An unexpected enigma in our data are the apparent antitumorigenic effect of EPR-A (control *versus* EPR-A; pooled data; $P = 0.06$) that seems antithetical to its protumorigenic effects when coadministered with either piroxicam or sulindac. It is possible that activation of the various EP receptors may differentially modulate tumorigenesis such that some receptors promote tumor growth, whereas others promote tumor regression. For example, Lehnert *et al.* (68) reported that 16,16-dimethyl-PGE_2 significantly attenuated carcinogen-induced small intestinal tumors in rats. Moreover, it was reported recently that 16,16-dimethyl-PGE_2 reduced tumor number in $Apc^{Min/+}$ mice by 20–50% after i.p. administration three times per week for 12 weeks (67). This is in agreement with research linking increases in cAMP levels and protein kinase A activity to alterations in proliferation and differentiation in several cancer cell lines including an antiproliferative effect on some colon cancer cell lines, both *in vitro* and *in vivo* (69, 70). Additionally, activation of EP2, EP3, or EP4 receptors has been associated with cAMP-mediated growth inhibition of B lymphocytes, NIH-3T3 cells, and mesangial cells *in vitro*, whereas 17-phenyl-trinor-PGE_2 or other EP1 agonists stimulated proliferation (71–73). Perhaps stimulation of the EP1 receptor helps to maintain tumor integrity, whereas activation of one or more of the other EP receptors, *i.e.*, EP4, is involved in the attenuation of tumor number in this model. Therefore, our EPR-A mixture containing both an EP1/EP3 agonist (17-phenyl-trinor-PGE_2) and an EP2–4 agonist (16,16-dimethyl-PGE_2) may be acting antithetically.

In summary, inhibition of PGE_2 biosynthesis accounts, at least in part, for the antitumorigenicity of NSAIDs. Furthermore, we show that NSAID treatment results in a higher apoptosis:mitosis ratio and lower $[Ca^{2+}]_i$ *in vivo*, and these effects are mediated, in part, by inhibition of PGE_2 biosynthesis. Because the EPR-A mixture used for these studies contained ligands for all four of the EP receptors, we are unable to definitively ascribe the effects on tumorigenesis to any one receptor or combination of receptors. However, previous research on receptor expression patterns and roles of select subtypes along with the data presented in this paper suggest that EP1 may be involved in mediating the proliferative effects of PGE_2 on intestinal tumorigenesis in this model. Additional investigation will be required to conclusively determine which EP receptor subtype(s) are responsible for the observed effects and whether these might prove to be dichotomous.

ACKNOWLEDGMENTS

We thank Dr. Joseph Portanova of Pharmacia (St. Louis, MO) for generously providing us the 2B5 monoclonal anti-PGE_2 antibody and technical assistance in its use.

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