Sulindac, a non-steroidal anti-inflammatory prodrug, is metabolized into pharmacologically active sulfide and sulfone derivatives. Sulindac sulfide, but not sulindac sulfone, inhibits cyclooxygenase (COX) enzyme activities, yet both derivatives have growth inhibitory effects on colon cancer cells. Microarray analysis was used to detect COX-independent effects of sulindac on gene expression in human colorectal cancer cells. Spermidine/spermine N\(^1\)-acetyltransferase (SSAT) gene, which encodes a polyamine catabolic enzyme, was induced by clinically relevant sulindac sulfone concentrations. Northern blots confirmed increased SSAT RNA levels in these colon cancer cells. Deletion analysis and mutational studies were done to map the sulindac sulfone-dependent response sequences in the SSAT 5'-flanking sequences. This led us to the identification of two peroxisome proliferator-activated receptor (PPAR) response elements (PPREs) in the SSAT gene. PPRE-2, at +48 bases relative to the transcription start site, is required for the induction of SSAT by sulindac sulfone and is specifically bound by PPAR\(_\gamma\) in the Caco-2 cells as shown by transfection and gel shift experiments. PPRE-1, at −323 bases relative to the start site, is not required for the induction of SSAT by sulindac sulfone but can be bound by both PPAR\(\alpha\) and PPAR\(_\gamma\). Sulindac sulfone reduced cellular polyamine contents in the absence but not in the presence of verapamil, an inhibitor of the export of monoacetyl diamines, inhibited cell proliferation and induced apoptosis. The induced apoptosis could be partially rescued by exogenous putrescine. These data suggest that apoptosis induced by sulindac sulfone is mediated, in part, by the COX-independent, PPAR-dependent transcriptional activation of SSAT, leading to reduced tissue polyamine contents in human colon cancer cells.

Numerous epidemiological, animal, and in vitro studies indicate that non-steroidal anti-inflammatory drugs (NSAIDs\(^1\)) have antitumorigenic activities against colorectal cancer (1–4).

Sulindac, an NSAID, inhibits colorectal carcinogenesis in rodent models (5, 6) and causes regression of adenomas (7, 8) in patients with familial adenomatous polyposis coli. NSAIDs work by inhibiting cyclooxygenases (COXs) of which there are at least two distinct forms, COX-1 and COX-2. Physiologically sulindac is metabolized into sulfide- or sulfone-containing derivatives. The sulfide derivative inhibits colon carcinogenesis by inhibiting COX-1 and COX-2 enzyme activities (9). However, sulindac sulfone also inhibits chemical carcinogenesis in rodents but by a mechanism that cannot be explained solely by the inhibition of prostaglandin synthesis (10, 11), yet both derivatives inhibit growth and induce apoptosis in a variety of human tumor-derived cell lines (12, 13). Sulindac sulfone, at clinically relevant concentrations ranging from 35 \(\mu M\) (in humans) to around 150 \(\mu M\) (in mice), has been shown to have chemopreventive effects on colon cancer (12, 14–16).

One of the COX-independent mechanisms of action of sulindac and its metabolites is to act as ligands for peroxisome proliferator-activated receptors (PPARs). PPARs are nuclear hormone receptors that bind to sequence-specific DNA response elements known as PPREs as a heterodimer with RXR\(\alpha\) and can regulate gene expression. There are three PPAR isotypes, \(\alpha\), \(\gamma\), and \(\delta\), present in humans. There is evidence that arachidonic acid metabolites like 15-deoxy-\(\Delta^{12,14}\)-PGJ\(_2\) can serve as activating ligands for PPARs (17, 18). Further PPAR\(\gamma\) can act as a potential tumor suppressor (19–21), while PPAR\(\alpha\) can act as a potential oncogene (22, 23) in colon cancer. Sulindac can bind PPAR\(\gamma\) and PPAR\(\alpha\) as their ligands and lead to their activation, which can have either a positive or a negative effect on gene transcription (22, 24).

NSAIDs, like piroxicam, aspirin, and indomethacin, have been shown to exert their chemopreventive action by affecting the polyamine metabolism in colorectal cancer (25–27). The polyamines putrescine, spermidine, and spermine are abundant polycations in eukaryotic cells that are often elevated in neoplastic cells when compared with normal cells and tissues (28). The polyamine levels are tightly regulated by the biosynthetic enzyme ornithine decarboxylase (ODC) and the catabolic enzyme spermidine/spermine \(N^1\)-acetyltransferase (SSAT) in cells. High levels of polyamines lead to rapid proliferation (29), while lower levels of polyamines have been shown to promote apoptosis (30, 31) and inhibit cell growth (32). Because of the effects of the polyamines on apoptosis and proliferation, regulation of the expression of these enzymes has been an area of intense research (33–38). The effects of one of the NSAIDs, indomethacin, on polyamine metabolism involve suppression of ODC and induction of SSAT, thereby decreasing polyamine pools in colon cancer cells (25). However, it is not known how NSAIDs induce SSAT nor has a role for polyamines in NSAID actions, such as induction of apoptosis, been investigated.
this study, we investigated the effects of sulindac sulfone on SSAT and polyamine metabolism and asked whether polyamines were involved in sulindac sulfone-induced apoptosis of colon cancer cells.

EXPERIMENTAL PROCEDURES

Materials

All cell culture reagents, DNA-modifying enzymes, TRIZol® reagent (Total RNA Isolation reagent), and LipofectAMINE reagent were purchased from Invitrogen. Ciglitazone and Wy-14463 were purchased from Biomol Research Laboratories. GW9662 and cPGI was purchased from Cayman Chemical. Sulindac sulfone was purchased from ICN Biomedicals, Inc. The anti-PPAR antibody PA3-820 was purchased from Affinity Bioreagents, Inc. Verapamil was purchased from Sigma.

Plasmids

Expression plasmids pSG5-xPPARγ, pSG5-xPPARα, and pSG5-xP-PARα were generously given by Dr. Liliane Michailik (Institut de Biologie Animale, Lausanne, Switzerland). The expression plasmid pCMX-hRXRαKpn was kindly provided by Dr. Ronald Evans (The Salk Institute for Biological Studies, La Jolla, CA). Full-SSAT-luc, having a 3,493-kb-long 5′-flanking sequence of the human SSAT gene was cloned into a promoterless pGL2-basic vector (Promega, Madison, WI) as previously reported (39). 197-SSAT-luc, having 283 nucleotides of the 5′-flanking region of SSAT promoter, was made from Full-SSAT-luc using polymerase chain reaction and subcloned into pGL2-basic vector. 348pPre-SSAT-luc has the PPRE at -48 replaced by a random sequence from the SSAT 5′ promoter region, which has no putative transcription factor binding sites as determined by TRANSFAC data base analysis.

Cell Culture and Transfections

The Caco-2 cell line was purchased from American Type Culture Collection (ATCC) (Manassas, VA) at passage 12 and was maintained in minimum essential α-medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin solution. The human colon cancer cell line HCT-116 was maintained as a monolayer culture in McCoy's 5A medium supplemented with 10% fetal bovine serum plus 1% penicillin/streptomycin solution. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO2. All cell culture supplies were from Invitrogen.

Transient transfections were performed using LipofectAMINE reagent according to the manufacturer's protocol. Briefly, 5 × 10^4 cells were seeded in a 6-well plate and cultured in normal medium for 24 h. Each well was transfected with 1 μg of firefly luciferase reporter construct along with 0.2 μg of pCMV-β-galactosidase expression plasmid, which acted as a transfection efficiency control. After 6 h of incubation with LipofectAMINE-DNA complex, cells were supplemented with complete medium having 20% fetal bovine serum and 2% penicillin/streptomycin solution and grown overnight. Afterward the medium was removed, and cells were refed with medium along with various concentrations of sulindac sulfone or its vehicle, dimethyl sulfoxide (Me2SO), for 48 h.

For PPAR studies, triple transient transfections were performed using the same protocol as above. Each well was transfected with 1 μg of firefly luciferase reporter construct with or without 0.5 μg of expression plasmid for xPPARγ, xPPARα, or xP-Parα and hRXRα. 0.2 μg of pCMV-β-galactosidase expression plasmid was cotransfected into each well to monitor transfection efficiency. After 6 h of incubation with LipofectAMINE-DNA complex, cells were supplemented with complete medium having 20% fetal bovine serum and 2% penicillin/streptomycin solution and grown overnight. On the following day, the medium was removed, and cells were refed with medium along with the appropriate PPAR activator or its vehicle, Me2SO, for 48 h.

All transfections were performed in triplicates unless stated differently. All transfected cells were washed once with phosphate-buffered saline and lysed, and luciferase activities were measured using 10 μl of cell extract and 50 μl of luciferase reagent (Promega). β-Galactosidase activity was measured using the β-galactosidase assay kit (Invitrogen) according to the manufacturer’s protocol.

dDNA Microarray Analysis

Probe Preparation—The microarray chip was made as described in detail before (40). The chip has ~5,300 human genes; >3,000 are known genes, and the remainder are expressed sequence tags as determined by Unigene. A list of the clones on the arrays is available upon request.

Target Preparation—Microarray analysis was done as described before (41). In short, the RNeasy total RNA kit (Qiagen, Valencia, CA) and protocol was used to isolate total RNA from the Caco-2 cells treated with either vehicle or sulindac sulfone. Fluorescent first strand cDNA was made using the Micromax Direct cDNA microarray system (PerkinElmer Life Sciences) following the manufacturer’s protocols. cDNA from sulindac sulfone-treated cells were Cy5 (Amersham Biosciences)-labeled, while vehicle-treated cells were Cy3 (Amersham Biosciences)-labeled. Labeled cDNA from two reactions (one Cy5-labeled and one Cy5-labeled) was combined and purified on a Microcon-50 column, lyophilized, resuspended in hybridization buffer (2 × SSC, 0.1% SDS, 100 ng/μg Cot1 DNA, 100 ng/μg oligo(dA)), denatured by boiling for 2.5 min, and added to a denatured (2-min boil, slide in double distilled water, plunge into room temperature ethanol, spin dry at 500 × g) microarray. A coverslip (22 × 22 mm) was applied, and the array was placed in a hybridization chamber (catalog number HYB-63, GeneMachine) at 62 °C for 18 h. Following hybridization, slides were washed and then scanned for Cy3 and Cy5 fluorescence using an Axon GenePix 4000 microarray reader (Axon Instruments, Foster City, CA) and quantitated using GenePix software. The analysis was done three independent times with the RNA of sulindac sulfone-treated Caco-2 cells.

RNA Isolation and Analysis

Total RNA was obtained from cells by extraction using TRIZol reagent and used for Northern blotting as described previously (42, 43). Membranes were hybridized with 32P-labeled cDNA encoding for human SSAT and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data are expressed as the ratio of the integrated densities of 32P-labeled hybridization bands for the SSAT and GAPDH genes.

In Vitro Transcription/Translation

cDNAs for xPPARα, xPPARγ, and hRXRα were transcribed and translated in vitro from the pSG5-xPPARα, pSG5-xPPARγ, pSG5-xPPARγ, and pCMX-hRXRαKpn plasmids, respectively. The TntC coupled reticulocyte lysate system (Promega) was used according to the manufacturer’s instructions. Translation products were verified by SDS-polyacrylamide gel electrophoresis.

Gei Electromobility Shift Assays

Nuclear extracts were prepared from Caco-2 cells essentially as described previously (44). To study the binding of nuclear hormone receptors to the putative PPRE, two double-stranded oligonucleotides, PPRE-2 and PPRE-1, spanning nucleotides −2 to +35 and −304 to −336, respectively, of the SSAT 5′ sequence, were 32P-labeled with polynucleotide kinase (Promega). A 15-μl reaction containing 0.5 ng of PPRE-2 probe and 5 μg of nuclear extract or 0.5–1 μl of in vitro transcription reaction was incubated for 20 min at 25 °C and 15 min at 4 °C in a buffer containing 20 mM HEpes (pH 8), 60 mM KCl, 1 mM dithiothreitol, 10% glycerol, and 2 μg of poly(dl-dc). The DNA-protein complexes were resolved from the free probe by electrophoresis at 4 °C on a 5% polyacrylamide gel in 1× Tris borate-EDTA buffer, pH 8. Double-stranded oligonucleotides composed of the following sequences were used for gel shift analysis: PPRE-2 (w), 5′-AGAAAAAGAACGAGGTCACCTTGGCGGGGGGGTCC-3′; PPRE-1 (w), 5′-CCGCTACATCCGGCGAGG- tttaccttggtcatggtggttc-3′; PPRE-2mut (m), 5′-AGAAAAAAGAAGATgAATcGTTgAAGGGGGCCGTG-3′; PPRE-1mut (m), 5′-CGCTACATCCGGCGAGGttttaccttggtcatggtggttc-3′. The PPRE sequence is underlined, and the mutated bases are shown by lowercase letters.

Immunoblotting

Western blots were done for COX-2 as described elsewhere (42). COX-2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and were used at 1:5,000 dilutions, respectively. All Western blots were repeated three times, and a representative blot was chosen for presentation.

Cell Number and Viability Determinations

Caco-2 cells were seeded at a concentration of 1 × 10^5 cells/100-mm culture plate. The cells were grown for 24 h before they were refed with a new medium and treated with sulindac sulfone, 1 μM putrescine, both, or the vehicle Me2SO. The cells were then harvested after 0, 1,
Cells were removed from the monolayer by treatment with trypsin (1,500 units/ml, Calbiochem)-EDTA (0.7 mM) and counted using a hemocytometer. A sample of the cell suspension was combined in a 1:1 volume ratio with trypan blue dye (Invitrogen), and at least two independently prepared suspensions were counted (two counts each) on a hemocytometer. Viability was determined by the percentage of cells able to exclude the trypan blue dye.
Apoptosis Assay: Plasma Membrane Phosphatidylserine Binding Assay

Caco-2 cells were seeded at a concentration of 1\times10^6 cells/100-mm culture plate. Cells were grown for 24 h before they were refed with a new media and treated with sulindac sulfone, 1 mM putrescine, both, or vehicle. The cells were then harvested after 0, 1, 2, 4, and 6 days postdrug exposure. Cells were removed from the monolayer by treatment with trypsin and counted using a hemocytometer. 5\times10^5 cells were pelleted down for the apoptosis staining. The procedure for staining with the ApoAlert® Annexin V kit (Clontech) was based on the manufacturer’s protocol. Briefly, the cells were resuspended in 200 \mu l of kit 1× binding buffer. To each tube, 5 \mu l of the Annexin V/fluorescein isothiocyanate binding buffer (20 \mu g/ml in Tris-NaCl) and 10 \mu l of the kit propidium iodide (50 \mu g/ml in 1× binding buffer) were added. Each tube was gently mixed and incubated for 15 min at room temperature in the dark. The volume was then brought up to 500 \mu l by adding 1× binding buffer. Cells were analyzed using a BD Biosciences FACScan flow cytometer.

**SSAT and ODC Enzyme Activity Determination**

For enzyme activities, cells were grown overnight and then treated with various concentrations of sulindac sulfone or its vehicle. Cells were harvested after 48 h of treatment and washed in cold phosphate-buffered saline. The radiochemical assay of the SSAT activity was performed by estimation of labeled N\textsuperscript{1}-acetylspermidine synthesized from [14C]acetyl-CoA and unlabeled spermidine as described elsewhere (45).

**Fig. 2. Sulindac sulfone, at lower concentrations, induces SSAT.** A. 2 \times 10^6 cells were seeded in a 150-mm culture plate and grown for 24 h. After 24 h, the cells were treated with 600 \mu M sulindac sulfone for 24 or 48 h and harvested, and total RNA was extracted as described under “Experimental Procedures.” Top, 20 \mu g of RNA was loaded onto the gel from cells treated with either vehicle (V) or sulindac sulfone (Sulfone) for 24 or 48 h. Each lane represents a separate experiment. Bottom, values of SSAT expression after being normalized to GAPDH. B. Caco-2 cells were transfected with the Full-SSAT-luc reporter constructs and then treated with either vehicle (open bar) or the indicated concentrations of sulindac sulfone for 48 h. Relative light units were calculated after normalizing to the protein and \beta-galactosidase activities in the cell lysates. The result is an average from three different experiments. *, p < 0.05.
The ODC enzyme activity was measured by evaluating the release of \(^{14}\text{CO}_2\) from L-[\(^{14}\text{C}\)]ornithine as described elsewhere (46). The fold change was calculated by dividing the enzyme activity for the sample by the vehicle. The enzyme assays were done in triplicates.

**Polyamine Analysis**

Cell extracts were prepared in 0.1 \(\text{N HCl}\) (4 \(\times\) \(10^7\) cells/900 \(\mu\)l). After sonication, the preparation was adjusted to 0.2 \(\text{N HClO}_4\), and the supernatant was analyzed by reverse-phase high performance liquid chromatography with 1,7-diaminoheptane as an internal standard (47). Protein was determined by BCA assay (48).

**Statistical Analysis**

All transient transfection experiments were performed in triplicates and were repeated at least three times. Cell growth assays, apoptosis assays, and Northern blots were done at least three times. Representative experiments or mean values \(\pm\) S.D. are shown. Statistical differences were determined by Student's \(t\) test. A \(p\) value of \(<0.05\) was considered significant.

**RESULTS**

Sulindac Sulfone Leads to Cell Growth Inhibition and Induction of Cell Death in Caco-2 Cells—Sulindac and its derivatives have been shown to either inhibit cell proliferation or induce apoptosis in colon cancer cells (49–51). We wanted to see whether sulindac sulfone, at doses that are clinically relevant, have any growth-suppressive effects on Caco-2 cells. We did cell counting using a hemocytometer to estimate cell proliferation and found that sulindac sulfone at concentrations of 50 \(\mu\)M and
Role of Polyamines in Sulindac Sulfone-induced Apoptosis

Sulindac Sulfone Induces SSAT Leading to Decreased Polyamine Levels in Caco-2 Cells—DNA microarray analysis of 5,300 genes with the cDNA prepared from total RNA from the Caco-2 human colorectal cancer cells treated with 600 μM sulindac sulfone, which is the dose necessary to reduce colony formation by 50% (IC₅₀ dose), showed altered expression of several genes compared with the control-treated Caco-2 cells. One of these genes was SSAT whose expression was induced 3.94 ± 0.64-fold in treated Caco-2 cells as compared with the controls (p < 0.06). The induction in SSAT mRNA expression was confirmed by Northern analysis (Fig. 2A). We next wanted to determine whether clinically relevant concentrations of sulindac sulfone have any effect on SSAT expression. We found that sulindac sulfone at concentrations of 100 μM and above led to an induction in SSAT mRNA (data not shown). To determine whether this induction of SSAT expression is at the level of SSAT transcription, we did transient transfection experiments using the Full-SSAT-luc reporter promoter construct, which has 3.53 kb of the SSAT 5’ promoter flanking region in front of the luciferase gene, and then treated the cells with various concentrations of sulindac sulfone. Sulindac sulfone at concentrations of 100 μM or greater led to an induction in the Full-SSAT-luc promoter activity in the Caco-2 cells after 48 h of incubation (Fig. 2B). These data suggest that sulindac sulfone increases the SSAT gene expression at the level of transcription. Next we wanted to see whether this induction in SSAT promoter activity and SSAT RNA affects the activity of SSAT enzyme resulting in altered cellular polyamine levels. Sulindac sulfone led to a 2-fold induction in the SSAT activity, which was correlated with a 2-fold reduction in the intracellular levels of spermine and spermidine with the treatment of sulindac sulfone for 48 h (data not shown).

Sulindac Sulfone Is Acting in a COX-2-independent Manner to Induce SSAT—Sulindac sulfone has been shown to have both COX-dependent and COX-independent mechanisms of action (10, 11). To elucidate the mechanism by which sulindac sulfone is inducing SSAT we used HCT-116 cells, which have no detectable COX-2 protein levels as compared with the Caco-2 cells (Fig. 3A). We expected to see lower PGE2 levels in these cells due to the reduction in COX-2 protein. HCT-116 cells had an approximately 10-fold reduced PGE2 concentration as compared with Caco-2 cells (Fig. 3B). To determine whether sulindac sulfone is acting in a COX-dependent manner, we hypothesized that sulfone would have no effect on SSAT promoter in HCT-116 cells. On the contrary, we found that sulindac sulfone led to an induction in the Full-SSAT-luc promoter construct in HCT-116 cells after 48 h of treatment (Fig. 3C). Next treatment of HCT-116 cells with sulindac or its metabolites sulindac sulfide and sulindac sulfone showed that both sulindac and sulindac sulfone, but not sulindac sulfide, induced SSAT RNA in HCT-116 cells (Fig. 3D). This indicated that SSAT induction by sulindac sulfone involves a mechanism that is not dependent on the COX-2 inhibition. To further test whether sulindac sulfone is acting in a COX-2-independent manner, we added 20 μM PGE₂ to Caco-2 cells and measured SSAT RNA after 48 h of treatment. Addition of PGE₂ led to an increase in the intracellular PGE₂ levels as measured by a PGE₂ enzyme-linked immunosorbent assay (data not shown). If sulindac sulfone is acting in a COX-dependent manner, then the addition of PGE₂ should lead to a reduction in the SSAT RNA. On the contrary, we found that addition of 20 μM PGE₂
FIG. 5. Sulindac sulfone induces SSAT through activation of PPARs in the Caco-2 cells. A, Caco-2 cells were transfected with the PPRE-Luc reporter construct along with the β-galactosidase plasmid. The cells were then treated for 48 h with either vehicle (open bar) or 600 μM sulindac sulfone, 20 μM ciglitazone (CG), 20 μM cPGI, 200 μM Wy14463, and 10 μM 15-d-PGJ₂ (all black bars). Normalized luciferase activities are shown as mean ± S.D. (n = 3) and are expressed as -fold inductions relative to the activity in the presence of vehicle alone. Asterisks indicate statistical difference from activity of the reporter construct treated with vehicle alone (*, p < 0.05). B, cells were grown overnight and then treated with vehicle (open bar) or 600 μM sulindac sulfone, 20 μM ciglitazone (CG), 20 μM cPGI, and 200 μM Wy14463 for 48 h. Total RNA was analyzed by probing for SSAT and GAPDH. -fold induction was calculated by dividing normalized sample values by the control. The result is an average from three different experiments. *, p < 0.05. RLU, relative luciferase units.
did not produce any changes in the SSAT RNA or the activity of SSAT promoter constructs by 48 h (data not shown) indicating that sulindac sulfone is acting in a PGE\textsubscript{2}-independent manner to induce SSAT gene expression in colon cancer cells.

The Human SSAT Gene Contains a Functional PPRE—To determine the sulindac sulfone-dependent, but COX-2 inde-
pendent, response elements in the SSAT gene, portions of the SSAT 5′-flanking sequences were tested for their ability to mediate sulindac sulfone-induced transcription of a reporter gene. Five luciferase constructs were used, each containing portions of the SSAT 5′-flanking region linked to a promoterless firefly luciferase gene. Full-SSAT-luc, 659-SSAT-luc, 358-SSAT-luc, 197-SSAT-luc, and 48ppre-SSAT-luc contained 3.53, 0.74, 0.44, 0.28, and 0.1 kb, respectively, of the SSAT 5′-flanking sequence (Fig. 4A). In one set of experiments, all of these constructs as well as the promoterless pGL2-basic control con-

**Fig. 7.** Sulindac sulfone induction of SSAT requires PPARγ acting on PPRE-2. A, Caco-2 cells were transfected with the 48ppre-SSAT-luc, Δ48ppre-SSAT-luc, or 197-SSAT-luc reporter constructs along with the β-galactosidase plasmid. The cells were then treated for 48 h with either vehicle (open bars) or 300 μM sulindac sulfone (black bars). Normalized luciferase activities are shown as mean ± S.D. (n = 3) and are expressed as -fold inductions relative to the activity in the presence of vehicle alone. Asterisks indicate statistical difference from activity of the reporter construct treated with vehicle alone (*, p < 0.05). B, Caco-2 cells were transfected with the 197-SSAT-luc or Full-SSAT-luc reporter constructs along with the β-galactosidase plasmid. The cells were then treated for 48 h with either vehicle (open bars), 300 μM sulindac sulfone (black bars), or sulindac sulfone and GW9662 (gray bars). Normalized luciferase activities are shown as mean ± S.D. (n = 3) and are expressed as -fold inductions relative to the activity in the presence of vehicle alone. Asterisks indicate statistical difference from activity of the reporter construct treated with vehicle alone (*, p < 0.05). RLU, relative luciferase units.
flanking region of the SSAT promoter, only one of which is in the 197-SSAT-luc and 48ppre-SSAT-luc construct, as initially identified using a transcription factor search analysis (TRANSFAC data base). PPRE-1 is present at −323, and PPRE-2 is present at +48 respective to the transcriptional start site in the SSAT gene. To study the involvement of PPARs in the induction of SSAT gene expression, PPAR expression was determined in the Caco-2 cells. Caco-2 cells express both PPARα and PPARγ proteins as detected by Western and Northern blot analysis (data not shown). Next, since PPARs function as receptors, we wanted to determine whether PPARs are functional in Caco-2 cells. Transient transfection experiments were done to determine the functionality of PPARs and their role in the regulation of SSAT. Caco-2 cells were transfected with the tk-PPRE−Luc plasmid and then treated with sulindac sulfone and an array of PPAR activators. To further characterize the responsiveness of these PPREs to the various PPAR subtypes, all five SSAT promoter reporter constructs were transfected into Caco-2 cells and assayed for luciferase activity in the presence and absence of various PPARs and their activators. The expression plasmid for RXRα was transfected to all the cells along with the β-galactosidase plasmid, which acted as the transfection efficiency control. The activators used were ciglitazone (PPARγ activator), cPGI (PPARδ activator), and Wy14463 (PPARα activator). As shown in Fig. 6A, cells transfected with PPARγ and treated with ciglitazone demonstrated a 3.5–4.5-fold increase in transcription of the reporter constructs having as low as 0.1 kb of the 5′-flanking sequence. PPARγ alone was not able to activate transcription of these promoter constructs. PPARγ was also able to activate transcription in these plasmids, but it could not induce either 197-SSAT-luc or 48ppre-SSAT-luc plasmid, which lack the PPRE-1 sequence (Fig. 6B). Finally the PPARγ subtype was unable to positively regulate transcription of any of the reporter constructs in the presence of its activator, but PPARγ alone was able to induce Full-SSAT-luc by 2-fold (Fig. 6C).

**PPARγ Is Involved in Regulating SSAT Expression by Sulindac Sulfone**—Based on the previous results showing that sulindac sulfone induces PPARs and that the induction by sulindac sulfone is retained in the 48ppre-SSAT-luc construct, which has only the PPRE-2, we wanted to test whether PPRE-2 plays an important role in sulindac sulfone-induced activation of SSAT gene. For this, we made another promoter construct, Δ48ppre-SSAT-luc, which has the same sequence as 48ppre-SSAT-luc but has a deleted PPRE-2. Caco-2 cells were transfected with either of these plasmids or the 197-SSAT-luc and treated with sulindac sulfone for 48 h. Sulindac sulfone led to a significant increase in the transcription of the PPRE-containing constructs but did not affect transcription of the PPRE-deleted construct (Fig. 7A). Further, to test whether PPRE-2 is
acted upon by an activated PPARγ, we used GW9446, a PPARγ antagonist. Caco-2 cells were transfected with either 197-SSAT-luc or Full-SSAT-luc and treated with either sulindac, a combination of sulfone and GW9446, or their vehicle, Me2SO, for 48 h. GW9446 was able to totally abolish the induction of 197-SSAT-luc by sulindac sulfone but could only partially do it for the Full-SSAT-luc (Fig. 7B). These data suggest that sulindac sulfone induces SSAT transcription by activation of PPARγ, which then binds to the PPRE-2.

PPARs and RXRs Bind as Heterodimers to the SSAT PPRE—To determine whether PPARs bind to the PPRE as heterodimers with RXRs, gel shift assays were performed with a double-stranded oligonucleotide containing either PPRE-1 (w2) or PPRE-2 (w) and in vitro translated PPAR proteins. Since PPARs bind DNA as a heterodimer with RXRs, all reaction mixtures had PPARs along with RXRα. As shown in Fig. 8A, only PPARγ-RXRα-DNA could bind to the PPRE-2 but not to the PPRE-2mut. A 20-fold excess of unlabeled PPRE-2, but not PPRE-2mut, oligonucleotide was able to compete for this binding, indicating that PPARγ is binding specifically to PPRE-2. Gel shifts done using the PPARγ-RXRα along with the RXRα showed no binding with the PPRE-2-containing wild-type oligonucleotide (data not shown). Furthermore, nuclear extracts from Caco-2 cells were able to form an in vitro complex with the PPRE-2 (Fig. 8A). To test the specificity of the protein-DNA interactions, a PPAR-specific antibody was added to the in vitro protein-DNA complex. This led to a supershift, which is seen as a slower migrating band (Fig. 8A). Gel shift assays were also done using the PPRE-1 probe (w2) along with the in vitro translated proteins. As shown in Fig. 8B, all PPARs could bind to the PPRE-1. Competition assays using 20-fold excess unlabeled PPRE-1 (w2) showed that all the PPAR isotypes bind with equal specificity to the PPRE-1.

Sulindac Sulfone Reduces Polyamines by Increased Export—We have demonstrated that, in Caco-2 cells, there is an increase in SSAT RNA and an increase in SSAT promoter-regulated transcription when treated with sulindac sulfone. This induction of SSAT is correlated with a 2-fold reduction in the intracellular levels of spermine and spermidine (Fig. 9) when treated with 600 μM sulindac sulfone for 48 h. To determine whether the reduction in intracellular polyamine levels is due to increased export of acetylated polyamines from the cell, we used verapamil, which has been shown to inhibit the diamine exporter specific for diamine polyamines (52). Verapamil at a concentration of 100 μM alone led to increased polyamines in the cell with around 2-fold accumulation of N1-acetylspermidine in the cell. When verapamil was added along with sulindac sulfone, it led to a 2–2.5-fold induction in levels of polyamines and a 3.5-fold induction in the levels of N1-acetylspermidine (Fig. 9). This result showed that sulindac sulfone caused reduction in intracellular polyamine levels due to their increased excretion from the cell. Intracellular polyamines can be decreased by either inducing SSAT, a catabolic enzyme, or by suppressing ODC, a biosynthetic enzyme. It has been shown before that indomethacin, an NSAID, can decrease polyamine levels by inducing SSAT and decreasing ODC enzyme activity (25). Based on this observation, we wanted to see the effects of sulindac sulfone on ODC in Caco-2 cells. We found that concentrations of sulindac sulfone that induce SSAT and decrease polyamines in Caco-2 cells did not have any effect on the expression of either ODC RNA or ODC enzyme activity (data not shown).

Sulindac Sulfone-induced Death of Caco-2 cells Can Be Rescued by Exogenous Putrescine—The effect of sulindac sulfone on polyamines could suggest that sulindac sulfone is acting via a polyamine-dependent mechanism to exert its effects on cell growth, apoptosis, or both in colon cancer cells. To test this hypothesis, we treated cells with 1 mM putrescine to increase intracellular polyamine levels. Putrescine was able to replenish the intracellular polyamines in these cells as detected by high performance liquid chromatography. All three major polyamines, putrescine, spermine, and spermidine were elevated in the combined treated (sulfone and putrescine) cells as compared with the sulindac sulfone-only treated cells (Fig. 9A) after 4 days in culture. As shown before, sulindac sulfone led to an inhibition of cell proliferation that was not rescued by exogenous putrescine in a span of 6 days (Fig. 9B). However, 1 mM putrescine was able to prevent cell death caused by sulindac sulfone by around 50% in a span of 6 days (Fig. 9C). To test whether the cell death caused by sulindac sulfone is due to apoptosis, we detected the changes in the position of phosphatidylserine in the cell membrane using an Annexin V kit. We observed that, after 6 days, sulindac sulfone-induced cell death was due to apoptosis, and putrescine was able to prevent the apoptosis caused by sulindac sulfone by around 50% (Fig. 9D).

DISCUSSION

Sulindac sulfone, but not sulindac sulfide, induces SSAT, a gene encoding an enzyme involved in polyamine catabolism and export in colon cancer cells. The induction of SSAT gene
transcription occurs at clinically relevant doses of sulindac sulfone and involves the activation of PPARs. SSAT induction by sulindac sulfone is associated with a decrease in intracellular polyamines. This decrease can be blocked by verapamil, an inhibitor of the diamine exporter (52), which exports polyamines acetylated by SSAT. Sulindac sulfone also induces apoptosis in these colon cancer cells, in part, via a polyamine-dependent mechanism as replenishment of the intracellular polyamine pools by exogenous putrescine is able to partially rescue this apoptosis. These results suggest that sulindac sulfone is activating SSAT expression, which in turn leads to reduced intracellular polyamine contents, which in turn leads to increased apoptosis (Fig. 10). In this study, we have identified two PPRE sequences in the 5' region of the human SSAT gene that are similar to the consensus sequences for previously identified PPREs. PPRE-1 is at −323 bases, while PPRE-2 is at +48 bases relative to the transcription start site. Further we demonstrate that SSAT induction by sulindac sulfone requires sulindac sulfone-induced activation of PPARγ, which can then bind to the PPRE-2 in the SSAT gene.

Animal and in vitro studies indicate that sulindac and sulindac sulfone inhibit colorectal cancer by mechanisms that cannot be explained solely by inhibition of prostaglandin formation (12, 16). Studying the mechanism of induction of SSAT by sulindac sulfone revealed a COX-independent action in Caco-2 cells based on two pieces of evidence. First, adding exogenous PGE2 does not influence the SSAT expression, and second, sulindac sulfone is able to induce SSAT in HCT-116, a cell line that lacks any detectable COX-2 protein and has very low levels of PGE2. Induction of SSAT by sulindac sulfone appears to occur at the level of transcription, which is consistent with the results that SSAT promoter activity and SSAT RNA levels are induced after exposure to sulindac sulfone (Fig. 11). NSAIDs like indomethacin can reduce intracellular polyamine levels by inducing SSAT and inhibiting ODC enzyme activity in Caco-2 cells (25). We found that sulindac sulfone has no observed effect on the expression of ODC RNA and enzyme activity in the Caco-2 cells, suggesting that the changes in polyamine pools are a result of modulation of SSAT expression.

There are many potential transcription factor binding sites
FIG. 11. Mechanism of induction of lowering of polyamines by sulindac sulfone in Caco-2 colon cancer cells.

Increased polyamines, as found in neoplastic cells, inhibit apoptosis. Cells regulate intracellular polyamine pools by regulating their biosynthesis or their catabolism by SSAT. Sulindac sulfone reduces intracellular polyamine pools by inducing SSAT, which leads to an increased export of the polyamines from the cells. Induction of SSAT by sulindac sulfone involves a COX-independent but PPAR-dependent mechanism in the Caco-2 cells. There are two PPREs in the SSAT gene. Sulindac sulfone induces SSAT by activation of PPARγ, which can then bind to the PPRE-2 in the SSAT promoter. PPRE-1 is not involved in the induction of SSAT by sulindac sulfone but can bind to PPARγ and PPARα. Sulindac sulfone can induce SSAT by acting on other transcription factor response elements upstream of the PPRE-1.

Identified in the SSAT promoter when it was originally cloned (39). We used various SSAT 5′-flanking sequence deletion constructs to map the SSAT gene, which is responsive to the sulindac sulfone-induced activation. This led us to the 0.1-kb segment of the SSAT gene (−88 to +88) that was still responsive to sulindac sulfone-induced activation. Searching for potential response elements in this 0.1-kb sequence led us to identify a putative PPRE sequence. Based on the role of PPARs in colon cancer (20–23), the relationship between sulindac and PPARs (22, 24), and the existence of PPRE in the SSAT promoter region, the possibility that sulindac sulfone is inducing SSAT via activating PPARs was tested. The likelihood that the observed induction of SSAT in these cells by sulindac sulfone involves transcriptional regulation by PPARs was substantiated by both the reporter promoter construct analysis and through the use of specific PPAR activators. Sulindac sulfone induces expression of the tk-PPREγ-Luc plasmid in Caco-2 cells, while ciglitazone (an activator of PPARγ) and cPGI (an activator of PPARδ) treatments resulted in a significant increase in SSAT RNA and promoter activity. Both of these experiments, although different, indicate that PPAR activation is one of the mechanisms by which sulindac sulfone is inducing SSAT in these cells. This effect on PPARs is not the only mechanism of SSAT induction by sulindac sulfone as PPAR activation cannot induce SSAT to the levels as done by sulindac sulfone. There are other Sp1, AP-1, and cAMP-response element transcription factor binding sites in the 5′ promoter flanking region of the SSAT gene that could be involved in the regulation of SSAT by sulindac sulfone.

To characterize the putative PPRE-2 found at +48 in the SSAT gene, transfection experiments using the various SSAT 5′-flanking deletion constructs along with the PPAR and RXRα proteins were done. The results shown here demonstrate the identification of one more PPRE, PPRE-1, in the SSAT promoter at −323 bases relative to the SSAT transcription start site. Further, by transfection experiments, we show that PPRE-1 can be bound by both PPARδ and PPARγ in the Caco-2 cells, but PPRE-2 is bound only by the PPARγ. By mutating PPRE-2 and using the PPARγ antagonist, it was confirmed that only PPRE-2 is bound by PPARγ and is required for the induction of SSAT by sulindac sulfone. Gel shifts using in vitro translated PPARs and RXRα showed that PPARδ/RXRα binds to the PPRE at −323 site, while PPARγ/RXRα binds at both +48 and −323 sites.

The effects of sulindac sulfone on preventing colon cancer are likely mediated by stimulating cellular apoptotic pathways or by inhibiting cellular proliferation (9, 10, 53). High levels of polyamines lead to rapid proliferation (29), while lower levels of polyamines have been shown to promote apoptosis (30, 31) and inhibit cell growth (32). Based on the fact that polyamines and sulindac sulfone have opposite effects on proliferation and apoptosis we questioned whether sulindac sulfone-induced cell death is due to polyamine depletion in the Caco-2 cells. The results demonstrate that sulindac sulfone induces cell death, which can be partially inhibited by replenishing the intracellular polyamine levels by exogenous putrescine or other polyamines (data not shown). Further sulindac sulfone-induced cell death is primarily due to apoptosis in these cells. Replenishing the intracellular polyamine levels was not able to rescue the inhibition of cell proliferation caused by sulindac sulfone. These results indicate that induction of apoptosis and inhibition of cell proliferation by sulindac sulfone are two distinct
pathways in the Caco-2 cells. Putrescine is able to partially rescue apoptosis without having any effect on proliferation. This is not a new result as it has been shown that, in ODC-knockout mice, ODC is an essential gene (54). Immunohistochemical analysis showed that knocking out ODC has no effect on proliferation, but there is an increased DNA breakage associated with apoptosis in the blastocysts. This indicates a possible role of polyamines in apoptosis but not in proliferation in these mice.

NSAIDs appear to be working by a mechanism involving SSAT induction and by decreasing intracellular polyamine levels in colon cancer cells. We have recently reported that aspirin induces SSAT and lowers intracellular putrescine and spermidine without affecting ODC activity in colon cancer cells (27). Others have found that indomethacin, piroxicam, and sulindac work by regulating genes in polyamine metabolism, ultimately leading to decreased polyamines (25, 26, 55). Recently it has been shown that exogenous polyamine reverses sulindac-induced toxicity in colon cancer cells (55). Results presented here suggest that one mechanism for the antitumor properties of sulindac sulfone is to act in a COX-independent but PPAR-dependent way to induce SSAT gene expression in Caco-2 colon tumor cells.

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