Sulindac Sulfone Inhibits K-ras-dependent Cyclooxygenase-2 Expression in Human Colon Cancer Cells[1]

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ABSTRACT

Both the sulfide and sulfone metabolites of sulindac, a nonsteroidal anti-inflammatory drug, display anticarcinogenic effects in experimental models. Sulindac sulfide inhibits cyclooxygenase (COX) enzyme activities and has been reported to suppress *ras*-dependent signaling. However, the mechanisms by which sulindac sulfone suppresses cancer growth are not as defined. We studied the effects of these sulindac metabolites in human colon cancer-derived Caco-2 cells that have been transfected with an activated K-*ras* oncogene. Stable transfected clones expressed high levels of COX-2 mRNA and protein, compared with parental cells. K-*ras*-transfected cells formed tumors more quickly when injected into severe combined immunodeficiency disease mice than parental cells, and this tumorigenesis was suppressed by treatment with sulindac. Sulindac sulfone inhibited COX-2 protein expression, which resulted in a decrease in prostaglandin synthase E_2 production. Sulindac sulfide had little effect on COX-2 in this model, but did suppress prostaglandin synthase E_2 production, presumably by inhibiting COX enzyme activity. These data indicate that the sulfide and sulfone derivatives of sulindac exert COX-dependent effects by distinct mechanisms.

INTRODUCTION

The COX pathway has been under intense investigation as a target for the treatment and prevention of colorectal cancer. Several studies have reported a 40–50% decrease in mortality from colorectal cancer with prolonged use of NSAIDs[3] (1–4). Sulindac, an inhibitor of COX-1 and COX-2, has been reported to reduce the size and number of colorectal tumors in FAP patients as well as in animal models of FAP (5). Sulindac is metabolized into sulfone and sulfide derivatives. Both derivatives are known to inhibit cell growth by the induction of apoptosis (6). The sulfide metabolite has been shown to have COX enzyme inhibitory activity, whereas the sulfone metabolite lacks this effect (7, 8). Recent studies have shown that the sulfone can prevent tumor formation in rodent models of chemical carcinogenesis without inhibiting COX enzyme activity or lowering prostaglandin levels (9, 10). The sulfone, which is now referred to as exisulind (Aptosyn) is currently being developed for the treatment of FAP patients as well as for other cancer chemopreventive and therapeutic indications.

The first evidence linking an activated *ras* oncogene to up-regulation of COX-2 was one in which an activated H-*ras* gene was transfected into rat intestinal epithelial cells (11). Treatment with the COX-2 selective inhibitor, SC58125, suppressed growth in these cells and induced apoptosis. An activated Ha-*ras* transfected into rat-1 fibroblasts caused an increase in transcription and in the half-life of

the COX-2 message, which appeared to be modulated by the MAPK pathway (12). Selective inhibition of the MAPK kinase by PD98059 caused a delay in COX-2 induction at both the mRNA and the protein levels. This suggests that the MAPK signaling cascade may mediate COX-2 expression.

Sulindac sulfide has been shown previously to bind ras to inhibit the recruitment of raf to the plasma membrane and thus directly inhibit *ras* signaling (13). In addition, sulindac sulfone and certain NSAIDs (*i.e.*, piroxicam and sulindac) have been shown previously to have a proportionately greater inhibitory effect on carcinomas harboring mutations in either K- or H-*ras* (14, 15). To determine whether sulindac sulfone can affect *ras*-dependent signaling, we investigated the effect of this drug on the K-*ras*-dependent expression of COX-2 in a human colon cancer-derived cell line.

MATERIALS AND METHODS

Cell Culture and Transfections. Caco-2 cells were maintained in MEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All cell culture supplies were obtained from Life Technologies, Inc., Rockville, MD. K-*ras* cDNA was purchased from American Type Culture Collection (Rockville, MD) and ligated into the multiple cloning site of a pCDNA3 mammalian expression vector (Invitrogen Corp., Carlsbad, CA) containing a cytomegalovirus promoter and a *neomycin resistance* gene. The pCDNA3-K-ras plasmid was transfected into Caco-2 cells using the calcium phosphate transfection technique as described in the literature (16). The K-*ras*-activated clones were maintained under selection with 350 μg/ml G418. Sulindac (the sulfoxide form), sulindac sulfide, and sulindac sulfone were purchased from ICN Biochemicals, Inc., Aurora, OH, and obtained as a generous gift from G. A. Piazza (Cell Pathways, Inc., Horsham, PA). Previous work from our laboratory indicated that the dose to reduce colony formation by 50% (IC_{50} dose) in both parental and K-*ras*-transfected clones was 120 μM for sulindac sulfide, 600 μM for sulindac sulfone, and 400 μM for sulindac sulfoxide (17).

PCR Assay for K-*ras* Mutations. Caco-2 cells and K-*ras*-activated clones were lysed in buffer containing 100 mM NaCl, 10 mM Tris-HCl (pH 8), 25 mM EDTA, 0.5% SDS, and 100 μg/ml proteinase K and incubated at 37°C for 16 h. DNA was isolated with two extractions in phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated on ice in 3 M sodium acetate and 100% ethanol. The pellet was washed in 70% ethanol and further purified in 100 μg/ml RNase, 0.5% SDS, and 100 μg/ml proteinase K. Another phenol:chloroform:isoamyl alcohol extraction was performed, the DNA pellet was precipitated with ethanol and then resuspended in TE (10 mM Tris · HCl · 1 mM EDTA) buffer. DNA concentration was determined by UV spectrophotometric methods. PCR was performed with specific primers (Life Technologies, Inc.) to create a BstNI restriction site at codon 12 of the human K-*ras* gene. The upstream primer sequence used was 5′: AAA CTT GTG GTA GTT GGA CCT; the downstream primer was 5′: TTG TTG GAT CAT ATT CGT CC. Mutation at codon 12 of K-*ras* alters the BstNI site, preventing restriction enzyme cleavage. PCR products were run out on a 4% NuSieve:agarose (3:1) gel stained with ethidium bromide.

Immunoblotting. Subconfluent cells were lysed on ice in radioimmunoprecipitation assay buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 30 μg/ml aprotinin, 100 mM sodium orthovanadate, and 10 mg/ml phenylmethylsulfonyl fluoride) and electrophoresed on a 10% SDS-PAGE gel. Proteins were transferred to nitrocellulose membrane. Membranes were blocked in Blotto A, probed with the appropriate antibodies, and developed by the enhanced chemiluminescence system (Amersham, Arlington Heights, IL).

COX-1 and COX-2 antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, and used at 1:250 and 1:5000 dilutions, respectively. All Western blots were repeated three times and a representative blot was chosen for presentation.

Northern Blotting. RNA was isolated from frozen cell pellets using Trizol:chloroform (5:1; Life Technologies, Inc.) extractions. RNA was further purified in isopropanol and washed in 75% ethanol. RNA was run out on a 1% agarose/formaldehyde gel in MOPS [3-(N-morpholino)propanesulfonic acid] buffer and transferred to nylon. The COX-2 cDNA probe (Oxford Biomedical Research, Inc., Oxford, MI) was labeled using the RTS RadPrime DNA labeling system (Life Technologies, Inc.) and [α^{32}P]-dCTP. The probe was purified with G-50 Sepharose columns (Boehinger Mannheim, Indianapolis, ID) and quantitated with a scintillation counter. The membrane was hybridized to the probe overnight at 42°C and then washed three times ($2\times$ SSC-0.1%SDS for 5 min at room temperature, then for 20 min at room temperature, and then $0.5\times$ SSC-0.1% SDS for 30 min at 65°C). Membranes were prehybridized again and then hybridized to glyceraldehyde-3-phosphate dehydrogenase (0.75 kb *Pst*I-*Xba*I fragment) as a loading control. Autoradiograms were quantitated by densitometric analysis (ImageQuant; Molecular Dynamics, Sunnyvale, CA).

PGE$_2$ Production. Cells were seeded 10^4/well on 96-well plates and treated with drug or vehicle 24 h later. Serum-free medium was supplemented with 15 μM arachidonic acid (Sigma, St. Louis, MO) after 24 h of drug treatment for 1 h prior to medium collection for the PGE$_2$ kit (Amersham, Arlington Heights, IL).

Animal Model. Caco-2- or K-*ras*-activated Caco-2 cells (clone 60) were injected subdermally into four areas of the flank of SCID mice at 1×10^6 cells/injection. There were three to four mice/group. The mice were fed 167 parts/million sulindac in AIN93G diet (Teklad, Indianapolis, IN). The injection sites were monitored once a week until tumors appeared; tumors were measured twice a week. The animals were sacrificed at 100 days because of tumor burden.

Statistics. Assessment of statistical differences for Figs. 3 and 4 were determined by ANOVA. A $P < 0.05$ was considered statistically significant.
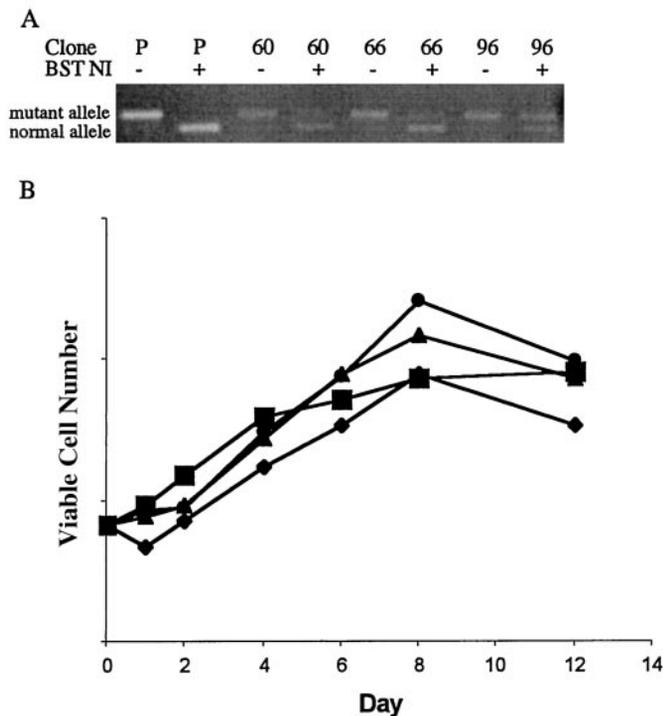

Fig. 1. Characterization of K-ras-activated Caco-2 cells. *A*, Caco-2 parental cells (*P*) and K-*ras* transfectants (K-*ras*-overexpressing clones 60, 66, and 96) were tested for normal and mutant alleles of K-*ras* with the use of a PCR-RFLP technique. PCR products were digested with BstNI; mutation at codon 12 prevents BstNI cleavage. *B*, Caco-2 parentals and K-*ras*-transfected clones were harvested at the indicated times and analyzed for viable cell number as determined by dye exclusion. Parentals (♦), 60 (●), 66 (▲), 96 (■).
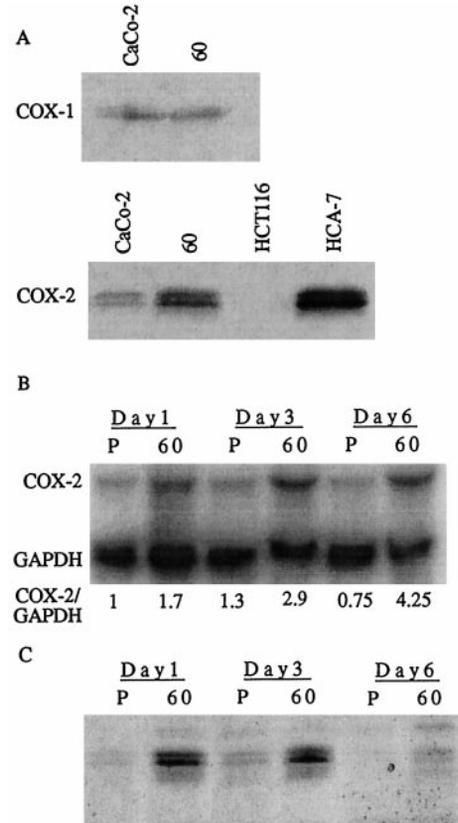

Fig. 2. Effect of activated K-*ras* on COX expression. *A*, Western blot analysis of COX-1 and COX-2 in Caco-2 cells and K-*ras*-activated 60 clones after 48 h in culture. HCT 116 cells served as a negative control for COX-2, and HCA-7 cells are a positive control because they constitutively express COX-2. *B*, Northern blot analysis of COX-2 for Caco-2 and 60 cells at 1-, 3-, and 6-day time points. Amounts of COX-2 mRNA were expressed in a ratio to GAPDH levels, normalized to parentals on day 1. *C*, time course of COX-2 protein levels in parental Caco-2 cells (*P*) and in K-*ras*-activated cells (60) at days 1, 3, and 6 of growth.

RESULTS

Characterization of K-*ras*-activated Clones. Caco-2 cells were used for stable transfection of $p21^{Val12}$ K-*ras* because they express normal K-*ras* (15). To better elucidate K-*ras* overexpression, we used a PCR-RFLP technique to determine whether the transfectants overexpressed the normal or mutant alleles. We introduced a BstNI restriction enzyme cut site using specific primers and then cut the PCR product; the enzyme cuts if the cells are expressing the normal allele, but it will not cut if the cells are expressing the mutant allele. The data shows that the clones with the highest expression (transfected clone numbers 60, 66, and 96) displayed both normal and mutant alleles, whereas the parental cell line expressed only normal K-*ras* (Fig. 1*A*). This shows that the transfected cells do indeed express the mutant K-*ras* gene but does not provide information regarding the level of the normal or the mutant allele being expressed. Because transfection with H-*ras* changes the growth characteristics of Caco-2 cells (18), we examined the growth of the K-*ras* transfectants and compared them with the parental line. After 12 days in culture, there was no significant difference in growth kinetics between the parental cell line and the K-*ras*-activated clones (Fig. 1*B*). This may indicate that both normal and mutant alleles are being overexpressed simultaneously in the transfectants.

COX Expression in K-*ras*-activated Caco-2 Cells. We measured COX-2 protein and RNA levels in the K-*ras*-activated clones and in the parental Caco-2 cell line. The level of COX-2 protein was increased in the K-*ras*-activated cells relative to the parental cell line,
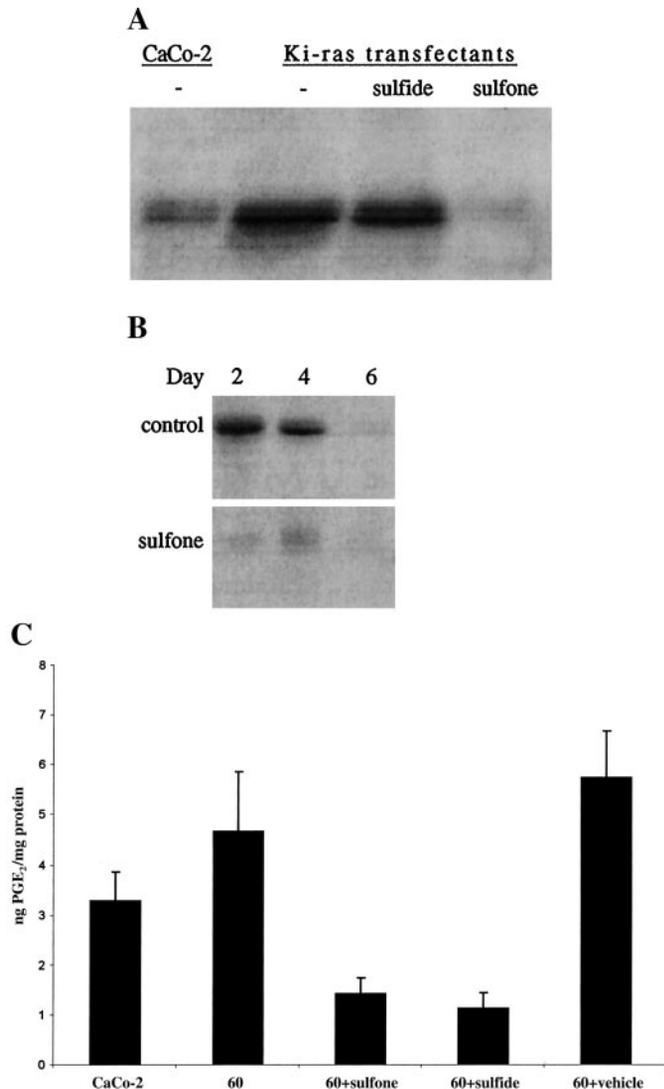
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Fig. 3. Effects of sulindac sulfone on COX-2 expression and PGE$_2$ production. *A*, Western blot analysis of COX-2 in Caco-2 cells and K-*ras* transfectants. K-*ras* transfectants were treated for 24 h with 120 μM sulfide or 600 μM sulfone. *B*, COX-2 protein levels for K-*ras*-transfected Caco-2 cells in the presence of 600 μM sulindac sulfone on days 2, 4, and 6. *C*, extracellular PGE$_2$ levels in Caco-2 cells, K-*ras* transfectants, and K-*ras* transfectants treated with 600 μM sulfone or 120 μM sulfide for 24 h. The vehicle used was DMSO. *P* for vehicle-treated cells compared with sulfone- or sulfide-treated cells are $P = 0.02$ and $P = 0.003$, respectively.

significantly inhibited PGE$_2$ production in K-*ras*-activated cells (data not shown). Sulindac sulfone at 600 μM and sulfide at 120 μM displayed similar inhibitory effects on prostaglandin synthesis (Fig. 3*C*). In addition, sulfone significantly inhibited COX-2 protein (and mRNA, data not shown), whereas the sulfide at its IC$_{50}$ dose did not affect protein levels (Fig. 3*A*). This finding suggests that the sulfone can indirectly affect prostaglandin synthesis by affecting COX-2 protein expression. By contrast, an IC$_{50}$ dose of sulindac sulfide suppresses PGE$_2$ levels, presumably by inhibiting COX enzyme activity, but does not affect COX-2 protein expression. This is the first demonstration that sulindac sulfone can exert COX-dependent effects on cell behaviors, specifically by suppressing K-ras-dependent signaling of COX-2 protein expression.

Sulindac inhibited tumor formation in SCID mice injected with K-*ras*-activated Caco-2 cells (Fig. 4). Caco-2 cells and the K-*ras*-activated transfectants showed no difference in growth characteristics *in vitro* (Fig. 1*B*) but displayed differences in tumor formation when injected in immune-compromised mice. *K-ras* activation caused an increase in the rate of tumor formation over the nontransfected cell line, and sulindac greatly inhibited this tumorigenesis. Measurable tumors became apparent in untreated mice after 35 days, whereas sulindac-treated mice did not present with tumors until after day 60. By day 100, sulindac had prevented tumor growth by 60% compared with the control group.

DISCUSSION

The data presented here indicate that sulindac and its sulfide and sulfone metabolites inhibit tumorigenesis in colon-derived cells by at least two distinct mechanisms. One mechanism involves the suppression of prostaglandin synthesis by COX-1 and/or COX-2 enzyme activity (8). The second mechanism, shown here for the sulfone metabolite, involves inhibition of the K-ras-dependent signaling of genes affecting tumorigenesis. Sulindac sulfone has been shown to affect the signaling pathways influencing the expression of genes other than *COX-2*, as shown here. Yamamoto *et al.* (19) have reported that this drug inhibits NF-κB activation in colon cancer and other cell types. We have observed an increase in promoter activity in the polyamine catabolic enzyme, spermidine/spermine N^1-acetyltransferase when K-ras-activated Caco-2 cells are treated with sulfone.[4] These observations suggest that sulfone is acting to modulate the K-ras-dependent signaling of a variety of genes influencing cellular processes. Recently, it has been reported that sulindac sulfone affects β-catenin signaling by inhibition of cyclic GMP-dependent phosphodiesterases and activation of protein kinase G (20).

NSAIDs are known to exert their effects by COX-dependent and -independent mechanisms (21). COX-dependent mechanisms would include those situations in which NSAIDs interact directly with COX proteins to inhibit enzyme activity and those in which agents, such as sulindac sulfone, suppress COX protein expression. This latter mechanism does not involve direct enzyme/inhibitor interactions, but only requires that formation of the target enzyme is prevented. COX-independent mechanisms could involve inhibition of signaling pathways affecting genes other than *COX-1* or *COX-2*.

In the studies reported here, we compared the effects of IC$_{50}$ concentrations of the sulfide (120 μM) and sulfone (600 μM) metabolites of sulindac on the K-ras-dependent expression of *COX-2*. The increased potency of sulindac sulfide may reflect the ability of this metabolite to both suppress COX enzyme activity and inhibit signaling, whereas sulindac sulfone only inhibits signaling. This suggests

but no difference was observed in COX-1 protein (Fig. 2*A*). There is a similar increase in COX-2 mRNA levels in the K-*ras*-activated clones (Fig. 2*B*). This data suggests that an activated K-*ras* increases the expression of COX-2 mRNA and protein. The increase in expression of COX-2 mRNA and protein was transient, peaking at 2–3 days in culture and decreasing over time (Fig. 2*B* and 2*C*). This transient effect is irrespective of K-*ras* status, as would be expected because *COX-2* is an inducible rather than constitutive gene.

Effects of Sulindac and its Sulfide and Sulfone Metabolites on *COX-2* Expression, PGE$_2$ Production, and Tumorigenesis. Because K-*ras*-activated Caco-2 cells exhibited an elevation in COX-2 mRNA and protein, we expected to observe an increase in PGE$_2$ production in K-*ras*-transfected cells compared with the parental Caco-2 cells. K-*ras*-activated cells showed a 30% increase in PGE$_2$ synthesis over the parental cells (Fig. 3*C*). PGE$_2$ production was next measured in cells treated for 24 h with sulindac and its sulfide and sulfone metabolites at doses previously established to inhibit Caco-2 colony formation by 50%. Treatment with 400 μM sulindac sulfoxide

[4] N. A. Ignatenko, N. Babbar, and E. W. Gerner. K-ras-dependent suppression of spermidine/spermine N^2-acetyltransferase gene expression, manuscript in preparation.
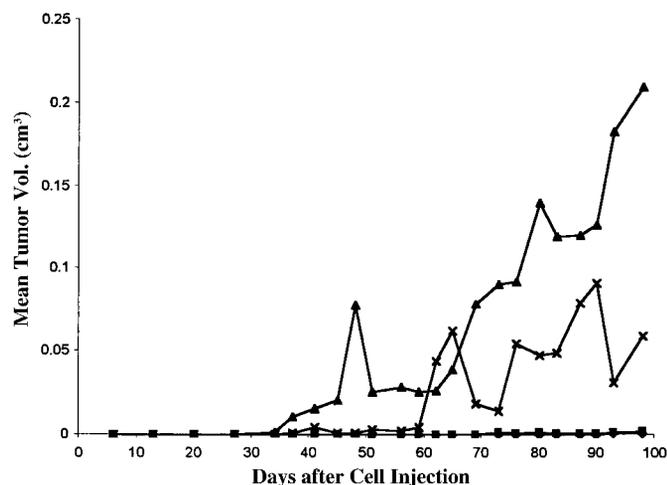
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Fig. 4. Effects of sulindac on tumor formation of Caco-2 and K-*ras*-transfected cells injected into SCID mice. 1×10^6 cells in 0.2 ml PBS were injected at 4 sites subdermally, and 167 parts/million sulindac were administered in the diet. Tumor growth was measured twice a week. Caco-2 (■), Caco-2 + sulindac (●), 60 (▲), 60 + sulindac (✕). *P*s were calculated at 100 days using ANOVA. *P* was <0.001 for Caco-2 and 60 cells. *P* = 0.01 for 60 and 60 + sulindac.

that higher concentrations of sulindac sulfide may also inhibit the signaling of COX-2 protein expression. Hermann *et al.* (13) have reported that sulindac sulfide can bind to ras in cell-free studies. Future studies will address whether the sulfone directly binds to ras in cells.

The reduction in PGE$_2$ in the sulindac-treated, K-*ras*-activated Caco-2 cells may be attributable to its effects on COX-1 or COX-2 or both. The distinction cannot be made by measuring prostaglandin levels. We hypothesize that sulindac sulfone is acting to reduce prostaglandin levels in our Caco-2 model by a K-ras-dependent mechanism acting selectively on COX-2, compared with COX-1. We favor this interpretation, because we observed no change in COX-1 protein levels in the K-*ras*-activated cells (Fig. 2*A*), and sulindac sulfone does not appear to affect COX-1 protein levels measured by Western blot (data not shown).

The data presented here provide additional evidence for the role of COX-2 expression in colon carcinogenesis, where up to 50% of large adenomas and adenocarcinomas contain activating mutations in K-*ras* (22). We showed that an activated K-*ras* oncogene leads to an up-regulation of COX-2 expression in human adenocarcinoma cells. Previous work has shown that activated H-*ras* leads to up-regulation of COX-2 in rodent cells (12). However, H-*ras* is not expressed in human colonic epithelium, whereas K-*ras* mutations occur with high frequency in colorectal cancers (~50%) and are found in nearly 100% of pancreatic cancers (22–24). K-*ras* has been shown to be the only *ras* family member to be essential for development in mice (25). Thus it is possible that, in humans, K-*ras* may have functions unique from H-*ras*, as has been shown for mice (25).

This study describes a novel COX-dependent effect of the NSAID sulindac, namely the inhibition of K-ras-dependent signaling of COX-2 protein expression by the sulfone metabolite of this drug. These data demonstrate that sulindac can inhibit K-ras-dependent colon tumorigenesis in this Caco-2 cell model system. The mechanisms responsible for suppression of tumor formation may involve both the direct inhibition of COX enzyme activity, by sulindac sulfide, and the suppression of signaling pathways, by sulindac sulfone, affecting the expression of genes which are required for tumor growth.

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