

Inhibition of Extracellular Signal-regulated Kinase 1/2 Phosphorylation and Induction of Apoptosis by Sulindac Metabolites¹

Pamela L. Rice,² Ryan J. Goldberg, Evan C. Ray, Linda J. Driggers, and Dennis J. Ahnen

Department of Medicine, University of Colorado Health Sciences Center, Denver, Colorado 80262 [P. L. R., D. J. A.], and Department of Veterans Affairs Medical Center, Denver, Colorado 80220 [P. L. R., R. J. G., E. C. R., L. J. D., D. J. A.]

ABSTRACT

Regular use of nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin and sulindac is associated with a decreased mortality from colorectal cancer. Sulindac causes regression of precancerous adenomatous polyps and inhibits the growth of cultured colon cell lines. Whereas induction of apoptotic cell death is thought to account for the growth inhibitory effect of sulindac, less is known about its biochemical mechanism(s) of action. Sulindac is metabolized *in vivo* to sulfide and sulfone derivatives. Both the sulfide and sulfone metabolites of sulindac as well as more potent cyclic GMP-dependent phosphodiesterase inhibitors were shown to cause inhibition of extracellular signal-regulated kinase (ERK)1/2 phosphorylation at doses (40–600 μM) and times (1–5 days) consistent with the induction of apoptosis by the drugs. Treatment of HCT116 human colon cancer cells with the specific mitogen-activated protein kinase kinase, U0126 (5–50 μM) resulted in a time- and dose-dependent inhibition of ERK1/2 phosphorylation, and induction of apoptosis. U0126 treatment (20 μM) increased basal apoptosis, and potentiated the apoptotic effect of sulindac sulfide and sulindac sulfone. These results suggest that the inhibition of ERK1/2 phosphorylation is responsible for at least part of the induction of programmed cell death by sulindac metabolites. Inhibition of ERK1/2 activity may, therefore, be a useful biochemical target for the development of chemopreventive and chemotherapeutic drugs for human colon cancer.

INTRODUCTION

Colorectal cancer is the second leading cause of cancer death in the United States (1). A large body of animal model and human epidemiological and clinical intervention data indicate that NSAIDs³ have chemopreventive activity for colorectal cancer. Numerous case-control and cohort studies show an inverse relationship between ingestion of NSAIDs and development of colon cancer (2). NSAIDs, including sulindac, inhibit adenoma formation in the *Min*-mouse, an animal model of human FAP (3) and also inhibit azoxymethane-induced colorectal cancer in rats and mice (4–6). Sulindac treatment also causes regression of existing colonic polyps and prevents formation of new polyps throughout the colon in patients with FAP (7–11). The striking regression of colonic neoplasms by sulindac is one of the most dramatic examples of the power of a chemopreventive agent not only to prevent but also to reverse the process of carcinogenesis.

Sulindac is a prodrug that is rapidly metabolized to two major derivatives; the sulfide metabolite is an active NSAID that inhibits both COX-1 and COX-2 and, thereby, inhibits prostaglandin synthesis (12). By contrast, the sulfone metabolite is not an NSAID because it

does not inhibit either COX-1 or COX-2 and does not inhibit prostaglandin synthesis (6, 12, 13). Both sulindac and its sulfone metabolite have chemopreventive activity; both drugs prevent tumor formation in chemically induced animal models of colon (4–6), breast (14, 15), and lung cancer (16, 17). These observations originally suggested that COX inhibition was not necessary for the chemopreventive activity of some of the NSAIDs and related compounds.

Several lines of evidence suggest that the biological mechanism of the chemopreventive action of sulindac is the selective induction of apoptosis, or programmed cell death, in neoplastic cells. Both sulindac sulfide and sulindac sulfone induce apoptosis in many cancer cell lines *in vitro* (12, 18–23). Sulindac treatment also increases the rate of apoptosis in adenomas from FAP patients 3-fold, with no apparent effect on cell proliferation (24). Whereas apoptosis primarily accounts for the growth inhibitory effects of sulindac, less is known about the biochemical mechanisms of apoptotic induction by sulindac. NSAIDs are classically defined by their ability to inhibit COX-1 and -2. However, NSAIDs including sulindac can induce apoptosis in cell lines such as HCT15 that have no COX-1 or -2 expression (13). In addition, the sulfone metabolite of sulindac, which does not inhibit COX-1 or -2, induces apoptosis of cancer cells *in vitro* and prevents tumor formation in animal models of colon (6), breast (14, 15), and lung cancer (16, 17).

Sulindac sulfone and related compounds inhibit cGMP, increase cGMP concentrations, and increase PKG activity in colon cancer cell lines with a dose and time dependence that suggests that cGMP inhibition is the proximate mediator of growth inhibition and apoptosis of these drugs (25). PKG is known to affect several signal transduction pathways that could mediate apoptosis. However, the mechanism of apoptotic cell death that follows cGMP inhibition by these drugs is not known.

Mutation of the *K-ras* proto-oncogene is a common, early event in colon cancer development (26). Activated Ras recruits c-Raf to the plasma membrane where it activates MEK1/2, which then phosphorylates its only known substrate, ERK1/2. Activation of ERK1/2 culminates in phosphorylation of transcription factors responsible for regulating genes that enhance cell proliferation and protect cells from apoptosis (27). Cancer cells with *ras* mutations appear to be more susceptible to the growth inhibitory effects of sulindac than those with wild-type *ras*. In a rat model of azoxymethane-induced colonic neoplasia, sulindac preferentially inhibited the growth of tumors with *K-ras* mutations from 90 to 36% of all tumors (28). Similarly, sulindac sulfone preferentially suppressed the number of mammary tumors containing *H-ras* mutations in a methylnitrosourea-induced model of rat mammary carcinogenesis (15). Sulindac sulfide has been reported to physically bind to Ras protein *in vitro*, and this interaction prevented binding of c-Raf to Ras (29). In addition, PKG can phosphorylate c-Raf and inhibit its interaction with Ras (30). Taken together, these data suggest the hypothesis that the biochemical mechanism of apoptosis by sulindac metabolites may be attributable to effects on Ras signaling pathways.

As a downstream effector of Ras signaling, ERK1/2 phosphorylation is often up-regulated in cancers harboring *ras* mutations. Colon tumors displayed especially high levels of ERK1/2 phosphorylation in

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² To whom requests for reprints should be addressed, at Division of Gastroenterology, A009–151 School of Medicine, University of Colorado Health Sciences Center, 4200 East Ninth Avenue, Denver, CO 80262.

³ The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; FAP, familial adenomatous polyposis; COX, cyclooxygenase; cGMP, cyclic GMP-dependent phosphodiesterase; PKG, protein kinase G; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase.

a screening study of >100 tumors (31). Inhibition of ERK1/2 phosphorylation *in vitro* by a synthetic MEK1/2 inhibitor, PD184352, decreased soft agar growth and inhibited the transformed phenotype of colon 26 cells. *In vivo*, PD184352 suppressed the growth of mouse and human colon tumor xenografts (32). We, therefore, tested the hypothesis that sulindac metabolites cause apoptosis by down-regulating ERK1/2 signaling in human colon cancer cells. This was determined by Western blotting with phospho-specific antibodies raised against ERK1/2 and MEK1/2. Both of the sulindac metabolites caused the inhibition of ERK1/2 phosphorylation at times and doses consistent with cleavage of caspase-3 and -7 and nuclear morphology indicative of apoptotic cell death. To further examine the role of ERK1/2 down-regulation and apoptotic induction, colon cancer cells were treated with the MEK1/2 inhibitor, U0126. U0126 substantially inhibited ERK1/2 phosphorylation, induced apoptosis alone, and potentiated apoptosis induced by both of the sulindac metabolites. These results indicate that sulindac metabolites inhibit ERK1/2, and this inhibition is sufficient to cause programmed cell death in human colon cancer cells, which suggests that this is one mechanism of the chemopreventive effect of sulindac.

MATERIALS AND METHODS

Materials. Cell culture media and fetal bovine serum were purchased from Mediatech (Herndon, VA), antibiotic/antimycotic solution (penicillin/streptomycin/fungizone) from Life Technologies, Inc. (Grand Island, NY), and tissue culture plates from Falcon (Franklin Lakes, NJ). Primary antibodies raised against phosphorylated ERK1/2 and total ERK1/2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); primary antibodies against phosphorylated MEK1/2, total MEK1/2, cleaved caspase-3 and cleaved caspase-7 were purchased from New England Biolabs (Beverly, MA). Horseradish peroxidase-conjugated antimouse, antigoat, and antirabbit secondary antibodies were purchased from Santa Cruz Biotechnology; Immobilon-P membranes were obtained from Millipore (Bedford, MA), chemiluminescent visualization reagents from NEN (Boston, MA), and X-ray film from Pierce (Rockford, IL). The MEK1/2 inhibitor, U0126, was purchased from Alexis Biochemicals (San Diego, CA). Sulindac sulfide, sulindac sulfone, CP248, and CP461 were generous gifts from Cell Pathways, Inc. (Horsham, PA).

Tissue Culture. HCT116 human colon cancer cells were purchased from American Type Cell Culture (Manassas, VA) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin/fungizone solution. Medium was replaced two or three times per week, and cells were passaged at subconfluency. The cells were grown in a humidified atmosphere of 5% CO₂-95% air. Cells were plated and grown to 80–100% confluency before treatment with vehicle or drug in the experiments described below.

Morphological Apoptosis Assay. Apoptosis and viability were quantified by staining cells with acridine orange and ethidium bromide, then by assaying for nuclear morphology, a hallmark of apoptosis (33, 34). For each determination, three separate 100-cell counts were scored. Apoptosis was expressed as a percentage calculated from the number of cells with apoptotic nuclear morphology divided by the total number of cells examined. Statistics reported in text are averages of at least three experiments, determined from triplicate wells for each experiment ± SE; *P*s were calculated using Student's *t* test.

Western Blot Analyses. For Western blot analysis, cells were scraped from plates, pelleted, resuspended in lysis buffer [15 mM Tris; 2 mM EDTA; 50 mM 2-mercaptoethanol; 20% glycerol; 0.1% Triton X-100; 1 mM phenylmethylsulfonyl fluoride; 1 mM sodium fluoride; 1 mM sodium orthovanadate; and 1 μg/ml each aprotinin, leupeptin, and pepstatin (pH 7.5)], incubated 10 min on ice, then sonified for 12 s on ice. Lysates were centrifuged at 10,000 rpm (14,000 × *g*) for 10 min at 4°C, and supernatant was collected. Protein concentrations were determined by the method of Lowry *et al.* (35). Lysates were prepared for SDS-PAGE, and 50 μg total protein were separated and electrotransferred overnight onto Immobilon-P polyvinylidene fluoride membranes (Millipore). Blots were blocked for 30 min in Tris-buffered normal saline with 1% (w/v) dry milk and 0.05% Tween 20, then incubated with phospho-ERK1/2 (1 μg/ml), phospho-MEK1/2 (1:5000), cleaved caspase-3 (1:1000), or cleaved caspase-7 (1:1000) primary antibody overnight at 4°C.

Immunoreactive protein was detected by incubating blots with horseradish peroxidase-conjugated secondary antibody for 1 h followed by chemiluminescent substrate for 1 min. Immunoreactive proteins were visualized by exposure to film. Quantitation of protein levels was determined by densitometry using a computerized visual imaging system (Bio-Rad, Hercules, CA). After the first Western blot using phospho-specific antibodies, the membranes were stripped for 30 min in 10% sodium dodecyl sulfide, 67 mM Tris (pH 6.7), and 0.8% 2-mercaptoethanol, while rocking at 50°C, and washed twice for 10 min each in Tris-buffered normal saline with 0.05% Tween 20. Blots were then reblocked and probed with pan-ERK1/2 (1 μg/ml) or pan-MEK1/2 (1:5000) primary antibodies as described above. Independent experiments validated that this stripping procedure did not lead to loss of signal.

RESULTS

Sulindac Metabolites Inhibit ERK1/2 Phosphorylation in Human Colon Cancer Cells. Sulindac sulfide treatment of HCT116 cells resulted in a time- and dose-dependent induction of apoptosis (Fig. 1A), as determined by examining nuclear morphology after staining with acridine orange and ethidium bromide. Low levels of apoptosis were seen at 24 h at the highest dose (120 μM) of sulindac sulfide; both doses resulted in progressively increased levels of apoptosis at 48, 72, and 120 h. Sulindac sulfide caused a similar time- and dose-dependent inhibition of ERK1/2 phosphorylation (Figs. 1B and 2A and B), as determined by Western blotting with a monoclonal antibody raised against phosphorylated ERK1 (the same peptide sequence is present in phosphorylated ERK2). Significant inhibition of

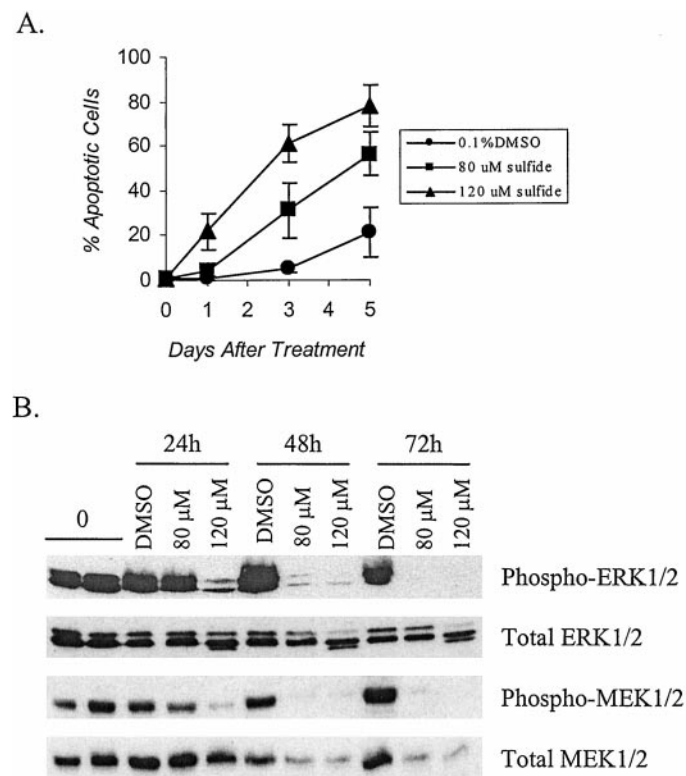


Fig. 1. In A, sulindac sulfide induces apoptosis of colon cancer cells. HCT116 cells were grown to confluency and treated with vehicle (0.1% DMSO) or with 80 or 120 μM sulindac sulfide. Cells were harvested 0, 1, 3, and 5 days after treatment, and apoptosis was quantified after staining with acridine orange and ethidium bromide. Apoptotic cells were determined by nuclear morphology. Three individually treated samples were counted per point. Error bars, SE. In B, sulindac sulfide inhibits phosphorylation of ERK1/2 and MEK1/2. HCT116 cells were treated with 0.1% DMSO or with 80 or 120 μM sulindac sulfide and harvested at 0, 24, 48, and 72 h after treatment. Cell lysates were prepared for SDS-PAGE and analyzed by Western blotting with phospho-specific ERK1/2 or phospho-specific MEK1/2 antibody, stripped, and re-probed with pan-ERK1/2 or pan-MEK1/2 antibody.

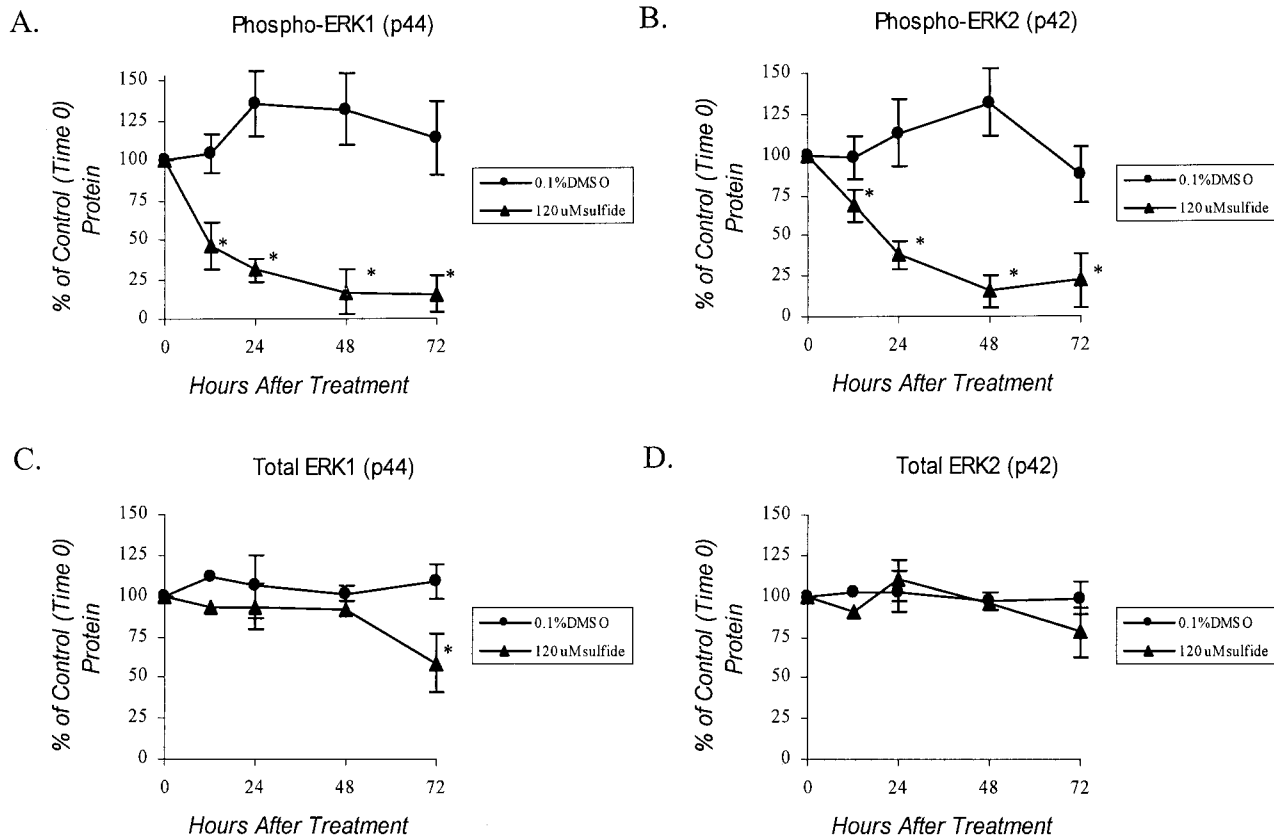


Fig. 2. Sulindac sulfide inhibits phosphorylation of ERK1 (p44; A) and ERK2 (p42; B) proteins, but not total ERK1 (p44; C) or total ERK2 (p42; D) protein. Line graphs, the mean and SE from at least three independent experiments. *, $P < 0.05$ versus day 0 control.

ERK1 and ERK2 phosphorylation occurred by 12 h and persisted through 72 h after treatment ($P < 0.05$). Decreased phospho-ERK1/2 was detected by 12 h, but not earlier than 8 h after sulindac sulfide treatment (data not shown). Phosphorylation of both ERK1 (p44) and ERK2 (p42) was reduced to similar extents. The inhibitory effect of sulindac on ERK1/2 activity was confirmed using an *in vitro* ERK1/2 kinase assay using Elk-1 as a substrate (data not shown). The amount of total ERK1/2 protein expression was similar to vehicle-treated cells except at 72 h of treatment, when ERK1 was significantly reduced compared with vehicle control ($P < 0.05$; Figs. 1B and 2, C and D).

Like the NSAID sulindac sulfide, the non-NSAID sulindac sulfone also caused a dose- and time-dependent induction of apoptosis that was detectable at the higher dose (600 μM) by 24 h, with progressively increased apoptotic levels with both drug doses at 72 and 120 h (Fig. 3A). Sulindac resulted in inhibition of phospho-ERK1/2 expression by 12 h of drug treatment that persisted through 72 h (Figs. 3B and 4A and B), without decreased expression of total ERK1/2 protein, except for total ERK1 at 72 h after treatment (Figs. 3B and 4, C and D). The ERK1/2 inhibitory effect of sulindac sulfone was confirmed with an *in vitro* ERK1/2 kinase assay using Elk-1 as a substrate (data not shown). Similar apoptotic induction and ERK1/2 inhibition were seen for both sulindac sulfide and sulindac sulfone using two additional human colon cancer cell lines, HT29 and SW480 (data not shown).

In addition to ERK1/2, we also examined the phosphorylation state of two other MAP kinases, JNK and p38, by Western blotting with phospho-specific antibodies. No consistent changes in the phosphorylation of JNK or p38, or in the expression of total JNK or p38 proteins were detected after treatment with either sulindac sulfide or sulindac sulfone metabolites (data not shown). In some individual experiments, increased levels of phospho-JNK were detected; how-

ever, the drugs could induce apoptotic cell death in the absence of any apparent change in JNK phosphorylation.

Sulindac Metabolites Inhibit MEK1/2 Phosphorylation in Human Colon Cancer Cells. The only characterized upstream activator of ERK1/2 is MEK1/2 (27). We, therefore, determined whether sulindac metabolites inhibit ERK1/2 phosphorylation by inhibiting its upstream activator, MEK1/2. Like ERK1/2, MEK1/2 is activated by phosphorylation, and phospho-specific antibodies were used to assay MEK1/2 activity by Western blotting. Sulindac sulfide (Fig. 1B) and sulindac sulfone (Fig. 3B) inhibited MEK1/2 phosphorylation with a time and dose dependence similar to that of ERK1/2 inhibition, which indicated that sulindac metabolites inhibit ERK1/2 phosphorylation by acting upstream of, and not at the level of, ERK1/2. Unlike total ERK1/2 expression, total MEK1/2 expression, as determined by Western blotting with pan-MEK1/2 antibody, was decreased after treatment with sulindac sulfide (Fig. 1B) or sulindac sulfone (Fig. 3B). However, inhibition of MEK1/2 phosphorylation, apparent by 12 h after treatment, preceded the loss of total MEK1/2 protein, which occurred after 24 h of treatment.

More Potent cGPD E Inhibitors CP248 and CP461 Inhibit ERK1/2 Phosphorylation. The more potent inhibitors of cGPD E, CP248 and CP461, induce apoptosis of cancer cells at substantially lower doses than do sulindac sulfide and sulindac sulfone (25). To determine whether ERK1/2 inhibition and apoptotic induction were common mechanisms of cGPD E-inhibitory agents, colon cancer cells were treated with CP248 and CP461 and analyzed for phospho-ERK expression. CP248 (5 and 10 μM) and CP461 (10 and 50 μM) caused a dose- and time-dependent induction of apoptosis (data not shown) and inhibition of ERK1/2 phosphorylation (Fig. 5). This suggests that inhibition of ERK1/2 phosphorylation may be a common mechanism of action of this class of antineoplastic agents, and that the effect is not

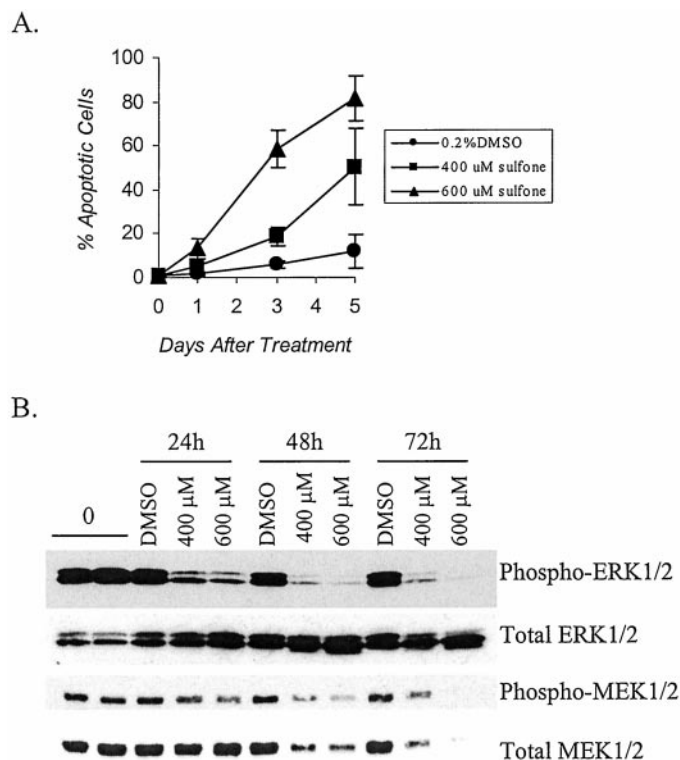


Fig. 3. In A, sulindac sulfone induces apoptosis of human colon cancer cells. HCT116 cells were treated with vehicle (0.2% DMSO) or with 400 or 600 μM sulindac sulfone and harvested at 0, 1, 3, and 5 days after treatment. Apoptotic induction was determined by staining cells with acridine orange and ethidium bromide. Apoptotic cells were determined by nuclear morphology. Samples were done in triplicate; error bars, SE. In B, sulindac sulfone inhibits phosphorylation of ERK1/2 and MEK1/2. HCT116 cells were treated with 0.2% DMSO or with 400 or 600 μM sulindac sulfone and were harvested at 0, 24, 48, and 72 h after treatment. Cell lysates were prepared for SDS-PAGE and analyzed by Western blotting with phospho-specific ERK1/2 or phospho-specific MEK1/2 antibody; they were then stripped and reprobed with pan-ERK1/2 or pan-MEK1/2 antibody.

limited to sulindac metabolites alone. There was a similar level of ERK1/2 inhibition by CP248 and CP461 compared with sulindac doses that induced equivalent apoptotic responses.

Sulindac Metabolites Activate Cleavage of Caspase-3 and Caspase-7. Sulindac sulfide (120 μM) and sulindac sulfone (600 μM) both induced cleavage of caspase-3 and caspase-7 (Fig. 6) at times preceding morphological apoptosis. Cleavage of caspase-3 and -7 was detectable as early as 12 h after treatment, whereas changes in nuclear morphology were usually not apparent until at least 24 h and were maximal at 5 days after treatment. Cleavage of both caspase-3 and caspase-7 occurred at similar times and to the same extent in both sulindac sulfide- and sulfone-treated cells. In all of the experiments, inhibition of ERK1/2 phosphorylation was seen concomitant with caspase cleavage.

U0126 Stimulates Apoptosis and Potentiates Apoptosis Induced by Sulindac Metabolites. To determine whether inhibition of ERK activity alone could induce apoptosis in colon cancer cells, an additional method of suppressing ERK phosphorylation was sought. U0126 is a highly specific inhibitor of MEK1/2 that has been used extensively as a tool to manipulate ERK1/2 signaling (36). HCT116 cells were treated with 0, 10, 25, or 50 μM U0126 and were harvested after 30 min, 24 h, and 48 h after treatment. At all of the doses, U0126 completely blocked ERK phosphorylation by 30 min (Fig. 7). A persistent dose-dependent inhibition of ERK phosphorylation was seen at 24 and 48 h after treatment. At these doses, U0126 induced cleavage of caspase-3 and caspase-7 at 48 h (Fig. 7B), which indicated that persistent down-regulation of ERK1/2 phosphorylation is able to

induce apoptosis. Apoptosis was confirmed by nuclear morphology (data not shown).

Next, we determined the effect of U0126 on sulindac metabolite-induced ERK1/2 inhibition and apoptosis. HCT116 cells were pre-treated with vehicle (0.1% DMSO) or 20 μM U0126 and, after 30 min, were treated with vehicle (0.1% DMSO), 80 μM sulindac sulfide, or 200 μM sulindac sulfone. Western blotting of phosphorylated ERK1/2 was performed to confirm inhibition by U0126. U0126 caused substantial inhibition of ERK1/2 phosphorylation from 30 min (data not shown) to 48 h after treatment (Fig. 8A). Total ERK1/2 protein levels were not decreased in U0126-treated compared with control lysates. U0126 treatment alone induced apoptosis of HCT116 cells and potentiated the apoptotic effect of sulindac sulfide and sulindac sulfone (Fig. 8B).

DISCUSSION

Sulindac has a dramatic chemopreventive effect on the colonic adenomas of patients with familial polyposis (7–11). There is evidence that the biological mechanism of this chemopreventive effect is the induction of apoptosis in neoplastic cells, but the biochemical mechanism of this apoptotic effect is incompletely understood. NSAIDs inhibit COX-1 and -2 and thereby decrease prostaglandin synthesis. In this study, however, we have shown that both the active NSAID metabolite of sulindac (sulindac sulfide) and the non-NSAID metabolite (sulindac sulfone) selectively inhibit the MEK1/2-ERK1/2 arm of the Ras signaling pathway at doses and times consistent with apoptotic induction. The comparable inhibitory effects of both of the sulindac metabolites indicate that these effects are independent of COX inhibition. Furthermore, we have shown that ERK1/2 inhibition by U0126, a highly selective MEK1/2 inhibitor that is structurally unrelated to NSAIDs, also induces apoptosis in colon cancer cells. These studies indicate that ERK1/2 inhibition alone is sufficient to induce apoptosis in colon cancer cells and suggest that down-regulation of ERK1/2 may be a mechanism of the apoptotic cell death induced by sulindac.

Although apoptotic induction of cultured cells by chemical inhibition of ERK1/2 has been described in other systems (37), to our knowledge, this is the first report that ERK1/2 inhibition can cause apoptotic cell death in cultured human colon cancer cells. ERK activation is commonly thought to protect many different types of cells from apoptosis, predominantly in neuronal models of growth factor withdrawal (37). One mechanism by which cancer cells maintain their growth advantage may be via activation of antiapoptotic pathways such as ERK1/2. This may be particularly relevant to colon cancer because mutation of *K-ras* occurs commonly during colon cancer development, and these mutations result in activation of the ERK1/2 arm of the Ras signaling pathway (26). ERK1/2 activity is higher in colon cancer cells with mutant *K-ras* than in those with wild-type *K-ras* (38), and *ras* mutant cell lines are differentially sensitive to the apoptotic effects of NSAIDs (39). Cells that have become dependent on high levels of *K-ras* signaling for survival may be more affected by inhibition of ERK1/2. Our results indicate that persistent inhibition of the pro-proliferative ERK1/2 pathway is sufficient to cause the colon cancer cells in culture to undergo apoptotic cell death.

Our results are also the first indication that inhibition of ERK1/2 signaling plays a role in sulindac-induced apoptosis. Both of the sulindac metabolites inhibit ERK1/2 phosphorylation at times and doses that are consistent with apoptotic induction, without causing an equivalent decrease in total ERK1/2 protein expression. The time course of effects indicate that the onset of ERK inhibition occurs before the earliest morphological evidence of apoptotic cell death and

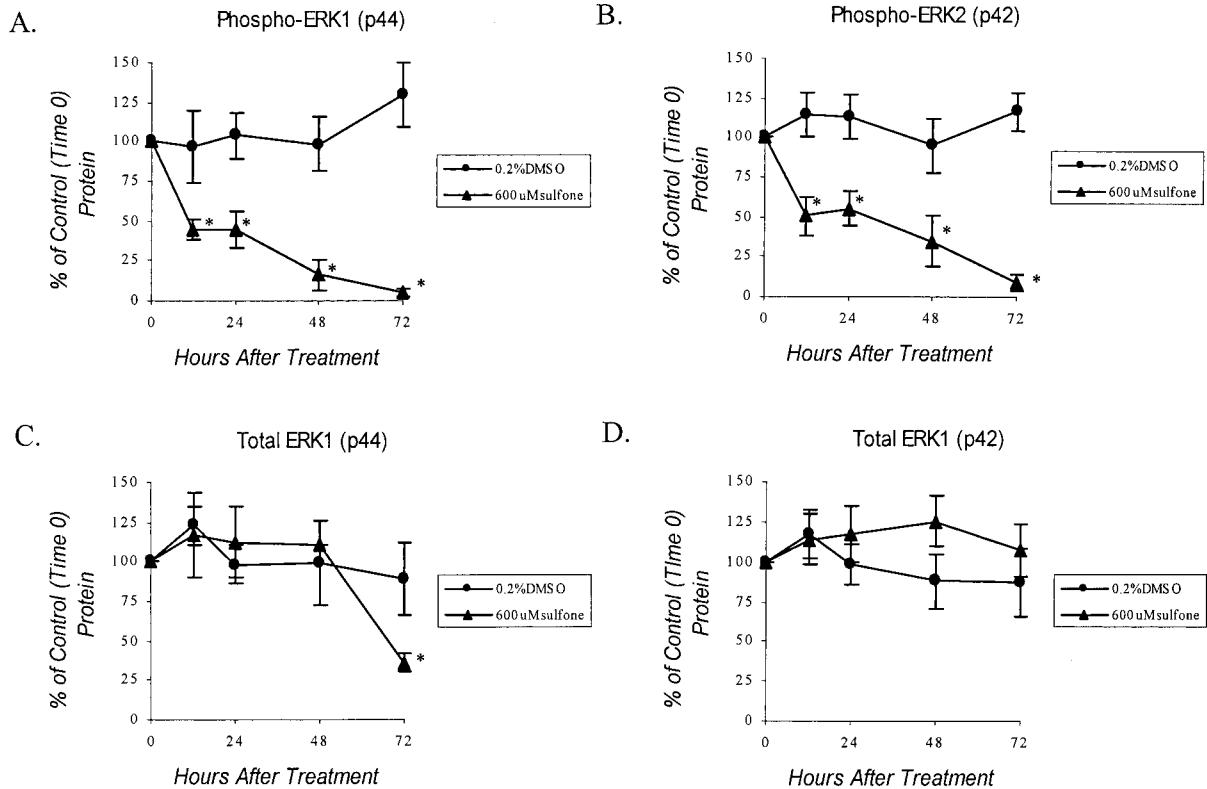


Fig. 4. Sulindac sulfone inhibits phosphorylation of ERK1 (p44; A) and ERK2 (p42; B) proteins, but not total ERK1 (p44; C) or total ERK2 (p42; D) protein. Line graphs, the mean and SE from at least three independent experiments. *, $P < 0.05$ versus day 0 control.

concomitant with the earliest evidence of caspase cleavage. These results suggest that ERK inhibition may not be the result of apoptotic cell death and that it occurs in a time frame such that it could be inducing the apoptotic process.

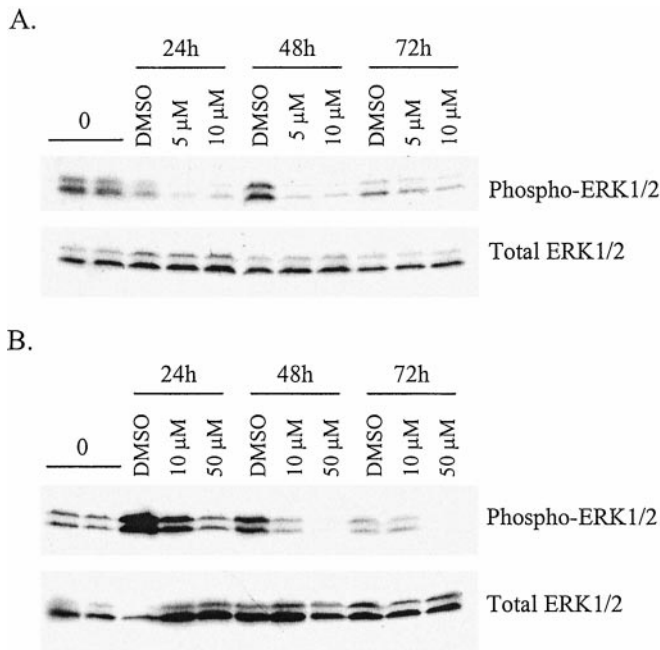


Fig. 5. Potent cGPDE inhibitors inhibit phosphorylation of ERK1/2. HCT116 cells were treated with 0.1% DMSO or with 5 μ M or 10 μ M CP248 (A) and 0.1% DMSO or 10 μ M or 50 μ M CP461 (B), and were harvested 0, 24, 48, and 72 h after treatment. Cell lysates were prepared for SDS-PAGE and analyzed by Western blotting with antibodies specific for phospho-ERK1/2 or phospho-MEK1/2; they were then stripped, and reprobbed with total ERK1/2 or total MEK1/2 antibody.

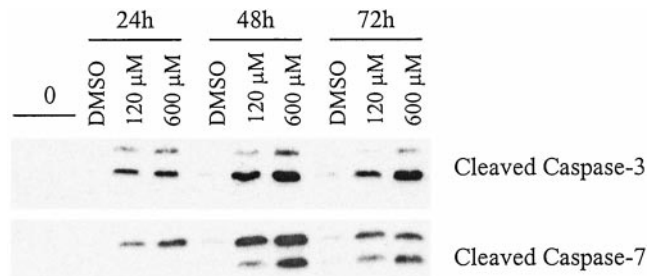


Fig. 6. Sulindac metabolites induce cleavage of caspase-3 and caspase-7. HCT116 cells were treated with 0.2% DMSO or with 120 μ M sulindac sulfide or 600 μ M sulindac sulfone and were harvested 24, 48, and 72 h after treatment. Cell lysates were prepared for SDS-PAGE and analyzed by Western blotting with antibodies specific for cleaved caspase-3 and cleaved caspase-7.

The observation that sulindac metabolites inhibit phosphorylation of MEK1/2, the upstream activator of ERK1/2, suggests that sulindac metabolites inhibit ERK1/2 activity by decreasing MEK1/2-dependent phosphorylation. These effects are likely to be at the level of MEK1/2 or upstream. The ability of sulindac sulfide to bind Ras protein *in vitro* and inhibit association with c-Raf (29), the upstream activator of MEK1/2, is a possible mechanism of the effects of sulindac on ERK1/2. The decrease in total MEK1/2 protein at later times indicates that sulindac induces proteolytic cleavage or inhibits the transcription or translation of MEK1/2 protein.

cGPDE is a direct biochemical target for non-NSAID apoptotic agents such as sulindac sulfone, CP248, and CP461 (25). We found that several of the cGPDE inhibitors have in common the ability to inhibit ERK activity at doses that induce apoptotic cell death. PKG activation that follows cGPDE inhibition by sulindac-like compounds may result in down-regulation of ERK1/2 signaling. Of interest, c-Raf-1 is a substrate for activated PKG, and PKG-mediated phos-

phorylation of c-Raf-1 results in the inhibition of Raf activity and dissociation from Ras (30). The inhibition of c-Raf-1 activity would down-regulate MEK1/2 and, therefore, ERK1/2 signaling.

Treatment of colon cancer cells with U0126 led to a profound transient inhibition of ERK1/2 phosphorylation followed by a dose-related persistence of the effect that was associated with the induction of apoptotic cell death. Thus, to the extent that U0126 has selective effects on MEK and ERK, this result suggests that ERK inhibition is sufficient to induce apoptotic cell death in colon cancer cells. The time course suggests that the duration of ERK inhibition may be important for the apoptotic effect in that both the sulindac metabolites and the higher doses of U0126 led to comparable ERK inhibition that lasted 24–48 h and led to the induction of apoptotic cell death. U0126 substantially potentiated the apoptotic effect of both of the sulindac metabolites, which suggests that these compounds together lead to greater inhibition of ERK1/2-mediated signaling. However, the level of ERK1/2 inhibition did not correlate well with the level of apoptotic cell death by these structurally unrelated compounds. For example, 20 μM U0126 and 200 μM sulindac sulfone induced similar amounts of apoptosis, whereas U0126 was much more effective at inhibiting ERK1/2 phosphorylation than sulindac sulfone (Fig. 8). This implies that sulindac metabolites also have effects on additional apoptotic pathways, which have an additive or synergistic effect on the induction of apoptotic cell death. Inhibition of ERK1/2 may, therefore, account for part, but not all, of the apoptotic effect of sulindac. Sulindac affects additional signaling pathways that may induce apoptotic cell death, including cGPD (25), nuclear factor- κB (40), and lipoxigenase (41). It is possible that some of these separate biochemical effects are in fact interdependent. Future studies will address possible interactions between ERK1/2 and these alternate biochemical targets of sulindac.

Establishing the mechanism of apoptotic induction by sulindac and its metabolites is an important step in understanding the potent chemopreventive activity of this drug in colonic adenomas. The results presented in this paper provide direct evidence of a link between one biochemical effect of sulindac (ERK1/2 inhibition) and the induction of apoptosis by the drug. Although it is possible, even likely, that sulindac affects more than one apoptotic pathway, we believe that we have identified ERK1/2 inhibition as at least one of the biochemical mechanisms of apoptotic activation by sulindac and its metabolites. Chemopreventive drugs that maintain this ERK inhibitory activity may be more potent than those that have more transient effects.

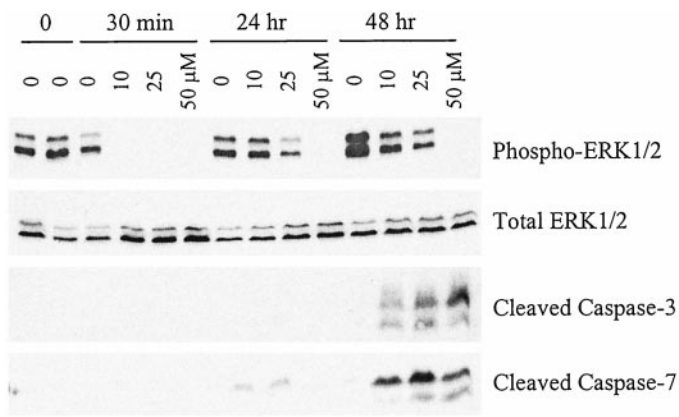


Fig. 7. U0126 inhibits ERK1/2 phosphorylation and induces cleavage of caspase-3 and caspase-7. HCT116 cells were grown to confluency and treated with 0.1% DMSO or with 10, 25, or 50 μM U0126 and were harvested 30 min, 24 h, and 48 h after treatment. Cell lysates were prepared for SDS-PAGE and were analyzed by Western blotting with antibodies specific for phosphorylated ERK1/2, total ERK1/2, and cleaved caspase-3, and cleaved caspase-7.

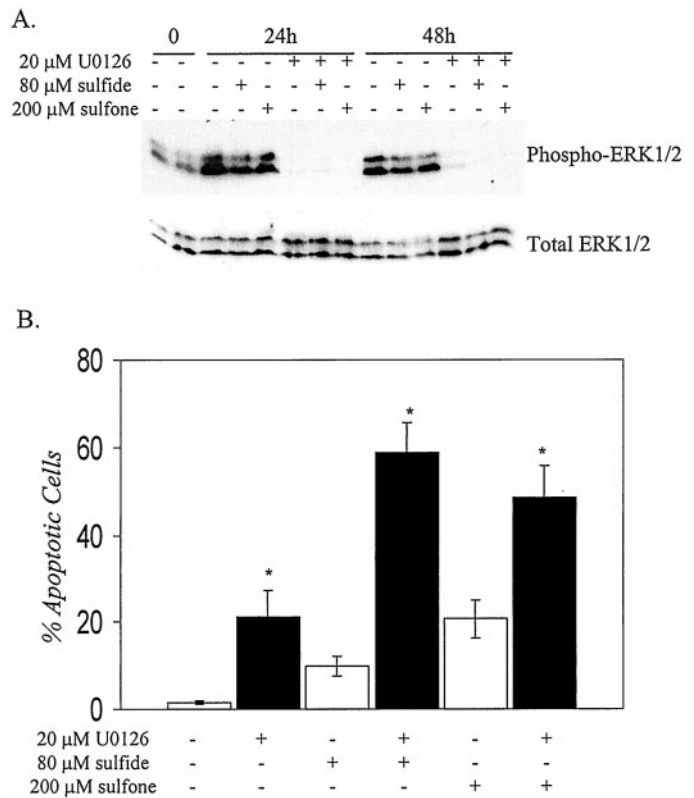


Fig. 8. U0126 potentiates the effects of sulindac metabolites on ERK1/2 phosphorylation (A) and apoptosis (B) in human colon cancer cells. In A, HCT116 cells were grown to confluency and pretreated with 0.1% DMSO or 20 μM U0126. After 30 min, cells were harvested or treated with 0.1% DMSO, 80 μM sulindac sulfide, or 200 μM sulindac sulfone and were harvested 24 and 48 h after treatment. Cell lysates were prepared for SDS-PAGE and analyzed by Western blotting. B, induction of apoptosis as determined by nuclear morphology after staining with acridine orange and ethidium bromide. Bars, mean and SE from three separately treated wells harvested three days after treatment. *, $P < 0.05$ for U0126 + sulindac- compared with sulindac-treated cells.

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