Sulindac independently modulates extracellular signal–regulated kinase 1/2 and cyclic GMP–dependent protein kinase signaling pathways

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Abstract
Colorectal cancer is the second leading cause of cancer mortality in the United States. Substantial human and animal data support the ability of nonsteroidal anti-inflammatory drugs to cause regression of existing colon tumors and prevent new tumor formation. The mechanism by which the nonsteroidal anti-inflammatory drug sulindac prevents tumor growth is poorly understood and seems complex as sulindac can modulate several growth-related signaling pathways. Sulindac metabolites simultaneously (a) increase cellular cyclic GMP and subsequently activate cyclic GMP–dependent protein kinase (PKG); (b) activate c-jun NH2-terminal kinase (JNK); (c) inhibit extracellular signal–regulated kinase 1/2 (ERK1/2); and (d) decrease β-catenin protein expression at times and doses consistent with apoptosis. The purpose of this study was to determine if PKG, ERK1/2, JNK, and β-catenin are independent targets for sulindac in vitro. Pharmacologic activation of PKG with YC-1 increases JNK phosphorylation and induces apoptosis in colon cancer cells without modulating ERK1/2 phosphorylation or β-catenin protein expression. Inhibition of ERK1/2 with U0126 induces apoptosis but fails to activate JNK phosphorylation or down-regulate β-catenin protein expression. Cotreatment with U0126 and YC-1 synergistically increases apoptosis in colorectal cancer cells and recapitulates the effects of sulindac treatment on ERK1/2, JNK, and β-catenin. These results indicate that sulindac metabolites modulate ERK1/2 and PKG pathways independently in colon cancer cells and suggest that the full apoptotic effect of sulindac is mediated by more than one pathway. Using similar combinatorial approaches in vivo may provide more effective, less toxic chemopreventive and chemotherapeutic strategies. Such therapies could dramatically reduce the incidence and death rate from colorectal cancer.

Introduction
Colorectal cancer is the second leading cause of cancer death in the United States (1), with >5% of the population expected to develop this disease in their lifetime. Novel strategies for the prevention of colorectal cancer are necessary to reduce this burden, both in terms of healthcare-related costs and loss of productivity. Nonsteroidal anti-inflammatory drugs, including sulindac, have the potential ability to prevent and reverse colorectal cancer development (2). Unfortunately, significant toxicity limits chronic use of nonsteroidal anti-inflammatory drugs for the prevention of colorectal cancer. The mechanism(s) by which sulindac prevents colon tumor growth are complex and incompletely understood. Although nonsteroidal anti-inflammatory drugs are classically defined by the ability to inhibit the activity of the cyclooxygenase (COX) enzymes, inhibition of COX-1 and COX-2 per se does not adequately explain the growth inhibitory effects of sulindac in vitro or in vivo. For example, the sulfone metabolite of sulindac does not inhibit COX enzymatic activities, yet prevents cancer cell growth in vitro and in vivo (3). In addition, nonsteroidal anti-inflammatory drugs, including sulindac, inhibit proliferation of cancer cells that do not express COX-1 or COX-2 (4). Several specific COX-independent biochemical targets have been proposed for sulindac, including inhibition of cyclic GMP–dependent phosphodiesterases 2 and 5 (5), inhibition of extracellular signal–regulated kinase 1/2 (ERK1/2; ref. 6), activation of c-jun NH2-terminal kinase (JNK; ref. 6), and down-regulation of β-catenin (5). A better understanding of the biochemical mechanisms mediating the antitumor effects of sulindac could lead to the development of more effective and widely tolerated chemopreventive strategies, the use of which could effectively reduce the incidence and burden of colorectal cancer.

Apoptosis is thought to mediate inhibition of tumor cell growth by sulindac in vitro and in vivo. Inhibition of ERK1/2 (7), activation of JNK (8), and inhibition of β-catenin protein expression (9) are required for apoptosis of colorectal cancer cells by sulindac and related compounds in vitro. Inhibition of cyclic GMP–dependent phosphodiesterases 2 and 5 leads to cellular cyclic GMP

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accumulation and subsequent activation of cyclic GMP-dependent protein kinase (PKG). PKG activation leads to phosphorylation and activation of JNK in colorectal cancer cells (8) and β-catenin is a substrate for PKG in vitro (5). Phosphorylation of β-catenin by PKG in theory could lead to ubiquitination and subsequent proteasomal degradation. In some cell types, PKG can phosphorylate c-Raf-1, which inhibits downstream signaling by mitogen-activated protein kinase/ERK kinase (MEK)/ERK (10). PKG activation could therefore mediate all the biochemical effects shown to be required for apoptosis by sulindac (i.e., inhibition of ERK1/2, activation of JNK, and inhibition of β-catenin protein expression).

The purpose of this study is to determine if PKG, ERK1/2, JNK, and β-catenin are mutually exclusive or codependent signaling pathways modulated by sulindac in human colorectal cancer cells. We report that activation of PKG by YC-1 induces apoptosis and activates JNK, with no consistent effect on ERK1/2 phosphorylation or β-catenin protein expression. Inhibition of ERK1/2 with U0126 induces apoptosis but does not affect JNK activation or β-catenin expression. Apoptosis by sulindac sulfide, but not by YC-1, was blocked in cells expressing a constitutively active MEK1 gene. The ability of both sulindac sulfide and YC-1 to activate JNK was not affected by constitutively active MEK1. Cotreatment with YC-1 and U0126 together induced apoptosis to a greater degree than with either compound alone, augmented ERK1/2 inhibition and JNK1 activation, and decreased β-catenin protein expression. Together these results indicate that ERK1/2 and PKG pathways are independently modulated by sulindac metabolites but may have common downstream effects that contribute to apoptosis. Combination therapy using both ERK1/2 inhibition and PKG activation represents an attractive strategy against colorectal cancer development and warrants further study.

Materials and Methods

Materials

U0126 and YC-1 were purchased from Calbiochem (San Diego, CA), sulindac sulfide was from LKT Laboratories (St. Paul, MN), and sulindac sulfone was a generous gift from Cell Pathways, Inc. (Horsham, PA). Cell culture media and fetal bovine serum were purchased from Mediatech (Herndon, VA), antibiotic/antimycotic solution was from Life Technologies, Inc. (Grand Island, NY), and tissue culture plates were from Falcon (Franklin Lakes, NJ). Primary antibodies were purchased from the following sources: phosphorylated ERK1/2, total ERK1/2, phosphorylated JNK, and total JNK from Santa Cruz Biotechnology (Santa Cruz, CA), β-catenin from Transduction Laboratories (Lexington, KY), full-length and cleaved caspase-3 from Cell Signaling Technology (Beverly, MA), and β-actin antibody from Novus Biologicals (Littleton, CO). Horseradish peroxidase–conjugated antimouse and antirabbit secondary antibodies were from Santa Cruz Biotechnology, Immobilon-P membranes were from Millipore (Bedford, MA), chemiluminescent visualization reagents were from NEN (Boston, MA), and X-ray film was from Pierce (Rockford, IL).

Tissue Culture

HT29 and HCT116 human colon cancer cells were purchased from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin/fungizone solution. Medium was replaced two to 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% to 100% confluency before treatment. HT29-R4F and HT29-pCEP clones were created as described (7). HT29-R4F clones stably express a constitutively active human MEK1 gene whereas HT29-pCEP clones are empty vector controls. These clones were maintained as the HT29 parental cells, except medium was supplemented with 600 μg/mL hygromycin B to select for stable gene expression.

Morphologic Apoptosis Assay

Apoptosis and viability were quantified as previously described (6) by staining cells with acridine orange and ethidium bromide, then analyzing for nuclear morphology, a hallmark of apoptosis. For each determination, adherent and floating cells were harvested together and three separate 100 cell counts were scored per sample. Apoptosis was expressed as a percentage calculated from the number of cells with apoptotic nuclear morphology divided by the total number of cells examined.

Flow Cytometry

Cell cycle analysis was done by the University of Colorado Cancer Center Flow Cytometry Core Laboratory. Cells were trypsinized 24 hours after treatment, washed in PBS, pelleted, resuspended in Krishan stain (11), and allowed to stand 24 hours in stain before analysis. Analysis was done using a Beckman Coulter Epics XL flow cytometer. Doublets were excluded from the analysis using the peak versus integral gating method. ModFit LT software (Verity Software House, Topsham, ME) was used for cell cycle analysis.

Western Immunoblotting

At time of harvest, cells were scraped from the plates, washed with ice-cold PBS, and pelleted at 2,400 × g for 5 minutes. Care was taken to keep tissue samples as cold as possible during the entire procedure. After aspirating the supernatant, the cells were washed twice with ice-cold PBS, removing and discarding the supernatant each time. The cell pellet was then lysed in cell extraction buffer [10 mmol/L Tris (pH 7.4), 100 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L NaF, 20 mmol/L Na3P2O7, 2 mmol/L Na2VO4, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride, 60 μg/mL aprotinin, 10 μg/mL leupeptin, 1 μg/mL pepstatin] for 30 minutes on ice, vortexing at 10-minute intervals. Lysates were then centrifuged at 18,000 × g for 10 minutes at 4°C and supernatants were collected. Protein concentrations of lysates were determined by the method of Lowry (12).
Lysates were prepared for SDS-PAGE and 50 μg of total protein were separated and electrotransferred overnight onto Immobilon-P polyvinylidene fluoride membranes (Millipore). Nonspecific binding was blocked for 30 minutes in Tris-neutral saline with 1% (w/v) dry milk and 0.05% Tween 20, then incubated with primary antibodies raised against phospho-ERK1/2(pY204), ERK1/2, phospho-JNK(pT183, pY185), JNK, β-catenin, cleaved caspase-3, or β-actin primary antibodies for 1 hour while rocking at 4°C. Immunoreactive protein was detected by incubating blots with horseradish peroxidase–conjugated secondary antibody for 1 hour followed by chemiluminescent substrate for 1 minute. Immunoreactive proteins were visualized by exposure to radiographic film. In some experiments, the membranes were stripped for 30 minutes in 10% SDS, 67 mmol/L Tris (pH 6.7), and 0.8% 2-mercaptoethanol while rocking at 50°C, and washed twice for 10 minutes each in Tris-neutral saline with 0.05% Tween 20. Blots were then reblocked and probed with ERK1/2, JNK, or β-actin primary antibodies as described above. Independent experiments validated that this stripping procedure did not lead to loss of signal. Densitometry was done using Un-Scan-It Software (Silk Scientific, Orem, UT).

Results

Sulindac Metabolites Simultaneously Inhibit ERK1/2 Phosphorylation, Increase JNK Phosphorylation, and Decrease β-Catenin Protein Expression

Sulindac sulfide and sulindac sulfone both inhibited ERK1/2 phosphorylation at 24 and 48 hours following drug treatment in HCT116 cells (Fig. 1). Sulindac metabolites increased JNK phosphorylation at 24 hours, but not at 48 hours, after treatment. Phospho-JNK was detected at ~46 and 54 kDa, with the 46-kDa band more prominently expressed in most experiments. Expression of β-catenin protein was inhibited at 24 and 48 hours after drug treatment. Under these conditions, cleavage of caspase-3, an indicator of apoptosis, was apparent at 48 hours of drug treatment. The apoptotic effect was confirmed by examining nuclear morphology after staining cells with acridine orange and ethidium bromide (data not shown). Sulindac treatment had no consistent effect on the expression of total ERK1/2, total JNK, or actin proteins at these times. Similar results were obtained in experiments with HT29 and SW480 colon cancer cells (data not shown). At concentrations sufficient to induce apoptosis, sulindac metabolites therefore modulate ERK1/2, JNK, and β-catenin signaling pathways simultaneously. JNK activation is transient whereas inhibition of ERK1/2 and β-catenin proteins is sustained.

Inhibition of ERK1/2 Phosphorylation with U0126 Induces G1 Cell Cycle Arrest and Apoptosis

U0126 treatment for up to 72 hours caused a dose-dependent induction of apoptosis in HT29 human colon cancer cells (Fig. 2A). U0126 at 25 and 50 μmol/L caused a significant increase in the percentage of apoptotic cells, as determined after staining cells with acridine orange and ethidium bromide. HT29 cells were also analyzed by flow cytometry to examine the effects of U0126 on cell cycle progression. U0126 treatment for 24 hours resulted in an increased number of cells in the G1 phase of the cell cycle, with fewer cells in S phase (Fig. 2B). U0126 treatment induced similar levels of apoptosis (6) and G1 cell cycle arrest (data not shown) in HCT116 colorectal cancer cells.

To confirm the biochemical activity of U0126 in treated cells, we examined HT29 cell lysates for expression of phospho-ERK1/2, the downstream effector of MEK1/2. U0126 caused a dose-dependent inhibition of phospho-ERK1/2 expression (Fig. 3), which was seen as early as 30 minutes (data not shown) and lasted up to 48 hours. Treatment with U0126 induced apoptosis at concentrations sufficient to inhibit ERK1/2 phosphorylation for a sustained period, up to 48 hours.

Next we determined if inhibition of ERK1/2 could mediate the other biochemical effects that we observed with sulindac (i.e., activation of JNK and down-regulation of β-catenin proteins). U0126, at times and doses that inhibited phospho-ERK1/2 and induced apoptosis, did not...
affect the expression of phospho-JNK, total JNK, or β-catenin proteins (Fig. 3). Detection of cleaved caspase-3 by Western blotting confirmed results obtained with the morphologic apoptosis assay (Fig. 2A). Similar results were obtained in HCT116 cells (6).

**Activation of PKG by YC-1 Induces Apoptosis, S-Phase Arrest, and Activation of JNK**

YC-1 is a potent activator of soluble guanylate cyclase (13), which increases production of cyclic GMP and activates PKG. One downstream effect of PKG activation in colorectal cancer cells is increased phosphorylation of JNK (8). We therefore used YC-1 as a biochemical tool for studying the effects of PKG activation on ERK1/2, JNK, and β-catenin protein expression in colorectal cancer cells. Treatment of HT29 cells with 1-5 μmol/L YC-1 for 48 hours induced a dose-dependent increase in morphologic apoptosis (Fig. 4A). Drug-treated cells were also analyzed for cell cycle progression after 24 hours. Treatment with 5 μmol/L YC-1 induced an accumulation of HT29 cells in the S phase of the cell cycle. YC-1 also induced apoptosis and S-phase arrest in HCT116 cells (data not shown). Whereas this increase in S phase could be interpreted as cell proliferation, we saw no indication that YC-1 increased cell number using the 3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay (data not shown), and after 48 hours the plates treated with YC-1 contained obviously fewer cells than the vehicle-treated plates when examined under an inverted microscope (data not shown).

To confirm the expected biochemical activity of YC-1, we examined JNK phosphorylation by Western blotting (8). At concentrations that induced apoptosis, YC-1 increased phospho-JNK (p46) expression 24 hours after treatment (Fig. 5). Increased phosphorylation of JNK was not apparent at 48 hours after YC-1 treatment. At these same concentrations, YC-1 had no effect on the expression of total JNK, phospho-ERK1/2, total ERK1/2, β-catenin, or actin proteins (Fig. 5). Expression of cleaved caspase-3 was apparent at 24 and 48 hours after YC-1 treatment, confirming our morphologic apoptosis results (Fig. 4A). Similar results were obtained in HCT116 cells (data not shown).

**Stable Expression of Constitutively Active MEK1 Does Not Affect YC-1-Induced Apoptosis or Cell Cycle Arrest**

Expression of a constitutively active MEK1 gene blocks the ability of sulindac to induce apoptosis and inhibit phospho-ERK1/2 expression in HT29 cells (7). Because our results indicated that ERK1/2 phosphorylation does not...
play a role in YC-1-induced apoptosis, we predicted that stable expression of a constitutively active MEK1 gene in HT29 cells would not affect apoptosis or cell cycle arrest by YC-1. The ability of YC-1 to induce apoptosis and S-phase cell cycle arrest was therefore assessed in stable clones of HT29 cells that express the activated MEK1(R4F) gene versus control clones that express an empty vector (pCEP). HT29-pCEP (empty vector control) and HT29-R4F (activated MEK1) clones were grown to 80% to 100% confluency and treated with 0, 5, or 10 μmol/L YC-1 in DMSO (0.1% final concentration). Cells were harvested after 72 hours of drug treatment and prepared for analysis of apoptosis after staining with acridine orange and ethidium bromide. YC-1 induced apoptosis equivalently in HT29-R4F clones and HT29-pCEP control clones (Fig. 6A), indicating that MEK/ERK signaling does not contribute to YC-1-induced apoptosis. In addition, after 24 hours of drug treatment, YC-1 induced an accumulation of cells in S phase in both vector control HT29-pCEP (Fig. 6B) and MEK1-activated HT29-R4F clones (Fig. 6C), similar to that seen in the parental HT29 cells (Fig. 4B). Similar results were obtained with additional clones of HT29-pCEP and HT29-R4F.

Constitutively Active MEK1 Blocks Apoptosis and ERK1/2 Inhibition, but not JNK Activation, by Sulindac Sulfide

HT29-R4F cells are resistant to apoptosis and ERK1/2 inhibition by sulindac sulfide and sulindac sulfone (7). We next examined expression of JNK and β-catenin proteins in lysates of sulindac-treated HT29-R4F and HT29-pCEP clones. Sulindac sulfide (160 μmol/L) induced apoptosis in HT29-pCEP control cells but not in HT29-R4F cells (Fig. 7A). Inhibition of ERK1/2 phosphorylation and cleavage of caspase-3 occurred in response to sulindac sulfide in the control HT29-pCEP cells but not in the resistant HT29-R4F cells (Fig. 7B). However, activation of JNK phosphorylation and inhibition of β-catenin protein expression occurred in both the HT29-pCEP and HT29-R4F cells after treatment with sulindac sulfide (Fig. 7B). Similar results were obtained with additional clones of HT29-pCEP and HT29-R4F.

Cotreatment with U0126 and YC-1 Potentiates Cell Death

HT29 cells were grown to confluency and treated with concentrations of YC-1 and U0126, which alone cause...
moderate apoptosis. Treatment with 4 μmol/L YC-1 or 25 μmol/L U0126 failed to significantly increase apoptosis after 48 hours of treatment in HT29 cells (Fig. 8A). However, in cells cotreated with 4 μmol/L YC-1 and 25 μmol/L U0126 together, a highly significant increase in apoptosis occurred compared with vehicle-treated, YC-1-treated, or U0126-treated cells (Fig. 8A). Cell lysates were prepared to examine protein expression by Western blotting (Fig. 8B). Phospho-ERK1/2 expression was moderately decreased by U0126 treatment at 24 hours, but not at 48 hours, whereas YC-1 had no effect at either time point. Cotreatment with YC-1 and U0126 failed to significantly increase apoptosis after 48 hours in HT29 cells (Fig. 8A). However, in cells cotreated with 4 μmol/L YC-1 and 25 μmol/L U0126 together, a highly significant increase in apoptosis occurred compared with vehicle-treated, YC-1-treated, or U0126-treated cells (Fig. 8A).

**Discussion**

In this article, we present data indicating that sulindac metabolites simultaneously down-regulate MEK1/2 and ERK1/2, activate PKG and JNK, and down-regulate β-catenin in colorectal cancer cells. Individually, these results have been reported in the literature by our laboratory (6, 14) and others (5, 8); however, this is the first time that the relationship between these effects has been investigated in sulindac-treated colorectal cancer cells. The selective MEK1/2 inhibitor U0126 inhibited ERK1/2 and induced apoptosis without affecting expression of JNK or β-catenin proteins. Similarly, the selective PKG activator YC-1 activated JNK and induced apoptosis without affecting ERK1/2 or β-catenin protein levels. Cotreatment with U0126 and YC-1 potentiated apoptotic cell death of human colon cancer cells and reconstituted all of the biochemical effects seen with sulindac treatment (down-regulation of ERK1/2, activation of JNK, and down-regulation of β-catenin). Expression of a constitutively active MEK1 gene blocked sulindac-induced apoptosis and ERK1/2 inhibition, but not JNK activation or β-catenin inhibition induced by sulindac sulfide. The ability of YC-1 to induce apoptosis and activate JNK was not altered by expression of constitutively active MEK1. Together these results indicate that ERK1/2, JNK, and β-catenin pathways are independently regulated in colorectal cancer cells and that simultaneous modulation of these multiple pathways may, at least in part, explain the potent growth inhibitory effects seen with sulindac treatment (down-regulation of ERK1/2, activation of JNK, and down-regulation of β-catenin). Expression of a constitutively active MEK1 gene blocked sulindac-induced apoptosis and ERK1/2 inhibition, but not JNK activation or β-catenin inhibition induced by sulindac sulfide. The ability of YC-1 to induce apoptosis and activate JNK was not altered by expression of constitutively active MEK1. 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with previous studies. Pharmacologic inhibition of ERK1/2 signaling induces G₁ arrest and/or apoptosis in many cancer cell lines (6, 15). In some cell types, ERK1/2 regulates expression of G₁ checkpoint genes including cyclin D₁, p21Cip1, and p27Kip1 (16). The regulation of apoptosis by ERK1/2 has also been described. ERK1/2 regulates the expression and activities of several Bcl-2 family proteins that control mitochondrial events leading to apoptosis. ERK1/2 activation leads to increased expression of antiapoptotic Bcl-2 (17) and Bcl-X₇ (18) and inactivation of proapoptotic Bad (19) and Bim (20, 21) by phosphorylation, events that block loss of mitochondrial membrane potential and release of cytochrome c, which are required for activation of the apoptotic machinery. Whereas it is possible that U0126 has ERK1/2–independent effects, our cell cycle and apoptosis results correlated well with the inhibition of ERK1/2 phosphorylation, suggesting that ERK1/2 inhibition is causing these biological effects. We have previously shown that down-regulation of ERK1/2 phosphorylation is both necessary and sufficient for apoptosis of colorectal cancer cells by nonsteroidal anti-inflammatory drugs (6, 7). Pharmacologic inhibitors of MEK/ERK signaling are being actively studied in vivo and preclinical data indicate that inhibition of this pathway alone is sufficient to inhibit growth of colon tumor xenografts (15). ERK1/2 is activated in many different tumors, including the three major causes of cancer mortality, colorectal, lung, and breast cancers (22), thereby making it an attractive target for cancer prevention and therapy.

Sulindac metabolites inhibit cyclic GMP–dependent phosphodiesterase and activate PKG, and this mechanism has been proposed to be responsible for the apoptotic effect of these drugs (5). YC-1 selectively activates soluble guanylyl cyclase to increase cellular cyclic GMP levels (13). Increased cyclic GMP activates PKG, which has several downstream effects including activation of JNK in colorectal cancer cells (8). Activation of JNK may be the mechanism by which YC-1 induces apoptosis of colorectal cancer cells (8) although additional cyclic GMP–dependent pathways may also play a role. In our studies, YC-1 induced both JNK activation and apoptosis in colorectal cell lines. Expression of constitutively active MEK1 in HT29 cells does not affect JNK activation or β-catenin down-regulation by sulindac sulfide. Vector control (pCEP) or activated MEK1 (R4F) cells were grown to 80% to 100% confluency, then treated with 0 or 160 μmol/L sulindac sulfide in 0.1% DMSO. A, induction of apoptosis after 72 h of drug treatment as determined by examining nuclear morphology of cells stained with acridine orange and ethidium bromide. Columns, mean (n = 3 independently treated samples); bars, SE. Data were analyzed by unpaired t test. *, P < 0.001, versus vehicle-treated control. B, Western blotting: cells were harvested 24 h after treatment with DMSO or sulindac sulfide and lysates were prepared for Western blotting of phospho-ERK1/2, total ERK1/2, phospho-JNK, total JNK, β-catenin, and cleaved caspase-3 proteins. Representative of duplicate experiments. C, densitometric analysis of Western blotting. Columns, mean (n = 3 independently treated samples); bars, SE. Data were analyzed by unpaired t test. *, P < 0.05, versus vehicle-treated control. Representative of duplicate experiments.
cancer cells but did not affect ERK1/2 signaling. In some cell types, PKG can phosphorylate c-Raf-1, which inhibits downstream signaling by MEK/ERK (10). However, this does not seem to hold true in colorectal cancer cells as YC-1 treatment activates JNK phosphorylation while having no effect on ERK1/2 phosphorylation. It therefore seems that sulindac metabolites inhibit ERK1/2 by a PKG/JNK-independent mechanism in colorectal cancer cells.

We found that YC-1 treatment induced the accumulation of colorectal cancer cells in the S phase of the cell cycle. To our knowledge, this has not been reported previously. This S-phase arrest was not peculiar to a single cell type as it occurred in both HT29 and HCT116 colorectal cancer cell lines. At similar concentrations, YC-1 has been reported to induce G0/G1 arrest in human hepatocellular carcinoma (23) and human umbilical vein endothelial cells (24). The mechanism by which YC-1 induces cell cycle arrest at different phases in different cell types is unknown but may depend on the individual cell type and/or culture conditions, including culture density and serum concentration.

Sulindac metabolites down-regulate β-catenin protein expression and activation of PKG has been proposed as the mechanism for this effect (5). Isolated PKG can phosphorylate β-catenin protein in vitro (5), suggesting that activation of PKG by sulindac metabolites could be the mechanism mediating β-catenin down-regulation in colorectal cancer cells. However, we found that the PKG activator YC-1 had no effect on β-catenin protein expression up to 48 hours following drug treatment. These results suggest that sulindac metabolites inhibit β-catenin protein expression in a PKG-independent manner. Although it is possible that YC-1 affects β-catenin protein function without altering total protein expression, it did not mimic the sulindac-induced down-regulation of total β-catenin. In contrast to the results obtained with either compound alone, cotreatment with U0126 and YC-1 decreased β-catenin protein expression, recapitulating the effect seen with sulindac metabolites. Simultaneous inhibition of ERK1/2 and activation of PKG may thus both be required for the degradation of β-catenin protein by sulindac metabolites. We have previously reported that β-catenin down-regulation seen with sulindac metabolites is mediated in part by proteasomal degradation and in part as a consequence of caspase activation (14). The inhibition of β-catenin expression by cotreatment with U0126 and YC-1 may therefore be in part a consequence of caspase activation in apoptotic cells, as it was seen at drug concentrations that did induce apoptosis.

Expression of constitutively active MEK1 in HT29 cells blocked the ability of sulindac metabolites to inhibit ERK1/2 phosphorylation and induce apoptosis (7). In the current study, we extend these observations to show that sulindac sulfide still activates JNK and inhibits β-catenin expression in HT29-R4F cells that are resistant to ERK1/2 inhibition and apoptosis. It has also been reported that expression of a dominant-negative JNK gene blocks apoptosis by sulindac metabolites (8); however, the ability of sulindac to inhibit ERK1/2 and β-catenin was not examined in this study. Together, these results suggest that inhibition of ERK1/2 phosphorylation and activation of JNK phosphorylation, which alone are sufficient to induce apoptosis of human colorectal cancer cells, are both required for apoptosis induced by sulindac metabolites. Perhaps this is because sulindac treatment does not inhibit ERK1/2 or activate JNK to a sufficient level for apoptosis to occur, but rather takes advantage of synergistic effects of moderate but simultaneous modulation of both pathways.

In conclusion, we show data indicating that ERK1/2, JNK, and β-catenin biochemical pathways are independently regulated in human colon cancer cells by sulindac metabolites. Sulindac displays complex biochemical effects on these pathways, suggesting that combination therapies may be necessary to fully exploit the therapeutic potential of sulindac in colorectal cancer.
in colorectal cancer cells likely because it can modulate several signaling pathways simultaneously. Whereas modulation of any single pathway does not adequately explain the growth inhibitory effects of sulindac, we show that simultaneous selective inhibition of ERK1/2 with U0126 and activation of PKG with YC-1 reconstitute the complex biochemical effects reported to be required for apoptosis of colorectal cancer cells by sulindac metabolites. Deciphering these intricate and potentially related pathways will help distinguish between the cellular events required for growth inhibition by sulindac and those drug effects that are irrelevant or even contrary to chemoprevention. In addition, understanding cell signaling pathways that regulate proliferation and apoptosis of colorectal cancer cells will provide additional targets for development as anticancer therapeutics. The results presented here help explain the complexity of the biochemical effects of sulindac in vitro and may lead to the development of successful combination therapies against colorectal cancer using highly selective agents such as MEK1/2 inhibitors and PKG activators in vivo.

References

Sulindac independently modulates extracellular signal–regulated kinase 1/2 and cyclic GMP–dependent protein kinase signaling pathways


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