Sulindac metabolites induce caspase- and proteasome-dependent degradation of β-catenin protein in human colon cancer cells

Pamela L. Rice,[1,2] Jennifer Kelloff,[3] Holly Sullivan,[2] Linda J. Driggers,[3] K. Scott Beard,[3] Scott Kuwada,[4] Gary Piazza,[5] and Dennis J. Ahnen[1,2,3]

[1]Department of Medicine, University of Colorado Health Sciences Center, Denver, CO; [2]University of Colorado Cancer Center, Denver, CO; [3]Department of Veterans Affairs Medical Center, Denver, CO; [4]Division of Gastroenterology, University of Utah, Salt Lake City, UT; and [5]Institute for Drug Development, San Antonio, TX

Abstract

Colorectal cancer (CRC) is the second leading cause of cancer death in the USA. Accumulation of β-catenin protein is nearly ubiquitous in colon adenomas and cancers, presumably due to mutations in the *APC* or *β-catenin* genes that inhibit proteasome-dependent degradation of β-catenin protein. Substantial clinical, epidemiological, and animal evidence indicate that sulindac and other non-steroidal anti-inflammatory drugs (NSAIDs) prevent the development of CRC. The mechanisms by which sulindac exerts its potent growth inhibitory effects against colon tumor cells are incompletely understood, but down-regulation of β-catenin has been suggested as one potential mechanism. The goal of this study was to determine the mechanism of β-catenin protein down-regulation by sulindac metabolites. Treatment of human colon cancer cell lines with apoptotic concentrations of sulindac metabolites (sulindac sulfide, sulindac sulfone) induced a dose- and time-dependent inhibition of β-catenin protein expression. Inhibition of proteasome activity with MG-132 partially blocked the ability of sulindac sulfide and sulindac sulfone to inhibit β-catenin protein expression. Pretreatment with the caspase inhibitor z-VAD-fmk blocked morphological signs of apoptosis as well as caspase cleavage, and also partially prevented β-catenin degradation by sulindac metabolites. These effects occurred in cells with bi-allelic *APC* mutation (SW480), with wild-type *APC* but mono-allelic *β-catenin* mutation (HCT116) and in cells that lack expression of either COX-1 or -2 (HCT15). These results indicate that loss of β-catenin protein induced by sulindac metabolites is COX independent and at least partially due to reactivation of β-catenin proteasome degradation and partially a result of caspase activation during the process of apoptosis. (Mol Cancer Ther. 2003;2:885–892)

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer deaths in the United States (1). A large body of animal model, human epidemiological, and clinical intervention data indicates that nonsteroidal anti-inflammatory drugs (NSAIDs) have chemopreventive activity against CRC. The mechanisms for this effect are not completely understood but may involve down-regulation of β-catenin protein. β-Catenin is a multifunctional protein that is located in at least three distinct cellular compartments and performs at least two separate biochemical functions. Normally, β-catenin serves as a component of the cytoskeleton in differentiated epithelial cells, participating in a multi-protein complex at the plasma membrane where it binds E-cadherin to the actin cytoskeleton (2). In normal cells, free cytosolic β-catenin is rapidly phosphorylated at multiple serine and threonine sites near the NH_2-terminal region of the protein by a multi-protein complex including adenomatous polyposis coli (APC), glycogen synthase kinase 3-β (GSK-3β), and axin/conductin (3). These phosphorylation events target β-catenin for ubiquitination and subsequent degradation by the proteasome (4).

The development of CRC is almost invariably associated with cytoplasmic and nuclear accumulation of β-catenin protein (5). Accumulation of β-catenin protein is thought to occur via mutation of the β-catenin binding site of *APC* (6), or by mutation of the GSK-3β phosphorylation site of *β-catenin* (7). These mutations decrease the normal ubiquitination and proteasomal degradation of β-catenin protein. β-Catenin subsequently accumulates in the cytosol and translocates to the nucleus, where it regulates the Tcf/Lef family of transcription factors (8, 9). Transcription of Tcf/Lef regulated genes, including cyclin D1 (10), c-Myc (11), and PPARδ (12), is thought to support the transformed phenotype and provide a growth advantage to the colon tumor cell.

The observations that accumulation of β-catenin protein is a nearly ubiquitous event during CRC development (5), and that the Tcf/Lef transcriptional activation that results from β-catenin accumulation may drive cells toward cancer development, make prevention of β-catenin accumulation an attractive target for chemopreventive agents. It has been hypothesized that sulindac sulfone and related compounds induce phosphorylation of β-catenin by inhibiting cyclic GMP phosphodiesterases (cGPDEs), increasing cGMP

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Requests for reprints: Pamela L. Rice, Department of Medicine, A009-151, School of Medicine, University of Colorado Health Sciences Center, Denver, CO 80262. Phone: (303) 399-8020 ext. 3629; Fax: (303) 393-5145. E-mail: pamela.rice@uchsc.edu

levels, and activating protein kinase G (PKG) (13). Phosphorylation of β-catenin by PKG provides a possible alternative to the APC/GSK-3β-dependent ubiquitination and proteasomal degradation pathway for β-catenin. While NSAIDs and cGPDE inhibitors both are capable of down-regulating β-catenin protein expression *in vitro* (13–16) and *in vivo* (17–19), neither the mechanism of this down-regulation, nor its role in the growth inhibitory actions of sulindac are completely understood.

The induction of apoptosis, or programmed cell death, is thought to be a major biological mechanism of growth inhibition by NSAIDs and related compounds in cells in culture (13, 20), in animal models (21–25), and in human adenomas in patients with familial adenomatous polyposis (FAP) (26). Induction of apoptotic cell death involves activation of the caspase family of proteases. Activation of caspases by proteolysis targets the cleavage of cellular proteins that contain consensus cleavage sites specific to the individual caspase. Consensus cleavage sites for caspase-3, -6, and -8 have been identified in the β-catenin protein (27), and β-catenin cleavage occurs following induction of apoptosis by sodium butyrate, trichostatin A, staurosporine (28), and paclitaxel (29). Thus, in addition to proteasomal degradation, caspase-mediated cleavage of β-catenin could be an alternative mechanism by which sulindac metabolites down-regulate cellular β-catenin levels during apoptotic cell death of human colon cancer cells. Caspase-dependent cleavage would suggest that down-regulation of β-catenin protein is not the mechanism by which sulindac metabolites induce apoptosis, but rather is a consequence of activation of the cellular apoptotic machinery.

In this study, we determine whether the COX-inhibitory sulfide and non-COX-inhibitory sulfone metabolites of sulindac inhibit β-catenin protein expression by the following mechanisms: (*a*) reactivation of proteasome-dependent degradation and/or (*b*) activation of caspase-dependent degradation during apoptotic cell death. We provide evidence that sulindac down-regulates β-catenin expression by both proteasome- and caspase-dependent mechanisms. These results indicate that degradation of β-catenin by sulindac metabolites is COX independent, partly dependent on the proteasome, and at least partly a consequence of activation of the cellular apoptotic machinery.

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 β-catenin were purchased from Transduction Laboratories (Lexington, KY), cleaved caspase-3 from Cell Signaling Technology (Beverly, MA), and anti-actin primary and horseradish peroxidase-conjugated secondary antibodies from Santa Cruz Biotechnology (Santa Cruz, CA). Immobilon-P membranes were obtained from Millipore (Bedford, MA), chemiluminescent visualization reagent from NEN (Boston, MA), and X-ray film from Pierce (Rockford, IL). Sulindac sulfide, MG-132, clastolactacystin, Caspase Inhibitor I (z-VAD-fmk), and Caspase Inhibitor III (Boc-Asp-OMe-fmk) were purchased from Calbiochem (San Diego, CA), and sulindac sulfone was a generous gift from Cell Pathways Inc. (Horsham, PA).

Tissue Culture

SW480, HCT116, HT29, and HCT15 human colon cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin-fungizone solution. Media were replaced 2× per week and cells were passaged at subconfluency. The cells were grown in a humidified atmosphere of 5% CO_2-95% air. For all experiments, cells were plated and grown to 80–100% confluency and media replaced the day before treatment with experimental drugs.

β-Catenin Immunofluorescence

SW480 cells were plated at equal density in chamber slides and grown to semi-confluence. Sulindac sulfide (0, 80, or 120 μM in 0.1% DMSO final concentration) was added to cultures for 24, 48, and 72 h. At harvest, chamber slides were washed once with PBS, fixed in 10% formalin for 5 min, and washed again with PBS. Slides were then covered with 10% normal goat serum for 15 min at room temperature followed with a 1:250 dilution of mouse monoclonal anti-β-catenin antibody (Transduction Laboratories) for 60 min. After washing 3× in PBS, slides were incubated in a 1:200 dilution of fluorescein-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 min, washed 3× in PBS, and mounted in Vectashield (Vector Laboratories, Burlingame, CA). As a negative control, parallel cells were incubated with an equal concentration (1 μg/ml) of mouse monoclonal antibody to rabbit MHC Class I instead of anti-β-catenin primary antibody. The stained slides were photographed, coded, and the percentage of cells displaying nuclear β-catenin was recorded by an observer blinded to the treatment conditions of the cultures. Three individual counts of 100 cells/sample were scored for each sample, and three individually treated samples counted for each treatment group.

Western Blotting

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, 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 collected. Protein concentrations were determined by the method of Lowry *et al.* (30). Lysates were prepared for SDS-PAGE and 50 μg total protein separated and electro-transferred overnight onto Immobilon-P polyvinylidene fluoride (PVDF) membranes. Blots were

blocked for 30 min in TNS with 1% (w/v) dry milk and 0.05% Tween 20, then incubated with primary antibodies overnight at 4°C. Immunoreactive proteins were 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 X-ray film.

Statistical Analysis

Data are represented in tables and graphs as mean ± SE, and analyzed by one-way ANOVA with a Newman-Keuls post hoc test (GraphPad Software, San Diego, CA). A minimum of three replicates was performed for each experiment. Significance was accepted at $P < 0.05$.

Results

Sulindac Sulfide Inhibits Expression of Nuclear β-Catenin in Colon Cancer Cells

To test the ability of the active NSAID metabolite of sulindac, sulindac sulfide, to prevent the accumulation of nuclear β-catenin in human colon cancer cells, SW480 cells were treated with 120 μM sulindac sulfide and immunocytochemical labeling of β-catenin protein performed. Sulindac sulfide treatment induced apoptotic cell death, determined by staining cells with acridine orange and ethidium bromide, and subsequent examination of nuclear morphology, as previously described (20) (data not shown). Control SW480 cells treated with vehicle (0.1% DMSO) displayed strong nuclear and diffuse cytosolic staining for β-catenin protein (Fig. 1A). Sulindac sulfide, at concentrations that induced apoptotic cell death, caused a decrease in the percentage of cells displaying nuclear β-catenin reactivity (Fig. 1B, Table 1). The loss of nuclear β-catenin was detected as early as 24 h and was maximal at 72 h after treatment with 120 μM sulindac sulfide. Sulindac sulfide treatment (0, 80, and 120 μM) caused a dose-dependent decrease in the percentage of SW480 cells expressing nuclear β-catenin after 72 h of drug treatment, and a dose-dependent increase in the number of cells undergoing apoptotic cell death (Table 1).

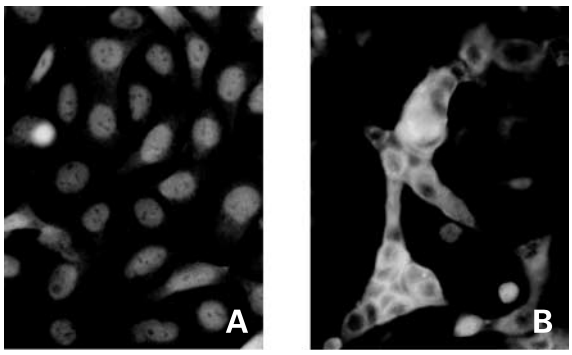

Figure 1. Sulindac sulfide inhibits expression of nuclear β-catenin in SW480 cells. SW480 colon cancer cells were plated in chamber slides and grown to subconfluency before treatment with 120 μM sulindac sulfide. Twenty-four hours after treatment, chamber slides were fixed and immunofluorescent labeling of β-catenin protein performed. **A,** control cells (0.1% DMSO). **B,** sulindac sulfide (120 μM)-treated cells. Magnification for each representative picture, ×400 (from three separate experiments).

Table 1. Effect of sulindac sulfide on nuclear β-catenin and apoptosis in SW480 cells (72 h after treatment)

Sulindac Sulfide (μM)	% Nuclear Positive Cells (±SD)	% Apoptosis (±SD)
0	94.43 ± 2.12	2.67 ± 0.58
80	72.85 ± 14.76*	17.00 ± 4.58*
120	47.82 ± 9.20**	26.67 ± 4.51**

*$P < 0.01$ *versus* 0 μM.
**$P < 0.001$ *versus* 0 μM.

Sulindac Metabolites Induce Dose- and Time-Dependent Loss of Total β-Catenin Protein in Colon Cancer Cells

To determine if sulindac sulfide caused a loss of total cellular β-catenin, we examined β-catenin protein expression in SW480 lysates after treatment with vehicle (0.1% DMSO), 120 or 160 μM sulindac sulfide. We detected β-catenin protein at M_r ~90,000, as previously reported (13). Treatment of SW480 cells with sulindac sulfide, at concentrations that induced apoptotic cell death, caused a dose- and time-dependent decrease of β-catenin protein, as detected by Western blotting of cell lysates using antibody raised against human β-catenin (Fig. 2A). Loss of β-catenin protein was detected as early as 24 h, and was maximal at 72 h after treatment with sulindac sulfide. Western blotting was performed for total cellular actin protein as a loading control (Fig. 2A), and confirmed that loss of β-catenin protein expression was not due to generalized protein loss occurring during apoptotic cell death.

The HCT116 human colon cancer cell line has no *APC* mutations but harbors a somatic *β-catenin* mutation coding for a 3-bp deletion which results in the removal of one amino acid (serine 45) in the coded protein (7). Ser45 is one site for APC/GSK-3β-dependent phosphorylation, a key event in targeting the β-catenin protein for ubiquitination and proteasomal degradation. If phosphorylation of serine 45 is essential for proteasomal degradation of β-catenin induced by sulindac metabolites, the HCT116 cell line would be expected to be resistant to these effects. Like the SW480 cell line, treatment of HCT116 cells with apoptotic concentrations of sulindac sulfide (NSAID metabolite) or sulindac sulfone (non-NSAID metabolite) resulted in a time-dependent loss of β-catenin protein, as measured by Western blotting (Fig. 2B). A lower concentration of sulindac sulfone was required to induce apoptosis in HCT116 cells compared to SW480 cells. We found that 400 μM sulindac sulfone induced a similar degree of apoptosis in HCT116 cells compared to 600 μM in SW480 cells, consistent with prior reports (20, 31). These results indicate that GSK-3β-dependent phosphorylation at serine 45 is not required for sulindac-induced down-regulation of

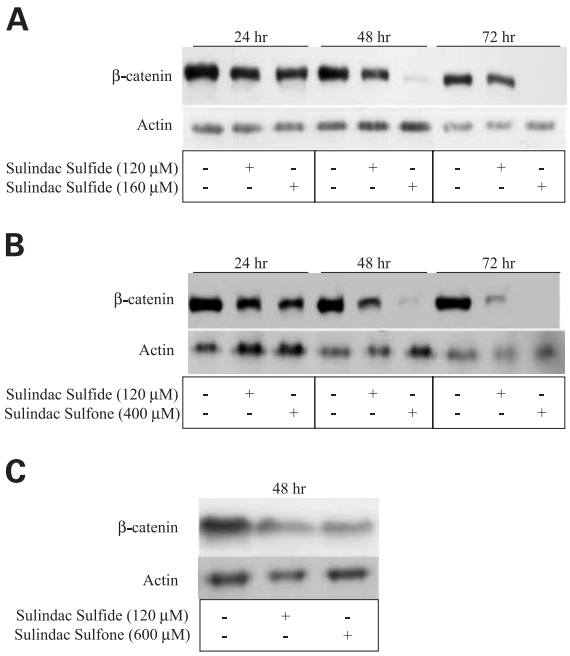

Figure 2. Sulindac sulfide induces time- and dose-dependent inhibition of β-catenin protein expression in human colon cancer cells. **A,** SW480 cells were grown to confluency then treated with 0.1% DMSO (vehicle), 120 or 160 μM sulindac sulfide and harvested after 24, 48, and 72 h. **B,** HCT116 cells, which express mutant β-catenin protein, were grown to confluency then treated with 120 μM sulindac sulfide, 400 μM sulindac sulfone, or vehicle (0.2% DMSO). Cells were harvested after 24, 48, and 72 h. **C.** HCT15 cells, which do not express COX-1 or COX-2, were grown to confluency then treated with 120 μM sulindac sulfide, 600 μM sulindac sulfone, or vehicle (0.2% DMSO) and harvested after 48 h. At time of harvest, lysates were prepared for Western blotting with antibodies raised against β-catenin and actin proteins.

β-catenin. Similar results were obtained using additional human colon cancer cell lines, including HCT15 (Fig. 2C) and HT29 (data not shown). Of note, the HCT15 cell line does not express mRNA and protein for either COX-1 or COX-2 (32), indicating that degradation of β-catenin induced by sulindac sulfide is independent of COX inhibition, the classic target of NSAIDs. Supporting the COX independency of this effect, sulindac sulfone, which does not inhibit COX-1 or -2 activities and is therefore not considered a NSAID, down-regulates β-catenin protein and induces apoptosis in colon cancer cells (Fig. 2, B and C). We have found similar results using the NSAIDs indomethacin and resveratrol (data not shown), indicating that down-regulation of β-catenin is a common effect of NSAIDs, and not limited to sulindac metabolites.

Cleavage of Caspase-3 Precedes Loss of β-Catenin Protein Expression

Inhibition of β-catenin protein expression was detectable as early as 24 h after treatment with sulindac metabolites. At this time, morphological signs of apoptosis are apparent, suggesting that the observable loss of β-catenin protein could occur subsequent to activation of the apoptotic cell death machinery. We therefore examined lysates prepared before 24 h of drug treatment, to compare the timing of caspase-3 cleavage and β-catenin expression following sulindac treatment in HCT116 cells. Sulindac treatment induced cleavage of caspase-3 into M_r ~13,000 and ~17,000 major fragments (20). Treatment of HCT116 cells with apoptotic concentrations of sulindac sulfide (120 μM) and sulindac sulfone (400 μM) induced cleavage of caspase-3 as early as 8 h following treatment (Fig. 3). However, at these relatively early times, no significant inhibition of β-catenin protein expression was detected (Fig. 3), indicating that at least part of the loss of β-catenin occurs subsequent to activation of effector caspases during apoptosis.

Caspase Inhibition Protects Cells from β-Catenin Degradation by Sulindac Metabolites

Because β-catenin is a substrate for caspases (27), and inhibition of β-catenin protein by sulindac appears to occur after activation of caspases (Fig. 3), we determined if loss of β-catenin protein following treatment with sulindac metabolites was a consequence of caspase activation. Pretreatment with 25 μM caspase inhibitor I (z-VAD-fmk), a broad specificity inhibitor of caspase activity, prevented cleavage of caspase-3 by sulindac sulfide (Fig. 4) and sulindac sulfone in HCT116 cells (Fig. 5) and SW480 cells (Fig. 6). In addition, pretreatment with z-VAD-fmk blocked morphological signs of apoptosis, including nuclear shrinkage and blebbing, determined by analyzing nuclear morphology after staining with acridine orange and ethidium bromide (data not shown). Under these conditions, caspase inhibition prevented complete degradation of β-catenin induced by sulindac sulfide (Fig. 4) and sulindac sulfone (Figs. 5 and 6). Analysis of at least three independent experiments in HCT116 cells pretreated with z-VAD-fmk showed a statistically significant protective effect of caspase inhibition on down-regulation of β-catenin protein by sulindac sulfide (Fig. 4B) and sulindac sulfone (Fig. 5B). Pretreatment with a different broad specificity inhibitor, caspase inhibitor III (Boc-Asp-OMe-fmk), also protected cells against β-catenin degradation by sulindac metabolites (data not shown).

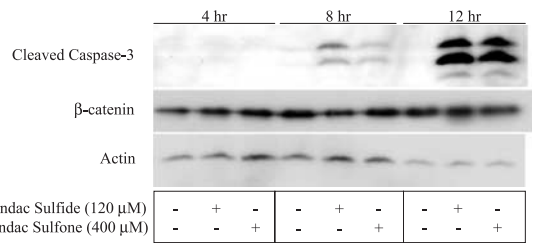

Figure 3. Cleavage of caspase-3 occurs before loss of β-catenin protein expression in HCT116 colon cancer cells. HCT116 cells were grown to confluency and treated with 120 μM sulindac sulfide, 400 μM sulindac sulfone, or vehicle (0.2% DMSO). Cells were harvested 4, 8, and 12 h after treatment and lysates prepared for Western blotting with antibodies raised against cleaved caspase-3, β-catenin, and actin proteins.

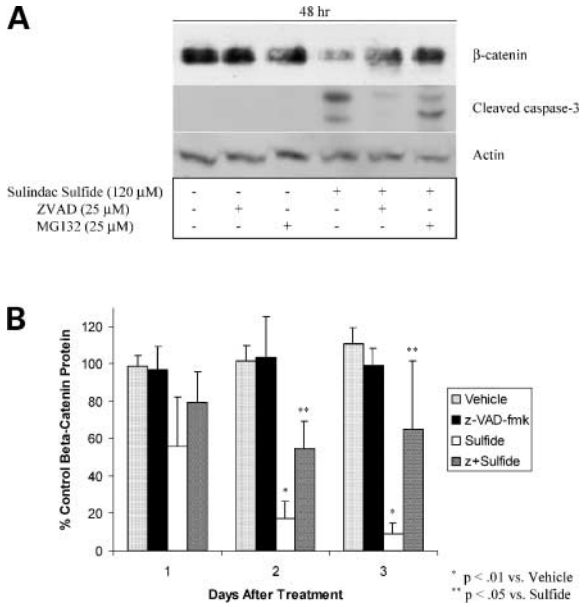

Figure 4. **A,** pretreatment with caspase inhibitor or proteasome inhibitor prevents loss of β-catenin protein induced by sulindac sulfide. HCT116 colon cancer cells were grown to confluency and pretreated with 25 μM caspase inhibitor (*z-VAD-fmk*), 25 μM proteasome inhibitor (*MG-132*), or vehicle (0.1% DMSO) for 60 min before treatment with 120 μM sulindac sulfide or vehicle (0.1% DMSO). Cells were harvested 48 h after drug treatment and lysates prepared for Western blotting with antibodies raised against β-catenin and actin proteins. **B,** summary of means and standard errors from multiple experiments using the caspase inhibitor, z-VAD-fmk, and sulindac sulfide in HCT116 cells.

Proteasomal Inhibition by MG-132 Protects Cells from *β*-Catenin Degradation by Sulindac Metabolites

Cellular accumulation of β-catenin is normally prevented by APC/GSK-3β-dependent phosphorylation, ubiquitination, and degradation by the proteasome. We therefore determined if loss of cellular β-catenin protein in HCT116 cells following sulindac sulfide treatment was mediated by proteasome-dependent degradation. Proteasome activity was inhibited by MG-132, a potent, reversible inhibitor of the 26*S* proteasome (33). Concentrations of MG-132 were chosen that were sufficient to increase jun NH_2-terminal kinase (JNK) phosphorylation, consistent with inhibition of proteasome activity as reported in the literature (34). We failed to see activation of JNK by MG-132 at concentrations less than 25 μM (data not shown), and 50 μM MG-132 caused substantial apoptotic death of colon cancer cells. We therefore used 25 μM MG-132 for all subsequent experiments. Pretreatment of HCT116 cells with 25 μM MG-132 for 60 min substantially inhibited loss of β-catenin protein induced by 120 μM sulindac sulfide after 48 h treatment (Fig. 4). Co-treatment with clasto-lactacystin, another proteasome inhibitor, as well as lower concentrations of MG-132 had similar, albeit less dramatic protective effects against β-catenin loss (data not shown). Treatment with MG-132 alone did not increase basal expression of β-catenin protein. Similar to these results, pretreatment with 25 μM MG-132 prevented β-catenin degradation induced by the non-NSAID metabolite, sulindac sulfone, in HCT116 (Fig. 5) and SW480 cells (Fig. 6). MG-132 did not affect cleavage of caspase-3 by sulindac metabolites (Figs. 4–6).

Discussion

The results presented in this paper are the first to demonstrate that both the NSAID metabolite (sulindac sulfide), as well as the non-NSAID metabolite (sulindac sulfone) of sulindac, inhibit expression of nuclear and total cellular β-catenin protein in cultured human colon cancer cells. Sulindac sulfide reduced the amount of nuclear β-catenin expression in a dose- and time-dependent manner consistent with induction of apoptotic cell death. Prior studies have shown that sulindac sulfone (13), as well as the NSAID indomethacin (14–13, 15), induce loss of cytoplasmic and nuclear β-catenin protein in human colon cancer cell lines. We observed that this effect occurs in HCT15 cells, which lack expression of COX-1 and -2. Taken together, these data indicate that β-catenin down-regulation is a common consequence of cell treatment with both the classic NSAIDs (sulindac sulfide, indomethacin, resveratrol) and the cGPDE inhibitors (sulindac sulfone, CP461, CP248), and that it is a

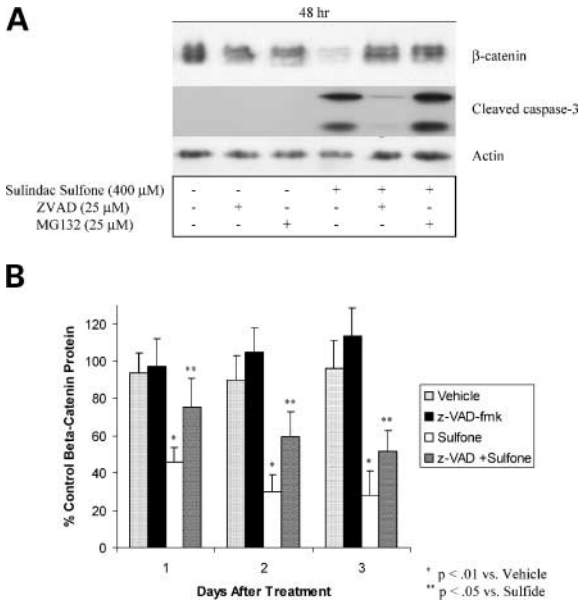

Figure 5. **A,** pretreatment with caspase inhibitor or proteasome inhibitor prevents loss of β-catenin protein induced by sulindac sulfone. HCT116 colon cancer cells were grown to confluency and pretreated with 25 μM caspase inhibitor (*z-VAD-fmk*), 25 μM proteasome inhibitor (*MG-132*), or vehicle (0.1% DMSO) for 60 min before treatment with 400 μM sulindac sulfone or vehicle (0.2% DMSO). Cells were harvested 48 h after drug treatment and lysates prepared for Western blotting with antibodies raised against β-catenin and actin proteins. **B,** summary of means and standard errors from multiple experiments using the caspase inhibitor, z-VAD-fmk, and sulindac sulfone in HCT116 cells.

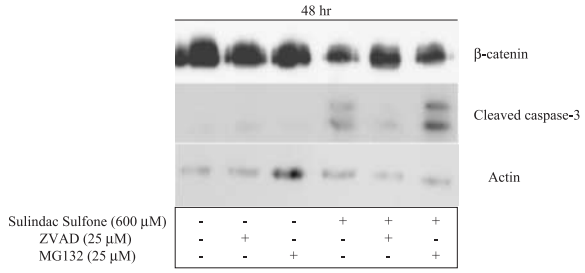

Figure 6. Pretreatment with caspase inhibitor or proteasome inhibitor prevents loss of β-catenin protein induced by sulindac sulfone. SW480 colon cancer cells were grown to confluency and pretreated with 25 μM caspase inhibitor (z-VAD-fmk), 25 μM proteasome inhibitor (*MG-132*), or vehicle (0.1% DMSO) for 60 min before treatment with 600 μM sulindac sulfone or vehicle (0.2% DMSO). Cells were harvested 48 h after drug treatment and lysates prepared for Western blotting with antibodies raised against β-catenin and actin proteins.

COX-independent effect. A number of non-COX targets of NSAIDs have been described but β-catenin down-regulation is one of few targets that has been confirmed *in vivo*. In the *Min* mouse model, sulindac treatment reduced expression of β-catenin protein in intestinal adenomas (17). In the rat azoxymethane model, indomethacin treatment inhibited nuclear, but not cytoplasmic β-catenin protein expression in colon tumors (19). The finding of down-regulation of β-catenin by NSAIDs *in vivo* highlights the relevance of cell culture models to elucidate the biochemical mechanisms responsible for β-catenin degradation by NSAIDs and related compounds.

Sulindac metabolites down-regulated β-catenin protein at doses and times similar to those required to induce apoptotic cell death. However, we found evidence of detectable activation of caspase-3 and morphological signs of apoptosis before observable β-catenin down-regulation. In addition, caspase inhibition partially blocked the β-catenin degradation by sulindac metabolites, indicating that caspase activation is required for sulindac metabolites to maximally down-regulate β-catenin expression. Inhibition of caspase activity did not return β-catenin protein levels to control levels, indicating that caspases are not completely responsible for loss of β-catenin protein expression by sulindac metabolites. While caspase-dependent cleavage of β-catenin has been described during apoptosis induced by unrelated compounds (28, 29), we provide the first evidence that caspases mediate down-regulation of β-catenin induced specifically by sulindac metabolites. A recent publication failed to detect caspase-dependent degradation of β-catenin by sulindac sulfone using a caspase-3 selective inhibitor (16), suggesting that activity other than caspase-3 may be responsible for degradation of β-catenin by sulindac sulfone. Caspase-mediated cleavage of β-catenin is thought to contribute to the dismantling of adherens junctions, and subsequent dissociation of cell-cell contacts during apoptosis (35, 36).

Although our results indicate that part of the down-regulation of β-catenin protein appears be a consequence of caspase activation, and thus not the mechanism by which NSAIDs induce apoptosis, they do not exclude the possibility that changes in β-catenin protein expression, localization, phosphorylation, and/or Tcf/Lef transcriptional activity precede caspase activation and contribute to apoptosis. Supporting a role for β-catenin in sulindac-induced apoptosis, overexpression of an NH_2-terminal truncated β-catenin gene conferred resistance to apoptosis induced by sulindac sulfone in SW480 cells (16). It would therefore be of interest to examine β-catenin phosphorylation and transcription of Tcf/Lef-dependent genes including *cyclin D1* and c-*myc* at times preceding caspase activation. Because β-catenin regulates Tcf/Lef-dependent expression of genes involved in the cell cycle (*e.g.*, cyclin D1), it is also possible that β-catenin inhibition is involved in cell cycle arrest, but not apoptosis by sulindac metabolites.

We also found evidence for proteasome-dependent degradation of β-catenin by both the sulfide and sulfone metabolites of sulindac. Sulindac sulfone, the non-NSAID metabolite of sulindac, has been previously reported to induce proteasome-dependent degradation of β-catenin (13, 15), but ours is the first report that the sulfide metabolite shares this property. Interestingly, one prior study reported that indomethacin caused degradation of β-catenin by a proteasome-dependent pathway in SW480, but not HCT116 cells (15). The resistance of the HCT116 line to indomethacin-induced β-catenin degradation was attributed to its expression of mutant β-catenin that could not be proteasomally degraded. In the current study, we did not detect any differences between SW480 and HCT116 cell lines with respect to either proteasome- or caspase-dependent degradation of β-catenin by either sulindac sulfide or sulindac sulfone. The differences between these two studies is not clear but may be due to structural differences between sulindac metabolites and indomethacin, or individual experimental conditions including the specific proteasome inhibitor used, drug concentrations, and incubation times. Our data suggest that both sulindac metabolites induce proteasome-dependent degradation of β-catenin by a mechanism that does not require APC-dependent phosphorylation. The ability of proteins other than APC and GSK-3β to mediate β-catenin phosphorylation and proteasomal degradation *in vivo* is possible. For example, PKG is activated in cancer cells by treatment with sulindac metabolites, and PKG can phosphorylate β-catenin *in vitro* (13, 15). This has been proposed as the mechanism for β-catenin degradation by sulindac sulfone and related compounds. It remains possible that other protein kinases could phosphorylate additional sites of β-catenin protein, leading to ubiquitination and proteasomal degradation.

One functional consequence of APC mutation and accumulation of cellular β-catenin levels is the subsequent translocation of β-catenin to the nucleus, where it interacts with Tcf/Lef family transcription factors. Binding of β-catenin to Tcf/Lef results in activation of Tcf-responsive elements and transcription of Tcf-regulated genes, including c-*myc* (11), *cyclin D1* (10), *PPARδ* (12), c-*jun*, and *fra*-1 (37). The ability of sulindac sulfide to decrease nuclear localization of β-catenin likely explains the ability of

sulindac metabolites to inhibit Tcf/Lef transcriptional activity (12). Down-regulation of β-catenin protein by NSAIDs may be functionally equivalent to expression of wild-type *APC*. Reintroduction of functional APC protein by transfection (38), or treatment with NSAIDs such as sulindac sulfide both result in apoptotic cell death, indicating that perhaps NSAIDs exert their chemopreventive effects by reactivating the APC pathway in colon cancer cells. Likewise, functional APC and sulindac treatment both inhibit transcription of the Tcf/Lef-regulated gene PPARδ (12). The β-catenin binding domain of APC was sufficient to induce loss of nuclear β-catenin, decrease Tcf/Lef transcriptional activation, suppress proliferation, and induce apoptosis of human colon cancer cells (39). Whether reintroduction of APC stimulates proteasome- and/or caspase-dependent down-regulation of β-catenin protein, similar to sulindac treatment, is unknown.

The relevance of the current *in vitro* observations to *in vivo* chemoprevention by NSAIDs and related compounds requires further study. The concentrations of sulindac metabolites used in this *in vitro* study are higher than has been measured *in vivo* in the plasma of human subjects (up to approximately 50 μM depending on dose and schedule) (40), and are also higher than required for the inhibition of sulindac's known biochemical targets *in vitro*, COX and cGPDE. However, it is difficult to directly compare short-term, high-dose *in vitro* experiments with longer term human and animal studies. The intracellular concentration of active drug has not been measured in either the *in vitro* or *in vivo* systems. Protein binding of drug, absorption efficiency, and enteric metabolism of drugs *in vivo* could substantially affect the intracellular concentration of metabolites in the gastrointestinal lumen (40). More potent cGPDE inhibitors that are structurally related to sulindac sulfone (CP461, CP248) induce apoptosis and cause β-catenin degradation in the nanomolar and low micromolar range (16), concentrations that can be achieved *in vivo*. This suggests that the current observations are not due to a non-specific effect of high drug doses. In the current study, doses that have been shown by several laboratories to induce a relevant biological effect on colon cancer cells (growth inhibition and apoptosis) were used. Most importantly, we are studying biochemical (β-catenin down-regulation) and biological effects (apoptosis) known to occur during sulindac treatment *in vivo*. Like chemically induced animal models in which high doses of carcinogens are used to induce multiple primary tumors in 100% of animals in short time periods, we have designed our *in vitro* studies to examine early biochemical events mediating the induction of apoptosis by sulindac in shorter time periods.

Our results indicate that at least part of the degradation of β-catenin by NSAIDs and related compounds is a consequence of, rather than a cause of, apoptotic cell death. Several other COX-independent targets for sulindac have been described *in vitro*, including cGPDEs (13, 15), extracellular regulated kinase 1/2 (ERK1/2) (20), JNK (41), NFkB (42), and PPARδ (12). Inhibition of cGPDE, activation of PKG, activation of JNK, and inhibition of ERK1/2 all occur at times before down-regulation of β-catenin protein, and therefore may be alternative mechanisms by which sulindac metabolites induce apoptosis.

Together our results indicate β-catenin degradation by sulindac metabolites to be partially a consequence of caspase activation during apoptosis and partially dependent on proteasomal degradation. Caspases may cleave β-catenin to dismantle cell contacts during apoptosis, while the proteasome may function to inhibit cytosolic and nuclear β-catenin and thereby inhibit transcription of Tcf/Lef regulated genes. Due to the timing and caspase-dependent nature, down-regulation of β-catenin protein does not appear to be the mechanism by which sulindac induces apoptosis. Whether or not (*a*) β-catenin down-regulation or (*b*) apoptotic death mediates any or all of sulindac's growth inhibitory effects *in vivo* is an important area for future studies.

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Pamela L. Rice, Jennifer Kelloff, Holly Sullivan, et al.

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