

# Sulindac Sulfide Inhibits Epidermal Growth Factor-induced Phosphorylation of Extracellular-regulated Kinase 1/2 and Bad in Human Colon Cancer Cells<sup>1</sup>

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## ABSTRACT

Colorectal cancer is the second leading cause of cancer death in the United States. Nonsteroidal anti-inflammatory drugs including sulindac are promising chemopreventive agents for colorectal cancer. Sulindac and selective cyclooxygenase (COX)-2 inhibitors cause regression of colonic polyps in familial polyposis patients. Sulindac induces apoptotic cell death in cancer cells *in vitro* and *in vivo*. In tumor cells, activation of extracellular-regulated kinase (ERK) 1/2 results in phosphorylation of several ERK1/2 effectors, including the proapoptotic protein Bad. Phosphorylation of Ser112 by ERK1/2 inactivates Bad and protects the tumor cell from apoptosis. Sulindac metabolites and other nonsteroidal anti-inflammatory drugs selectively inhibit ERK1/2 phosphorylation in human colon cancer cells. In this study we show that epidermal growth factor (EGF) strongly induces phosphorylation of ERK1/2 and Bad in HT29 colon cancer cells. EGF-stimulated phosphorylation of ERK and Bad is blocked by pretreatment with U0126, a selective MAP kinase kinase (MKK)1/2 inhibitor. Similarly, pretreatment with sulindac sulfide blocks the ability of EGF to induce ERK1/2 and Bad phosphorylation, but also down-regulates total Bad but not ERK1/2 protein levels. The ability of sulindac to block ERK1/2 signaling by the EGF receptor may account for at least part of its potent growth-inhibitory effects against cancer cells.

## INTRODUCTION

CRC<sup>3</sup> is the second most common cause of cancer death in the United States with 148,300 cases and 56,600 deaths in 2001 (1). Epidemiological evidence suggests that regular use of NSAIDs such as aspirin or sulindac is associated with a decreased death rate for CRC and a lower prevalence of colorectal adenomas (2). In patients with FAP, several studies have shown that sulindac treatment causes marked regression of existing colonic polyps and prevents formation of new polyps (3–7). More recently, the selective COX-2 inhibitor celecoxib has been reported to cause a modest regression of colonic adenomas in patients with FAP (8). Sulindac, non-COX-inhibiting sulindac metabolites, and selective COX-2 inhibitors all prevent tumor formation in a variety of animal models of CRC (9–12). The regression of neoplasms by sulindac is a striking example of the potential for a chemopreventive agent to inhibit and even reverse the process of carcinogenesis.

Whereas evidence supporting the ability of sulindac to inhibit tumor formation is strong, the biochemical mechanisms by which sulindac metabolites cause growth inhibition are not clear. NSAIDs are defined by their ability to enzymatically inhibit the activity of

COX-1 and -2 proteins. COX-1 is constitutively expressed in many normal tissues, whereas COX-2 expression is normally absent but can be induced by various stimuli including growth factors. COX-2, but not COX-1, is overexpressed in colonic adenomas and CRC (13). COX enzymes convert arachidonic acid to prostaglandins, which contribute to neoplastic progression by stimulating cell division, motility, and angiogenesis (13).

In addition to COX inhibition, other biochemical targets have been suggested for sulindac, including ERK1/2 (14), cyclic GMP-dependent phosphodiesterase (15), nuclear factor  $\kappa$ B (16), peroxisome proliferator-activated receptor  $\delta$  (17), and c-Jun NH<sub>2</sub>-terminal kinase (18). We described recently the ability of both sulindac metabolites to inhibit phosphorylation and activity of ERK1/2 at times and doses consistent with induction of apoptotic cell death (14). We found that inhibition of ERK1/2 signaling alone is sufficient to induce apoptosis in human colon cancer cells (14). Use of a selective MKK1/2 inhibitor *in vivo* inhibits growth of human colon tumor xenografts (19), indicating that inhibition of the ERK1/2 signaling pathway alone is sufficient to block tumor growth. Activation of ERK1/2 in neoplastic cells results in phosphorylation of downstream effectors that inhibit apoptosis and stimulate cell proliferation. In some cell systems, ERK1/2-dependent phosphorylation of the proapoptotic protein Bad at serine 112 results in dissociation of Bad from Bcl-2 and Bcl-X<sub>L</sub>, allowing these antiapoptotic proteins to prevent mitochondrial events required for apoptosis (20).

Several lines of evidence indicate that EGFR signaling is important in CRC progression. Ligands that activate the EGFR, including EGF and TGF- $\alpha$ , are known to stimulate proliferation of both normal and neoplastic intestinal epithelial cells (21). In intestinal epithelial cells, signaling via the MKK1/2-ERK1/2 pathway is required for the growth-promoting effects of TGF- $\alpha$  (22) and activated Ras (23, 24). The *Min* mouse carries a germ-line mutation of *APC* (adenomatous polyposis coli), the same gene mutated in the human disease, FAP. The *Min* mouse spontaneously develops numerous intestinal polyps and is a model for human FAP. Targeted deletion of the EGFR on a *Min* mouse background dramatically reduces the incidence of intestinal polyps (25), suggesting that EGFR signaling is required for adenoma formation in this model. Combination therapy using NSAIDs and EGFR blockers has a synergistic effect over either compound alone in the *Min* model (26). Based in part on this pre-clinical data, EGFR antibodies are currently being used clinically for the treatment of CRC (27).

The ability of NSAIDs to inhibit some aspects of EGFR activity has been described previously. Sulindac sulfide and indomethacin both inhibit TGF- $\alpha$  induced prostaglandin production and thymidine incorporation in RIE-1 cells (21), indicating that NSAIDs can modulate an activity of the EGFR. In addition, indomethacin, ibuprofen, and aspirin all block EGF-induced Ca<sup>2+</sup> influx in CACO-2 cells (28). The biochemical mechanisms by which NSAIDs might interact with EGFR signaling were not addressed in these studies.

On the basis of these reports, we hypothesized that sulindac inhibits the pro-proliferative and antiapoptotic effects of EGFR activation in colon cancer cells by down-regulating ERK1/2 phosphorylation and downstream signaling. In this paper we describe the ability of EGF to

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<sup>3</sup> The abbreviations used are: CRC, colorectal cancer; NSAID, nonsteroidal anti-inflammatory drug; FAP, familial adenomatous polyposis; ERK, extracellular-regulated kinase; EGFR, epidermal growth factor receptor; TGF, transforming growth factor; COX, cyclooxygenase; MKK, MAP kinase kinase.

induce phosphorylation of MKK1/2, ERK1/2, and Bad(Ser112) in human colon cancer cells. We show that Bad(Ser112) phosphorylation by EGF is mediated by ERK1/2 signaling. Sulindac sulfide, in addition to inhibiting basal MKK1/2, ERK1/2, and Bad(Ser112) phosphorylation, also blocks EGF-induced activation of MKK1/2, ERK1/2, and Bad(Ser112). Unexpectedly, sulindac sulfide also led to a decrease in total Bad protein, but not ERK1/2 or MKK1/2. Inhibition of total Bad protein expression is not dependent on ERK1/2 inhibition and may be related to the effects of sulindac on other signaling pathways. Together these results additionally support inhibition of EGFR signaling as a major biochemical mechanism of action for sulindac.

## MATERIALS AND METHODS

**Materials.** Cell culture medium 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 from Santa Cruz Biotechnology (Santa Cruz, CA); primary antibodies against phosphorylated MKK1/2, total MKK1/2, phosphorylated Bad(Ser112), total Bad, and cleaved caspase-3 were from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated antimouse, antigoat, and antirabbit secondary antibodies were 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). Sulindac sulfide, the MKK1/2 inhibitor (U0126), and Caspase Inhibitor III [Boc-Asp(OMe)-CH<sub>2</sub>F] were from Calbiochem (San Diego, CA).

**Tissue Culture.** HT29, HCT116, and SW480 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% CO<sub>2</sub>-95% air. Cells were plated and grown to 80–100% confluency before treatment.

**Morphological Apoptosis Assay.** Apoptosis and viability were quantified as described previously (14), by staining cells with acridine orange and ethidium bromide, then assaying 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. Apoptosis was expressed as a percentage calculated from the number of cells with apoptotic nuclear morphology divided by the total number of cells examined.

**Western Immunoblotting.** For Western blot analysis, cells were scraped from plates, pelleted, resuspended in lysis buffer [15 mM Tris; 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 collected. Protein concentrations were determined by the method of Richter *et al.* (29). Lysates were prepared for SDS-PAGE, and 50 μg total protein separated and electrotransferred overnight onto Immobilon-P polyvinylidene difluoride membranes (Millipore). Nonspecific binding was blocked for 30 min in Tris-neutral saline with 1% (w/v) dry milk and 0.05% Tween 20, then incubated with phospho-ERK1/2 (1 μg/ml), phospho-MKK1/2 (1:5,000), phospho-Bad(Ser112; 1:1000), or cleaved caspase-3 (1:1,000) primary antibodies overnight while rocking 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 radiographic film. In some experiments, the membranes were stripped for 30 min in 10% SDS, 67 mM Tris (pH 6.7), and 0.8% 2-mercaptoethanol while rocking at 50°C, and washed twice for 10 min each in TNS with 0.05% Tween 20. Blots were then reblocked and probed with pan-ERK1/2 (1 μg/ml), pan-MEK1/2 (1:5,000), or pan-Bad (1:1,000) primary antibodies as described above. Independent experiments validated that this stripping procedure did not lead to loss of signal.

## RESULTS

**EGF Induces Phosphorylation of MKK1/2, ERK1/2, and Bad in HT29 Human Colon Cancer Cells.** HT29 human colon cancer cells were grown to confluency in medium containing 10% fetal bovine serum then switched to serum-free medium for 24 h before addition of EGF. After 24 h in serum-free medium, addition of 10–100 ng/ml EGF to the culture medium induced phosphorylation of MKK1/2, ERK1/2, and Bad(Ser112) proteins after 5 and 30 min of treatment, and the induction was no longer present at 24 h (Fig. 1). Maximal phosphorylation of MKK1/2 and ERK1/2 was seen at 5 min after EGF treatment, whereas phosphorylation of Bad(Ser112) was equivalent at 5 and 30 min. Expression of total MKK1/2, ERK1/2, and Bad proteins was not increased by EGF treatment (Fig. 1). These results indicate that EGF stimulates phosphorylation of the ERK1/2 signaling cascade, without similarly effecting total protein expression. EGF, at concentrations from 1–100 ng/ml, consistently induced MKK1/2, ERK1/2, and Bad(Ser112) phosphorylation from 5 to 60 min after treatment in HT29 cells (Fig. 1; data not shown).

**Sulindac Sulfide Blocks EGF-induced Phosphorylation of MKK1/2, ERK1/2, and Bad Proteins.** Sulindac sulfide and sulindac sulfone inhibit basal levels of phospho-MKK1/2 and phospho-ERK1/2 at times and doses consistent with induction of apoptotic cell death (14). We next determined whether pretreatment with sulindac could prevent EGF-induced phosphorylation of the ERK1/2 signaling cascade. HT29 cells were grown to confluency and serum-deprived for 24 h before drug treatment. Treatment with 10 ng/ml of EGF induced phosphorylation of MKK1/2, ERK1/2, and Bad(Ser112) after 5 min of treatment (Fig. 2). Pretreatment of HT29 cells for 24 h with 80 μM sulindac sulfide, the NSAID metabolite of sulindac, inhibited basal MKK1/2 and ERK1/2 phosphorylation, and blocked the ability of EGF to induce phosphorylation of MKK1/2, ERK1/2, and Bad(Ser112) proteins (Fig. 2). The basal expression of phospho-Bad(Ser112) was relatively low in HT29 cells, but sulindac sulfide inhibited expression of phospho-Bad(Ser112) additionally. Sulindac sulfide treatment did not significantly affect levels of total MKK1/2 or ERK1/2 proteins (Fig. 2). In contrast, expression of total Bad protein was dramatically inhibited after 24 h of sulindac sulfide treatment.

Treatment with 80 μM sulindac sulfide induced apoptotic cell death, as determined by examining nuclear morphology after staining with acridine orange and ethidium bromide, and cleavage of caspase-3

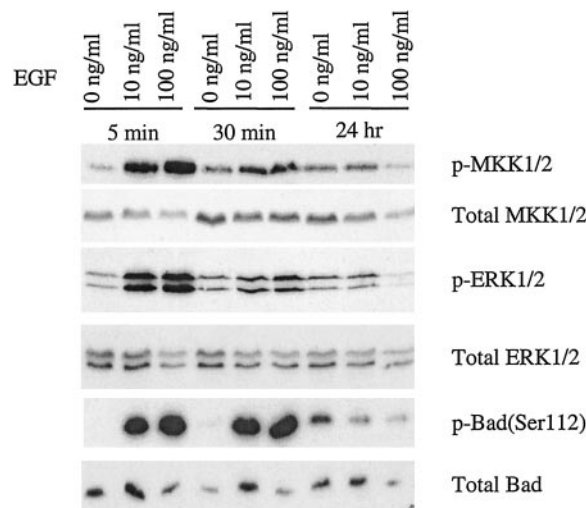


Fig. 1. EGF induces MKK1/2, ERK1/2, and Bad(Ser112) phosphorylation. HT29 cells were serum deprived for 24 h then treated with 0, 10, or 100 ng/ml EGF. Cells were harvested 5 min, 30 min, and 24 h after EGF treatment, and lysates prepared for Western immunoblotting.

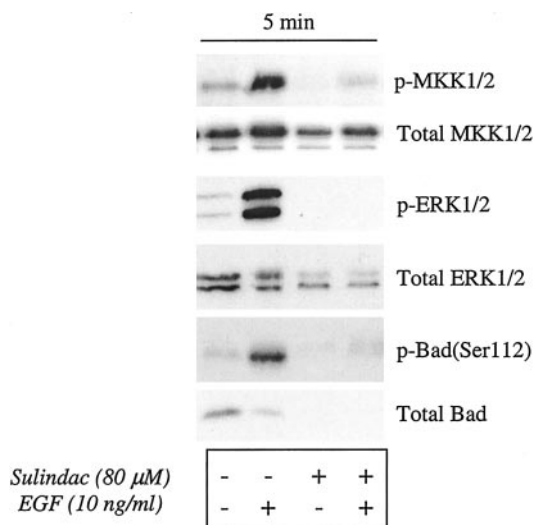


Fig. 2. Sulindac sulfide blocks EGF-induced phosphorylation of MKK1/2, ERK1/2, and Bad(Ser112). HT29 cells were serum-deprived for 24 h before pretreatment with 80  $\mu$ M sulindac sulfide or vehicle. After 24 h cells received 10 ng/ml EGF or vehicle. Cells were harvested 5 min after EGF treatment and lysates prepared for Western immunoblotting.

(data not shown), consistent with our previous report (14). Treatment with EGF had no effect on morphological apoptosis or caspase-3 cleavage induced by sulindac sulfide (data not shown). The ability of sulindac sulfide to inhibit MKK1/2, ERK1/2, and Bad(Ser112) phosphorylation, as well as total Bad protein was also observed in other colon cancer cell lines, including HCT116 and SW480 (data not shown). In each of these cell lines, sulindac sulfide induced apoptotic cell death at times and doses consistent with down-regulation of ERK1/2 signaling and inhibition of Bad protein expression.

**The MKK1/2 Inhibitor, U0126, Blocks EGF-induced Phosphorylation of ERK1/2 and Bad Proteins.** We reported previously that treatment of colon cancer cells with U0126, a selective inhibitor of MKK1/2, potently inhibits ERK1/2 phosphorylation and induces apoptotic cell death, indicating that inhibition of ERK1/2 alone is sufficient to induce apoptosis of colon cancer cells (14). To determine whether ERK1/2 activation mediates EGF-induced phosphorylation of Bad(Ser112), HT29 cells were pretreated with 10  $\mu$ M U0126 for 60 min before addition of 10 ng/ml EGF. As seen in Figure 3, U0126 pretreatment inhibited basal and EGF-induced phosphorylation of ERK1/2 and Bad(Ser112), but not MKK1/2 (U0126 inhibits MKK1/2 activity but not its phosphorylation). U0126 and EGF treatment did not affect expression of total MKK1/2, ERK1/2, or Bad at these time points. Loss of total Bad protein, as seen after sulindac sulfide treatment, did not occur even after 1–3 days of U0126 treatment, at doses that are sufficient to induce caspase cleavage and morphological apoptosis (data not shown).

**Loss of Total Bad Protein Is Not a Consequence of Caspase Activation.** Treatment with apoptotic concentrations of sulindac sulfide for 24 h led to loss of total Bad protein as well as EGF-induced Bad phosphorylation (Fig. 2). The decrease in total Bad protein expression after sulindac sulfide treatment was an unexpected result. To confirm this result, we performed Western immunoblotting of sulindac sulfide-treated cells using an antibody raised against full-length Bad protein (Santa Cruz Biotechnology) and found the same result (data not shown). Because Bad can colocalize at the mitochondrial membrane, we also examined particulate samples for membrane translocation of Bad. Whereas Bad protein was detected in these particulate samples, sulindac sulfide caused loss of membrane-bound

as well as cytosolic Bad (data not shown), indicating loss of total cellular Bad protein.

To determine whether inhibition of ERK1/2 and Bad phosphorylation, and loss of total Bad protein were consequences of caspase activation in cells undergoing apoptosis, SW480 cells were pretreated for 60 min with 25  $\mu$ M Caspase Inhibitor III [Boc-Asp(OMe)-CH<sub>2</sub>F] before addition of 200  $\mu$ M sulindac sulfide. At this dose, sulindac sulfide induced apoptotic cell death, as shown by cleavage of caspase-3 (Fig. 4), and confirmed by nuclear morphology after staining cells with acridine orange and ethidium bromide (data not shown). Similar to HT29 colon cancer cells, SW480 cells treated with apoptotic concentrations of sulindac sulfide had dramatically reduced expression of phospho-ERK1/2, phospho-Bad(Ser112), and total Bad proteins (Fig. 4). Pretreatment with 25  $\mu$ M Caspase Inhibitor III blocked caspase-3 cleavage (Fig. 4) and morphological apoptosis (data not shown) induced by sulindac sulfide. However, pretreatment with Caspase Inhibitor III did not prevent loss of phospho-ERK1/2, phospho-Bad(Ser112), or total Bad protein expression (Fig. 4), indicating that these biochemical events are not a consequence of caspase cleavage.

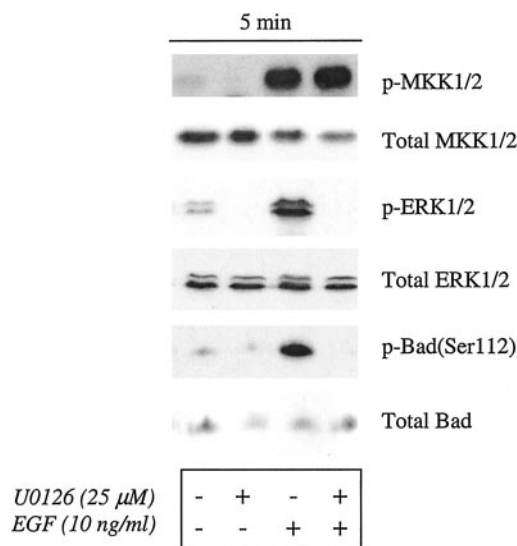


Fig. 3. U0126 blocks EGF-induced phosphorylation of MKK1/2, ERK1/2, and Bad(Ser112). HT29 cells were serum-deprived for 24 h before pretreatment with 10  $\mu$ M U0126 or vehicle. After 60 min cells received 10 ng/ml EGF or vehicle. Cells were harvested 5 min after EGF treatment and lysates prepared for Western immunoblotting.

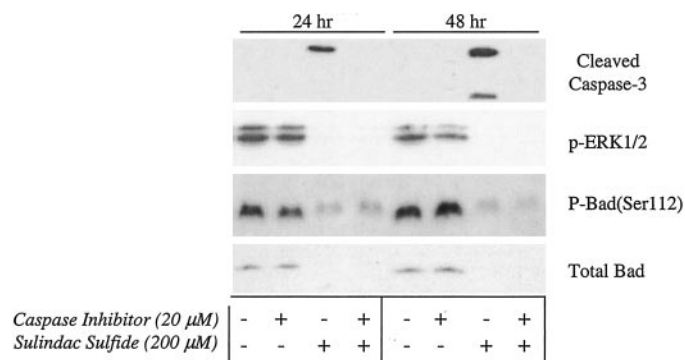


Fig. 4. Down-regulation of phospho-ERK1/2, phospho-Bad(Ser112), and total Bad proteins is not dependent on caspase activation. SW480 colon cancer cells were pretreated for 60 min with 25  $\mu$ M Caspase Inhibitor III or vehicle. Cells then received 200  $\mu$ M sulindac sulfide or vehicle. Cells were harvested 24 and 48 h after sulindac treatment, and lysates prepared for Western immunoblotting.

## DISCUSSION

We have reported previously that sulindac metabolites inhibit basal MKK1/2 and ERK1/2 phosphorylation, and that inhibition of ERK1/2 is sufficient to induce apoptotic cell death (14). The results presented in this paper confirm the ability of NSAIDs to inhibit ERK1/2 signaling in colon cancer cells and link this effect to the EGFR signaling pathway. We found that EGF stimulates phosphorylation of ERK1/2 and Bad(Ser112) in colon cancer cells, a prosurvival pathway in other cell types. EGF-induced phosphorylation of Bad(Ser112) was dependent on ERK1/2 activation.

Substantial evidence exists linking EGFR signaling to the process of colonic carcinogenesis. Our results suggest that EGF activates a prosurvival pathway in colon cancer cells by inhibiting Bad(Ser112) phosphorylation. EGF receptors are present on both normal and neoplastic colonic cells, and overexpression of EGF receptor agonists including EGF and TGF- $\alpha$  is thought to contribute to colon cancer progression (21). As a result, the EGFR has become a biochemical target for chemopreventive and chemotherapeutic agents against colon cancer. Treatment of human colon cancer xenografts with the EGFR inhibitor EKI-785 produced a dose-dependent inhibition of tumor growth in nude mice (25). Transfer of the *Apc(Min)* allele onto a homozygous *Egfr*(knockout) background resulted in a 90% reduction in intestinal polyp number relative to *Apc(Min)* mice carrying a wild-type *EGFR* allele (25). Combination therapy using both an EGFR antagonist and sulindac inhibited polyp growth in *Min* mice to a greater extent than either agent alone (26). It has been suggested that this synergistic effect is because of the combination of down-regulation of COX-2 expression by EGFR blockade and inhibition of COX-2 activity by NSAIDs. Our results suggest an alternative or additional reason for such synergy. We found that sulindac sulfide strongly inhibits EGF-induced activation of MKK1/2 and ERK1/2. Thus, the combination of an EGFR antagonist and an NSAID may provide additive inhibition of the same signaling pathway.

Activation of ERK1/2 leads to phosphorylation of several downstream effectors, including protein kinases and transcription factors responsible for regulating genes that enhance cell proliferation and protect from apoptosis (30). ERK1/2-dependent phosphorylation of transcription factors, including c-Fos, c-Jun, and c-Myc, has been directly linked to promotion of cell proliferation (30). The biochemical links between ERK1/2 signaling and prevention of apoptosis are less well defined. Our results suggest that one biochemical link may be regulation of the activity of Bad, one of the Bcl-2 family members. The Bcl-2 family proteins can be roughly divided into proapoptotic (Bad, Bax, and Bak) and antiapoptotic (Bcl-2 and Bcl-X<sub>L</sub>) members. The function of the proapoptotic members is to bind to the antiapoptotic members, which facilitates binding to mitochondrial membranes, pore formation, loss of the mitochondrial membrane potential, release of cytochrome *c*, and activation of caspases (31). It was first shown in MC/9 and FD-CP1 cells that MKK1/2 signaling could mediate phosphorylation of Bad at Ser112 (20). Phosphorylation at Ser112 inactivates Bad by sequestering it in the cytosol and preventing its dimerization with Bcl-X<sub>L</sub> (20). We describe the ability of sulindac sulfide to inhibit EGF-mediated phosphorylation of Bad(Ser112) in human colon cancer cells. Sulindac sulfide also inhibited basal levels of Bad(Ser112) phosphorylation. Inhibition of ERK1/2 activity by U0126 was sufficient to block EGF-induced phosphorylation of Bad(Ser112). Thus, our data suggest that sulindac sulfide blocks Bad phosphorylation by inhibiting MKK1/2 and ERK1/2.

The inhibition of Bad(Ser112) phosphorylation by sulindac sulfide is consistent with induction of apoptosis, as phosphorylation at this site inhibits Bad and protects cells from apoptosis (31, 32). However,

the decrease in total Bad protein expression by sulindac sulfide was an unexpected result, as overexpression of dephosphorylated Bad has been shown to enhance apoptosis in several cell lines (33, 34). We are unaware of previous evidence for the ability of an apoptotic-inducing agent, such as sulindac, to inhibit protein expression of Bad. The coexistence of down-regulation of both phospho-Bad(Ser112) and total Bad protein expression by sulindac sulfide is predicted to have opposing effects on apoptosis. Because of these conflicting results, the relevance of phospho-Bad expression, in relation to induction of apoptotic cell death of colon cancer cells by sulindac, is open to question. Future studies will examine the time course of phospho-Bad(Ser112) and total Bad expression to determine when these changes occur after sulindac treatment.

Decreased expression of total Bad protein does not occur when colon cancer cells are treated with concentrations of U0126 that induce apoptosis, and down-regulate phospho-ERK1/2 and phospho-Bad(Ser112) expression, even after 1–3 days of drug treatment.<sup>4</sup> Therefore, inhibition of total Bad protein expression is an ERK-independent mechanism of sulindac, and may be related to the effects of sulindac on other signaling pathways, such as protein kinase G<sup>15</sup> or c-Jun NH<sub>2</sub>-terminal kinase (18). It is possible that sulindac inhibits transcription or translation of Bad, or perhaps induces Bad degradation by noncaspase-dependent proteolysis. The function of the Bad protein is regulated by phosphorylation of residues in addition to Ser112. Phosphorylation at Ser112, Ser136, or Ser155 inhibits the proapoptotic activity of Bad by preventing it from binding and sequestering proapoptotic proteins such as Bcl-X<sub>L</sub>. More recently a separate Bad phosphorylation site was identified at Ser128 (35). Phosphorylation at Ser128 enhances the proapoptotic activity of Bad, even when Bad is phosphorylated at Ser136, an antiapoptotic site (35). Sulindac may induce changes in one or more of the phosphorylation sites of Bad before induction of the apoptotic machinery.

Whereas this is the first report indicating that sulindac sulfide inhibits Bad phosphorylation and total protein expression, NSAIDs including sulindac have been shown to affect other Bcl-2 family members. Sulindac sulfide and indomethacin inhibit expression of the antiapoptotic Bcl-X<sub>L</sub> protein in colon cancer cells by a mechanism that is not defined (36). In this same report, targeted deletion of the proapoptotic gene *Bax* rendered cells completely resistant to apoptosis by sulindac and indomethacin. In addition, sulindac sulfide and sulindac sulfone have been shown to down-regulate Bcl-2 protein expression in SW480 cells (29). Because ERK1/2 can regulate Bcl-2 (37) and Bcl-X<sub>L</sub> (38) protein expression in other cell types, it is possible that NSAIDs down-regulate expression of these proteins by their ability to inhibit ERK1/2 signaling. Sulindac alters the expression and activation of several pro- and antiapoptotic Bcl-2 family members, perhaps shifting the balance in favor of the proapoptotic members. Regulation of the Bcl-2 family of proteins may be the ultimate mechanism by which sulindac and other NSAIDs induce apoptosis in cancer cells.

In summary, we have described the ability of EGF to stimulate phosphorylation of MKK1/2, ERK1/2, and Bad(Ser112) in human colon cancer cells. Phosphorylation of Bad(Ser112) induced by EGF is dependent on MKK1/2 activity in these cells. Sulindac sulfide, in addition to inhibiting basal expression of MKK1/2, ERK1/2, and Bad(Ser112) phosphorylation, completely blocks the ability of EGF to induce activation of this pathway. This likely occurs by the ability of sulindac to inhibit ERK1/2 signaling. Treatment with sulindac, but not U0126, down-regulates total Bad protein expression, an effect that may mitigate the apoptotic effects of sulindac. The biochemical mech-

<sup>4</sup> Unpublished observations.

anism by which sulindac sulfide inhibits EGFR and ERK1/2 signaling is unknown, but may be mediated by its ability to bind to Ras protein *in vitro* (39), and thereby block downstream signaling. Together these results strengthen the role of ERK1/2 as a biochemical mechanism by which sulindac, and perhaps additional NSAIDs, induce apoptotic cell death *in vitro* and inhibit cancer cell growth *in vivo*.

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