Nonsteroidal Anti-inflammatory Drugs Induce Colorectal Cancer Cell Apoptosis by Suppressing 14-3-3ε

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Abstract

To determine the role of 14-3-3 in colorectal cancer apoptosis induced by nonsteroidal anti-inflammatory drugs (NSAIDs), we evaluated the effects of sulindac on 14-3-3ε protein expression in colorectal cancer cells. Sulindac sulfide inhibited 14-3-3ε proteins in HT-29 and DLD-1 cells in a time- and concentration-dependent manner. Sulindac sulfone at 600 μmol/L inhibited 14-3-3ε protein expression in HT-29. Indomethacin and SC-236, a selective cyclooxygenase-2 (COX-2) inhibitor, exerted a similar effect as sulindac. Sulindac suppressed 14-3-3ε promoter activity. As 14-3-3ε promoter activation is mediated by peroxisome proliferator–activated receptor δ (PPARδ), we determined the correlation between 14-3-3ε inhibition and PPARδ suppression by NSAIDs. Sulindac sulfide inhibited PPARδ protein expression and PPARδ transcriptional activity. Overexpression of PPARδ by adenoviral transfer rescued 14-3-3ε proteins from elimination by sulindac or indomethacin. NSAID-induced 14-3-3ε suppression was associated with reduced cytosolic Bad with elevation of mitochondrial Bad and increase in apoptosis which was rescued by Ad-PPARδ transduction. Stable expression of 14-3-3ε in HT-29 significantly protected cells from apoptosis. Our findings shed light on a novel mechanism by which NSAIDs induce colorectal cancer apoptosis via the PPARδ/14-3-3ε transcriptional pathway. These results suggest that 14-3-3ε is a target for the prevention and therapy of colorectal cancer.

Introduction

Human and animal studies have shown that nonsteroidal anti-inflammatory drugs (NSAIDs) suppress colorectal tumorigenesis (1). Population-based epidemiologic studies have shown that use of aspirin and NSAID reduces risk of colorectal cancer (2, 3). Animal experiments provide evidence for control of colorectal cancer by NSAIDs such as sulindac and selective cyclooxygenase-2 (COX-2) inhibitors (4, 5). Results from clinical trials have documented the efficacy of sulindac and selective COX-2 inhibitors in reducing the size and numbers of colon adenomatous polyps in patients with familial adenomatous polyposis, a precancerous condition (6, 7). The action of NSAIDs on controlling colorectal cancer is attributed to the inhibition of COX-2 (8), which is overexpressed in a majority of patients with colorectal cancer (9, 10). NSAID could also exert its action on colorectal cancer in a COX-2–independent manner (11). It is well documented that NSAIDs, notably sulindac, indomethacin, and selective COX-2 inhibitors exert their actions by inducing colorectal cancer cell apoptosis (12, 13). The mechanism by which these agents induce apoptosis is not entirely clear. It was proposed that NSAID-induced apoptosis might be mediated by down-regulation of peroxisome proliferator–activated receptor-δ (PPARδ; ref. 14). Several studies have shown that PPARδ is overexpressed in colorectal cancer and PPARδ is activated by prostacyclin generated by colorectal cancer cells (15). Activation of PPARδ accelerates intestinal adenoma growth whereas genetic disruption of PPARδ decreases the tumorigenicity of human colorectal cancer cells (16, 17). It has been reported that PPARδ plays an important role in cellular resistance to apoptotic insults (18, 19). Activation of PPARδ was reported to be associated with increased expression of phosphoinositide-dependent kinase-1 and activation of Akt (20). Akt is recognized as a key signaling molecule for transmitting survival messages. We have recently reported that PPARδ activation by prostacyclin or L-165041, a synthetic PPARδ ligand, increases the expression of 14-3-3 proteins, especially the 14-3-3ε isoform (21). Promoter analysis identifies three PPAR response elements (PPRE) located between −1426 and −1477 of the 14-3-3ε promoter region. Deletion of these PPREs abrogates the protective effect of prostacyclin or L-165041 (21). Our previous findings suggest that PPARδ-mediated 14-3-3ε up-regulation plays a crucial role in endothelial cell resistance to apoptosis (21). It is unclear whether 14-3-3ε is involved in colorectal cancer cell survival. We postulated in this study that NSAIDs induce apoptosis by reducing 14-3-3ε via suppressing PPARδ. Reduction in 14-3-3ε protein levels results in mitochondrial Bad translocation and colorectal cancer cell apoptosis. The results show that sulindac sulfide, sulindac sulfone, indomethacin, and SC-236 suppressed HT-29 14-3-3ε protein and promoter activity, which correlated with the suppression of PPARδ protein expression. The reduction in 14-3-3ε levels was accompanied by enhanced mitochondrial Bad translocation and HT-29 apoptosis.

Materials and Methods

Cell culture and reagents. HT-29 and DLD-1 cells were obtained from American Type Culture Collection (Manassas, VA). HT-29 were maintained in DMEM and DLD-1 were maintained in RPMI 1604 supplemented with 10% fetal bovine serum and penicillin-streptomycin at 37°C in a humidified 5% CO2 atmosphere. Cell culture media and antibiotics were purchased from BRL, Life Technologies (Grand Island, NY). Sulindac sulfide and sulindac sulfone were obtained from ICN Biomedicals, Inc. (SOLON, OH). Indomethacin, SC-236, MG-132, and cell-permeable caspase 3 inhibitor, ac-DVED aldehyde (DEVD-CHO), were from Calbiochem (San Diego, CA). We used 10 μmol/L of MG-132 and 50 μmol/L of DEVD-CHO, which were reported to block proteasome and caspase 3 activities, respectively (22, 23).

Recombinant adeno viral vectors. Replication-defective recombinant adenoviruses were generated by homologous recombination and amplified in 293 cells as described previously (24, 25). The recombinant viruses were purified by CsCl density gradient centrifugation and the virus titers were determined by a plaque assay as previously described (24). Ad-PPARδ was

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HT-29 cells were transfected with Ad-GFP (green fluorescent protein) or Ad-PPARδ at a multiplicity of infection (moi) of 50 for 48 h before treatment with indomethacin or sulindac sulfide.

**Preparation of mitochondrial fraction.** HT-29 cells treated with indicated reagents (sulindac sulfide, 160 μmol/L; sulindac sulfone, 300 μmol/L; indomethacin, 800 μmol/L; or SC-236, 40 μmol/L) for 8 h were washed thrice with PBS and harvested by centrifugation. Mitochondrial fractions were prepared by a mitochondria isolation kit (Sigma, St. Louis, MO) as described previously (26). The mitochondrial and cytosolic fractions were purified by two-step gradient centrifugation and stored at −80°C. Mitochondrial Bad protein level was analyzed by Western blotting. Heat shock protein 60 (Hsp60) was used as a mitochondrial marker.

**Immunoprecipitation.** HT-29 cells treated with the indicated reagents were harvested and lysed in 1× radioimmunoprecipitation assay buffer (Upstate, Charlottesville, VA) with cocktail protease inhibitors (Sigma). Lysate proteins (200 μg) were immunoprecipitated with a human 14-3-3ε antibody. The immunoprecipitated complex was pulled down with protein A/G-agarose (Santa Cruz Biotechnology, Santa Cruz, CA). After washing five times, the proteins were analyzed by Western blotting using a Bad antibody.

**Western blot analysis.** Cells were lysed in radioimmunoprecipitation assay buffer containing protease inhibitors. Protein concentrations were determined by a Bio-Rad (Hercules, CA) protein assay kit. The percentage of apoptotic cells was determined by counting at least 200 cells.

**Results**

**Suppression of 14-3-3ε by sulindac sulfide and COX-2 inhibitors.** To evaluate the effect of sulindac on 14-3-3ε protein levels, we treated HT-29 with sulindac sulfide at 0 to 160 μmol/L for 4 to 24 h. 14-3-3ε proteins were analyzed by Western blotting. Sulindac sulfide did not have an effect at 4 h of treatment, suppressed 14-3-3ε at 8 h only at 160 μmol/L, and exerted a concentration-dependent inhibition of 14-3-3ε at 24 h of treatment (Fig. 1A). Sulindac sulfone had no effect at 300 μmol/L but suppressed 14-3-3ε expression at 600 μmol/L (Fig. 1B). Indomethacin, a nonselective COX inhibitor, or SC-236, a selective COX-2 inhibitor, also concentration-dependently inhibited 14-3-3ε protein expression at 600 μmol/L (Fig. 1A).

**Statistical analysis.** ANOVA was used to analyze statistical differences among multiple groups. P < 0.05 was considered to be statistically significant.

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**Figure 1.** NSAIDs suppressed 14-3-3ε expression. HT-29 cells were treated with (A) sulindac (Sul) sulfide for 4 to 24 h, (B) sulindac sulfone for 24 h, or (C) indomethacin (Indo) or SC-236 for 24 h. D, DLD-1 cells were treated with sulindac sulfide for 24 h. 14-3-3ε proteins were determined by Western blotting. Actin was used as a control. Representative of three independent experiments with similar results.
levels (Fig. 1C). Sulindac sulfide suppressed 14-3-3ε protein levels in DLD-1 with a pattern similar to that of HT-29 (Fig. 1D).

Inhibition of 14-3-3ε promoter activity by sulindac sulfide. To determine whether sulindac sulfide inhibits 14-3-3ε at the transcription level, we transfected HT-29 with a human 14-3-3ε promoter construct (-1628/+24) and treated the transfected cells with various concentrations of sulindac sulfide for 24 h. Sulindac sulfide concentration-dependently inhibited 14-3-3ε promoter activity (Fig. 2A). Sulindac-induced 14-3-3ε protein suppression was not influenced by MG-132, a proteasome inhibitor, or DEVD-CHO, a caspase 3 inhibitor (Fig. 2B). Taken together, these results suggest that sulindac suppresses 14-3-3ε primarily at the transcriptional level.

Restoration of 14-3-3ε by adenoviral PPARδ transfer. We have recently discovered that 14-3-3ε transcription is mediated by PPARδ (21). We were, therefore, interested in learning whether sulindac-induced 14-3-3ε suppression involves PPARδ. HT-29 cells were treated with sulindac sulfide (160 μmol/L) or indomethacin (800 μmol/L) for 24 h and PPARδ proteins were analyzed by Western blots. PPARδ levels became undetectable following sulindac sulfide treatment (Fig. 2C). We were, therefore, interested in learning whether sulindac-induced 14-3-3ε suppression involves PPARδ. HT-29 cells were treated with sulindac sulfide (160 μmol/L) or indomethacin (800 μmol/L) for 24 h and PPARδ proteins were analyzed by Western blots. PPARδ levels became undetectable following sulindac sulfide treatment (Fig. 2C). We were, therefore, interested in learning whether sulindac-induced 14-3-3ε suppression involves PPARδ. 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treated with or without sulindac sulfide or indomethacin by a 14-3-3 antibody and analyzed Bad in the immunoprecipitate with a Bad antibody. The results show that in the absence of NSAIDs, most Bad were localized to the cytosol, whereas Hsp60 was predominantly detected in the mitochondrial fraction with a trace quantity detected in the cytosolic fraction (Fig. 5A). Bad was detected at a very low level in mitochondrial fraction (Fig. 5A). Sulindac and indomethacin enhanced mitochondrial Bad (Fig. 5A) and suppressed binding of Bad to 14-3-3q (Fig. 5B). SC-236 enhanced mitochondrial Bad to an extent comparable to sulindac sulfide and indomethacin (Fig. 5A). These results suggest that sulindac, indomethacin and SC-236 induce Bad translocation to mitochondria as a consequence of severe 14-3-3q suppression.

**Rescue of NSAID-induced apoptosis by Ad-PPARδ and 14-3-3ε.**
Sulindac sulfide and indomethacin induced HT-29 apoptosis as evidenced by an increase in Hoechst-positive cells. Apoptosis was reduced by transduction of cells with Ad-PPARδ (Fig. 5C). To ascertain the role of 14-3-3ε in NSAID-induced HT-29 apoptosis, we prepared a HT-29 cell line overexpressing 14-3-3ε (HT-2914-3-3ε) and a control cell line (HT-29CTR; Fig. 6A). In HT-29 control cells, sulindac suppressed 14-3-3ε proteins (Fig. 6A) accompanied by a large increase in apoptosis (Fig. 6B). Overexpression of 14-3-3ε proteins in HT-2914-3-3ε cells (Fig. 6A) prevented apoptosis by >50% (Fig. 6B).

**Discussion**
Our findings show for the first time that sulindac and other NSAIDs drastically suppress 14-3-3ε expression in colorectal cancer cells, resulting in reduced Bad sequestration in cytosol, increased mitochondrial Bad, and enhanced apoptosis. The 14-3-3 family comprises at least seven members in mammalian cells (30). They share sequence homology and biochemical properties, interact with more than 100 cellular proteins, and have diverse biological activities (31). They bind phosphorylated Bad, thereby sequestering Bad in cytosol and suppressing Bad-induced mitochondrial injury and the consequential apoptosis via the intrinsic (mitochondrial) apoptotic pathway (32). It was reported in endothelial cells that among the 14-3-3 members, 14-3-3q is expressed in abundance, and its cellular level is regulated and is crucial in protecting cells from oxidative damage (21). The results in this study show that 14-3-3q is constitutively expressed in HT-29 and DLD-1 cells and is a target of NSAID-induced apoptosis. Sulindac, indomethacin, and SC-236 suppress 14-3-3ε expression in HT-29 accompanied by increased translocation of Bad to mitochondria and apoptosis. Stable overexpression of 14-3-3ε alleviates sulindac-induced apoptosis. Taken together, these results strongly suggest that the suppression of 14-3-3ε is a major mechanism by which NSAIDs induce colorectal cancer apoptosis.
14-3-3ε transcription requires PPARγ and is regulated by the level of PPARγ, as evidenced by increased 14-3-3ε proteins in Ad-PPARγ-transduced cells. Our results are consistent with a previous report that sulindac suppresses PPARγ transcription activity (14), and provide new data which attribute reduced PPARγ transcription activity to the suppression of PPARγ protein expression. The results show a good correlation between sulindac- and indomethacin-induced 14-3-3ε and PPARγ suppressions. Furthermore, PPARγ overexpression by Ad-PPARγ transduction rescues 14-3-3ε from suppressions by sulindac sulfide at 80 μmol/L or indomethacin at 400 μmol/L. These data indicate that sulindac and other NSAIDs suppress PPARγ expression, thereby blocking PPARγ-mediated 14-3-3ε transcription. Although Ad-PPARγ abrogates 14-3-3ε suppression by sulindac and indomethacin at 80 and 300 μmol/L, respectively, it only partially corrects 14-3-3ε levels suppressed by higher concentrations of sulindac (160 μmol/L) or indomethacin (800 μmol/L). Lack of complete correction by Ad-PPARγ may be due to the following reasons. First, activated PPARγ promotes transcription by forming a heterodimer with RXR nuclear receptor (14). Overexpression of PPARγ alone without concurrent RXR overexpression may, therefore, be inadequate to exert maximal transcriptional activation. Second, sulindac and other NSAIDs have diverse COX-2–dependent and COX-2–independent effects on signaling transduction and gene expression. Lack of a complete rescue of 14-3-3ε suppression and cell apoptosis may suggest that PPARγ is not the only pathway via which NSAIDs suppress 14-3-3ε. Furthermore, differential effects of Ad-PPARγ (50 moi) on rescuing 80 versus 160 μmol/L sulindac-induced 14-3-3ε suppression may reflect dose-dependent effects on diverse cellular functions. Lastly, lack of a correlation between the magnitude of PPARγ overexpression and 14-3-3ε rescue could also be attributed to the requirement of PPARγ posttranslational modification for its full transactivation activity. Because neither MG-132 nor DEVD-CHO blocks the 14-3-3ε–suppressing effect of sulindac, it is unlikely that the reduction in 14-3-3ε protein levels are due to degradation by ubiquitin-proteasome or caspase 3.

PPARγ expression is regulated by the β-catenin-Tcf (T cell factor) transcriptional program (14). The wild-type adenomatous polyposis coli tumor suppressor gene product binds and targets β-catenin for degradation, thereby suppressing PPARγ expression (14). Adenomatous polyposis coli mutation results in increased β-catenin, which translocates to the nucleus and complexes with Tcf to form an active transcription factor that binds to specific responsive elements on PPARγ and up-regulates PPARγ expression in familial adenomatous polyposis (14, 33). It is reported that sulindac and celecoxib inhibit β-catenin expression and/or the transcriptional activity of the β-catenin/Tcf complex (34, 35). It is, therefore, possible that sulindac, SC-236, and other NSAIDs suppress PPARγ expression through the β-catenin/Tcf transcription pathway.

Sulindac and other NSAIDs may induce colorectal cancer cell apoptosis by COX-2–dependent and COX-2–independent mechanisms (36–39). COX-2 is overexpressed in colorectal cancer and COX-2–derived prostaglandin I₂ (PGI₂) has been shown to activate PPARγ in colorectal cancer cells (15). PPARγ activation confers resistance to apoptosis in colorectal cancer as well as a number of non–cancer cells such as endothelial, renal interstitial, cardiomyocytes and keratinocytes (18, 19, 21). Sulindac sulfide and selective COX-2 inhibitors may induce apoptosis by blocking COX-2 derived PGI₂ production, thereby suppressing PPARγ activation and the antiapoptosis action of PPARγ. COX-2–derived prostaglandin E₂ (PGE₂) up-regulates β-catenin expression (40) and NSAIDs may further suppress β-catenin–mediated PPARγ expression by blocking PGE₂ production. However, a recent report has shown that sulindac, selective COX-2 inhibitors, and a host of classic NSAIDs inhibit β-catenin by a COX-2–independent mechanism (41). Our results reveal that sulindac sulfone, which does not inhibit COX-2, is capable of inhibiting 14-3-3ε expression, suggesting that NSAIDs

**Figure 5.** NSAIDs induced Bad translocation to mitochondria and apoptosis. A, HT-29 cells were treated with sulindac sulfone (300 μmol/L), sulindac sulfide (160 μmol/L), indomethacin (800 μmol/L), or SC-236 (40 μmol/L) for 8 h. Mitochondrial and cytosolic fractions were isolated and Bad proteins were determined by Western blotting. Hsp60 was analyzed to validate the homogeneity of mitochondrial fraction. B, HT-29 cells were treated with sulindac sulfide (160 μmol/L) or indomethacin (800 μmol/L) for 8 h. Cell lysates were immunoprecipitated with a 14-3-3ε antibody. Bad proteins in the precipitate were analyzed by Western blotting. C, Ad-GFP and Ad-PPARγ transfected HT-29 cells were treated with 160 μmol/L of sulindac sulfide or 800 μmol/L of indomethacin for 24 h. Percentages of apoptotic cells were determined by Hoechst staining. Columns, mean of three independent experiments; bars, SD.
further reducing Bad sequestration leading to excessive Bad regulated kinase, thereby blocking Bad phosphorylation (45), and sequestration. Second, NSAIDs inhibit extracellular signal–regulated kinase (ERK) activation, thereby reducing Bad phosphorylation and enhancing Bad translocation to the mitochondria. In process 3, NSAIDs inhibit Bcl-XL and Bcl-2 protein levels. A common result of these processes is a reduced ratio of Bcl-XL and Bcl-2 to Bad and Bax at mitochondria, and an increased release of proapoptotic factors, notably Smac/DIABLO from mitochondria into the cytosol which contribute to caspase 3 activation and apoptosis.

**Figure 6.** 14-3-3q overexpression rescued HT-29 from apoptosis. HT-29 stably transfected with 14-3-3q (HT-2914-3-3q) and control (HT-29WT) were treated with or without sulindac sulfide (160 μmol/L) for 24 h. A, 14-3-3q-proteins were analyzed by Western blotting. B, apoptosis was analyzed by Hoechst staining. Columns, mean of three independent experiments; bars, SD. C, a schematic illustration of multiple biochemical processes via which NSAIDs induce apoptosis. Process 1 refers to a COX-2–dependent pathway in which NSAIDs inhibit COX-2–derived PGI2 and PGE2 productions, and thereby suppress PPARγ-mediated 14-3-3q overexpression rescued HT-29 from apoptosis. HT-29 stably transfected with 14-3-3q was analyzed by Hoechst staining. Columns, mean of three independent experiments; bars, SD.

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