15-LOX-1: a Novel Molecular Target of Nonsteroidal Anti-inflammatory Drug-Induced Apoptosis in Colorectal Cancer Cells


Background: Nonsteroidal anti-inflammatory drugs (NSAIDs) appear to act via induction of apoptosis—programmed cell death—as potential colorectal cancer chemopreventive agents. NSAIDs can alter the production of different metabolites of polyunsaturated fatty acids (linoleic and arachidonic acids) through effects on lipoxygenases (LOXs) and cyclooxygenases. 15-LOX-1 is the main enzyme for metabolizing colonic linoleic acid to 13-S-hydroxyoctadecadienoic acid (13-S-HODE), which induces apoptosis. In human colorectal cancers, the expression of this enzyme is reduced. NSAIDs can increase 15-LOX enzymatic activity in normal leukocytes, but their effects on 15-LOX in neoplastic cells have been unknown. We tested the hypothesis that NSAIDs induce apoptosis in colorectal cancer cells by increasing the protein expression and enzymatic activity of 15-LOX-1. Methods: We assessed 15-LOX-1 protein expression and enzymatic activity, 13-S-HODE levels, and 15-LOX-1 inhibition in association with cellular growth inhibition and apoptosis induced by NSAIDs (primarily sulindac and NS-398) in two colorectal cancer cell lines (RKO and HT-29). All P values are two-sided. Results: Sulindac and NS-398 progressively increased 15-LOX-1 protein expression in RKO cells (at 24, 48, and 72 hours) in association with subsequent growth inhibition and apoptosis. Increased 13-S-HODE levels and the formation of 15-hydroxyeicosatetraenoic acid on incubation of the cells with the substrate arachidonic acid confirmed the enzymatic activity of 15-LOX-1. Inhibition of 15-LOX-1 in RKO cells by treatment with caffeic acid blocked NS-398-induced 13-S-HODE production, cellular growth inhibition, and apoptosis (P = .007, P < .0001, and P < .0001, respectively); growth inhibition and apoptosis were restored by adding exogenous 13-S-HODE (P < .0001 for each) but not its parent compound, linoleic acid (P = 1.0 for each). Similar results occurred with other NSAIDs and in HT-29 cells. Conclusions: These data identify 15-LOX-1 as a novel molecular target of NSAIDs for inducing apoptosis in colorectal carcinogenesis. [J Natl Cancer Inst 2000;92:1136–42]

Nonsteroidal anti-inflammatory drugs (NSAIDs) are an extremely promising area of chemoprevention research in colorectal cancer and in other major cancers (1,2). Preventive effects of NSAIDs against colorectal cancer have been suggested by epidemiologic studies (3,4), in various experimental models (5,6), and in trials of patients with familial adenomatous polyposis (FAP) (7,8).

The loss of apoptosis contributes to tumorigenesis (9), and the induction of apoptosis by NSAIDs appears to mediate the observed effects of these agents in suppressing colorectal carcinogenesis in many in vitro and in vivo systems (10–17). Studies (14–17) have examined the relationship of NSAID-induced apoptosis to the inhibition of cyclooxygenase (COX) and prostaglandin synthesis, which was proposed initially as the major mechanism of NSAID chemopreventive effects. These studies (15–17) indicated, however, that NSAIDs can induce apoptosis independently of COX inhibition, suggesting that NSAIDs have other possible molecular targets.

In the classic eicosanoid-generation pathways, arachidonic acid is a substrate for both lipoxygenase (LOX) and COX enzymes to form various metabolites, such as prostaglandins and hydroxyeicosatetraenoic acids (HETEs) (5-, 8-, 12-, and 15-HETE) (18). These enzymes also can metabolize another substrate, linoleic acid, the predominant polyunsaturated fatty acid in the human diet (19). Linoleic acid is oxidized primarily by 15-LOX to produce 13-S-hydroxyoctadecadienoic acid (13-S-HODE) (20,21). The two known isoenzymes of 15-LOX are 15-LOX-1 and 15-LOX-2 (22), and only 15-LOX-1 has been found in the epithelium of the human colon (23).

Based on the data outlined above, we previously studied the expression of 15-LOX-1 protein and 13-S-HODE levels in clinical colorectal cancer samples, finding that both are reduced compared with paired normal colorectal epithelia (24). We also found that colorectal cancer cell lines lack 15-LOX-1 expression and that adding 13-S-HODE induced cell-cycle arrest and apoptosis (24). These data suggest that reduced 15-LOX-1 protein expression is potentially important for colorectal cancer development, in that the subsequent reduction in 13-S-HODE formation may allow neoplastic cells to escape apoptosis. Therefore, increasing 15-LOX-1 expression potentially could induce apoptosis in neoplastic cells. NSAID effects on 15-LOX in neo-

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plastic cells have remained unknown, although it has been shown that NSAIDs preferentially activate 15-LOX in normal human leukocytes (25,26). These data led to our present studies of NSAIDs to test the hypothesis that these agents induce apoptosis in colorectal cancer cells by increasing 15-LOX-1 protein expression and 13-S-HODE production.

**MATERIALS AND METHODS**

**Materials**

- Rabbit polyclonal antisem to human recombinant 15-LOX-1 and standards of recombinant 15-LOX-1 were from Drs. Mary Malkins and Elliot Sigal (Roche Bioscience, Palo Alto, CA) (27–29).
- Standard 13-S-HODE solution was obtained from Cayman Chemical, Inc. (Ann Arbor, MI).
- Antiprotease cocktail tablets were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN).
- 13-S-HODE enzyme-linked immunosorbent assay (ELISA) kits were obtained from Oxford Biomedical Research, Inc. (Oxford, MI). The specificity of the 13-S-HODE antibody has been well demonstrated previously (30).
- Caffeic acid and MK-886 were purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA).
- HT-29 colon cancer cells were obtained from the American Type Culture Collection (Manassas, VA), and RKO rectal cancer cells were provided by Dr. Michael Brattain (The University of Texas in San Antonio). Apo-BRDU kits were obtained from Phoenix Flow Systems, Inc. (San Diego, CA).
- We purchased sulindac from Sigma Chemical Co. (St. Louis, MO), NS-398 from Cayman Chemical, Inc., sulindac sulfate from LKT Laboratories, Inc. (St. Paul, MN), and indomethacin and ibuprofen from ICN Biomedicals, Inc. (Costa Mesa, CA). Other reagents, molecular grade solvents, and chemicals were obtained from regular commercial manufacturers or as specified below.

**Cell Cultures**

- We selected sulindac (an NSAID that can inhibit COX-1 and COX-2) and NS-398 for primary testing in our system because sulindac’s clinical activity against colon premalignancy (FAP) is established (7) and NS-398 is a selective COX-2 inhibitor, as is celecoxib, which is the only NSAID besides sulindac with established clinical activity in this setting (8). We added tests of indomethacin, sulindac sulfate, and ibuprofen for assessing consistency with results of the two main study NSAIDs. RKO and HT-29 cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin (100 IU/mL), and streptomycin (100 μg/mL) (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD).
- Cells were treated once with either 150 μM sulindac or 120 μM NS-398 [concentrations were selected on the basis of prior studies to induce apoptosis in colon cancer cell lines (15,17)] in 0.5% dimethyl sulfoxide when they reached 60%–80% confluence. The presence of dimethyl sulfoxide at that concentration did not affect cell growth in repeated experiments (data not shown). At the times and under the conditions specified below, cells were examined for 15-LOX-1 protein levels (by western blotting), for 13-S-HODE levels (by ELISA), for HETEs (by incubation with arachidonic acid and mass spectrometry), and for evidence of apoptosis as described in the following paragraphs.

To further assess the role of 15-LOX-1 in NSAID-induced growth inhibition and apoptosis, we next examined the influence of 15-LOX-1 inhibition on these NSAID effects. We used caffeic acid at a concentration of 2.2 μM. Caffeic acid is a very potent inhibitor of human 15-LOX, with an IC₅₀ (concentration that causes 50% inhibition of activity) of 0.8 μM (31), which is 5-, 38-, 625-, and 125-fold lower than the IC₅₀ for inhibiting 5-LOX, 12-LOX, or COX5 or for producing antioxidant effects, respectively (32,33).

RKO and HT-29 were treated with sulindac and NS-398, with and without the addition of caffeic acid. Similar experiments were performed with indomethacin, sulindac sulfate, and ibuprofen in concentrations that have been reported to induce apoptosis in colon cancer cell lines (15) (150 μM, 300 μM, and 1 mM, respectively).

To further assess whether the effects of 15-LOX-1 inhibition resulted from loss of 13-S-HODE production, 13-S-HODE or linoleic acid was added, as described previously (24), to NSAID-plus-caffeic-acid-treated cells. Later control experiments used a substitute for caffeic acid, MK-886, which is a selective inhibitor of a 5-lipoxygenase-activating protein (34), at a concentration of 53.3 μM.

**Western Blotting of 15-LOX-1 Protein**

Cells were grown for 12, 24, 48, or 72 hours after treatment with sulindac or NS-398, lysed with a lysis buffer (i.e., 0.05 M Tris–HCl [pH 8.0], 0.15 M NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl, and protease inhibitor cocktail [Boehringer Mannheim Biochemicals]), and harvested at 4 °C. Cell lysates were sonicated for 20 seconds four times and frozen at −70 °C until analyzed (35).

**Incubation With Arachidonic Acid**

Cells were cultured in 100-mm dishes and were treated with 150 μM of sulindac or 120 μM of NS-398 with or without caffeic acid (2.2 μM) at a subconfluent stage (70%). Forty-eight hours after treatment, cells were harvested and suspended in a lysis buffer solution (10 mM Tris–HCl [pH 7.4], 1 mM EDTA, 1% sodium dodecyl sulfate [SDS], and 400 mM NaCl). 13-S-HODE was extracted and measured as described previously (24), 13-S-HODE concentrations were expressed as nanograms per micrograms of crude protein.

**Liquid Chromatography/Mass Spectrometry**

**ELISA of 13-S-HODE**

13-S-HODE levels in cells were measured by ELISA after 48 hours of culture with sulindac or NS-398. Cells were harvested and suspended in a lysis buffer solution (10 mM Tris–HCl [pH 8.0], 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail [Boehringer Mannheim Biochemicals]) at 4 °C. Cells were sonicated for 20 seconds four times (35), and protein concentrations were measured by the method of Bradford (36). A volume that contained 1 mg of crude protein from each sample (621–786 μL) was completed to 1 mL with phosphate-buffered saline and then added to 1 mL of the reaction solution (100 mM Tris–HCl [pH 8.0] and 10 mM CaCl₂) to a final volume of 2 mL. Arachidonic acid was added to create a final concentration of 40 μM, and incubation was performed at 37 °C for 15 minutes. The reaction was stopped by adding 1 mL of citric acid (final pH = 2.5–3). The HETE metabolites of arachidonic acid were extracted three times with hexane/ethyl acetate (1:1) and stored at −70 °C until high-performance liquid chromatography (HPLC) analysis. The adequacy of extraction efficiency was assessed in experiments with internal standards of 11-HETE. Control experiments were performed with untreated cells and with medium alone (to assess auto-oxidation). Adding caffeic acid simultaneously with adding NSAIDs or at the time of incubation with arachidonic acid produced identical results.

**HETE products of arachidonic acid metabolism were detected by use of liquid chromatography/mass spectrometry (37). The instrument was a MicroMass Quattro Ultima (Micromass, Inc., Beverly, MA) connected to a Waters 2790 HPLC (Waters Corp., Milford, MA). The samples were introduced via an electrospray ionization inlet source operating in a negative-ion mode. The first m/z was set to m/z 319.4, which corresponds to the m/z value common for HETE products. The second quadrupole was set to the most abundant fragment ion of each HETE compound (m/z 219.2 for 15-HETE, 179 for 12-HETE, 155 for 8-HETE, 115 for 5-HETE, and 167 for 11-HETE [used as internal standard]). Therefore, the various HETE products were identified through their specific fragmentation patterns. Chromatographic separation for each of the HETEs was performed by use of an HPLC-analytic YMC column (YMC Inc., Milford, MA) (basic 5 μ 2 × 250 mm) maintained at a temperature of 40 °C. Mobile phases were water and methanol. Separation was achieved by use of a gradient of 70%–90% methanol for 15 minutes, then holding the percentage of methanol at 90% for an additional 3 minutes. Injection volume was 25 μL of extracted sample. With the use of the same conditions as used for the unknown samples, known quantities of each of the HETEs were spiked together into cell homogenates to produce standard curves for
quantification. The mass detector settings were as follows: capillary, 3.50 kV; cone, 50 V; source temperature, 120 °C; cone gas flow, 90 L/h; desolvation gas flow, 900 L/h; collision energy, 15 eV; and collision gas (argon), 2.3e⁻³ mbar.

Assessments of Apoptosis

Apoptosis was measured by DNA gel electrophoresis, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay, subdiploid fraction, and floating-cell ratio. Inverse light (phase-contrast) microscopy was used to assess gross evidence of apoptosis and to determine floating-cell ratio, which we and other investigators [17,24] have found to be a reliable indicator of NSAID-induced apoptosis in this system. Apoptosis induction in floating cells was confirmed with acridine orange staining (5 µg/mL), examined by fluorescence microscopy [17] and quantitated by flow cytometry [38,39]. For DNA gel electrophoresis [40], cells harvested 72 hours after NSAID treatment were lysed in 10 mM Tris–HCl (pH 8), 1 mM EDTA, 1% SDS, and 400 mM NaCl on ice. Cell lysates were centrifuged at 12,000g for 20 minutes at 4 °C, treated with ribonuclease (deoxyribonuclease free, 50 µg/mL; Boehringer Mannheim Biochemicals) at 37 °C for 1 hour, and later incubated at 50 °C for 2 hours with 0.2 mg/mL proteinase K. DNA was extracted with a 25 : 24 : 1 solution of phenol : chloroform : isoamyl alcohol, re-extracted with chloroform : isooamyl alcohol, and then precipitated with ethanol at −70 °C overnight. Precipitated DNA from an equal number of cells in each experiment was subjected to electrophoresis by use of 2% agarose gels and visualized by ethidium bromide staining. For the TUNEL assays and subdiploid fraction analyses, cells were harvested 72 hours after NSAID treatment and labeled (by use of an Apo-BRDU kit; Phoenix Flow Systems, San Diego, CA) with fluorescein-labeled deoxyuridine triphosphate and propidium iodide for fragmented apoptotic DNA [41]. Samples were processed according to the manufacturer’s protocol. We used flow cytometry to estimate the percentage of cells that stained with bromodeoxyuridine (i.e., apoptotic cells) by use of Coulter EPICS software (Coulter Corporation, Miami, FL). For cell-cycle distribution flow cytometric analyses, DNA content was measured with a Coulter ELITE flow cytometer, and a multicycle software program (Phoenix Flow System, Inc.) was used to produce histograms of DNA content frequency. Subdiploid DNA peaks were quantified from the cell-cycle distribution data to measure apoptosis.

Statistical Analysis

Data were analyzed by use of SAS software (SAS Institute, Cary, NC). One-way analysis of variance was used for comparing various outcome measures (e.g., 13-S-HODE levels) in different experimental conditions (e.g., NSAID with caffeic acid versus NSAID without). The mean values and 95% confidence intervals (based on asymptotic normal approximation) for the outcome variables are shown in relevant figures. All reported P values are two-sided and were considered to be statistically significant at the .05 level. P values for comparing the difference between groups (i.e., contrasts) are given after the Bonferroni adjustment for multiple comparisons.

RESULTS

NSAID Effects on 15-LOX-1 Expression and 13-S-HODE Levels

Sulindac and NS-398 increased 15-LOX-1 protein expression in RKO cells in a time-dependent fashion, whereas cells cultured without these NSAIDs did not express 15-LOX-1 (Fig. 1, A). The increased 15-LOX-1 expression appeared first at the 24-hour time point (and increased progressively at 48 and 72 hours) after NSAID treatment. Similar time-dependent results with NS-398 occurred in HT-29 cells, and sulindac sulfone also increased 15-LOX-1 protein expression in these cells but not until the 72-hour time point (Fig. 1, B).

The increased 15-LOX-1 protein was enzymatically active, as indicated by findings involving 15-LOX-1 products. Levels of endogenous 13-S-HODE increased in NSAID-treated RKO cells (Fig. 1, C). Also, 15-HETE increased markedly in NSAID-

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Fig. 1. Panel A: Nonsteroidal anti-inflammatory drug (NSAID) effects on 15-lipoxygenase-1 (15-LOX-1) protein expression in RKO cells. Cells were treated with NS-398 or sulindac and were cultured for 12, 24, 48, or 72 hours; they were then analyzed for 15-LOX-1 protein expression (120 µg of crude protein/sample) by western blotting. NS-398 and sulindac induced a time-dependent increase in 15-LOX-1 protein expression. Lane 1 = standard (S) positive control (human recombinant 15-LOX-1 protein, 40 ng); lane 2 = negative control (NC); lanes 3–6 = control experiment with untreated cells at 12, 24, 48, and 72 hours; lanes 7–10 = NS-398-treated cells at 12, 24, 48, and 72 hours; and lanes 11–14 = sulindac-treated cells at 12, 24, 48, and 72 hours. Panel B: NSAID effects on 15-LOX-1 protein expression in HT-29 cells are consistent with effects in RKO cells. Cells were treated with NS-398 and were cultured for 12, 24, 48, or 72 hours; they were then analyzed for 15-LOX-1 protein expression (50 µg of crude protein/sample) by western blotting. NS-398 induced a time-dependent increase in 15-LOX-1 protein expression. Lane 1 = positive control (human recombinant 15-LOX-1 protein, 20 ng); lane 2 = negative control; lanes 3–6 = control experiment with untreated cells at 12, 24, 48, and 72 hours; lanes 7–10 = NS-398-treated cells at 12, 24, 48, and 72 hours; and lanes 11–14 = sulindac sulfone-treated cells at 12, 24, 48, and 72 hours. Panel C: NSAID effects on 13-S-hydroxyoctadecadienoic acid (13-S-HODE) formation in RKO cells. Cells were treated with sulindac or NS-398 (NS) for 48 hours and harvested, and 13-S-HODE was extracted and measured by enzyme-linked immunosorbent assay. 13-S-HODE levels are expressed as nanograms per micrograms of crude protein. Consistent with the 15-LOX expression patterns (see panel A), 13-S-HODE levels were increased by NS and sulindac. Caffeic acid (CAF) inhibited these NSAID effects on 13-S-HODE. Values are means and 95% confidence intervals of the means. Prespecified contrasts: NS versus NS plus CAF, P = .007; sulindac versus sulindac plus CAF, P = .035.
treated RKO cells incubated with arachidonic acid (5-, 8-, and 12-HETE were minor metabolites) (data not shown).

Caffeic acid selectively inhibited the enzymatic activity of the NSAID-induced 15-LOX-1 protein. Caffeic acid blocked the 13-S-HODE-level increases produced by NS-398 (completely) and sulindac (partially) \((P = .007\) and \(P = .035\), respectively) in RKO cells (Fig. 1, C). Caffeic acid also inhibited the formation of only 15-HETE in NSAID-treated RKO cells incubated with exogenous arachidonic acid (data not shown). Our findings of the inhibition of 15- but not of 5-, 8-, or 12-HETE further indicated (see "Materials and Methods" section) that our concentration of caffeic acid (2.2 \(\mu M\)) was highly selective in inhibiting 15-LOX-1.

15-LOX-1 Inhibition (by Caffeic Acid) and NSAID-Induced Growth Inhibition and Apoptosis

Fig. 2, A, shows the time course of RKO cell counts at 0, 24, 48, and 72 hours for the control group and after cells were treated with NS-398, NS-398 plus caffeic acid, sulindac, and sulindac plus caffeic acid. At 72 hours, NS-398 and sulindac reduced the cell count by 47% and 58%, respectively. Blocking 15-LOX-1 enzymatic activity with caffeic acid (described above) blocked the RKO cell-count reduction completely in NS-398-treated cells and partially in sulindac-treated cells. All specified contrasts (see Fig. 2, A, legend) were highly statistically significant \((P < .0001)\). Similar statistically significant results occurred in HT-29 cells, for which the 72-hour time point is shown (Fig. 2, B). Morphological changes (cell loss) occurred in NSAID-treated RKO and HT-29 cells at 72 hours, and caffeic acid blocked these effects (Fig. 2, C). Several findings demonstrated that increased 15-LOX-1/13-S-HODE preceded growth inhibition/apoptosis (as shown, for example, in RKO cells in Figs. 1, A, and 2, A). Sulindac induced apoptosis in RKO and HT-29 cells, and this effect also was blocked by caffeic acid, as measured by TUNEL (Fig. 2, D) and floating-cell ratio (data not shown). NSAID-induced apoptosis and its inhibition by caffeic acid were confirmed by several other methods in RKO cells, including DNA ladder (Fig. 2, E) and subdiploid fraction (data not shown). Similar results were observed with ibuprofen and indomethacin (Fig. 2, E) and sulindac sulfone (data not shown). Caffeic acid (2.2 \(\mu M\)) did not affect cell growth or apoptosis in cells not treated with NSAIDs (data not shown).

Whereas 15-LOX-1 inhibition blocked apoptosis in NSAID-treated RKO cells, selective inhibition of 5-LOX by MK-886 markedly enhanced apoptosis (data not shown), further indicating the specific role of 15-LOX-1 in NSAID-induced apoptosis.

13-S-HODE Restoration of NSAID-Induced Growth Inhibition and Apoptosis Blocked by 15-LOX-1 Inhibition

Fig. 3, panels A (cell counts) and B (TUNEL), show that 13-S-HODE (135 \(\mu M\)) restored NSAID-induced growth inhibition and apoptosis blocked by caffeic acid in RKO cells \((P < .0001\) and \(P < .0001\), respectively), but equal amounts \((135\ \mu M)\) of linoleic acid did not \((P = 1.0\) and \(P = 1.0\), respectively). Supplementation with 13-S-HODE and linoleic acid in HT-29 cells had similar effects to those in RKO cells: i.e., 13-S-HODE, but not linoleic acid reversed caffeic acid effects on NSAID-induced cell growth inhibition (cell count) and apoptosis (measured as floating-cell ratio) (data not shown).

DISCUSSION

These results support our original study hypotheses: that NSAIDs increase 15-LOX-1 in colorectal cancer cells and that this increased expression is crucial to the induction (by NSAIDs) of apoptosis. The relationship between increased 15-LOX-1 protein expression and NSAID effects on cell growth and apoptosis is illustrated by our following results: 1) 15-LOX-1 protein expression was increased by NSAIDs in a time-dependent fashion in association with subsequent growth inhibition and apoptosis, and 2) 13-S-HODE cellular levels were increased markedly at 48 hours as growth inhibition and apoptosis became evident. This includes sulindac sulfone-induced 15-LOX-1 protein expression (although delayed) and apoptosis. Sulindac sulfone is a sulindac metabolite that has no substantial COX inhibitory effects and is weaker than sulindac in regard to apoptosis-inducing activity in vitro \((13)\) and colon carcinogenesis-suppression effects in vivo \((42)\). Our finding suggests that there may be a link between 15-LOX-1 and sulindac sulfone-induced apoptosis.

The specificity of 15-LOX-1 as a target for NSAID-induced apoptosis is supported by the following results: 1) NSAIDs markedly increased 15-HETE formation, whereas 5-, 8-, and 12-HETE remained minor metabolites; 2) the caffeic acid concentration used in our study inhibited only 15-LOX-1 (not 5-, 8-, or 12-LOX); 3) the inhibition of 15-LOX-1 blocked NSAID-induced apoptosis; 4) adding the 15-LOX-1 product 13-S-HODE (but not its parent compound, linoleic acid) restored apoptosis; and 5) in contrast to 15-LOX-1 inhibition, inhibition of 5-LOX by MK-886 increased apoptosis, which is consistent with other reports that the 5- and 12-LOX metabolites of arachidonic acid (leukotriene \(B_2\) and 12-HETE, respectively) enhance cell proliferation \((43,44)\) and block apoptosis \((45)\). We found that the inhibition of 15-LOX-1 reversed or reduced markedly apoptosis induced by other NSAIDs (sulindac sulfone, indomethacin, and ibuprofen) in addition to sulindac and NS-398. Therefore, these results indicate that the metabolism of linoleic acid into 13-S-HODE by 15-LOX-1 is mechanistically critical to the induction of apoptosis in colorectal cancer cells by NSAIDs. Based on our study of five different NSAIDs and two colorectal cancer cell lines, these results also suggest the general importance of this mechanism in NSAID prevention of human colorectal carcinogenesis.

The following studies support our findings that 15-LOX-1 and 13-S-HODE are critical to growth inhibition, apoptosis, and chemoprevention: 1) Transient transfection of an osteosarcoma cell line with 15-LOX-1 reduced cell proliferation \((27)\), 2) the addition of 15-LOX products induced apoptosis of lymphocytes \((46)\), 3) sodium butyrate-induced apoptosis in Caco-2-transformed colonic cells was associated with increased 15-LOX-1 expression \((35,47)\), and 4) linoleic acid conversion to 13-S-HODE inhibits mouse skin tumorigenesis \((48,49)\).

13-S-HODE has been linked to peroxisome proliferator-activated receptor-\(\gamma\) (PPAR-\(\gamma\)) activation and is one of the most potent natural ligands of PPAR-\(\gamma\) receptors \((50)\). PPAR-\(\gamma\) receptors are expressed in the colon \((51)\) and can be activated by NSAIDs \((52)\), and activation of PPAR-\(\gamma\) leads to cell differentiation and apoptosis in human colon cancer cell lines \((51,53)\). Therefore, 13-S-HODE binding to and activation of PPAR-\(\gamma\) might be an event in the signal-transduction pathway involved in 13-S-HODE-induced apoptosis.

Our previous findings suggested the hypothesis that decreased 15-LOX-1 expression and the subsequent reduction in
Fig. 2. 15-Lipoxygenase-1 (15-LOX-1) inhibition blocked nonsteroidal anti-inflammatory drug (NSAID)-induced cellular growth inhibition and apoptosis.

**Panel A:** Sulindac and NS-398 (NS) reduced the number of RKO cells after 48 and 72 hours of treatment, with the extent of growth inhibition dependent on time. Caffeic acid (CAF) attenuated or blocked this effect. Proliferation rates are represented as the numbers of attached cells at the time of harvesting. Values shown are the means and 95% confidence intervals (CIs) of the means of triplicate experiments. Prespecified contrasts at 72 hours: control versus NS, control versus sulindac, NS versus NS plus CAF, and sulindac versus sulindac plus CAF (all contrasts P<.0001). 

**Panel B:** Inhibition of 15-LOX-1 blocked NSAID-induced growth inhibition in HT-29 cells. HT-29 cells were treated with sulindac or NS with or without CAF and then cultured for 72 hours. Proliferation rates are represented as the number of attached cells at 72 hours. Values shown are the means and 95% CIs of triplicate experiments. Prespecified contrast: sulindac versus sulindac plus CAF P<.0001 for RKO and HT-29 cells. Similar results with floating-cell ratio were observed for both cell lines (data not shown).

**Panel C:** Light micrographs of RKO cells (top panel) and HT-29 cells (bottom panel) treated with NS± or a control experiment after 72 hours of treatment (original magnification ×200). Similar results were achieved with sulindac (data not shown).

**Panel D:** Sulindac-induced apoptosis was blocked by 15-LOX-1 inhibition in RKO and HT-29 cells (as measured by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling [TUNEL] at 72 hours). Values shown are the means and 95% CIs of triplicate experiments. Prespecified contrast: sulindac versus sulindac plus CAF P<.0001 for RKO and HT-29 cells. Similar results with floating-cell ratio were observed for both cell lines (data not shown).

**Panel E:** Inhibition of 15-LOX-1 blocked apoptosis induced by sulindac, NS, ibuprofen, or indomethacin in RKO cells, as measured by DNA laddering. The lanes are as follows: 1 = cells treated with sulindac, 2 = cells treated with NS, 3 = cells treated with ibuprofen, 4 = cells treated with indomethacin, 5 = cells treated with sulindac plus CAF, 6 = cells treated with NS plus CAF, 7 = cells treated with ibuprofen plus CAF, 8 = cells treated with indomethacin plus CAF, and 9 = untreated cells (control).
13-S-HODE production may be involved in colon cancer development (24). This hypothesis is supported by our presently reported data, which strongly suggest that the established positive clinical effects of NSAIDs in colonic carcinogenesis (7,8) may be mediated by the restoration of 15-LOX-1 enzymatic activity and subsequent induction of growth inhibition and apoptosis. Our studies indicate that 15-LOX-1 is a novel molecular target for the development of new treatment and prevention strategies for colorectal cancer.

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**Notes**

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