15-Lipoxygenase-1 Mediates Nonsteroidal Anti-Inflammatory Drug-induced Apoptosis Independently of Cyclooxygenase-2 in Colon Cancer Cells

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Abstract

We previously found that (a) 15-LOX-1 and its product 13-S-hydroxyoctadecadienoic acid (13-S-HODE) are decreased; and (b) NSAID-induced 15-LOX-1 expression is critical to NSAID-induced apoptosis in colorectal cancer cells expressing cyclooxygenase-2 (COX-2). We used the NSAIDs sulindac sulfone (COX-2-independent) and NS-398 (a COX-2 inhibitor) to assess NSAID up-regulation of 15-LOX-1 in relation to COX-2 inhibition during NSAID-induced apoptosis in the DLD-1 (COX-2-negative) colon cancer cell line. We found that: (a) NSAIDs up-regulated 15-LOX-1, which preceded apoptosis; and (b) 15-LOX-1 inhibition blocked NSAID-induced apoptosis, which was restored by 13-S-HODE but not by its parent, linoleic acid. NSAIDs can induce apoptosis in colon cancer cells via up-regulation of 15-LOX-1 in the absence of COX-2.

Introduction

Apoptosis appears to mediate the chemopreventive effects of NSAIDs (Refs. 1–6 and references contained within). NSAIDs can alter the production of different metabolites of polyunsaturated fatty acids (linoleic and arachidonic acids) through modulating LOXs and COXs (7). We found that the expression of 15-LOX-1, the main enzyme for metabolizing colonic linoleic acid to 13-S-HODE, is down-regulated in human colorectal cancer cells (8). We recently showed that NSAIDs induce 15-LOX-1 expression in COX-2-expressing colorectal cancer cells and that 15-LOX-1 up-regulation is critical to NSAID induction of apoptosis (9). There are data indicating that NSAID-induced apoptosis is unrelated to COX-2 inhibition (2–4, 6). Other data suggest that COX inhibition may increase LOX activities by a shift of substrate away from the COXs and toward the LOXs (10). This may explain why sodium butyrate can both up-regulate 15-LOX-1 and down-regulate COX-2 during induction of apoptosis in Caco-2 human colon cancer cells (11). Therefore, NSAID inhibition of COX-2 may indirectly up-regulate 15-LOX-1, thereby affecting apoptosis induction, by increasing the amount of substrate available to be metabolized by 15-LOX-1. To assess whether NSAID effects on 15-LOX-1 and apoptosis depend on COX-2 inhibition, we examined the effects of an active NSAID that does not inhibit COX-2 (and those of a NSAID COX-2 inhibitor) in a colon cancer cell line that lacks COX-2 expression.

Materials and Methods

Materials. Rabbit polyclonal antiserum to human recombinant 15-LOX-1 and standards of recombinant 15-LOX-1 were a gift from Drs. Mary Mulkins and Elliot Sigal (Roche Biosciences, Palo Alto, CA; Ref. 12). Standard solutions of 13-S-HODE, linoleic and arachidonic acids, and COX-1 antibody were obtained from Cayman Chemical, Inc. (Ann Arbor, MI). We obtained antiprotease cocktail tablets from Boehringer Mannheim, Inc. (Indianapolis, IN), a 13-S-HODE ELISA kit from Oxford Biomedical Research (Oxford, MI), and a 15-S-HETE enzyme immunoassay kit from Assay Designs Inc. (Ann Arbor, MI). Caffeic acid was purchased from BIOMOL Research Laboratories, Inc., (Plymouth Meeting, PA) and COX-2 antisera from Transduction Laboratories (Lexington, KY). DLD-1, HT-29, HCT-15, and SW620 cells were obtained from the American Type Culture Collection (Manassas, VA).

We purchased NS-398 from Cayman Chemical, Inc., (Ann Arbor, MI) and sulindac sulfone from KLT Laboratories, Inc., (St. Paul, MN). Other reagents, molecular grade solvents, and chemicals were obtained from regular commercial manufacturers or as specified below.

Western Blot Analysis to Detect COX-1 and COX-2. We examined the expression of COX-1 and COX-2 in various colon cell lines (DLD-1, SW620, HT-29, and HCT-15). Cells that were not treated by NSAIDs were harvested and lysed, and the protein concentration in the lysate was determined. Aliquots containing 30 μg of protein extracted from each cell line were then subjected to electrophoresis in 10–14% polyacrylamide slab gels. After transfer, blots were probed with COX-1 and COX-2 antibodies and processed by an enhanced chemiluminescence method, as described previously (9).

Cell Cultures. In cell cultures, sulindac sulfone was used for its COX-independent chemopreventive activity, and NS-398 for its selectivity for COX-2 inhibition. DLD-1 cells were grown in RPMI 1640 supplemented with 10% FBS, penicillin, and streptomycin (Life Technologies, Inc., Grand Island, NY). When cells reached 60–80% confluence, they were treated once with either 300 μM sulindac sulfone or 120 μM NS-398 in 0.5% DMSO (9). The presence of 0.5% DMSO did not affect cell growth in repeated experiments (data not shown). Cells were cultured and harvested for assays to evaluate 15-LOX-1 protein expression and apoptosis, as described in the following paragraphs.

We used caffeic acid at a concentration of 2.2 μM to inhibit 15-LOX-1, which we examined in respect to NSAID-induced apoptosis. We previously established the specificity of this concentration of caffeic acid for inhibiting 15-LOX-1 in colorectal cancer cells (9). DLD-1 cells were treated with sulindac sulfone and NS-398, with and without the addition of caffeic acid. To further assess whether the effects of 15-LOX-1 inhibition resulted from loss of 13-S-HODE production, 135 μM of 13-S-HODE or linoleic acid was added, as described previously (8), to NSAID-plus-caffeic-acid-treated cells. Although relatively high, our 135-μM concentration of 13-S-HODE was necessitated by our use of 10% FBS for supplementing the cell cultures, which is consistent and comparable with the 5–20% FBS described in the literature for other in vitro NSAID/colorectal cancer studies (2–4). We used 135 μM of 13-S-HODE to account for the substantial amount of albumin in 10% FBS (which is well known to strongly bind exogenously added 13-S-HODE; Ref. 13) and to allow bioavailability of 13-S-HODE to the DLD-1 cells. We previously reported...
dose-response effects of 13-S-HODE in other colorectal cancer cell lines (8), which were consistent with current dose-response effects in DLD-1 cells. For example, we saw equivalent effects of 135 μM of 13-S-HODE on growth inhibition of DLD-1 cells cultured with either 0.1% FBS or 10% FBS (mean ± SE, 99.5 ± 0.005% and 97.9 ± 0.11%, respectively) and additional 13-S-HODE growth-inhibition effects (in DLD-1 cell cultures containing 10% FBS) of 77.7 ± 0.86% (SE) for 13.5 μM, 48.35 ± 7.18% for 1.35 μM, and 4.63 ± 3.32% for 0.135 μM.

Western Blot Analysis of 15-LOX-1/COX-2 Protein.

Cells were grown for 12, 24, 48, or 72 h after treatment with sulindac sulfone or NS-398, lysed, sonicated, and kept frozen at −70°C until analyzed. Protein (50 μg) from each sample was subjected to electrophoresis on an 8% SDS-polyacrylamide gel under reducing conditions. After transfer, blots were probed with 15-LOX-1 or COX-2 primary antibody and processed by an enhanced chemiluminescence method, as described previously (9).

Immunooassay Quantitation of Endogenous 13-S-HODE and 15-S-HETE Production. DLD-1 cells were cultured for 48 h after treatment with sulindac sulfone or NS-398 and then lysed. 15-S-HETE and 13-S-HODE were extracted as described previously (9). Endogenous levels of 13-S-HETE and 13-S-HODE were measured using 15-S-HETE enzyme immunoassay and 13-S-HODE ELISA commercial kits according to the manufacturers’ protocols.

LC/MS after Incubation with Arachidonic or Linoleic Acid.

Cells were cultured in 150-mm dishes and were treated with 300 μM sulindac sulfone or 120 μM NS-398 at a subconfluent stage (70%). Forty-eight h after treatment, cells were harvested, lysed, sonicated, and incubated with either 125 μM arachidonic acid or 125 μM linoleic acid, and enzymatic activity, or metabolite production, was detected via LC/MS methods similar to those described previously (9).

Assessments of Apoptosis. Apoptosis was evaluated by several methods: DNA gel electrophoresis; microscopic examination to identify morphological changes associated with apoptosis; floating-cell ratio; staining with acridine orange; and flow-cytometric cell-cycle distribution analysis to determine sub-G1 fractions. Inverse-light (phase-contrast) microscopy was used to assess gross evidence of apoptosis and to determine floating-cell ratio, which we and others have found to be a reliable indicator of NSAID-induced apoptosis in this system (4, 9). Apoptosis induction in floating cells was confirmed by acridine orange staining (5 μg/ml) and by fluorescence microscopy. For DNA gel electrophoresis, cells were harvested 72 h after treatment (e.g., with sulindac sulfone, NS-398, or NS-398 plus caffeic acid) and lysed. DNA was extracted from an equal number of cells, precipitated, electrophoresed on 2% agarose gels, and visualized by ethidium bromide staining, as described previously (9). Cells also were stained with propidium iodide for determination of cell-cycle distribution by flow cytometric analyses, as described previously (9). Cell-cycle distribution data were used to calculate subdiploid DNA (sub-G1) peaks as a measure of apoptosis.

Results

NSAID Effects on 15-LOX-1 Expression. In DLD-1 cells that showed no expression of COX-1 or COX-2 protein (data not shown), sulindac sulfone and NS-398 up-regulated 15-LOX-1 protein expression, whereas cells cultured without these NSAIDs did not express 15-LOX-1 (Fig. 1). We detected the NS-398- and sulindac-sulfone-induced 15-LOX-1 protein expressions at different time points, 24 and 48 h, respectively (Fig. 1). The increases in 15-LOX-1 protein expression occurred before the induction of apoptosis. Incubation experiments with linoleic or arachidonic acid confirmed that the NSAID-induced 15-LOX-1 was enzymatically active (by LC/MS). NSAID treatment of DLD-1 cells significantly increased 13-S-HODE formation, when cells were incubated with linoleic acid (e.g., 2.7-fold in the case of NS-398 treatment). Caffeic acid in a concentration of 2.2 μM inhibited the enzymatic activity of NSAID-induced 15-LOX-1 (data not shown).

NSAID Effects on 13-S-HODE and 15-S-HETE Production. NSAID-induced expression of 15-LOX-1 increased the formation of 13-S-HODE but not of 15-S-HETE. NSAID treatment of DLD-1 cells increased endogenous 13-S-HODE levels by 2- to 3-fold at 48 h (control (mean ± SE), 5.96 ± 0.19 ng/μg protein; sulindac sulfone, 18.84 ± 3.16 ng/μg protein; NS-398, 11.56 ± 1.28 ng/μg protein). In contrast, the endogenous levels of 15-S-HETE were very low (<0.01 ng/μg protein in untreated cells), and NSAID treatment resulted in no increase in endogenous 15-S-HETE formation (data not shown).

Effects of 15-LOX-1 Inhibition on NSAID-induced Apoptosis.

Sulindac sulfone and NS-398 induced apoptosis in DLD-1 cells (confirmed by acridine orange staining), and caffeic acid blocked these effects (Fig. 2, A–C). We used caffeic acid at a concentration of 2.2 μM, which selectively inhibits 15-LOX-1 (9). We further confirmed our apoptosis-induction and -inhibition findings via quantitation of sub-G1 fractions of cells (using propidium iodide staining and flow cytometry analyses; Fig. 2D) and DNA fragmentation assays (Fig. 2E). Caffeic acid did not affect cell growth or apoptosis in cells not treated with NSAIDs (data not shown).

Effects of 13-S-HODE Supplementation on Cells Treated with NSAIDs and Caffeic Acid. We supplemented 13-S-HODE (the main product of 15-LOX-1) into DLD-1 cells treated with caffeic acid and either sulindac sulfone. 13-S-HODE (135 μM) induced apoptosis in these cells, as assessed by floating-cell ratio (Fig. 3A) and DNA laddering (Fig. 3B). In control experiments, we replaced 13-S-HODE with an equal concentration (135 μM) of its parent compound, linoleic acid, which, unlike 13-S-HODE, did not restore NSAID induction of apoptosis in DLD-1 cells (Fig. 3, A and B), Fig. 2B shows the cellular morphological changes consistent with these findings.

Discussion

Our present results show that NSAIDs up-regulate 15-LOX-1 expression in colon cancer cells independently of COX-2. The independence of the two events is confirmed by two of our findings. First, in DLD-1 cells, which lack COX-2 expression, NS-398 induced 15-LOX-1 expression. Second, sulindac sulfone, a sulindac derivative devoid of COX-inhibitory activity, induced 15-LOX-1 expression; and the induction of 15-LOX-1 expression was necessary for apoptosis induction by sulindac sulfone (as well as by NS-398). Because the NSAID up-regulation of 15-LOX-1 increased the formation of 13-S-HODE but not of 15-S-HETE (the other major product of 15-LOX-1), it is clear that the effects of 15-LOX-1 up-regulation on apoptosis are mediated through 13-S-HODE. These findings agree with previous reports that linoleic acid is the preferred substrate for human 15-LOX-1, which predominantly produces 13-S-HODE (14).
The crucial role of 15-LOX-1 up-regulation in sulindac sulfone-induced apoptosis was demonstrated by the blocking of apoptosis (via inhibition of 15-LOX-1) and restoration of apoptosis (via adding the 15-LOX-1 product 13-S-HODE, but not its parent compound, linoleic acid). Therefore, in contrast to COX inhibition [shown, in the reports of several others (2–4, 6) and in our present studies, to be unrelated to apoptosis], 15-LOX-1 expression is independently crucial to NSAID induction of apoptosis in colorectal cancer cells, whether expressing COX-2 (9) or not.

Therefore, NSAID-induced apoptosis in colorectal cancer cells appears to be unrelated to COX-2 inhibition, either directly or indirectly through a COX-2-inhibition-related substrate shift (discussed above in the “Introduction”). The lack of a substrate-shift relationship is further supported by our previous findings of NSAID-induced apoptosis via 15-LOX-1 up-regulation in HT-29 colon cancer cells (9), which recently were shown to express only enzymatically inactive COX-2 (15).

Our present findings: (a) support our prior results (9), which indicated that 15-LOX-1 is a specific, novel, and crucial molecular target for inducing apoptosis (whereas 5- and 12-LOX are involved in blocking apoptosis; Ref. 16); and (b) extend those findings by demonstrating that the role of 15-LOX-1 is independent of COX-2 inhibition, including independence from any competition with COX-2 for substrate. Our present—and previous (9)—finding that 15-LOX-1 up-regulation is critical to apoptosis and to growth inhibition is supported by others’ findings that: 15-LOX-1 transfection inhibits the growth of osteosarcoma cells (12); 15-LOX products induce apoptosis in lymphocytes (17); 13-S-HODE inhibits mouse-skin carcinogenesis (18); and sodium butyrate (11, 19) and interleukin 4 (20, 21) induce 15-LOX-1 apoptosis or growth inhibition in colorectal cancer cells.

The mechanisms for 15-LOX-1 up-regulation by NSAIDs remain unknown and deserve further investigation. Regulation of 15-LOX-1 expression has been observed at both the transcriptional (22) and posttranslational (23) levels. Another provocative area of 15-LOX-1 research involves the reported link between its product 13-S-HODE and peroxisome proliferator-activated receptor-γ activation, which may be an event in the signal transduction pathway involved in NSAID-induced apoptosis in colon cancer (24–26). Future mechanistic studies should provide insight into the biology of colorectal carcinogenesis and drug activity that will further the development of effective agents (e.g., targeting the direct mechanistic link with 15-LOX-1 up-regulation) for colorectal cancer prevention.

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References


Fig. 2. 15-LOX-1 inhibition blocks apoptosis induced by NSAIDs in DLD-1 cells. A. Sulindac sulfone (S sulfone) induced apoptosis (measured as floating-cell ratio) in a time-dependent manner, starting at 48 h after treatment; , inhibition of 15-LOX-1 by caffeic acid (CAF) blocked sulindac sulfone-induced apoptosis; ○, control. Values shown are the means ± SEs of triplicate experiments. Similar results were observed with NS-398 (NS) with and without CAF (data not shown). B. Light microscopy pictures (>200) DLD-1 cells after 72 h of treatment with: no treatment (Control); NS; NS with CAF; S sulfone; S sulfone with CAF; S sulfone with 13-S-HODE; and S sulfone with CAF and linoleic acid. S sulfone and NS induced morphological changes that indicate apoptosis, including cytoplasmatic and nuclear shrinkage. Inhibition of 15-LOX-1 by CAF blocked these morphological changes; 13-S-HODE (15-LOX-1 product) restored apoptosis, whereas its parent linoleic acid had no effect. C. Floating cells treated with S sulfone and NS show typical morphological changes of apoptosis 72 h after treatment. Cells were stained with acridine orange and examined by a fluorescence microscope (>200). Cells display chromatin condensation and nuclear fragmentation that are typical features of apoptosis. D. DLD-1 cells were treated with: no treatment (Control); NS, NS + CAF, S sulfone, and S Sulfone + CAF. Cells were cultured for 72 h, harvested, stained with propidium iodide, and assessed for sub-G1 fractions by flow cytometry. Values shown are the means ± SEs of triplicate experiments. Inhibition of 15-LOX-1 by CAF blocked apoptosis by S sulfone and NS. E. DLD-1 cells were treated as follows: Lane 2, untreated; Lane 3, NS; and Lane 4, NS and CAF. Cells were harvested 72 h later. DNA was extracted and analyzed by agarose gel electrophoresis. NS-treated cells show DNA fragmentation in the typical ladder pattern. Inhibition of 15-LOX-1 (Lane 4) blocked these changes. Lane 1, a 1-kb standard DNA ladder.

Fig. 3. 13-S-HODE restored NSAID-induced apoptosis blocked by 15-LOX-1 inhibition in DLD-1 cells. A. DLD-1 cells were treated as indicated on the x-axis and harvested after 72 h; floating and attached cells were counted. Values are means ± SEs. Sulindac sulfone (S Sulfone) increased the ratio of floating cells (as an indicator of apoptosis), whereas inhibition of 15-LOX by caffeic acid (CAF) blocked this effect. 13-S-HODE, but not its parent compound (linoleic acid [LA]), restored apoptosis. B. DLD-1 cells were treated with sulindac sulfone (Lane 2), sulindac sulfone with caffeic acid (Lane 3), sulindac-sulfone with caffeic acid and 13-S-HODE (Lane 4), and sulindac sulfone with caffeic acid and linoleic acid (Lane 5). DNA was extracted 72 h later, and analyzed by agarose gel electrophoresis. Sulindac sulfone-treated cells exhibited the typical DNA fragmentation for apoptosis. Inhibition of 15-LOX-1 by caffeic acid (Lane 3) blocked these changes, whereas 13-S-HODE was able to reestablish apoptosis in these cells (Lane 4). Linoleic acid had no effects on caffeic acid blocking of sulindac sulfone-induced apoptosis (Lane 5). Lane 1, DNA extracted from untreated cells (control experiment).
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