The Histone Deacetylase Inhibitor Suberoylanilide Hydroxamic Acid Induces Apoptosis via Induction of 15-Lipoxygenase-1 in Colorectal Cancer Cells

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ABSTRACT

Histone deacetylases (HDACs) mediate changes in nucleosome conformation and are important in the regulation of gene expression. HDACs are involved in cell cycle progression and differentiation, and their deregulation is associated with several cancers. HDAC inhibitors have emerged recently as promising chemotherapeutic agents. One such agent, suberoylanilide hydroxamic acid, is a potent inhibitor of HDACs that causes growth arrest, differentiation, and/or apoptosis of many tumor types in vitro and in vivo. Because of its low toxicity, suberoylanilide hydroxamic acid is currently in clinical trials for the treatment of cancer. HDAC inhibitors induce the expression of <2% of genes in cultured cells. In this study, we show that low micromolar concentrations of suberoylanilide hydroxamic acid induce the expression of 15-lipoxygenase-1 in human colorectal cancer cells. The expression of 15-lipoxygenase-1 correlates with suberoylanilide hydroxamic acid-induced increase in 13-5-hydroxyoctadecadienoic acid levels, growth inhibition, differentiation, and apoptosis observed with these cells. Furthermore, specific inhibition of 15-lipoxygenase-1 significantly reduced the suberoylanilide hydroxamic acid-induced effects. These novel findings are the first demonstration of a mechanistic link between the induction of 15-lipoxygenase-1 by a HDAC inhibitor and apoptosis in cancer cells. This result has important implications for the study of suberoylanilide hydroxamic acid and other HDAC inhibitors in the prevention and therapy of colorectal cancer and supports future investigations of the mechanisms by which HDAC inhibitors up-regulate 15-lipoxygenase-1.

INTRODUCTION

Histone deacetylases (HDACs) mediate changes in nucleosome conformation and are important in the regulation of gene expression (1). HDACs are involved in cell-cycle progression and differentiation, and their deregulation is associated with several cancers (2). HDAC inhibitors, such as trichostatin A and suberoylanilide hydroxamic acid, have emerged recently as promising chemotherapeutic agents (1, 3) because they have antitumor effects. They can inhibit cell growth, induce terminal differentiation, and prevent the formation of tumors in mice models (1, 4, 5). Suberoylanilide hydroxamic acid, in particular, has been shown to induce growth arrest, differentiation, and/or apoptosis in a variety of cell types (4–7) and inhibit tumor growth in vivo (6, 8–10). Furthermore, because of its low toxicity, suberoylanilide hydroxamic acid is currently in clinical trials for the treatment of solid and hematologic tumors (5, 11, 12).

Although suberoylanilide hydroxamic acid has been shown to cause cells to differentiate and undergo apoptosis, the mechanisms underlying these events are largely unknown. One previous study proposed that changes at the level of chromatin structure, induced by a HDAC inhibitor such as sodium butyrate (NaBt), leads to the expression of an unknown protein that facilitates the pathway by which mitochondria activate caspase-3 and trigger apoptotic death of cancer cells (13). Another study has showed that butyrate induces 15-lipoxygenase-1 (14). On the basis of these two studies, we hypothesized that 15-lipoxygenase-1 could be one such protein that activates the apoptotic pathway or commits the cells to apoptosis in the case of suberoylanilide hydroxamic acid. To assess whether suberoylanilide hydroxamic acid-induced growth inhibition, differentiation, and/or apoptosis involve 15-lipoxygenase-1, we examined whether suberoylanilide hydroxamic acid can induce 15-lipoxygenase-1 and whether suberoylanilide hydroxamic acid-induced apoptosis is dependent on 15-lipoxygenase-1 expression and function.

MATERIALS AND METHODS

Cell Culture. The human colorectal cell lines Caco-2, HCT-116, and SW-480 were obtained from the American Type Culture Collection and were grown at 37°C in a humidified 5% CO2/95% air atmosphere. The Caco-2 cells were grown in Eagle’s minimal essential medium (1X; Life Technologies, Inc., Carlsbad, CA) with l-glutamine supplemented with 15% fetal bovine serum, 1 mmol/L sodium pyruvate (Life Technologies, Inc.), and 1 mg/100 mL gentamycin (Life Technologies, Inc.). The HCT-116 and SW-480 cells were grown in RPMI media supplemented with 15% fetal bovine serum. Trypsin (Life Technologies, Inc.) was used to subculture cells.

Treatments with HDAC Inhibitors. NaBt (Sigma, St. Louis, MO) was dissolved in PBS, and suberoylanilide hydroxamic acid was dissolved in DMSO. Cells were treated from 0 to 4 days and at the doses indicated. Treatment groups were compared with vehicle controls. Caffeic acid, a 15-lipoxygenase-1 inhibitor, was used at a concentration of 2.2 μmol/L. The specificity of this concentration has been previously established for inhibiting 15-lipoxygenase-1 in colorectal cancer cells (15). Nordihydroguaiaretic acid, a general lipoxygenase inhibitor, was used at a concentration of 10 μmol/L. Cells were harvested at the various time points indicated.

Western Blot Analysis of 15-Lipoxygenase-1 Protein. In general, SDS-PAGE and Western blotting techniques were carried out as described previously (16). Briefly, treated and control cells groups were washed twice with ice-cold PBS and lysed in protein lysis buffer containing protease and phosphatase inhibitors. Cells were sonicated three times for 5 seconds each at 50% power for a total protein preparation. Protein content was quantified by the Bradford method as described previously (16). Aliquots of the protein preparation were heated to 70°C in protein sample buffer (Invitrogen, Carlsbad, CA) and separated by a 4 to 12% gradient gel (Invitrogen), according to the manufacturer’s instructions. Proteins were transferred onto nitrocellulose membrane (Invitrogen). Blots were blocked with 10% nonfat dry milk in 20 mmol/L Tris-buffered saline (pH 7.4) containing 0.05% Tween 20 (TBS-T) at 4°C and washed. The blots were then incubated in 1% milk in TBS-T with an appropriate primary antibody. Rabbit polyclonal antiserum to recombinant human 15-lipoxygenase-1 was a gift from Mary Mulkins and Elloit Sigal (Roche Bioscience, Palo Alto, CA) and was also generated by Lampire Biological Laboratories (Pipersville, PA). The 15-lipoxygenase-1 primary antibody was used at a dilution of 1:2000. Normal human tracheobronchial epithelial cells, as previously described (16), were used as a positive control for the expression of 15-lipoxygenase-1. Actin antibody (Sigma) was used at a dilution of 1:2000. Actin was analyzed as a control for protein loading. After washing, blots were incubated with an appropriate horseradish peroxidase-linked secondary antibody (Amersham, Piscataway, NJ). After reaction with chemiluminescence reagents (Amersham enhanced chemiluminescence system), bands were detected by exposure to film (Amersham).
ELISA Measurement of 13-S-Hydroxyoctadecadienoic Acid Levels. After treatment with suberoylanilide hydroxamic acid, cells were lysed in lysis buffer containing protease and phosphatase inhibitors. The protein concentration was determined by Bradford’s method. 13-S-Hydroxyoctadecadienoic acid levels were determined with an ELISA kit (Assay Design, Ann Arbor, MI) according to the manufacturer’s specifications. 13-S-Hydroxyoctadecadienoic acid was measured in treated and untreated cells. The protein concentration was measured by Bradford’s method. Alkaline phosphatase activity, a marker for differentiation, was used to assess differentiation. An alkaline phosphatase kit (Pointe Scientific, Inc., Lincoln Park, MI) was used according to the manufacturer’s specifications.

Cellular Proliferation. Cells were grown in 100-mm plates and treated with compounds of interest or vehicle at indicated concentrations and for 0 to 96 hours. Cells were harvested and counted at the indicated time points. Growth curves were plotted with the number of attached cells at the time of harvesting.

Cellular Differentiation. Cell differentiation was assessed by measuring the enzymatic activity of alkaline phosphatases. Cells were lysed in lysis buffer containing protease and phosphatase inhibitors. The protein concentration was measured by Bradford’s method. Alkaline phosphatase activity, a marker for differentiation, was used to assess differentiation. An alkaline phosphatase kit (Pointe Scientific, Inc., Lincoln Park, MI) was used according to the manufacturer’s specifications.

DNA Fragmentation Assay. After treatment of interest, floating and attached cells were harvested at the indicated time points. Washed cell pellets were resuspended in cell lysis buffer containing protease and phosphatase inhibitors. The cell lysates were centrifuged and then resuspended in cell lysis buffer [10 mmol/L Tris-HCl (pH 7.4), 10 mmol/L EDTA (pH 8.0), 0.5% TritonX-100] and incubated for 10 minutes at 4°C. Cell lysates were centrifuged and then resuspended in cell lysis buffer [10 mmol/L Tris-HCl (pH 7.4), 10 mmol/L EDTA (pH 8.0), 0.5% TritonX-100] and incubated for 10 minutes at 4°C. DNA was extracted from an equal number of cells and then was precipitated by etomidate and run on a 2% agarose gel as described previously (15). Agarose gels were stained with etomidate and DNA visualized by an UV transilluminater.

Caspase 3 Assay. After treatment of interest, floating and attached cells were harvested at the indicated time points. Cells were counted and an equal number of cells were used for each sample with a Caspase 3 kit (BD Biosciences Clontech, Palo Alto, CA) according to the manufacturer’s protocol.

RESULTS

Suberoylanilide Hydroxamic Acid Effects on 15-Lipoxygenase-1 Expression in Colon Cancer Cells. Three different colorectal cancer cell lines, Caco-2, SW-480, and HCT-116, were found to express 15-lipoxygenase-1 after treatment with suberoylanilide hydroxamic acid (data not shown). We have selected the Caco-2 cell line for all subsequent experiments to study the effect of 15-lipoxygenase-1 on apoptosis because of its ability to undergo terminal differentiation. The induction of 15-lipoxygenase-1 by suberoylanilide hydroxamic acid was both dose and time dependent (Fig. 1). Caco-2 cells were treated with varying concentration of suberoylanilide hydroxamic acid ranging from 0.1 to 10 μmol/L for 24 hours. An increase in 15-lipoxygenase-1 was observed at concentrations as low as 1 μmol/L but was strongly expressed at 2.5 μmol/L and higher. The cells were also treated with 2.5 μmol/L suberoylanilide hydroxamic acid for 0 to 96 hours, and 15-lipoxygenase-1 expression was determined (Fig. 1B). 15-Lipoxygenase-1 was observed as early as 12 hours, and expression increased at 24 hours and remained at later time points.

Suberoylanilide Hydroxamic Acid Effects on 13-S-Hydroxyoctadecadienoic Acid Formation. 13-S-Hydroxyoctadecadienoic acid is the primary metabolite of 15-lipoxygenase-1. To determine whether the suberoylanilide hydroxamic acid-induced expression of 15-lipoxygenase-1 also increased 13-S-hydroxyoctadecadienoic acid levels, 13-S-hydroxyoctadecadienoic acid was measured in treated and untreated cells. Treatment of Caco-2 cells with suberoylanilide hydroxamic acid for 24 hours increased endogenous 13-S-hydroxyoctadecadienoic acid levels by 2-fold compared with untreated control cells (Fig. 2). Furthermore, the 13-S-hydroxyoctadecadienoic acid levels were reduced upon addition of 15-lipoxygenase-1 inhibitors caffeic acid (2.2 μmol/L; ref. 15) or the more general lipoxygenase inhibitor nordihydroguaiaretic acid (10 μmol/L).

Effect of 15-Lipoxygenase Inhibition on Cell Growth. Caco-2 cells were treated with suberoylanilide hydroxamic acid or suberoylanilide hydroxamic acid plus caffeic acid for 0–96 h and cell growth examined. At 72 and 96 h, suberoylanilide hydroxamic acid reduced the cell count by 45% and 54% respectively compared with untreated control cells (Fig. 3). Inhibiting 15-lipoxygenase-1 activity with caffeic acid (2.2 μmol/L) attenuated the suberoylanilide hydroxamic acid-induced cell count reduction. Caffeic acid alone did not affect cell growth (data not shown).

Fig. 1. Suberoylanilide hydroxamic acid (SAHA) effects on 15-lipoxygenase-1 (15-LOX-1) expression in Caco-2 cells. Western blot analysis of cell lysates demonstrated dose- and time-dependent induction of 15-LOX-1 by SAHA. Actin is a control for the amount of protein loaded. Thirty micrograms of total protein were loaded per lane. The data show represents one of three separate experiments with similar results. A. Western blot analysis of cell lysates shows dose-dependent expression of 15-LOX-1 after treatment with SAHA for 24 hours. Lane 1, 15-LOX-1 standard; Lanes 2–8, SAHA-treated cells at 0.1, 0.5, 1, 2.5, 5, 10, and 25 μmol/L, respectively. B. Western blot analysis of cell lysates shows time-dependent expression of 15-LOX-1 after treatment with 2.5 μmol/L SAHA. Lane 1, 15-LOX-1 standard; Lanes 2–9, SAHA-treated cells at 0, 3, 6, 12, 24, 48, 72, and 96 hours, respectively.

Fig. 2. Suberoylanilide hydroxamic acid (SAHA) effects on 13-S-hydroxyoctadecadienoic acid (13-S-HODE) formation. 13-S-Hydroxyoctadecadienoic acid is the primary metabolite of 15-lipoxygenase-1. To determine whether the suberoylanilide hydroxamic acid-induced expression of 15-lipoxygenase-1 also increased 13-S-hydroxyoctadecadienoic acid levels, 13-S-hydroxyoctadecadienoic acid was measured in treated and untreated cells. Treatment of Caco-2 cells with suberoylanilide hydroxamic acid for 24 hours increased endogenous 13-S-hydroxyoctadecadienoic acid levels by 2-fold compared with untreated control cells (Fig. 2). Furthermore, the 13-S-hydroxyoctadecadienoic acid levels were reduced upon addition of 15-lipoxygenase-1 inhibitors caffeic acid (2.2 μmol/L; ref. 15) or the more general lipoxygenase inhibitor nordihydroguaiaretic acid (10 μmol/L).
Effects of 15-Lipoxygenase-1 Inhibition on Suberoylanilide Hydroxamic Acid-induced Cellular Differentiation and Apoptosis.

To determine whether 15-lipoxygenase-1 is involved in suberoylanilide hydroxamic acid-induced differentiation, alkaline phosphatase activity, a marker for differentiation, was measured in Caco-2 cells treated with suberoylanilide hydroxamic acid or suberoylanilide hydroxamic acid plus caffeic acid for 0 to 96 hours. Treatment of the cells with suberoylanilide hydroxamic acid resulted in an increase in alkaline phosphatase activity over time compared with untreated control cells (Fig. 4A). Inhibition of 15-lipoxygenase-1 activity with caffeic acid attenuated this effect.

To determine whether 15-lipoxygenase-1 also plays a role in suberoylanilide hydroxamic acid-induced apoptosis, DNA fragmentation assays were used to assess apoptosis in cells treated with suberoylanilide hydroxamic acid or suberoylanilide hydroxamic acid plus caffeic acid. At 72 hours, DNA laddering was observed for cells treated with suberoylanilide hydroxamic acid (Fig. 4B). Inhibition of 15-lipoxygenase-1 with caffeic acid blocked the suberoylanilide hydroxamic acid-induced DNA laddering. No laddering was observed for untreated control cells or for cells treated with caffeic acid alone. Similar results were observed at 96 hours of treatment (data not shown). Suberoylanilide hydroxamic acid-induced apoptosis and its inhibition by caffeic acid were also confirmed by measuring caspase 3 activity. At 72 hours, caspase 3 activity of Caco-2 cells treated with suberoylanilide hydroxamic acid increased over 3.7-fold compared with untreated control cells (Fig. 4C). Inhibition of 15-lipoxygenase-1 activity with caffeic acid attenuated the caspase 3 activity. Similar results were observed at 96 hours of treatment (data not shown).

DISCUSSION

The present study is the first to establish that HDAC inhibitor-induced apoptosis and 15-lipoxygenase-1 expression are linked mechanistically. We found that the HDAC inhibitor suberoylanilide hydroxamic acid induced 15-lipoxygenase-1 expression in a dose- and time-dependent manner and induced growth arrest and apoptosis. This is the first study to show a relationship between suberoylanilide hydroxamic acid and 15-lipoxygenase-1 expression. Inhibition of 15-lipoxygenase-1 with 2.2 μmol/L caffeic acid, a concentration shown to be specific for 15-lipoxygenase-1 inhibition, resulted in the attenuation of the suberoylanilide hydroxamic acid-induced effects of growth inhibition, apoptosis, and, possibly, differentiation. The induction of 15-lipoxygenase-1 expression, which is suppressed in colon tumor cells, likely will play an important role in the potential clinical
effects of suberoylanilide hydroxamic acid in colorectal cancer prevention and therapy.

Suberoylanilide hydroxamic acid is one of a number of chemically diverse agents, including the short chain fatty acid NaBT, that can inhibit HDAC activity. NaBT is an extensively studied low-potency HDAC inhibitor that has antitumor activity and can induce differentiation and apoptosis in various cancer cell lines. In human colorectal carcinoma Caco-2 cells, Kamitani et al. (14) found that NaBT induces the expression of 15-lipoxygenase-1 and differentiation and apoptosis. Although this study provided the first evidence that a HDAC inhibitor can up-regulate 15-lipoxygenase-1 expression in human colorectal carcinoma cells, it did not establish a mechanistic link between 15-lipoxygenase-1 and HDAC inhibitor-induced differentiation and apoptosis. The authors did speculate that 15-lipoxygenase-1 induction may have been associated with differentiation and apoptosis after treatment with 5 mmol/L NaBT. Kamitani et al. (14) used the nonspecific lipoxygenase inhibitor nordihydroguaiaretic acid to assess the relationship of 15-lipoxygenase-1 with differentiation and apoptosis. Furthermore, nordihydroguaiaretic acid also can influence the redox state of cells. Because nordihydroguaiaretic acid can influence other lipoxygenases (besides 15-lipoxygenase-1) and the redox state, the authors concluded that the importance of 15-lipoxygenase-1 expression in the mechanism of NaBT-induced differentiation and apoptosis was not clear. Building on these findings, our presently reported results based on the specific inhibition of 15-lipoxygenase-1 show for the first time that HDAC inhibitor-induced apoptosis is mechanistically linked to 15-lipoxygenase-1. We also used the more clinically relevant HDAC inhibitor suberoylanilide hydroxamic acid at clinically achievable concentrations.

A broad spectrum of transformed cells are sensitive to suberoylanilide hydroxamic acid-induced growth inhibition in in vitro and in vivo studies (1). Suberoylanilide hydroxamic acid is in phase I and II clinical trials for the treatment of various cancers and has shown antitumor activity at doses that are well tolerated by patients (11, 12). These preclinical results and the clinical trials show that suberoylanilide hydroxamic acid targets transformed cells in preference to normal cells (9).

The inhibition of HDAC by suberoylanilide hydroxamic acid occurs through a direct interaction with the catalytic site of the enzyme as shown by X-ray crystallography studies (17). Inhibition of HDAC activity results in the accumulation of acetylated histones H2a, H2b, H3, and H4. The result of HDAC inhibition is believed not to have a generalized effect on the genome but rather only effects the transcription of a small subset of the genome (18). Evidence provided by DNA microarrays with malignant cell lines cultured with a HDAC inhibitor shows that there are a small (1 to 2%) number of genes of which the products are altered (7, 18, 19). From this study and others (14), it appears that 15-lipoxygenase-1 is among the few genes altered by HDACs in colorectal carcinoma cells.

We have investigated the effect of suberoylanilide hydroxamic acid on human colorectal cancer cell lines. We have shown that suberoylanilide hydroxamic acid can induce 15-lipoxygenase-1 expression in colorectal cancer cells and that inhibition of 15-lipoxygenase-1 can attenuate the effects of suberoylanilide hydroxamic acid in these cells. These findings indicate an important role for 15-lipoxygenase-1 in mediating suberoylanilide hydroxamic acid-induced and, more generally, HDAC inhibitor-induced growth inhibition, differentiation, and apoptosis in colorectal cancer cells. Furthermore, suberoylanilide hydroxamic acid can induce 15-lipoxygenase-1 at concentrations in the low micromolar range in comparison to NaBT, which has been reported to induce 15-lipoxygenase-1 with 5 mmol/L concentration range. Although appearing to have antitumor activity, NaBT has been hampered in its clinical development by a short half-life (5 minutes) and the inability to achieve a therapeutic level in plasma. The fact that suberoylanilide hydroxamic acid can induce 15-lipoxygenase-1 in the 1 to 2 mmol/L concentration range is of clinical relevance and may also explain in part the success of suberoylanilide hydroxamic acid as a therapeutic agent in treatment of colon cancer.

The present study shows that the induction of 15-lipoxygenase-1 expression is involved mechanistically in suberoylanilide hydroxamic acid-induced growth arrest and apoptosis in colorectal cancer cells. Establishing this mechanistic link emphasizes the importance of future investigations of the mechanisms by which HDAC inhibitors such as suberoylanilide hydroxamic acid up-regulate 15-lipoxygenase-1. These mechanisms remain unknown and could occur at the transcriptional and/or posttranslational levels because 15-lipoxygenase-1 regulation occurs at both levels (20, 21). Additional mechanistic studies of 15-lipoxygenase-1 regulation in colorectal cancer cells potentially will advance the development of effective agents for colorectal cancer prevention and therapy.

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