ESE-1/EGR-1 pathway plays a role in tolfenamic acid-induced apoptosis in colorectal cancer cells

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Abstract

Nonsteroidal anti-inflammatory drugs (NSAIDs) are known to prevent colorectal tumorigenesis. Although antitumor effects of NSAIDs are mainly due to inhibition of cyclooxygenase activity, there is increasing evidence that cyclooxygenase-independent mechanisms may also play an important role. The early growth response-1 (EGR-1) gene is a member of the immediate-early gene family and has been identified as a tumor suppressor gene. Tolfenamic acid is a NSAID that exhibits anticancer activity in a pancreatic cancer model. In the present study, we investigated the anticancer activity of tolfenamic acid in human colorectal cancer cells. Tolfenamic acid treatment inhibited cell growth and induced apoptosis as measured by caspase activity and bioelectric impedance. Tolfenamic acid induced EGR-1 expression at the transcription level, and analysis of the EGR-1 promoter showed that a putative ETS-binding site, located at −400 and −394 bp, was required for activation by tolfenamic acid. The electrophoretic mobility shift assay and chromatin immunoprecipitation assay confirmed that this sequence specifically bound to the ETS family protein epithelial-specific ETS-1 (ESE-1) transcription factor. Tolfenamic acid also facilitated translocation of endogenous and exogenous ESE-1 to the nucleus in colorectal cancer cells, and gene silencing using ESE-1 small interfering RNA attenuated tolfenamic acid-induced EGR-1 expression and apoptosis. Overexpression of EGR-1 increased apoptosis and decreased bioelectric impedance, and silencing of endogenous EGR-1 prevented tolfenamic acid-induced apoptosis. These results show that activation of ESE-1 via enhanced nuclear translocation mediates tolfenamic acid-induced EGR-1 expression, which plays a critical role in the activation of apoptosis.

Introduction

Epidemiologic studies have reported an inverse correlation between colon cancer incidence and the use of nonsteroidal anti-inflammatory drugs (NSAIDs; ref. 1). As a result, the regular use of NSAIDs as a chemopreventive strategy for colorectal cancer is now generating a great deal of interest because NSAIDs inhibit cyclooxygenase (COX) and prostaglandin biosynthesis, a pathway strongly associated with colorectal carcinogenesis (2). NSAIDs induce cell cycle arrest and apoptosis during different stages of colorectal tumorigenesis (3–5) and inhibit angiogenesis, invasion, and metastasis in vivo (6, 7).

Tolfenamic acid has been broadly used for treatment of migraines and has shown fewer upper gastrointestinal side effects than other NSAIDs (8). There is recent evidence showing that tolfenamic acid also affects metastasis and tumorigenesis in pancreatic cancer models (9, 10). Tolfenamic acid reduces the expression of vascular endothelial growth factor and its receptor (9, 10), which are mediated in part by down-regulation of specificity proteins (Sp). Like other NSAIDs, tolfenamic acid acts by inhibiting COX and prostaglandin biosynthesis; however, the molecular basis for induction of apoptosis and the scope of its action in colorectal cancer has not been reported.

One potential molecular target is early growth response-1 (EGR-1), an immediate-early gene encoding a zinc finger transcription factor; EGR-1 regulates the expression of genes involved in growth control or survival (11). The EGR-1 gene is stimulated by many extracellular signaling molecules including cytokotic metabolites, hormones, growth factors, and neurotransmitters. The growth-promoting activity by EGR-1 has been observed in various human cancer models such as prostate (12), skin (13), and kidney (14). Various growth factors target the EGR-1 gene and mediate the mitogenic signaling cascade (15). Despite the discovery of EGR-1 as a growth-promoting protein, several reports have described EGR-1 as a proapoptotic protein. Indeed, EGR-1 is down-regulated in neoplasia and an array of tumor cell lines (16), and constitutive expression of EGR-1 suppressed growth and transformation in tumor cells (17). Recently, we and others reported that EGR-1 acts as a proapoptotic gene in human colorectal cancer cells.
cancer cells. EGR-1 is a target of chemopreventive compounds including NSAIDs, LY294002, peroxisome proliferator-activated receptor γ ligands (18–21), and dietary compounds such as epicatechin gallate and 1,1-bis(3-indolyl)-1-(p-substituted phenyl) methanes (22–24). In addition, EGR-1 mediates several proapoptotic proteins, including p33, phosphatase and tensin homologue, activating transcription factor 3, and NSAID-activated gene-1 (NAG-1), which appear to play a critical role in mediating apoptosis in human colorectal cancer cells (18–21, 25, 26).

Thus, the proapoptotic activity of EGR-1 may depend on the cell type and the nature of the cytotoxic stimulus.

The current study was done to elucidate whether tolfenamic acid affects human colorectal tumorigenesis. Here, we report, for the first time, that tolfenamic acid affects human colorectal tumorigenesis. The proapoptotic activity of EGR-1 may depend on the cell type and the nature of the cytotoxic stimulus.

**Materials and Methods**

**Materials**

Tolfenamic acid and SC-560 were purchased from Cayman Chemical, and 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furanone was described previously (20). All other NSAIIDs were purchased from Sigma-Aldrich. Antibodies for EGR-1 (sc-110), ESE-1 (sc-17306), actin (sc-1615), control small interfering RNA (siRNA; sc-37007), and ESE-1 siRNA (sc-37851) were purchased from Santa Cruz Biotechnology, and antibody for poly(ADP-ribose) polymerase (PARP) was purchased from Cell Signaling. Antibodies for V5 (R960-25) and Sp1 (07-124) were purchased from Invitrogen and Upstate Biotechnology, respectively. TRITC (1031-03) was purchased from Southern Biotech, and antibody for NAG-1 was described previously (27). All chemicals were purchased from Fisher Scientific unless otherwise specified.

** Constructs**

Full-length ESE-1 and ELF-1 cDNAs were amplified using ReadyMix Taq polymerase (Sigma) with the following primers: ESE-1 forward 5'-CCAACCTCATCTCTCCTTCCCCCTACC-3' and reverse 5'-GTTCGACTCTGGA-GAACCTTTCTCCTC-3' and ELF-1 forward 5'-GGAATT-TTTCTCATGGTGGTGGTGGG-3' and reverse 5'-AAAAGGTTGGGTGGTGGTGGG-3'. PCR was done for 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. The amplified products were subcloned into pcDNA3.1/V5-His-TOPO (Invitrogen) to generate V5-His-tagged proteins. EGR-1 (pcDNA3.1/EGR-1/NEO) expression vector was described previously (19). For deletion analysis of the EGR-1 promoter, pEGR1-1260/+35 (20) was serially deleted using the Erase-a-Base System (Promega) according to the manufacturer’s protocols. The internal deletion mutant clones for ETS-binding site (EBS), Sp1, SRF, and C/EBP of the EGR-1 promoter were generated with the pEGR1-1260/+35 wild-type clone using the QuickChange II site-directed Mutagenesis Kit (Stratagene).

**Cell Culture and Treatment**

Human colorectal carcinoma cell lines HCT-116, SW480, LoVo, and HT-29 were purchased from American Type Culture Collection. HCT-116 and HT-29 cells were maintained in McCoy’s 5A medium, and SW480 and LoVo cells were maintained in RPMI 1640 and Ham’s F-12 medium, respectively. All media were supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were incubated at 37°C under a humidified atmosphere of 5% CO2 until cells were 70% to 80% confluent. The cells were then treated with different concentrations of tolfenamic acid at different time points as indicated in the figure legends.

**Cell Proliferation**

A cell proliferation assay was done using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) as described previously (28). Briefly, cells were seeded at the concentration of 2,000 per well in 96-well tissue culture plates in four replicates and maintained overnight. The cells were then treated with 0, 1, 5, 10, 20, 30, and 50 μmol/L tolfenamic acid. At 0, 24, and 48 h after treatment, 20 μL CellTiter 96 Aqueous One solution was added to each well, and the plate was incubated for 1 h at 37°C. Absorbance at 490 nm was recorded in an ELISA plate reader (Bio-Tek Instruments).

**Caspase-3/7 Enzyme Activity**

Apoptosis was measured using Apo-ONE Homogeneous Caspase-Glo-3/7 Assay kit (Promega) according to the manufacturer’s protocol. After harvest of the cells using radioimmunoprecipitation assay buffer containing protease inhibitors, the cell lysates (50 μg protein) in 50 μL volume were mixed with 50 μL Caspase-Glo-3/7 reagent in 96-well plates and incubated at room temperature in the dark for 1 h. The luminescence was measured using a plate-reading luminometer (FLX800; Bio-Tek).

**Electrical Impedance Measurements**

The cellular electrical impedance method measures the frequency-dependent resistance and reactance of a cell-covered, thin-film gold electrode as a function of time. To perform these measurements, a data acquisition and analysis system was implemented using LabVIEW. A reference voltage source, with 50 Vrms reference signal via a series 1 MΩ resistor to the electrode array. A National Instruments SCXI-1331 switch made successive connections between the various working electrodes and the counter electrode of each array. A SR830 lock-in amplifier (Stanford Research) measured the electrode voltage. The input impedance of the lock-in amplifier was equivalent to a parallel resistor and capacitor combination of 10 MΩ and 10 pF, respectively. Direct measurements of the cell parasitic capacitances were made using a LCR meter and incorporated into a circuit model to estimate the impedance.
based on the lock-in voltage measurements (29). The preliminary naked scan also checked for any debris on electrodes as well as electrode defects. A 1 s naked scan, sampled at a rate of 32 Hz, was then done for the naked electrodes. The electrodes were then inoculated with 400 μL medium containing HCT-116 cells having a concentration of 10⁵ cells/mL. During the cellular attach scan, data were acquired at a rate of 32 Hz for 2 s using a 30 ms filter time constant and a 12 dB/decade roll off. Averages and SD estimates were obtained from the sampled data points. During the experiments, cell-inoculated electrodes were kept in a cell culture incubator that maintained the temperature at 37°C and the CO₂ level at 5%.

**Transient Transfection and Luciferase Assay**

Transient transfection was done using Lipofectamine (Invitrogen) according to the manufacturer’s instructions. The cells were plated in 12-well plates at a concentration of 2 × 10⁵ per well and left overnight. The cells were then transfected with the EGR-1 promoter (0.5 μg DNA) and the pRL-null vector (0.05 μg DNA) for 5 h. For the cotransfection experiment, 0.25 μg of the EGR-1 promoter and 0.25 μg of the expression vector were cotransfected with 0.05 μg of the pRL-null vector. The cells were fed fresh medium (McCoy’s 5A medium with 10% fetal bovine serum) overnight and treated with DMSO or 30 μmol/L tolfenamic acid for 24 h. After cell harvest using 1/2 luciferase lysis buffer, luciferase activity was determined and normalized to the pRL-null luciferase activity using a dual luciferase assay kit (Promega).

**RNA Interference**

HCT-116 cells were transfected with control siRNA or ESE-1 siRNA at a concentration of 100 nmol/L, using TransIT-TKO transfection reagent (Mirus), as described previously (28). After a 24 h transfection, the cells were serum starved overnight and then treated with DMSO or 30 μmol/L tolfenamic acid for 2 h. For EGR-1 interference, sense EGR-1 oligo (5'-ZECGGGCGCGGGAACFOT-3') and antisense EGR-1 oligo (5'-AEZGTTCCCCCGCGCCCGCCA-3') were synthesized by Invitrogen and transfected using TransIT-TKO transfection reagent (Mirus) at a concentration of 200 nmol/L. After 24 h transfection, the cells were serum starved overnight and treated with DMSO or 30 μmol/L tolfenamic acid for 2 h.

**RNA Isolation and Reverse Transcription-PCR**

Total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. Total RNA
(1 μg) was reverse transcribed with an iScript cDNA kit (Bio-Rad) according to the manufacturer’s instructions. PCR was carried out using ReadyMix Taq polymerase (Sigma) with primers for human EGR-1 and GAPDH as follows: EGR-1 forward 5′-CTGCGACATCTGTGGAA-GAA-3′ and reverse 5′-TGTCCTGGGAGAAAAGGTTG-3′ and GAPDH forward 5′-GGGCTGCTTTTAACTCTGGT-3′ and reverse 5′-TGCCAGGTITTTCTAGACGG-3′. PCR was done at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min for 25 cycles (EGR-1 and NAG-1) or 19 cycles (GAPDH).

Western Blot Analysis and Immunofluorescence

Western blot and immunofluorescence were done as described previously (28, 30). For immunofluorescence, the cells were incubated with primary antibody for V5tag protein, 1× binding buffer, 2.5% glycerol, 5 mmol/L MgCl2, 50 ng/μL poly(dI-dC), and 0.05% NP-40 at room temperature for 20 min. For competition assay, nuclear extracts were preincubated with the unlabeled oligonucleotide (10 μmol/L) and mutant 5′-AGCACCGCATGACGAGCATGGAAGCAGCGCATG-3′. Electrophoretic mobility shift assay (EMSA) was done using the LightShift Chemiluminescent EMSA kit according to the manufacturer’s protocols (Pierce). Briefly, biotin-labeled oligonucleotide (10 n mol/L) was incubated with nuclear extract (5 μg protein), 1× binding buffer, 2.5% glycerol, 5 mmol/L MgCl2, 50 ng/μL poly(dl-dC), and 0.05% NP-40 at room temperature for 20 min. For supershift assay, nuclear extracts were precubated with 0.6 μg antibodies (ESE-1, Sp1, and IgG) in 1× binding buffer (Promega) for 15 min before binding reactions. DNA-protein complexes were resolved by 5% nondenaturing polyacrylamide gel and transferred to a nylon membrane followed by chemiluminescent nucleic acid detection according to the manufacturer’s protocols.

Chromatin Immunoprecipitation Assay

HCT-116 cells were plated on a 100 mm culture dish and serum starved overnight. Cells were treated with DMSO or 30 μmol/L tolfenamic acid for 2 h and fixed with 1% formaldehyde at 37°C for 10 min. The fixed cells were scraped into conical tubes, pelleted, and lysed in lysis buffer [1% SDS, 10 mmol/L EDTA, 50 mmol/L Tris-HCl, 0.5% sodium deoxycholate, and 1× protease inhibitor cocktail (Roche)]. The nuclei were washed with cold saline and centrifuged, and the DNA was precipitated with 200 μL absolute ethanol. The DNA-protein complex was sheared by sonication, and the samples were diluted with 2× ChIP dilution buffer (Cell Signaling Technology). The samples were incubated with IgG-agarose beads (Cell Signaling Technology) for 2 h at 4°C. The beads were collected by magnetic beads separation, and the DNA-protein complexes were eluted with 0.2 M NaOH and 1 M EDTA. The DNA-protein complexes were incubated with proteinase K for 1 h at 55°C and then extracted with phenol/chloroform/isoamyl alcohol. The DNA was precipitated with 100 μL 95% ethanol, dried, and resuspended in 100 μL TE buffer. The recovered DNA was used as the input control.

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(pH 8.0)] containing protease and phosphatase inhibitors as described (28). DNA was sheared to fragments by sonication four times for 10 s at 50% constant maximal power. The sonicated cell supernatant was diluted 10-fold in immunoprecipitate buffer (0.1% SDS, 1% Triton X-100, 0.1% sodium deoxycholate, 140 mmol/L NaCl), and 1% of diluted cell supernatant was kept as a control (input). The chromatin was precleared with protein A/G agarose slurry for 1 h at 4°C. The precleared supernatant was incubated with 50 μL protein A/G agarose, 20 μg heat-denatured salmon sperm DNA, 50 μg bovine serum albumin, and 10 μg antibodies against ESE-1 or IgG overnight at 4°C. The immunocomplexes were washed five times with wash buffer [20 mmol/L Tris (pH 8.0), 0.1% Triton-X, 150 mmol/L NaCl, 2 mmol/L EDTA], and eluted with elution buffer (1% SDS, 100 mmol/L NaHCO₃). Protein-DNA cross-links were reversed with 27 μL of 5 mol/L NaCl and 1 μL of 10 mg/mL RNase A at 65°C for 4 h and incubated with 2 μL of 20 mg/mL proteinase K at 45°C for 1 h. The DNA was purified by phenol extraction and ethanol precipitation. The region between −530 and −345 of the human EGR-1 promoter was amplified using the 5’-GGCCCACACGCACGCCACCTCCCGCTCCT3’ and 5’-CCACTCAATAAGGTGCTGCGTTGCG-3’ primers. The 186 bp products were resolved on a 2% agarose gel and visualized under UV light.

Figure 3. EBS located at −400 to −394 of the EGR-1 promoter is necessary for EGR-1 transcription induced by tolfenamic acid. A, structures of EGR-1 sequential deletion constructs and deletion promoter assay. The EGR-1 promoter fragments of a different length but with the same 3′-end were cloned into pGL3 Basic. HCT-116 cells were cotransfected with 0.5 μg of each reporter construct containing the EGR-1 promoter and 0.05 μg pRL-null vector using Lipofectamine. After growth overnight with fresh medium, the cells were treated with 30 μmol/L tolfenamic acid for 24 h. Luciferase activity was measured as a ratio of firefly luciferase signal/Renilla luciferase signal. Mean ± SD of three independent transfections. B, putative transcription binding sites within the −403 to +35 region in the EGR-1 promoter and luciferase assay with internal deletion clones. The boxes in the promoter represent the binding site of indicated transcription factors and are used for construction of internal deletion clones. HCT-116 cells were cotransfected with 0.5 μg of each internal deletion construct of the EGR-1 promoter and 0.05 μg pRL-null vector using Lipofectamine and treated with 30 μmol/L tolfenamic acid for 24 h. X axis, fold induction over vehicle as 1.0. Mean ± SD of three independent transfections. C, effect of ESE-1 overexpression on EGR-1 transactivation. HCT-116 cells were cotransfected with wild-type EGR-1 promoter (pEGR1-1260/+35 wild-type or internal deletion clone pEGR1-1260/+35ΔEBS) in the presence of empty (E), ESE-1, or ELF-1 expression vector using Lipofectamine. After growth overnight with fresh medium, the cells were treated with 30 μmol/L tolfenamic acid for 24 h. X axis, fold induction over vehicle as 1.0. Mean ± SD of three independent transfections. The overexpression of ELF-1 and ESE-1 was confirmed by Western analysis using V5 tag (GKPIPNPLLGLDST) antibody.
Statistical Analysis
Statistical analysis was done using a Student’s unpaired t test, with statistical significance set at $P < 0.05$, $P < 0.01$, and $P < 0.001$.

Results
Identification of Tolfenamic Acid as a Strong Inducer of EGR-1 and NAG-1
Several studies indicate that EGR-1 plays a role as a tumor suppressor gene (16, 17, 25, 26), and EGR-1 mediates proapoptotic activity of the NAG-1 by NSAIDs (20). To identify a potent NSAID that induces the two proapoptotic EGR-1 and NAG-1 genes in human colorectal cancer cells, we screened induction responses in HCT-116 cells by conventional (diclofenac, ibuprofen, aspirin, tolfenamic acid, and naproxen) and COX-2 [SC-236, 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furanone, and celecoxib] or COX-1 (SC-560) selective inhibitors. Because EGR-1 is an early induced gene (20, 21), we initially treated cells with 30 μmol/L NSAIDs for 6 h. As shown in Fig. 1A, the expression of EGR-1 and NAG-1 was obviously increased in the cells treated with tolfenamic acid and SC-560. Celecoxib also highly increased EGR-1 but not NAG-1 expression. Because induction of EGR-1 was more dramatic in tolfenamic acid-treated cells than in SC-560-treated cells, tolfenamic acid was selected for further study. EGR-1 protein began to increase at 1 h, reached a plateau at 2 h, and gradually decreased after 8 h, whereas after 30 μmol/L tolfenamic acid treatment, NAG-1 expression started to increase at 2 h and continued to increase until 24 h (Fig. 1B). Sp1 expression was decreased after 8 h, consistent with the data seen in pancreatic cancer cells (9, 10). We observed a significant increase in EGR-1 transcripts after 30 min and subsequent induction of NAG-1 transcripts in the cells treated with 30 μmol/L tolfenamic acid for 2 h. The in vivo DNA-protein complexes were cross-linked by formaldehyde treatment, and chromatin pellets were extracted and sonicated. The associated EGR-1 DNA was isolated as described inMaterials and Methods. The sequence of the human EGR-1 promoter region (−530/−345) was amplified by PCR primer pairs (arrows). The input represents PCR products obtained from 1% aliquots of chromatin pellets before immunoprecipitation.

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Statistical analysis was done using a Student’s unpaired t test, with statistical significance set at $P < 0.05$, $P < 0.01$, and $P < 0.001$.

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Tolfenamic Acid Suppresses Cell Growth and Increases Apoptosis in HCT-116 Cells

Because tolfenamic acid exhibited antitumorigenic activity in pancreatic cancer cells (9, 10), HCT-116 human colorectal cancer cells were incubated with 0, 1, 5, 10, 20, 30, and 50 μmol/L tolfenamic acid for 24 and 48 h, and cell proliferation was measured. As shown in Fig. 2A, HCT-116 cells treated with 20, 30, and 50 μmol/L tolfenamic acid significantly decreased cell growth after 24 and 48 h. Longer exposure of tolfenamic acid (48 h) also significantly inhibited cell growth at a lower concentration of tolfenamic acid (10 μmol/L). To further examine tolfenamic acid-induced apoptosis, dynamic and noninvasive cellular electrical microimpedance measurements were done. This method measures the frequency- and time-dependent resistance and reactance of a cell-covered, thin-film gold electrode and is very sensitive to apoptosis. As shown in Fig. 2B, 20 and 30 μmol/L tolfenamic acid decreased the normalized impedance components consistent with increasing numbers of apoptotic cells. Both resistance and reactance using vehicle-treated cells increased in cell-cell and cell-substrate adhesion. Tolfenamic acid-treated cells, however, produced normalized resistances and reactances that decreased after they reached their maximum. These changes are, therefore, represented as the induction of apoptosis (31, 32). To investigate the effects of tolfenamic acid on caspase-dependent apoptosis, HCT-116 cells were treated with 0, 1, 5, 10, 20, 30, and 50 μmol/L tolfenamic acid for 24 h, and caspase-3/7 activity and apoptosis-related cleaved PARP were measured by Western blot analysis. As shown in Fig. 2C, caspase-3/7 activity was slightly increased in the cells treated with 20 μmol/L tolfenamic acid and dramatically increased by 4.8- and 5.1-fold in cells treated with 30 and 50 μmol/L tolfenamic acid, respectively. In addition, PARP cleavage was dramatically increased in HCT-116 cells treated with 30 and 50 μmol/L tolfenamic acid with a slight increase in 20 μmol/L tolfenamic acid-treated cells (Fig. 2D).

Figure 5. Tolfenamic acid mediates translocation of ESE-1 into the nucleus, and siRNA-mediated inhibition of ESE-1 expression suppressed tolfenamic acid-induced EGR-1 expression and apoptosis. A, nuclear translocation of endogenous ESE-1. HCT-116 cells were serum starved overnight and then treated with 30 μmol/L tolfenamic acid for 2 h. Nuclear and cytosol fractions were isolated, and Western blot analysis was done for ESE-1, EGR-1, and actin antibodies. B, nuclear translocation of exogenous ESE-1. HCT-116 cells were transiently transfected with pcDNA3.1/V5-His/ESE-1 expression vector using Lipofectamine as described in Materials and Methods. After serum starvation overnight, the cells were treated with 30 μmol/L tolfenamic acid for 1 h. Nuclear and cytosol fractions were isolated, and Western blot was done for V5 and actin antibodies. C, immunohistochemistry. After transfection as described in B, the cells were serum starved overnight and treated with 30 μmol/L tolfenamic acid for 1 h. The cells were fixed and stained with anti-V5 antibody overnight and subsequently secondary anti-mouse TRITC conjugate (red). 4,6-Diamidino-2-phenylindole staining was used to visualize the nucleus of the cells (blue). Magnifications, ×400. Arrows, ESE-1 localization. D, effect of ESE-1 knockdown on tolfenamic acid-induced EGR-1 expression and apoptosis. HCT-116 cells were transfected with control siRNA (100 nmol/L) or ESE-1 siRNA (100 nmol/L) for 24 h using a Transit-TKO transfection reagent. After serum starvation overnight, the cells were treated with 30 μmol/L tolfenamic acid for 2 h (left) or for 24 h (right). Western blot analysis was done for ESE-1, EGR-1, PARP, and actin antibodies, and caspase-3/7 activity was measured as described in Materials and Methods. The same cell lysates were used to measure caspase-3/7 activity as described in Materials and Methods (bottom). Mean ± SD of three independent experiments.
Therefore, we decided to use 30 μmol/L tolfenamic acid for further experiments because this concentration induced both apoptosis and EGR-1/NAG-1 gene expression.

Tolfenamic Acid Induces EGR-1 at the Transcription Level

The effects of tolfenamic acid on EGR-1 expression at the transcriptional level were investigated using an EGR-1 promoter in transient transfection studies. HCT-116 cells were transfected with sense (S) or antisense (AS) oligo for human EGR-1 as described in Materials and Methods. The representative time-dependent normalized resistances and reactances shown here were scanned at 5.62 and 100 kHz, respectively. Filled symbols, cell measurements with DMSO; open symbols, cells treated with 30 μmol/L tolfenamic acid. For the sake of clarity, symbols are selectively marked.

Figure 6. EGR-1 induces apoptosis and mediates tolfenamic acid-induced apoptosis. A, apoptosis detection after EGR-1 overexpression. HCT-116 cells were transfectected with the empty or EGR-1 expression vector. PARP cleavage was measured by Western blot analysis (top) and caspase-3/7 activity was determined (bottom) as described in Materials and Methods. Mean ± SD of three independent experiments. B, effect of EGR-1 knockdown on tolfenamic acid-induced apoptosis. HCT-116 cells were transfected with sense (S) or antisense (AS) oligo for human EGR-1 as described in Materials and Methods. The representative time-dependent normalized resistances and reactances shown here were scanned at 5.62 and 100 kHz, respectively.

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The effects of tolfenamic acid on EGR-1 expression at the transcriptional level were investigated using an EGR-1 promoter in transient transfection studies. HCT-116 cells were transfected with different promoter constructs spanning the −1260 to +35 promoter regions as shown in Fig. 3A. Tolfenamic acid treatment increased luciferase activity by 5.6, 5.7, and 5.5-fold in cells transfected with pEGR1-1260/+35, pEGR1-836/+35, and pEGR1-403/+35, respectively, indicating that the −403 and +35 region of the promoter was sufficient for tolfenamic acid-induced EGR-1 transactivation. To further identify potential regulatory cis-acting elements that mediate the stimulatory effects of tolfenamic acid, the Transcription Element Search System4 was used to search for conserved transcription factor binding sites within the −403 and +35 region. The EGR-1 promoter (pEGR1-403/+35) contains multiple transcription factor binding sites including EBS, Sp1, SRF, and C/EBP (Fig. 3B, top). We constructed internal deletion clones lacking specific binding sites as indicated in Fig. 3B. HCT-116 cells were transfected with these deletion constructs and treated with DMSO or 30 μmol/L tolfenamic acid for the sake of clarity, symbols are selectively marked.

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D, expression of EGR-1, NAG-1, and COX-2 in other colorectal cancer cells. SW480, LoVo, and HT-29 cells were grown, serum starved overnight, and treated with 30 μmol/L tolfenamic acid, sulindac sulfide (SS) and SC-560 for 2 h. Western blot analysis was done for EGR-1, NAG-1, COX-2, and actin antibodies.

4 http://www.cbil.upenn.edu/cgi-bin/tess/tess

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24 h, and luciferase activity was measured. As shown in Fig. 3B, in cells transfected with wild-type pEGR1-1260/+35 promoter, tolfenamic acid increased luciferase activity by 4-fold, whereas tolfenamic acid-induced activity was significantly decreased in cells transfected with a promoter construct lacking an EBS1 (pEGR1-1260/+35AEBS1). Moreover, deletion of another EBS (pEGR1-1260/+35AEBS2) also showed a significant decrease of luciferase activity. Interestingly, deletion of a SRF site (pEGR1-1260/+35ΔSRF2) resulted in increased luciferase activity induced by tolfenamic acid. Other deletion constructs did not influence tolfenamic acid-induced luciferase activities. These data suggest that the EBS region of the EGR-1 promoter may play a role in tolfenamic acid-induced EGR-1 transactivation.

EBS is characterized by a 5'-GG(A/T)-3' DNA core motif, and all ETS proteins contain a highly conserved DNA-binding domain (ETS domain) and bind to this site as a monomer (33). To obtain further evidence that tolfenamic acid induces EGR-1 through EBS, we cotransfected the EGR-1 promoter constructs and expression vectors for ESE-1, ELF-1, and ETS-1 into HCT-116 cells and determined luciferase activity. Expression of transfected vectors was confirmed by Western blot analysis (Fig. 3C, right). ESE-1 expression caused a dramatic increase of tolfenamic acid-induced luciferase activity compared with empty vector-transfected cells. Interestingly, ESE-1 expression attenuated the increase of EGR-1 promoter activity in cells transfected with a construct lacking the EBS1 (−400 to −394), whereas the deletion clone lacking the EBS2 (−372 to −318) did not show dramatic attenuation of tolfenamic acid-induced activation (Fig. 3C), suggesting that EBS1 is most likely required for activation of the EGR-1 promoter induced by tolfenamic acid. ELF-1 expression decreased tolfenamic acid-induced EGR-1 promoter activity. We excluded the contribution of ELK-1 on tolenamic acid-induced EGR-1 expression because our reverse transcription-PCR and Western blot data showed that HCT-116 cells do not express ELK-1 (data not shown).

**Effect of Tolfenamic Acid on ESE-1 DNA-Binding Activity**

To examine whether the transactivation of the EGR-1 gene promoter is mediated by ESE-1, an EMSA was done using oligonucleotide probes derived from the EGR-1 promoter (−400 to −394) and nuclear extracts were prepared from HCT-116 cells treated with DMSO or tolfenamic acid for 2 h. As shown in Fig. 4A, tolfenamic acid treatment caused an induction of DNA-protein complex formation. Preincubation of nuclear extracts with 10×, 50×, and 100× excess unlabeled EBS oligonucleotide abolished the binding activity (Fig. 4B, lanes 3-5), whereas preincubation of nuclear extracts with unlabeled mutant EBS oligonucleotide did not compete with labeled oligonucleotides for binding (Fig. 4B, lanes 6-8), suggesting that the binding protein is specific for the EBS1 sequence. A supershift assay was done to confirm that ESE-1 protein binds to this site. Preincubation of nuclear extracts with ESE-1 antibody resulted in a supershift of the DNA-protein complexes, whereas antibodies for Sp1 and IgG did not affect the mobility of the DNA-protein complex, suggesting that ESE-1 is specifically bound to this site (Fig. 4C). Finally, a chromatin immunoprecipitation assay was done to confirm that ESE-1 binds to EBS1 in the EGR-1 promoter. As shown in Fig. 4D, immunoprecipitation of the chromatin-protein complex with ESE-1 amplified 186 bp of the PCR products, and tolfenamic acid treatment increased the binding affinity of ESE-1 to DNA. An aliquot (1%) of the total chromatin DNA was used for input. The chromatin immunoprecipitation assay was also done at 0, 30 min, 1 h, and 2 h time points, and we observed that the binding affinity increased as early as 30 min after treatment (data not shown). These results confirm that endogenous ESE-1 strongly binds to EBS on the EGR-1 promoter after treatment of HCT-116 cells with tolfenamic acid.

**Tolkenamic Acid Mediates Translocation of ESE-1 and EGR-1-Induced Apoptosis**

EGR-1 protein was strongly induced by tolfenamic acid at 2 h; however, ESE-1 protein levels were not changed by tolfenamic acid treatment for up to 24 h (data not shown). The activity of ESE-1 can be regulated by subcellular localization (34), therefore, effects of tolfenamic acid on nuclear translocation of ESE-1 were investigated. Figure 5A shows depleted cytosolic ESE-1 and increased expression of this protein in the nuclear fraction after tolfenamic acid treatment for 2 h. We also observed that tolfenamic acid-induced nuclear translocation of ectopically expressed ESE-1 in a manner similar to that shown for endogenous ESE-1 (Fig. 5B). To verify the Western blot data, we used immunofluorescence imaging to directly visualize localization of ESE-1 after tolfenamic acid treatment in cells transfected with an expression vector encoding V5-His epitope-tagged ESE-1. The result shows that the nuclear staining of ESE-1 was significantly increased in HCT-116 cells after treatment with 30 μmol/L tolfenamic acid for 1 h, confirming data obtained from the fractionation experiments (Fig. 5C). To determine the requirement of ESE-1 for EGR-1 expression, HCT-116 cells were transfected with control or ESE-1 siRNA and then treated with 30 μmol/L tolfenamic acid for 2 h. Immunoblotting of the lysates confirmed efficient knockdown of ESE-1 expression (Fig. 5D). Tolfenamic acid induced EGR-1 expression in control siRNA-transfected cells; however, ESE-1 siRNA transfection attenuated tolfenamic acid-induced EGR-1 expression (Fig. 5D, left). The same result was obtained from reverse transcription-PCR (data not shown). Two hours after tolfenamic acid treatment, a 1.6-fold higher caspase-3/7 enzyme activity was observed in control siRNA-transfected cells, whereas the activity was not changed in ESE-1 siRNA-transfected cells. To show the specific involvement of ESE-1 in tolfenamic acid-induced apoptosis, control or ESE-1 siRNA-transfected cells were treated with 30 μmol/L tolfenamic acid for 24 h, and caspase-3/7 enzyme activity and PARP cleavage were determined. As shown in Fig. 5D (right), 24 h after treatment with tolfenamic acid, high levels (5-fold) of caspase-3/7 enzyme activity were observed, indicative of cell death, whereas induction of caspase-3/7 enzyme...
activity (3.9-fold) was decreased in ESE-1 siRNA-treated cells (Fig. 5D, right). Similar results were obtained for PARP cleavage, where knockdown of ESE-1 decreased tolfenamic acid-induced PARP cleavage compared with cells transfected with control siRNA. Together, these results show that ESE-1 may contribute to tolfenamic acid-induced EGR-1 expression and cell death.

EGR-1 Contributes at Least in Part to Apoptosis by NAG-1 Expression in the Presence of Tolfenamic Acid

The involvement of EGR-1 in apoptosis was investigated in cells that overexpressed EGR-1. As shown in Fig. 6A, expression of EGR-1 increased PARP cleavage and caspase-3/7 enzyme activity. Interestingly, the proapoptotic protein NAG-1 was induced in EGR-1-overexpressing cells. In addition, suppression of endogenous expression of the EGR-1 gene using antisense oligonucleotide blunted tolfenamic acid-induced caspase-3/7 activity and NAG-1 expression, suggesting a linkage between EGR-1 expression, NAG-1 expression, and tolfenamic acid-induced apoptosis (Fig. 6B). We also measured electrical impedance after EGR-1 overexpression. Figure 6C shows that overexpression of EGR-1 dramatically reduced the normalized resistance and reactance. In addition, in the presence of EGR-1, tolfenamic acid decreased impedance components more dramatically, which is consistent with increasing numbers of apoptotic cells. These results show that EGR-1 mediates tolfenamic acid-induced apoptosis in HCT-116 cells.

Finally, to investigate whether tolfenamic acid modulates EGR-1-dependent NAG-1 expression in other human colorectal cancer cells, SW480, LoVo, and HT-29 cells were treated with tolfenamic acid, sulindac sulfide, and SC-560 for 2 h, and induction of EGR-1 and NAG-1 was determined. Tolfenamic acid increased EGR-1 and NAG-1 expression in SW480 (COX-2-null cells) and HT-29 (COX-2-expressing cells) but not in LoVo cells (COX-2-null cells). However, expression of EGR-1 and NAG-1 were not changed at 2 h after treatment of sulindac sulfide and SC-560 in any of the tested cells. These data indicate that NAG-1 induction by NSAIDs may be mediated in a cell context-dependent and COX-2-independent manner (Fig. 6D).

Discussion

Recent studies showed tolfenamic acid suppressed cell growth and angiogenesis in in vitro and in vivo models of pancreatic cancer (10). This response was mediated by decreased expression of Sp1, Sp3, and Sp4 proteins and subsequent down-regulation of Sp-dependent genes such as vascular endothelial growth factor and its receptor (9, 10). In this study, expression of Sp1 was also decreased after treatment with tolfenamic acid for 8 h, indicating that Sp1 down-regulation occurred and may contribute to tolfenamic acid-dependent antitumorigenic activity in colorectal cancer cells. However, Sp1 (Fig. 1A) and vascular endothelial growth factor (data not shown) expression did not change at earlier time points (6 h) in tolfenamic acid-treated cells. This suggests that tolfenamic acid affects antitumorigenic activity through Sp1 down-regulation at later time points (8-48 h), whereas tolfenamic acid induces apoptosis through EGR-1 induction at early time points (0-6 h).

ESE-1 protein belongs to the ETS family of transcription factors and is also identified as ERT (35), ELF3 (36), and ESEX (37). ESE-1 proteins contain a highly conserved ETS domain that recognizes the core motif GGAA(A/T). ESE-1 proteins are constitutively expressed in many types of epithelia, including lung and intestine (38), and regulate terminal differentiation of the epidermis (38, 39). ESE-1 have multiple functions in the transcriptional regulation of genes involved in epithelial differentiation and development of cancer (40), depending on cell context, and exact molecular mechanisms and their transcriptional targets need to be defined in cancer cells. The present study supports the antitumorigenic role of ESE-1 by facilitating cell growth arrest and inducing apoptosis in HCT-116 cells. We have also shown that knockdown of ESE-1 by RNA interference inhibited tolfenamic acid-induced cell death as assessed by caspase-3/7 enzyme activity and PARP cleavage, which is associated with decreased expression of EGR-1 (Fig. 5D). Furthermore, inhibition of EGR-1 attenuated cell death as well as NAG-1 expression by tolfenamic acid treatment (Fig. 6B). The main finding of the present study is that tolfenamic acid resulted in translocation of ESE-1 protein to the nucleus. This temporal pattern of nuclear ESE-1 translocation and binding of ESE-1 to the EGR-1 promoter nicely corresponded to tolfenamic acid-induced activation of EGR-1 gene expression at the transcriptional level. Under these experimental conditions, nuclear ESE-1 translocation was almost exclusively observed in cells 1 h after administration of tolfenamic acid. It is likely that ESE-1 has two distinct but separable biological functions, that is, apoptosis and transformation, which depend on the subcellular localization of this protein. Indeed, Prescott et al. reported that endogenous ESE-1 localize in the cytoplasm of human breast cancer cells; however, ectopically expressed ESE-1 in the nucleus resulted in the induction of apoptosis (34). These findings support our presumption that cytoplasmic accumulation of ESE-1 may be involved in the development of colon cancer, whereas nuclear translocation of ESE-1 enhances both EGR-1 transactivation and apoptosis. Interestingly, a recent study showed that p21-activated kinase 1 targets ESE-1 for phosphorylation, resulting in increased protein stability and higher accumulation in cells (41). Therefore, it is likely that tolfenamic acid may also trigger an upstream pathway followed by phosphorylation of ESE-1, which results in ESE-1 nuclear translocation. Future studies are needed to determine the molecular mechanism by which tolfenamic acid modifies ESE-1 by phosphorylation.

A combination of EMSA and chromatin immunoprecipitation assay of the binding of ESE-1 protein to the EGR-1 promoter has shown that the GGAA core sequence located in −400 to −394 is essential for the specific binding of ESE-1, indicating ESE-1 specificity in tolfenamic acid-induced
ECR-1 expression. However, we could not exclude the possibility that this EBS is not sufficient to fully activate the EGR-1 promoter, because the EGR-1 promoter assay using internal deletion clones is not enough to decrease the promoter activity to basal levels. Human EGR-1 promoter also contains the second EBS at −327 to 318 (EBS2). As shown in Fig. 3B, deletion on this site results in the marginal decrease of tolfenamic acid-induced luciferase activity, indicating that this site also contributes to the tolfenamic acid-induced EGR-1 expression. However, cotransfection experiments suggest that the EBS2 site is less significant than the EBS1 site in tolfenamic acid-induced EGR-1 expression (Fig. 3C).

The NAG-1, one of the transforming growth factor-β superfamily genes, has been reported as proapoptotic and antitumorigenic by our group and others (27, 42). Recently, we generated NAG-1 transgenic mice that overexpress human NAG-1 and have shown that these mice (NAG-Tg) are much less sensitive to carcinogens or genetic toxicity (43). The results support our previous study that EGR-1 mediates activation of NAG-1 gene expression by sulindac sulfide in human colorectal cancer cells (20). Thus, it appears that EGR-1 and NAG-1 play a significant role in the chemopreventive effect of NSAIDs in human colon or other cancers. On the other hand, tolfenamic acid induced both EGR-1 and NAG-1 expression in COX-2 wild-type (HT-29) and COX-2 mutant (SW480) cell lines, supporting that tolfenamic acid may affect tumorigenesis in a COX-2-independent manner. Although we did not observe that tolfenamic acid increased EGR-1 and NAG-1 expression in LoVo cells, another group has shown that treatment with NCX4040 (nitric oxide-releasing aspirin) induced NAG-1 expression in LoVo cells (44). These conflicting results may be explained by cell/NSAID specificity or duration of NSAID treatment.

Here, we report that tolfenamic acid inhibited proliferation and induced apoptosis in human colorectal cancer cells through a COX-independent pathway. This new pathway involved (a) activation of ESE-1 via nuclear translocation, (b) EGR-1 transactivation and apoptosis induction by ESE-1, and (c) NAG-1 transactivation and apoptosis induction by EGR-1.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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ESE-1/EGR-1 pathway plays a role in tolfenamic acid-induced apoptosis in colorectal cancer cells

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