Dual Function of Nonsteroidal Anti-Inflammatory Drugs (NSAIDs): Inhibition of Cyclooxygenase and Induction of NSAID-Activated Gene

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Received January 14, 2002; accepted February 8, 2002

This article is available online at http://jpet.aspetjournals.org

ABSTRACT

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used drugs for the treatment of inflammatory disease and have a chemopreventive effect on colorectal cancer. NSAIDs inhibit cyclooxygenase (COX)-1 and/or COX-2 activity, but the chemopreventive effect may be, in part, independent of prostanoid inhibition. NSAID-activated gene (NAG-1) was previously identified as a gene induced by some NSAIDs in cells devoid of COX activity. NAG-1 has proapoptotic and antitumor activity in vitro and in vivo. To determine whether the induction of NAG-1 by NSAIDs is influenced by COX expression, we developed COX-1- and COX-2-overexpressing HCT-116 cells. COX expression did not affect NSAID-induced NAG-1 expression as assessed by transient and stable transfection. Also, NAG-1 expression was not affected by PGE₂ and arachidonic acid, suggesting that NAG-1 induction by NSAIDs occurs by a prostanoid-independent manner. We also report that indomethacin increased NAG-1 expression in a number of cells from tissues other than colorectal. In conclusion, NSAIDs have dual function, induction of NAG-1 expression and inhibition of COX activity that occurs in a variety of cell lines.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most widely used drugs for the treatment of inflammatory diseases (Vane et al., 1998). Despite the different structures, these drugs share the same therapeutic properties. In varying doses, they alleviate swelling, redness, pain of inflammation, fever, and headache. Recently, the use of NSAIDs was linked to chemoprevention of colorectal cancer, and to a lesser extent, breast and lung cancer (Castonguay et al., 1998; Han et al., 1998; Taketo, 1998). For example, experimental studies revealed that NSAIDs are effective in reducing the number and size of colorectal polyps in human (Girardiello et al., 1993) and animal models (Mahmoud et al., 1998). In addition, epidemiological studies show a 40 to 50% reduction in mortality from colorectal cancer in individuals taking NSAIDs (Thun et al., 1993).

NSAIDs inhibit the two isoforms of prostaglandin H synthase, COX-1 and COX-2, the enzymes responsible for the formation of prostaglandins from arachidonic acid (AA).

COX-1 is constitutively expressed, whereas mitogens, tumor promoters, and growth factors regulate COX-2 expression (Herschman, 1996). Although many reports support the role of the inhibition of prostaglandins in anticarcinogenesis, some experimental evidence contradicts the concept that inhibition of prostaglandin synthesis plays a critical, but poorly understood, role in the antitumor effects of NSAIDs (Kopp and Ghosh, 1994; Hanif et al., 1996; Dong et al., 1997). For instance, the R-enantiomer of flurbiprofen, which does not inhibit COX, has chemopreventive activity in the mouse model of intestinal polyposis (Wechter et al., 1997) and prostate cancer (Wechter et al., 2000). Also, sulindac sulfone, which is not a COX inhibitor, inhibits azoxy-methane-induced colon tumors in rats (Piazza et al., 1997). Furthermore, non-COX-expressing cells, including human colorectal HCT-116 cells, were shown to undergo NSAID-induced apoptosis (Baek et al., 2001b). These studies suggest that NSAIDs can act via COX-dependent and COX-independent pathways.

Recently, we reported the identification of a cDNA (designated NSAID-activated gene, NAG-1) from a NSAID-induced library of human colorectal cells devoid of COX activity (Baek et al., 2001b). It has also been reported as placental transforming growth factor-β (Lawton et al., 1997), prostate-derived factor (Paralkar et al., 1998), macrophage inhibitory

ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; AA, arachidonic acid; NAG-1, nonsteroidal anti-inflammatory drug-activated gene-1; INDO, indomethacin; PGE₂, prostaglandin E₂; FBS, fetal bovine serum; HPLC, high-performance liquid chromatography; PG, prostaglandin; NS-398, N-[2-cyclohexyloxy-4-nitrophenyl]methane sulfonamide.
cytokine (Boovert et al., 1997), and as a placental bone morphogenetic protein (Hromas et al., 1997). NAG-1 is a divergent member of the transforming growth factor-β superfamily and expression of NAG-1 is increased and apoptosis is induced in colon cancer cells by treatment with several NSAIDs such as indomethacin (INDO), aspirin, and ibuprofen. The NAG-1 promoter has been characterized and many transcription factors are involved in the regulation of the NAG-1 gene (Baek et al., 2001a). NAG-1 basal expression is up-regulated by Sp1, Sp3, and COUP-TF1 transcriptional factors and by activators of the p53 tumor suppressor gene (Li et al., 2000; Tan et al., 2000). Furthermore, NAG-1 has been shown to have proapoptotic and antitumorigenic properties (Baek et al., 2001b). Thus, NAG-1 seems to be a common link between NSAIDs and their proapoptotic activity, and may provide new clues for explaining NSAID-induced antitumorigenesis.

The aim of the present study is to determine whether COX expression influences the stimulation of NAG-1 expression by NSAIDs and whether NSAID-induced NAG-1 expression is restricted to colorectal cells. Herein, we report that NAG-1 induction by NSAIDs is not altered by COX expression or the presence of PGE2 or AA. In addition, NSAIDs induce NAG-1 expression in a number of human cell lines.

Materials and Methods

Cell Lines and Reagents. Cell lines in this study were purchased from American Type Culture Collection (Manassas, VA). HCT-116, human colorectal carcinoma cells, were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS) and gentamicin. A649 human lung epithelial carcinoma cells were grown in RPMI 1640 medium supplemented with 10% FBS and gentamicin. MCF-7 cells were grown in Eagle's minimal essential medium with 10% FBS, and PC-3 cells were grown in DMEM/F-12 medium supplemented with 10% FBS and gentamicin. Diclofenac, piroxicam, and indomethacin were purchased from Sigma-Aldrich (St. Louis, MO), 5,5-diethyl-3-(3-fluorophenyl)-4-(4-methylsulfonylphenyl-2(5H)-furanone was a gift from Merck (Rahway, NJ), and NS-398 was purchased from Cayman Chemicals (Ann Arbor, MI). All NSAIDs were dissolved in dimethyl sulfoxide. Prostaglandins and AA were purchased from Cayman Chemicals and dissolved in ethanol.

RNA Isolation and Northern Analysis. Total RNAs were isolated as described previously (Baek et al., 2001b). For Northern blot analysis, 10 μg of total RNA was denatured and separated in a 1.2% agarose gel containing 2.2 M formaldehyde, and transferred to Hybond-N membrane (Amersham Biosciences, Piscataway, NJ). Blots were prehybridized in hybridization solution (Rapid-hyb buffer; Amersham Biosciences) for 1 h at 65°C followed by hybridization with NAG-1 cDNA fragments labeled with [α-32P]dCTP by random primer extension (Ambion, Austin, TX). After 4 h of incubation at 65°C, the blots were washed once with 2× standard saline citrate/0.1% SDS at room temperature and twice with 0.1× standard saline citrate/0.1% SDS at 65°C. Messenger RNA abundance was estimated by intensities of hybridization bands on autoradiographs using Scion Image (Scion Corporation, Frederick, MD). Equivalent loading of RNA samples was confirmed by hybridizing the same blot with a [α-32P]-labeled β-actin probe, which recognizes around 2-kb RNA.

Transient Transfection of Wild and Mutant COX cDNA into HCT-116 Cells. The full-length human COX-1 and COX-2 cDNAs were described previously (Hsi et al., 2000). The COX-1 Y384F mutant clone was generated by in vitro mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer's instructions. The primer sequences containing the mutation were 5'-GAGTCTAACCACATCTCTCCACT-GGCACCCCTCTC-3' for the forward, and 5'-GAGGGGTTGCCAGT-GGAAGAGTGGTTGAACTC-3' for the reverse primer. The COX-2 Y371F mutant clone was generated from pOSML/COX2Y371F vector (provided by Dr. Smith, Michigan State University, East Lansing, MI). The SalI fragment was isolated from pOSML/COX2Y371F and ligated into XhoI site in pCDNA3.1 vector (Invitrogen, Carlsbad, CA). Site-specific mutations (underlined) were confirmed by DNA sequencing. For the transient transfection, HCT-116 cells were plated in 100-mm plate at 1 × 10^6 cells/well in McCoy's 5A medium supplemented with 10% fetal bovine serum. After growth for 16 h, 10 μg of each plasmid was transfected by LipofectAMINE (Invitrogen) according to the manufacturer's protocol. Forty-eight hours after transfection, cells were harvested in the desired buffer.

Generation of COX-1-Overexpressing Cell Lines. HCT-116 cells were plated in a six-well plate and transfected with human COX-1 cDNA controlled by a cytomegalovirus promoter (pCDNA3.1) using LipofectAMINE according to the manufacturer's protocol (Invitrogen). The transfected cells were selected under 500 μg/ml G418 for 2 to 3 weeks, and several individual clones were isolated. After Western analysis, the clone HCT-116 COX-1 no. 4 was selected due to high COX-1 expression for further analysis.

Analysis of Arachidonic Metabolites by High-Pressure Liquid Chromatography. HCT-116 cells cultured in 10-cm² dishes were washed twice with phosphate-buffered saline. Cells were scraped and collected in 1 ml of lysis buffer (100 mM Tris-HCl, pH 8.0, 1 μg/ml leupeptin and pepstatin, and 0.5 mM phenylmethylsulfonyl fluoride). Cells were sonicated four times for 20 s at 50% power by a sonic dismembrator for total protein preparation. Protein content was quantified, and 0.8 mg of total cell lysate was used. The sonicates were then diluted with 1 ml of reaction buffer (100 mM Tris-HCl, pH 8.0, and 10 mM CaCl2) and incubated with [3H]Arachidonic acid (3 μCi, 25 μM) (PerkinElmer Life Sciences, Boston, MA) for 30 min at 37°C. After acidification to pH 3.5 with acetic acid, the reaction mixture was applied to a C18-PrepSep solid phase extraction column pretreated with methanol. The column was washed with acidified water, and the fatty acid compounds were eluted with methanol, evaporated to dryness, and reconstituted with methanol/water/acetic acid (60:39:1). Reverse phase HPLC analysis was performed using an UltraspHERE ODS column (5 μm; 4.6 × 250 mm; Beckman Coulter, Inc., Fullerton, CA). The solvent system consisted of a solution gradient (10% methanol and 0.01% acetic acid in water) at flow rate of 1.1 ml/min. Radioactivity was monitored using a flow scintillation analyzer (Packard Instrument Company, Inc., Downers Grove, IL) with EcoLume (ICN Biochemicals, Costa Mesa, CA) as the liquid scintillation mixture.

Results

Stable COX-1 Expression Is Not Associated with NAG-1 Expression and Induction. Previously, we reported that several NSAIDs induced NAG-1 expression in HCT-116 cells (Baek et al., 2001b). Most conventional NSAIDs, including diclofenac and piroxicam induced NAG-1 expression as well as apoptosis, whereas most COX-2-specific inhibitors, including 5,5-diethyl-3-(3-fluorophenyl)-4-(4-methylsulfonyl)phenyl-2(5H)-furanone and NS-398 did not induce NAG-1 or apoptosis in HCT-116 cells. Studies examining the increased expression of NAG-1 were done in HCT-116 cells, which are devoid of COX activity. Because most cells express COX-1 and/or COX-2, the presence of COX could alter NAG-1 induction by NSAIDs. To determine the influence of COX expression on NAG-1 induction, several stably COX-transfected HCT-116 cells were developed and isolated. The highest COX-1-overexpressing clone, HCT-116-COX-1 no. 4, was chosen for additional ex-
Fig. 1. NAG-1 induction by NSAIDs in COX-1-expressing HCT-116 cells. A, Western analysis of stable COX-1-overexpressing HCT-116 cells using COX-1 antibody. The 70-kDa COX-1 protein was detected in HCT-116 COX-1 no. 4 cells, whereas HCT-116 vector cells did not express COX-1. STD represents COX-1 standard from Cayman Chemicals (10 ng). B, HPLC analysis of COX-1 no. 4 cells. Cell lysates were prepared from HCT-116 vector and COX-1 no. 4 cells, as described under Materials and Methods. PGE₂ was used as a standard (STD). C, Northern analysis of HCT-116 vector and COX-1 no. 4 cells. Both cells were incubated with INDO for 24 h and Northern analysis was performed using NAG-1 as a probe. The hybridization signals were quantitated using Scion Image software. Levels of the NAG-1 transcript were normalized to the levels of β-actin transcripts and were represented relative to vehicle treatment.
periments. We could not isolate COX-2-overexpressing HCT-116 cells, probably due to growth arrest and/or apoptosis. Indeed, it has been reported that COX-2 overexpression causes cell growth arrest in cell culture systems (Trifan et al., 1999). The pCDNA3/NEO plasmid was also transfected into HCT-116 cells, to develop stable cell lines to be used as control cells. The HCT-116:COX-1 no. 4 cells highly express COX-1 protein compared with vector-transfected control cells (HCT-116:Vector), as determined by Western analysis (Fig. 1A). No significant differences in growth rate or morphology have been found between the two cell lines (data not shown). Next, we measured the formation of COX metabolites using reverse HPLC. As shown in Fig. 1B, HCT-116:COX-1 no. 4 cells produce significant amounts of prostaglandin E₂ and PGE₂, whereas no detectable metabolites were found in HCT-116:Vector cells, indicating that HCT-116:COX-1 no. 4 cells produce active COX-1 protein. To investigate whether stable COX-1 expression affects NAG-1 induction by INDO, Northern blot analysis was performed. Both cells produced the same amount of basal level NAG-1. Furthermore, INDO treatment induced NAG-1 expression in both cell lines in a dose-dependent manner (Fig. 1C), suggesting that NAG-1 expression and induction by NSAIDs are not affected by COX-1 present in the cells. Western analysis was also performed and was consistent with Northern blot analysis (data not shown). Therefore, the induction of NAG-1 by NSAIDs seems not to be altered by the presence of COX in cells and confirms that NSAID-induction of NAG-1 is independent of COX activity and PG formation.

Effect of Arachidonic Acid and PG Formation on NAG-1 Induction. Because COX-2 stable cell lines could not be established in HCT-116 cells, we performed transient transfection of COX-1 and COX-2 cDNA. In addition, two COX mutant clones were prepared and also transfected into HCT-116 cells. The tyrosine 384 of COX-1 and tyrosine 371 of COX-2 were mutated to phenylalanine. The tyrosine positions are required for the cyclooxygenase activity of COX-1 and COX-2. As shown in Fig. 2A, the transfection of wild-type and mutant COX in HCT-116 cells produced COX-1 or COX-2 protein only in transfected cells (lanes 3, 4, 5, and 6) as assessed by Western analysis. However, basal NAG-1 expression did not change with wild-type or mutant COXs being expressed, suggesting that COX expression and/or COX activity did not affect NAG-1 expression. These data are consistent with previous data using stable cell lines. AA is reported to induce apoptosis through the sphingomyelin pathway in HCT-116 cells (Chan et al., 1998). Thus, both COX-expressing cells and cells expressing mutant COX were incubated with AA after transfection for 8 h, and NAG-1 expression measured. As shown in Fig 2B, NAG-1 expression was not affected by AA treatment in either wild-type or mutant COX-expressing cells. Thus, the formation of PG or high cellular levels of AA does not affect NAG-1 expression.

PGE₂ Effect on NAG-1 Expression. Because PGE₂ is the major AA metabolite of the COX pathway, we treated HCT-116 cells with PGE₂ to examine NAG-1 expression. As shown in Fig. 3A, PGE₂ did not change NAG-1 expression over a broad range of concentrations, as assessed by Northern and Western analyses, providing further evidence that NAG-1 expression is prostaglandin-independent. In addition, different concentrations of diclofenac were added to PGE₂-treated HCT-116 cells to examine whether NAG-1 expression is altered in the presence of PGE₂. As shown in Fig. 3B, NAG-1 is still induced by diclofenac, suggesting NAG-1 induction by NSAIDs is prostaglandin-independent. We also treated HCT-116 cells with PGF₂α, and examined similar results (data not shown).

NAG-1 Induction by Indomethacin in Other Cell Lines. To determine whether NSAIDs can increase NAG-1

![Fig. 2. COX expression and AA effect on NAG-1 expression. A, wild-type and mutant forms of COX-1 and COX-2 plasmids were transfected into HCT-116 cells. After transfection, cells were grown for 2 days, and cell lysates were harvested. Western analysis was performed with 30 µg of total protein and COX-1, COX-2, and NAG-1 expression was measured. Lane 1, no DNA; lane 2, empty vector; lane 3, COX-1; lane 4, COX-2; lane 5, mCOX-1Y384F; and lane 6, mCOX-2Y371F. B, AA effects on NAG-1 expression. The transfected HCT-116 cells were treated with either vehicle (−) or 30 µM AA (+) for 8 h. The cell lysates were harvested and subjected to Western analysis using NAG-1 antibody. The figure shown here is representative of five different experiments.](https://jpet.aspetjournals.org/article/10.1124/jpet.1999.267.1129/)

![Fig. 3. Effect of prostaglandin E₂ on basal and NSAID-induced NAG-1 expression. A, HCT-116 cells were treated with various concentrations of PGE₂, and Northern and Western analyses were performed. V indicates DMSO (0.2%) treatment. B, HCT-116 cells were treated with either PGE₂ or a combination of PGE₂ and diclofenac. Western analysis was performed using NAG-1 and actin antibody. The hybridization signals were quantitated using Scion Image software. Levels of the NAG-1 expression were normalized to the levels of β-actin expression and are represented relative to vehicle treatment.](https://jpet.aspetjournals.org/article/10.1124/jpet.1999.267.1129/)
expression in cells other than human colorectal, breast epithelial adenocarcinoma (MCF-7), lung epithelial adenocarcinoma (A549), and prostate carcinoma (PC-3) cells were used to examine NAG-1 induction by NSAIDs. These cells were selected for this study because many reports suggest that NSAIDs may have chemopreventive effects on breast (Cotterchio et al., 2001), lung (Moody et al., 2001), and prostate cancer (Myers et al., 2001). In addition, MCF-7 cells constitutively express COX-1 (Liu and Rose, 1996), whereas A549 and PC-3 cells constitutively express COX-2 (Hsu et al., 2000; Tsubouchi et al., 2000). INDO induces NAG-1 in HCT-116 cells (Baek et al., 2001b), and thus was used to treat the different cell lines. As shown in Fig. 4, NAG-1 transcripts were induced by INDO in all cell lines tested. Thus, the ability of NSAIDs to increase the expression of NAG-1 is not restricted to human colorectal cells.

Discussion

NSAIDs are effective chemopreventive agents for a number of cancers, and it is generally presumed that this activity is due to their ability to inhibit prostaglandin synthesis. Although the chemopreventive and antitumorigenic activities of NSAIDs against colorectal and other human cancers are established, the molecular mechanisms responsible for these properties are not yet elucidated. A number of studies suggest that the chemopreventive properties may be independent of COX (Hanif et al., 1996; Piazza et al., 1997; Murphy et al., 1998; Trifan et al., 1999; Smith et al., 2000). We reported previously that the induction of the proapoptotic and antitumorigenic protein, NAG-1, is induced by NSAIDs (Baek et al., 2001b). NAG-1 seems to be an important target gene for NSAIDs and our results may provide new clues to aid in the understanding of how these drugs alter tumor development.

The induction of NAG-1 by NSAIDs was examined in the human colorectal cell line, HCT-116, which is devoid of COX activity. Herein, we provide evidence supporting the hypothesis that the expression of COX or the formation of PG in the cells did not alter the expression of NAG-1 induced by COX inhibitors. Studies with both stable and transiently transfected cells support this conclusion. Even though COX-2 is often highly expressed in tumors, COX-2 expression seems to not alter NAG-1 expression, despite the presence of this second target for these inhibitors. In addition, these experiments confirm the concept that NAG-1 expression by NSAIDs occurs independently from COX inhibition. In addition, prostaglandins, particularly PGE$_2$, do not affect NAG-1 expression. The effects of PGE$_2$ on cell proliferation and apoptosis are contradictory. PGE$_2$ can induce cellular proliferation (Sheng et al., 1997; Tjandrawinata et al., 1997), growth arrest and apoptosis (Brown et al., 1992), or have no effect (Qiao et al., 1995), depending on cell type and concentration. PGE$_2$ does not affect NAG-1 induction by NSAIDs in HCT-116 cells, suggesting that NAG-1 induction by NSAIDs occurs through a prostaglandin-independent pathway.

We observed that NAG-1 expression is not restricted to colorectal cells because NSAIDs can induce NAG-1 in MCF-7, A549, and PC-3 cells. NAG-1 induction by NSAIDs in different cell lines may apply to previous studies reporting that breast (Han et al., 1998), lung (Castonguay et al., 1998), and prostate (Palayoor et al., 1998) cell lines also undergo apoptosis by different NSAIDs. Recently, it was reported that the p53 tumor suppressor could induce NAG-1 (Li et al., 2000; Tan et al., 2000). Because HCT-116 and MCF-7 cells express wild-type p53, it is interesting to know whether p53 protein mediates NSAID-induced NAG-1 expression. As shown in Fig. 4, however, NSAID-induced NAG-1 expression was observed in PC-3 cells, which are p53 null (Herrmann et al., 1998; Akashi et al., 1999). In addition, the biochemical pathway for NSAID-induced apoptosis seems to not require p53 induction (Piazza et al., 1997). Therefore, taken together with previous reports, NSAIDs induce NAG-1 in both COX-1-positive (MCF-7) and COX-2-positive cells (MCF-7 and PC-3 cells), as well as in both p53-negative (PC-3) and p53-positive cells (HCT-116 and MCF-7 cells). Thus, the regulation of NAG-1 expression by NSAIDs occurs via COX- and p53-independent mechanisms. In addition, NAG-1 induction by NSAIDs in different cell lines gives us the opportunity to study the molecular mechanism of NSAID-induced antitumorigenic effects in breast, lung, and prostate cancer.

Several groups have proposed molecular mechanisms of NSAID-induced apoptosis. For example, indomethacin induces a 41-kDa mitogen-associated protein kinase (Fiorucci et al., 1997), and salicylates activate c-Jun N-terminal kinase or p38 mitogen-activated protein kinase (Schwenger et al., 1997). Furthermore, NSAIDs enhance glutathione S-transferase theta levels in rat colon (Van Lieshout et al., 1998), and the transcription factors nuclear factor-$\kappa$B and activator protein-1 are inhibited by aspirin, salicylate, and ibuprofen in vitro (Kopp and Ghosh, 1994; Dong et al., 1997; Stuhlmeier et al., 1999). Also, some NSAIDs serve as ligands for peroxisome proliferator-activated receptor transcription factors (Lehmann et al., 1997). However, none of these effects completely explain the COX-independent apoptotic effect of NSAIDs. Furthermore, many of these responses occur only at high, nonphysiological concentrations. In contrast, NAG-1 expression is observed at lower concentrations. For example, 5 $\mu$M sulindac sulfide induces NAG-1 at a concentration within the normal physiological concentration range observed for this COX inhibitor (Baek et al., 2001b).

In summary, NSAIDs are potent anti-inflammatory drugs that also have chemopreventive activity toward colon cancer. NAG-1 induction by NSAIDs was seen not only in colorectal cancer cells but also in different cancer cells. The present...
study may provide new clues to explain both the antitumorogenic and anti-inflammatory activity of NSAIDs in different cells and tissues.

Acknowledgments
We thank Drs. Linda Hsi, Hiroo Kawajiri, and Yuji Mishina (National Institute of Environmental Health Sciences) for comments and suggestions. We also thank Mark Geller for technical assistance.

References


