Cyclooxygenase Inhibitors Induce the Expression of the Tumor Suppressor Gene EGR-1, Which Results in the Up-Regulation of NAG-1, an Antitumorigenic Protein

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ABSTRACT

Nonsteroidal anti-inflammatory drugs (NSAIDs) have been shown to have chemopreventive activity, but the mechanisms involved are not clearly understood. Although NSAIDs inhibit cyclooxygenase activity, they also increase the expression of a divergent member of the transforming growth factor-β superfamily, termed NSAID-activated gene 1 (NAG-1), a protein with an antitumorigenic and proapoptotic activity that could in part be linked to the chemoprevention activity of NSAIDs. NAG-1 is induced by some NSAIDs, but the mechanisms responsible are not clear. In this report, we have identified a *cis*-acting element responsive to NSAIDs located within the −73 to −51 region of the NAG-1 promoter. This region contains overlapping EGR-1 and Sp1 bind-ing sites, and mutations in this region suggest that the transcription factors have an important role in NSAID-induced NAG-1 expression. EGR-1 was found to play a critical role in the induction of NAG-1 by sulindac sulfide and other NSAIDs. NSAIDs increase EGR-1 protein expression that occurs before the induction of NAG-1 expression, supporting the hypothesis that EGR-1 is necessary for NSAID-induced NAG-1 expression. Thus, NSAIDs induce the expression of EGR-1, a tumor suppressor gene, providing a novel mechanism to explain, in part, the antitumorigenic properties of some NSAIDs. NAG-1 seems to be an important downstream target protein of this transcription factor, EGR-1, and may mediate the chemopreventive activity of some NSAIDs.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in the treatment of inflammatory disease, and their anti-inflammatory effects are believed to result from inhibition of prostaglandin H synthase (also known as cyclooxygenase, COX). Two isoforms of prostaglandin H synthase, COX-1 and COX-2, are known; COX-1 is constitutively expressed in many tissues, whereas mitogens, tumor promoters, and growth factors up-regulate the expression of COX-2. COX-2 protein is also up-regulated in human colorectal tumors and regulates tumor growth in animal models (Taketo, 1998; Reddy and Rao, 2002; Ricchi et al., 2003). NSAIDs reduce the number and size of polyps in animal models, and epidemiological studies reveal a 40 to 50% reduction in mor-tality from colorectal cancer (Thun and Heath, 1995; Thun, 1996). Chemopreventive effects of certain NSAIDs seem to be mediated through both COX-dependent and -independent pathways. However, the target molecules that mediate chemopreventive effects are not elucidated. Identifying possible targets is important to understanding the mechanism of the chemopreventive actions. A number of molecular mechanisms responsible for the chemopreventive effects of NSAIDs have been proposed. One hypothesis is the obvious involvement of COX-2 inhibition, but it is clear that prostaglandin-independent mechanisms are also involved (Jones et al., 1999; McEntee et al., 1999; Baek et al., 2002b; Ferrandez et al., 2003; Rice et al., 2003). In either case, the mechanisms responsible for the chemopreventive activity are not clear. Changes in gene expression by COX inhibitors could provide an explanation for the activity. A number of studies with microarray and PCR-based subtractive hybridization have identified potential target genes (Baek et al., 2001b; Iizaka et

ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; TGF-β, transforming growth factor-β; NAG-1, nonsteroidal anti-inflammatory drug-activated gene-1; DMSO, dimethyl sulfoxide; PCR, polymerase chain reaction; DFU, 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furanone; LUC, luciferase; SC-560, 5-(4-chloro-phenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole.

al., 2002; Bottone et al., 2003). NAG-1 was identified in our laboratory and is a highly promising target gene because it is a proapoptotic and antitumorigenic protein, and its expression is highly induced by NSAIDs (Baek et al., 2001b). The human NAG-1 cDNA encodes a secreted protein with homology to members of the transforming growth factor-β superfamily and has been identified previously as macrophage inhibitory cytokine-1 (Bootcov et al., 1997), placental transformation growth factor-β (Li et al., 2000), prostate-derived factor (Paralkar et al., 1998), growth differentiation factor 15 (Bottner et al., 1999), and placental bone morphogenetic protein (Hromas et al., 1997). In mature intestinal epithelial cells, NAG-1 is expressed, but the expression is significantly reduced in human colorectal carcinoma samples and neoplastic intestinal polyps of *Min* mice (Kim et al., 2002). NAG-1 overexpression from a recombinant adenoviral vector results in an 80% reduction of MDA-MB-468 and MCF-7 breast cancer cell viability (Li et al., 2000), and treatment of prostate cancer cells with purified NAG-1 induces apoptosis (Liu et al., 2003). These data support the hypothesis that NAG-1 is linked to apoptosis and that its reduced expression may enhance tumorigenesis. NAG-1 expression is up-regulated independently of prostaglandin formation in human colorectal cancer cells by several NSAIDs (Baek et al., 2002b), In addition, antitumorigenic compounds such as resveratrol (Baek et al., 2002a), genistein (Wilson et al., 2003), diallyl disulfide (Bottone et al., 2002), retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (Newman et al., 2003), 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (Monks et al., 2003), and peroxisome proliferator-activated receptor-γ ligands induce NAG-1 expression (Baek et al., 2004). These findings suggest a critical role for NAG-1 in the chemopreventive activity of a number of unrelated chemopreventive chemicals.

The aim of the present study was to identify *cis*- and *trans*-acting elements that are responsible for NAG-1 induction by sulindac sulfide and other NSAIDs. During this investigation, we found NSAIDs to increase the expression of EGR-1, a transcription factor and tumor suppressor protein, suggesting that the chemopreventive activity of NSAIDs is mediated via this tumor suppressor protein. NAG-1 was characterized as a key downstream target of EGR-1.

Materials and Methods

Cell Lines and Reagents. Cell lines in this study were purchased from American Type Culture Collection (Manassas, VA). Human colorectal carcinoma cells, HCT-116, were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum and gentamicin. All NSAIDs in this study were purchased from Sigma-Aldrich (St. Louis, MO), except for sulindac sulfide, SC-560 (both from Cayman Chemical, Ann Arbor, MI), and [5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5*H*)-furanone] (DFU) (Merck, Whitehouse Station, NJ). All NSAIDs were dissolved in DMSO.

Western Blot Analysis. After reaching 60 to 80% confluence in 10-cm plates, the cells were treated at the indicated concentrations and times with each different NSAID in the absence of serum. The level of NAG-1 was evaluated using Western blot analysis with anti-human NAG-1 antibody reported previously (Baek et al., 2001b). Cell lysates were harvested, and 30 μg of total proteins was separated by 15% SDS-polyacrylamide gel electrophoresis and transferred for 1 h onto nitrocellulose membrane (ECL Hybond; Amersham Biosciences Inc., Piscataway, NJ). The blots were blocked for

1 h with 5% skim milk in Tris-buffered saline/0.05% Tween 20 and probed with anti-NAG-1 antibody (1:1000 dilution, 5% skim milk in Tris-buffered saline/0.05% Tween 20 at 4°C overnight). After washing, the blots were treated with horseradish peroxidase-conjugated secondary antibody for 1 h and washed several times. The signal was detected by the enhanced chemiluminescence system (Amersham) and autoradiography.

Transfection of Cells with the Luciferase Reporter System. HCT-116 cells were plated in six-well plates at 2×10^5 cells/well in McCoy's 5A media supplemented with 10% fetal bovine serum. After growth for 16 h, plasmid mixtures containing 1 μg of promoter linked to luciferase and 0.05 μg of *pRL-null* (Promega, Madison, WI) were transfected by LipofectAMINE (Invitrogen, Carlsbad, CA). For the cotransfection experiment, 0.5 μg of reporter vector and 0.5 μg of expression vector were transfected with 0.05 μg of *pRL-null* vector according to the manufacturer's protocol. After 48 h of transfection, the cells were harvested in 1× luciferase lysis buffer, and luciferase activity was determined and normalized to the *pRL-null* luciferase activity with a Dual Luciferase Assay Kit (Promega).

Construction of Plasmids. Several *NAG-1* promoter clones linked to luciferase have been reported previously (Baek et al., 2001a). The full-length *EGR-1* cDNA was generated by PCR from human universal QUICK-Clone cDNA (BD Biosciences Clontech, Palo Alto, CA) using the following primers: 5′-GACACCAGCTCTCCAGCCTGCTCGTC-CAGG-3′ (top strand) and 5′- TTCCCTTTAGCAAATTTCAATTGTC-CTGGG-3′ (bottom strand). The amplified products were cloned into pCR2.1 TOPO vector (Stratagene, La Jolla, CA) and followed by cloning into pCDNA3.1/NEO expression vector (Invitrogen). The Sp1 expression vector was described previously (Baek et al., 2001a). The EGR-1 promoter (-1260 to $+35$) linked to the luciferase gene (pEGR1260) was cloned by PCR from human Genomic DNA (Promega) with the following primers: 5′-CGGCTCGAGCGGGAGGAGGAGCGAGGAG-GCGGCGG-3′ (top strand, XhoI site was underlined) and 5′-CCCAAGCTTGGGCGGCGGCGGCTCCCCAAGTTCTGCGGC-3′ (bottom strand, HindIII site was underlined). After PCR amplification, the fragment was digested with XhoI and HindIII and ligated into pGLBasic3 luciferase vector. The deletion clones of *EGR-1* promoter were generated from pEGR1260 using ExoIII nuclease. All plasmids were sequenced for verification.

Real-Time PCR. HCT-116 cells were pretreated with vehicle or 5 μg/ml, followed by the treatment of 30 μM sulindac sulfide for 12 h. Cells were washed with phosphate-buffered saline, removed by scraping, and lysed using Nucleic Acid Purification Lysis solution (1×) (Applied Biosystems, Foster City, CA). Total RNA was isolated using ABI Prism 6100 Nucleic Acid PrepStation (Applied Biosystems) according to the manufacturer's recommendations, quantified, diluted with diethyl pyrocarbonate water, and stored at $-70°C$. Total RNA (50 ng) was denatured at 70°C for 10 min and then reverse-transcribed with oligo(dT) primers (1 μM) and Moloney murine leukemia virus reverse transcriptase (1.25 U/μl) in 10-μl reactions at 37°C for 1 h. Real-time fluorescence detection was carried with ABI Prism 7900 Sequence Detection System (Applied Biosystems). 18S rRNA was amplified with a probe dye VIC-MGB using TaqMan Universal PCR Master Mix 1×. NAG-1 was amplified using the following primers: forward, 5′-TGCCCGCCAGCTACAATC-3′; reverse, 5′-TCTTTGGCTAACAAGTCATCATAGGT-3′ (0.2 μM each) (Research Genetics, Huntsville, AL) with SYBR Green PCR Master Mix (1×) and cDNA (10 μl) in a final PCR reaction volume of 50 μl. PE 7900 amplification parameters were the following: denaturation at 94°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Reverse transcription and PCR reagents were purchased from Applied Biosystems.

Results

NAG-1 Induction Requires de novo Synthesis. NAG-1 is induced by several NSAIDs at the transcriptional level (Baek et al., 2001b). Sulindac sulfide is very potent, and thus,

this NSAID was used as a model for COX inhibitors. To confirm that NSAIDs induce NAG-1 at the protein level, HCT-116 cells were treated with two conventional NSAIDs (sulindac sulfide and indomethacin), a COX-1–specific inhibitor (SC-560), a COX-2–specific inhibitor (DFU), or a non-COX inhibitor (acetaminophen), and Western analysis was performed. As shown in Fig. 1A, sulindac sulfide highly induces NAG-1 protein expression, whereas indomethacin and SC-560 induce NAG-1 expression to a lesser extent. However, DFU and acetaminophen do not induce NAG-1 expression. This result is consistent with our previous report showing that NAG-1 mRNA expression is induced by sulindac sulfide and indomethacin but not by DFU and acetaminophen (Baek et al., 2001b). To examine whether NSAID-induced NAG-1 expression requires de novo synthesis, HCT-116 cells were pretreated with or without cycloheximide for 30 min, and then followed by incubation with 30 μM sulindac sulfide. As shown in Fig. 1B, NAG-1 mRNA was induced by sulindac sulfide treatment by 15.9-fold, but sulindac sulfide could not increase the level of NAG-1 mRNA in the presence of cycloheximide, suggesting that sulindac sulfide-induced NAG-1 expression requires de novo protein synthesis. These data are compatible with the notion that the increase in NAG-1 biosynthesis conferred by sulindac sulfide and presumably other NSAIDs requires, at least in part, de novo synthesis at the transcriptional level.

Effects of NSAIDs on the NAG-1 Promoter Activity. Because NSAIDs induce the expression of NAG-1 mRNA and protein, we examined whether *NAG-1* promoter activity is increased in the presence of several NSAIDs. A construct,
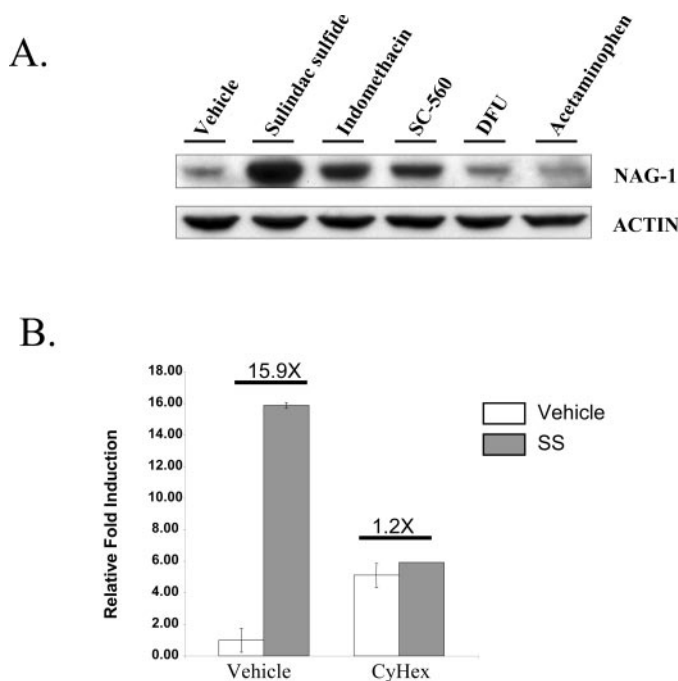

Fig. 1. NAG-1 expression is induced by several NSAIDs and requires de novo synthesis. A, HCT-116 cells were treated with vehicle (0.2% DMSO), sulindac sulfide (30 μM), indomethacin (100 μM), SC-560 (25 μM), DFU (100 μM), and acetaminophen (100 μM) for 24 h. After incubation, cells were harvested, and cell lysates were prepared for Western analysis. B, HCT-116 cells were treated with or without cycloheximide (5 μM) for 30 min, followed by the addition of 30 μM sulindac sulfide for 12 h. After incubation, total RNAs were prepared, and real-time PCR was conducted as described under *Materials and Methods*. CyHex, cyclohexamide.

pNAG1739/LUC, containing the −1739 to +41 NAG-1 promoter region and luciferase reporter gene was transfected into HCT-116 cells. The cells were then treated with different concentrations of NSAIDs and subsequently assayed for luciferase activity. As shown in Table 1, sulindac sulfide treatment was the most potent inducer of luciferase activity, with an estimated ED_{50} of 16 μM, followed by indomethacin, diclofenac, ibuprofen, piroxicam, naproxen, sodium salicylate, and aspirin. These data are very consistent with Northern and Western data reported previously (Baek et al., 2001b). In addition, celecoxib and acetaminophen did not increase the NAG-1 promoter activity (data not shown), which is consistent with previous data (Fig. 1A), showing that celecoxib and acetaminophen do not induce NAG-1 protein expression in HCT-116 cells.

Deletion Analysis of NAG-1 Promoter. To evaluate the importance of *cis*-acting elements regulating NSAID-inducible NAG-1 expression, the 3.5-kb *NAG-1* promoter and other deletion clones were transfected into HCT-116 cells and treated with the model NSAID, sulindac sulfide. As an internal control, the plasmid *pRL-null* (Promega) was used for adjusting transfection efficiency. As shown in Fig. 2A, a large increase in luciferase activity induced by sulindac sulfide treatment was observed with all constructs. These data suggest that there is a positive *cis*-acting element responsible for sulindac sulfide within −133-base pair *NAG-1* promoter region. As a negative control, the promoterless vector, *pGLBasic3*, was also transfected into HCT-116 cells and showed no significant luciferase activity after treatment of the cells with sulindac sulfide. A p53 binding site present in the *NAG-1* promoter at the +43 position responds to several dietary antitumorigenic compounds (Baek et al., 2002a; Wilson et al., 2003). To examine the importance of the p53 site in sulindac sulfide induced NAG-1 expression, we generated two constructs containing a p53 site at the +43 position in the *pNAG133*/+70 and *pNAG41*/+70. These constructs were transfected into HCT-116 cells and treated with sulindac sulfide, and the luciferase activity was measured. As shown in Fig. 2B, sulindac sulfide did not increase the activity of the *pNAG41*/+70 construct, which has the p53 site, but did increase the activity of the *pNAG133*/+70 construct. Thus the p53 site at +43 is not involved in the induction of NAG-1 expression by sulindac sulfide. These data are in agreement with a previous report that NSAID-induced NAG-1 expression is p53-independent (Baek et al., 2002b). The NSAID

TABLE 1
Effects of several NSAIDs on NAG-1 promoter activity

A construct, pNAG1739/LUC, was transfected into HCT-116 cells and treated with the indicated NSAIDs at different concentrations. As an internal control, *pRL-null* vector was cotransfected and used to adjust transfection efficiencies. The data shown is the average from three independent experiments. The estimated ED_{50} was calculated from the 50% induction of their maximum luciferase activity.

NSAIDs	ED_{50}
	μM
Sulindac sulfide	16
Indomethacin	47
Diclofenac	57
Ibuprofen	579
Piroxicam	1260
Naproxen	1578
Sodium salicylate	4505
Aspirin	5175

response element is thus located between −133 and −41 in the *NAG-1* promoter.

Sulindac Sulfide Response Element Is Located in −73 to −51 of NAG-1 Promoter Region. Three Sp1 binding sites, Sp1A, Sp1B, and Sp1C, are present in the −133 to −41 region of the *NAG-1* promoter (Baek et al., 2001a). To further define the responsible element for sulindac sulfide, internal deletion clones *pNAG133ΔSP1-I* and pNAG133ΔSP1-II were generated and transfected into HCT-116 cells. The pNAG133ΔSP1-I clone has a deletion in the Sp1A site, whereas pNAG133ΔSP1-II has a deletion in the Sp1B and Sp1C sites in the NAG-1 promoter region. It is interesting that Sp1B and Sp1C sites are over-lapped with two EGR-1 sites. As shown in Fig. 3A, the deletion mutant pNAG133ΔSP1-II showed no significant induction of luciferase activity after sulindac sulfide treatment, indicating that the sulindac sulfide response element is located in this region containing two Sp1 sites and two EGR-1 sites. To inves-tigate whether other NSAIDs also required this site for NAG-1 induction, pNAG133/LUC-transfected cells were treated with indomethacin, diclofenac, sulindac sulfide, or acetaminophen. As shown in Fig. 3B, all of the compounds except for acetamin-ophen increase the luciferase activity. In contrast, NSAID treat-ment of HCT-116 cells transfected with the pNAG133ΔSP1-II construct did not exhibit an increase in luciferase activity, sug-gesting that this site is critical for NAG-1 induction by NSAIDs, including sulindac sulfide. It should be noted that 30 μM sulin-dac sulfide was used, whereas 100 μM was used for the other NSAIDs.

Sp1, EGR-1, and Sulindac Sulfide-Induced NAG-1 Ex-pression. Sp1 and EGR-1 sites have a pivotal role in the sulindac sulfide-induced NAG-1 expression. We next deter-mined whether the expression of Sp1 and/or EGR-1 protein would alter sulindac sulfide-induced NAG-1 expression. An Sp1 or EGR-1 expression vector was cotransfected along with the pNAG133/LUC reporter vector into HCT-116 cells and the cells incubated with sulindac sulfide. Sp1 expression in the absence of sulindac sulfide increased the luciferase activity 1.5-fold, consistent with previous data (Baek et al., 2001a). However, sulindac sulfide treatment in the Sp1-transfected cells did not enhance the luciferase activity rel-ative to the sulindac sulfide-treated vector-transfected cells. In fact, a marginal reduction in the luciferase activity in the Sp1-transfected cells, 6.9- versus 5.5-fold, respectively (Fig. 4A), was observed. These data suggest that Sp1 expression
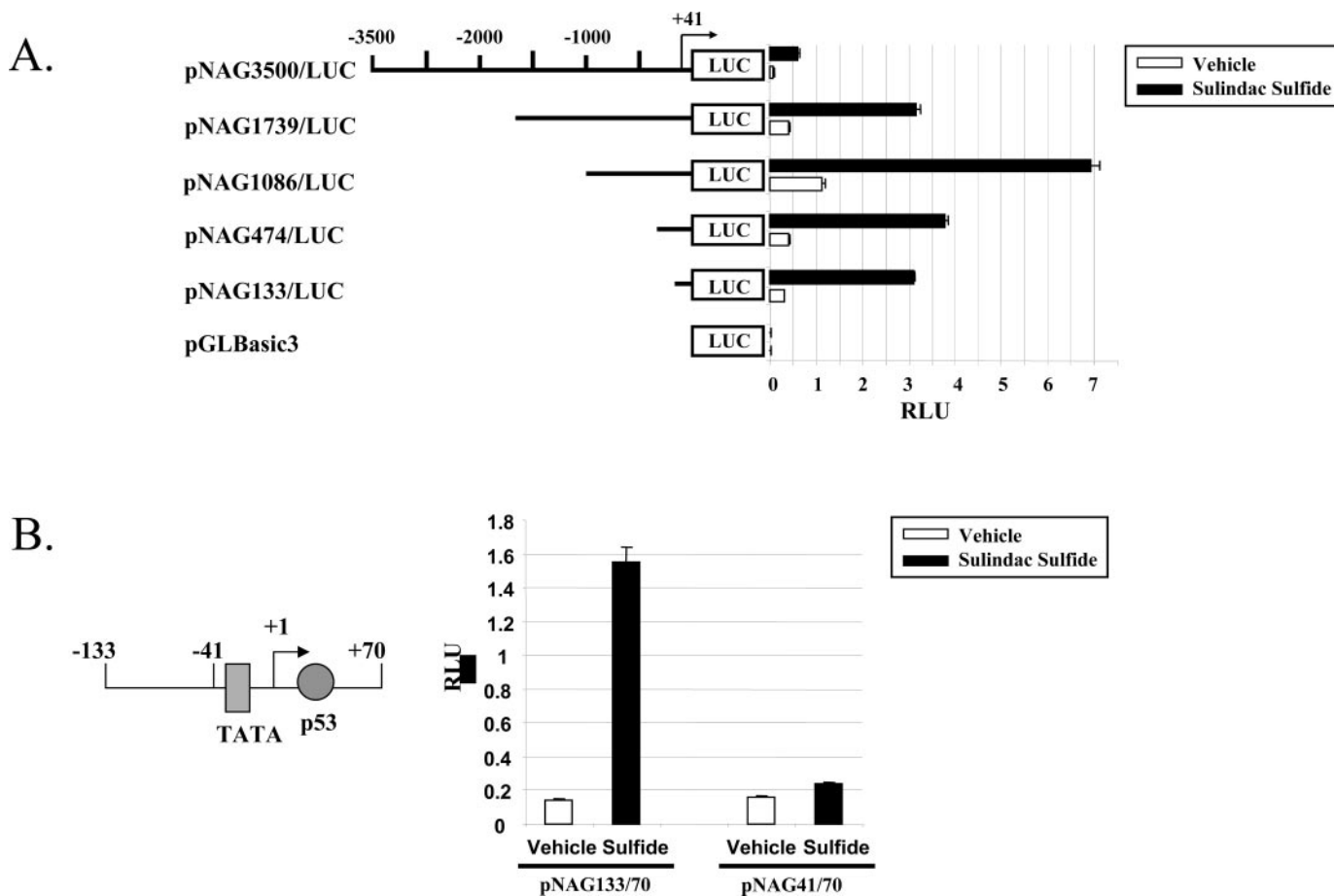

Fig. 2. NAG-1 promoter activity. A, the indicated promoter regions were fused to the luciferase reporter gene. Each construct (1 μg) was cotransfected with 0.1 μg of *pRL-null* (Promega) vector into HCT-116 cells using LipofectAMINE (Invitrogen), and the cells were treated with either vehicle (□) or 30 μM sulindac sulfide (■) in the absence of serum. After 24-h treatment, the promoter activities were measured by luciferase activity. Transfection efficiency for luciferase activity was normalized to the *Renilla reniformis* luciferase (*pRL-null* vector) activity. The *x*-axis shows relative luciferase unit (luciferase activity/*R. reniformis* unit). The results are the mean ± S.D. of three independent transfections. B The constructs, *pNAG133/+70* and *pNAG41/+70*, were transfected with *pRL-null* control vector. Both constructs contain the p53 binding site (left). The transfected HCT-116 cells were treated with either vehicle or 30 μM sulindac sulfide. Transfection efficiency for luciferase activity was normalized to the *R. reniformis* luciferase (*pRL-null* vector) activity. The *y*-axis shows relative luciferase unit (luciferase activity/*R. reniformis* unit). The results are the mean ± S.D. of three independent transfections.

may interfere with sulindac sulfide-induced NAG-1 expression. The promoters of many genes contain a GC box, which may share its binding proteins with Sp1 and EGR-1 (Khachigian et al., 1995; Raychowdhury et al., 2002; Davis et al., 2003). The NAG-1 promoter activity was measured after EGR-1 expression. The cells expressing EGR-1 were treated with sulindac sulfide, and a 2-fold increase luciferase activity (6.9- versus 12.6-fold) was observed. Thus, EGR-1 expression seems to be critical for sulindac sulfide-induced NAG-1 expression in HCT-116 cells. In contrast, Sp1 may compete with EGR-1 in controlling NAG-1 expression by sulindac sulfide. Therefore, to elucidate the relationship between Sp1 and EGR-1 expression, both Sp1 and EGR-1 were transfected with the pNAG133/LUC reporter vector, and luciferase activity was measured with and without sulindac sulfide treatment. As shown in Fig. 4B, the more EGR-1 is expressed, the more NAG-1 promoter activity is induced by sulindac sulfide. The expression of SP-1 protein attenuated the increase in NAG-1 expression by sulindac sulfide. These results indicate that EGR-1 is a key mediator of sulindac sulfide-induced NAG-1 expression, whereas Sp1 may inhibit the sulindac sulfide-induced NAG-1 expression, probably by competing for the same binding sites in the NAG-1 promoter region.

Expression of EGR-1 Is Induced by Sulindac Sulfide. Because EGR-1 plays an important role in sulindac sulfide-induced NAG-1 expression in HCT-116 cells at the transcrip-
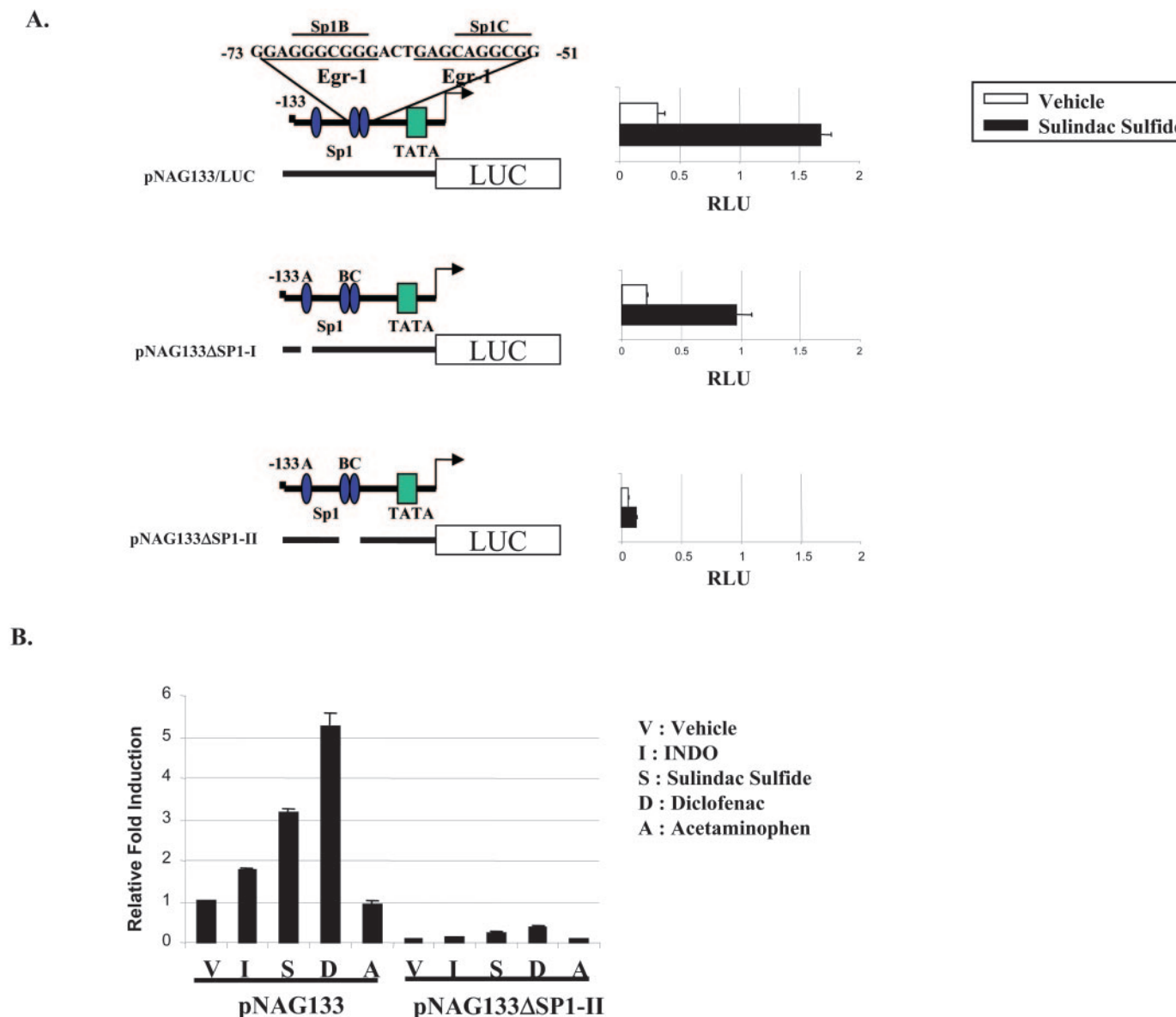

Fig. 3. Sp1/EGR-1 sites located in the NAG-1 promoter play an important role in sulindac sulfide-induced NAG-1 expression. A, effects of deletion of Sp1/EGR-1 binding sites on sulindac sulfide-induced activity of the NAG-1 promoter. The construction of NAG-1 promoter vectors with deletion mutations has been described previously (Baek et al., 2001a). Wild-type (*pNAG133/LUC*) or Sp1/Egr-1 site-deleted reporters (1 μg each) and pRL-null (0.1 μg) were cotransfected into HCT-116 cells. After 24-h treatment with vehicle or sulindac sulfide, the cell lysates were isolated and the luciferase activity was measured. The *x*-axis shows relative luciferase unit (Firefly luciferase/*R. reniformis* luciferase). The results are the mean ± S.D. of three independent transfections. B, Effects of deletion of Sp1/EGR-1 binding sites on the induction of the *NAG-1* promoter by several NSAIDs. The constructs, *pNAG133/LUC* and *pNAG133ΔSp1-II*, were transfected into HCT-116 cells, and the cells were treated with either vehicle (V, 0.2% DMSO), indomethacin (I, 100 μM), sulindac sulfide (S,30 μM), diclofenac (D,100 μM), or acetaminophen (A,100 μM). The luciferase activity was measured as described previously. The *y*-axis shows fold induction (over relative luciferase activity of pNAG133-transfected/vehicle-treated cells) as 1.0.

tion level, we sought to determine whether EGR-1 protein is altered during sulindac sulfide treatment in HCT-116 cells. HCT-116 cells were treated with 30 μM sulindac sulfide at the indicated times, and cell extracts were analyzed for EGR-1 by Western analysis. EGR-1 expression was increased with treatment with sulindac sulfide. An increase in protein was observed as early as 2 h and then decreased after 16 h of treatment (Fig. 5). In contrast, Sp1 protein expression was not changed during the sulindac sulfide treatment. NAG-1 expression was also increased by sulindac sulfide, but it was observed after the increase in EGR-1 expression. In contrast, incubation with the prodrug sulindac did not increase the expression of EGR-1, Sp1, or NAG-1 in HCT-116 cells. This result is consistent with previous reports that sulindac sulfide induces NAG-1 expression, whereas sulindac does not induce NAG-1 expression (Baek et al., 2001b). These data support the hypothesis that sulindac sulfide-induced NAG-1 expression requires de novo protein synthesis (Fig. 1B) and is mediated by EGR-1.
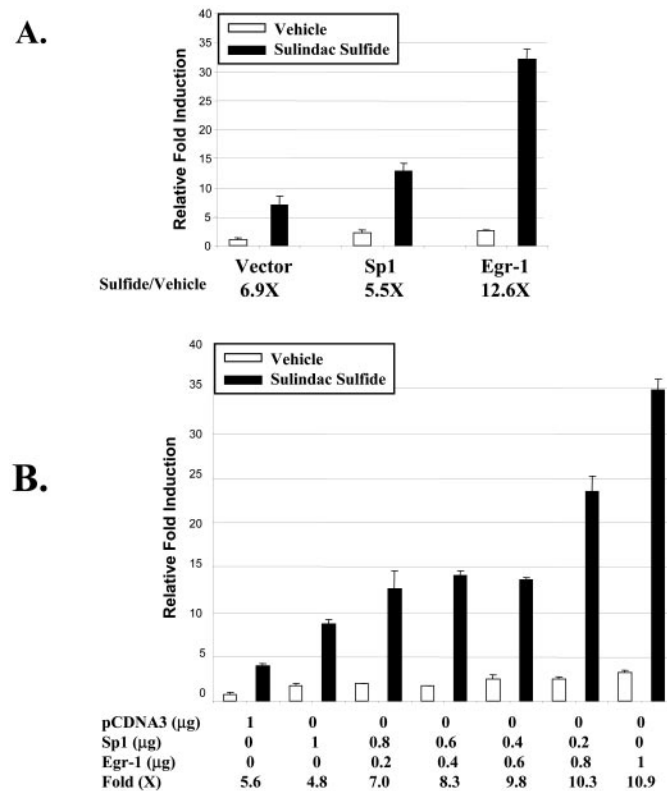
Fig. 4. EGR-1 transactivates *NAG-1* promoter activity. A, sulindac sulfide enhances the NAG-1 promoter activity by the coexpression of *EGR-1*. HCT-116 cells were cotransfected with 0.5 μg of the pNAG133/LUC construct and with 0.5 μg of *EGR-1*, *Sp1*, or the empty *pcDNA3* vector. Cells were treated with either vehicle or sulindac sulfide (30 μM) after transfection, and luciferase activity was assayed after 24-h treatment. As an internal control, pRL-null vector (0.1 μg) was used to adjust transfection efficiency. The *y*-axis shows fold induction (over relative luciferase activity of empty vector-transfected/vehicle-treated cells) as 1.0. The results shown here are the mean ± S.D. of three independent transfections. B, transactivation of *NAG-1* promoter activity by Sp1 and EGR-1. HCT-116 cells were transfected with the *pNAG133/LUC* (1 μg) construct and a combination of *EGR-1* and *Sp1* at the indicated amounts. The empty *pCDNA3.1* vector was used to keep total plasmid DNA constant. After transfection, cells were treated with vehicle or 30 μM sulindac sulfide, and luciferase activity was performed after 24 h and activity reported as fold increase (over relative luciferase activity of pCDNA3-transfected/vehicle-treated cells) as 1.0. The results shown here are the mean ± S.D. of three independent transfections.

EGR-1 Promoter Is Activated by NSAIDs. To confirm that sulindac sulfide induces EGR-1 at the transcription level, the EGR-1 promoter was cloned into the luciferase reporter vector. A plasmid, *pEGR1260/LUC* (Baek et al., 2004), was transfected into HCT-116 cells, and the luciferase activity was measured in response to several NSAIDs. As shown in Fig. 6A, sulindac sulfide greatly enhanced *EGR-1* promoter activity by 25-fold, whereas indomethacin, ibuprofen, and diclofenac were less effective in stimulating the *EGR-1* promoter activity. Aspirin, piroxicam, and naproxen marginally enhanced the EGR-1 promoter activity at the indicated concentrations. Sulindac sulfone, sulindac, acetaminophen, and DFU did not enhance the luciferase activity (data not shown). Thus, NSAID-induced *EGR-1* promoter activity and NSAID-induced *NAG-1* promoter activity show a similar pattern of responses to different NSAIDs. To determine whether enhanced EGR-1 production by NSAIDs results in the transactivation of EGR-1 target genes, a construct containing four copies of EGR-1 binding sites *pEBS1⁴luc* construct was transfected into HCT-116 cells, and the cells were treated with several NSAIDs. As shown in Fig. 6B, sulindac sulfide dramatically enhanced promoter activity, followed by diclofenac and indomethacin. These data indicate that NSAID induces the expression of a functionally active protein, EGR-1, that binds and transactivates EGR-1 target genes.

Discussion

Many epidemiological studies have reported a consistent 40 to 50% reduction in the risk of developing colorectal cancer associated with the use of NSAIDs (Thun and Heath, 1995; Thun, 1996; Thun et al., 2002). Although many reports suggest a clear relationship between NSAID usage and cancer chemoprevention, the exact molecular mechanism by which NSAIDs exert their antitumorigenic effect is not clear. A substantial body of data suggests that COX-2 overexpression promotes tumor progression, including resistance to apoptosis, increased invasiveness, and angiogenesis (Tsujii et al., 1998). Furthermore, COX-1 expression also plays an important role in tumorigenesis (Tiano et al., 2002). Thus, COX inhibition by NSAIDs probably plays an important role in antitumorigenesis. Other findings indicate that COX-independent mechanisms are also responsible for the chemopreventive activity of COX inhibitors. For instance, the *R*-enantiomer of flurbiprofen does not inhibit COX but has chemopreventive activity in the mouse model of intestinal polyposis (Wechter et al., 1997), prostate cancer (Wechter et al., 2000), and in vitro (Grosch et al., 2003). In addition, sulindac sulfone, which is not a COX inhibitor, inhibits azoxymethane-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). Thus, both COX-dependent and COX-independent pathways seem to be involved in the chemopreventive activity of NSAIDs.

NAG-1 was identified as a target gene for NSAIDs, and a significant increase in the expression of this protein was observed in cultured cells treated with some NSAIDs and mice treated with chemopreventive doses of the prodrug sulindac (Baek et al., 2001b; Kim et al., 2002). The changes in NAG-1 expression were not dependent on the inhibition of

COX but could provide a possible explanation for antitumorigenic activities independent of COX inhibition. NAG-1 is a unique member of the transforming growth factor-β superfamily with incompletely characterized biological activity, but studies with xenograft mouse models confirm that NAG-1 has antitumorigenic activity. The increase in NAG-1 expression results in the induction of apoptosis in several cancer cell lines, including human colorectal HCT-116 cells (Li et al., 2000; Tan et al., 2000; Baek et al., 2001b). NAG-1 expression is induced not only by NSAIDs, but also by several antitumorigenic compounds, including dietary chemicals, peroxisome proliferator-activated receptor-γ ligands, and p53 activators. NAG-1 contains a p53 binding site in the 5' upstream region (Li et al., 2000; Baek et al., 2001a), and several dietary compounds induce NAG-1 expression by increasing p53 expression (Baek et al., 2002a; Wilson et al., 2003). In

fact, NAG-1 was recently reported to be the most highly induced gene by p53 as measured with cDNA array technology (Robles et al., 2001). However, mutations commonly occur at the p53 tumor suppressor locus in many forms of cancer, including colorectal cancer. The increase in NAG-1 expression by NSAIDs is independent of p53, suggesting that NSAIDs could still increase NAG-1 expression in tumors with mutations in p53. In this report, we found that the regulation of NAG-1 by NSAIDs is mediated by the transcription factor EGR-1. Our findings indicate that an increase in EGR-1 expression occurs before NAG-1 expression and is required for the increased transcriptional activity of NAG-1 by NSAIDs (Fig. 5). An EGR-1 binding site was found in the NAG-1 promoter that overlaps with an Sp1 site. Thus, the transcriptional activity of NAG-1 depends on the balance of EGR-1 and Sp1 family members. Indeed, this site in the
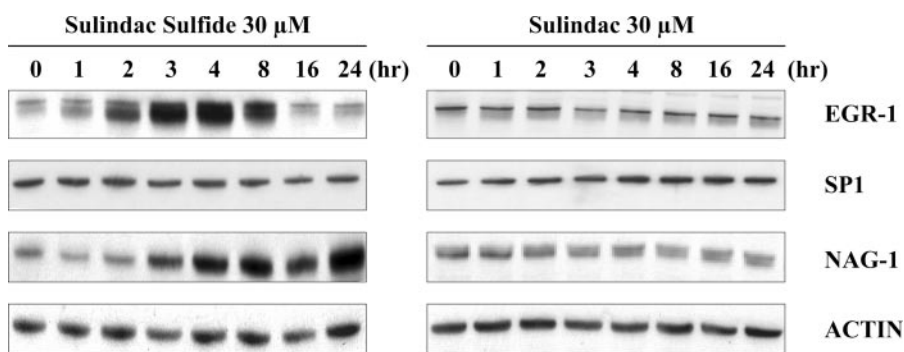

Fig. 5. EGR-1 induction occurs before NAG-1 induction. HCT-116 cells were treated with 30 μM sulindac sulfide (left) or 30 μM sulindac (right), and the expression of EGR-1, Sp1, NAG-1, and actin was measured by immunoblotting of cell extracts.
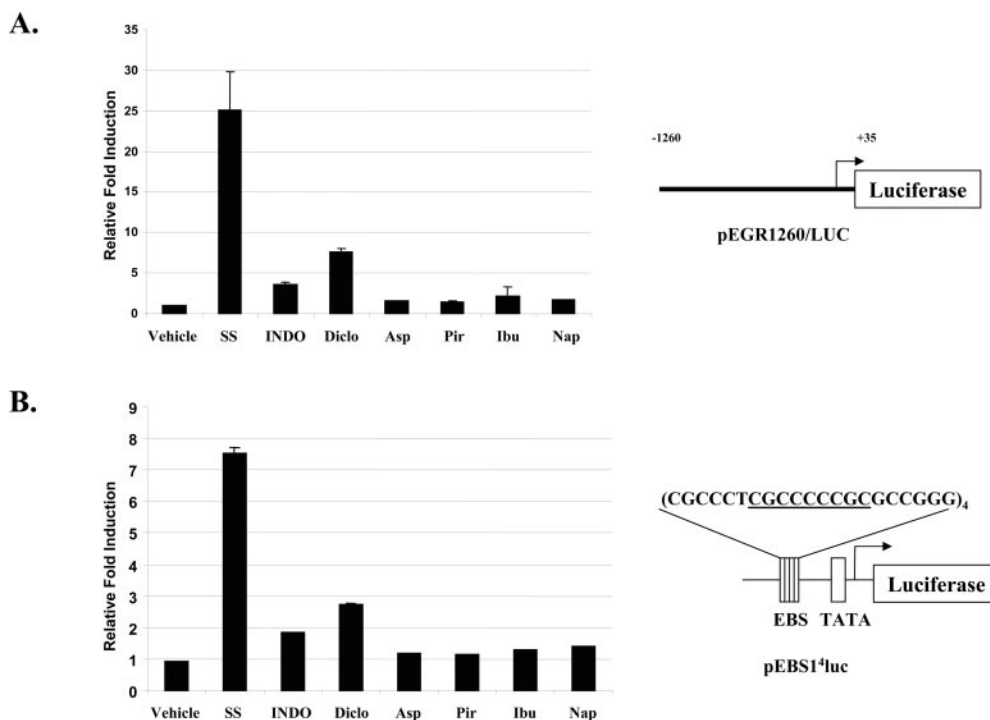

Fig. 6. Promoter activity of EGR-1 induced by NSAIDs. A, the promoter activity of *pEGR1260/LUC* is stimulated by several NSAIDs. HCT-116 cells were transfected with *pEGR1260/LUC* and then treated for 24 h with several NSAIDs in the absence of serum, and luciferase activity was measured. The internal control vector (*pRL-null*) was used to normalize for transfection efficiency. The data represent mean ± S.D. from three different experiments. The concentration of compounds used were vehicle, 0.2% DMSO; sulindac sulfide (SS), 30 μM; indomethacin (INDO), 100 μM; diclofenac (Diclo), 100 μM; aspirin (Asp), 5 mM; ibuprofen (Ibu), 1 mM; and naproxen (Nap), 100 μM. The *y*-axis shows fold induction (over relative luciferase activity of vehicle-treated cells) as 1.0. B, the *pEBS1⁴luc* construct (1 μg) was transfected into HCT-116 cells, and then transfected cells were treated with varying concentrations of the same compounds described above for 24 h. As an internal control, *pRL-null* vector (0.05 μg) was used to adjust for transfection efficiency. The results are the means ± S.D. of three independent transfections. The *y*-axis shows fold induction of Firefly luciferase activity/*R. reniformis* luciferase activity compared with that of vehicle-treated cells.

NAG-1 promoter was identified previously as a troglitazone-response element, and EGR-1 was shown to binds to this site as assessed by electrophoretic mobility shift assay (Baek et al., 2004). The expression of Sp1 is not altered in the presence of sulindac sulfide, whereas EGR-1 expression is increased. The expression of EGR-1 also increases NAG-1 transcription and will enhance sulindac sulfide-induced NAG-1 expression. Overlapping consensus sequences for EGR-1 and Sp1 have been described in the regulatory elements of numerous cytokine genes, including M-CSF (Srivastava et al., 1998) and IL-2 (Decker et al., 1998). Competition for DNA binding between the inducible product of EGR-1 and the constitutively produced Sp1 provides a well-defined means of transcriptional regulation. Troglitazone, a chemopreventive drug but not an NSAID, increases NAG-1 expression also via EGR-1 expression (Baek et al., 2004). In both cases, the expression of EGR-1 occurred before the increase in NAG-1 expression. The EGR-1 expression results in the transcriptional activation of the NAG-1 promoter and hence an increase in NAG-1 expression. However, the mechanisms responsible for the regulation of EGR-1 expression are different. Although sulindac sulfide dramatically increased the promoter activity of EGR-1, troglitazone does not. Troglitazone increased EGR-1 by altering the stability of EGR-1 RNA mediated by increased extracellular signal-regulated kinase 1/2 activity (Baek et al., 2003).

The EGR-1 transcription factor (also know as *NGFI-A, Zif268, krox24,* and *TIS8*) is a member of a transcription factor family that contains three zinc fingers and preferentially binds to the GC-rich DNA core sequence. *EGR-1* is also a member of the immediate early gene family and encodes a nuclear phosphoprotein involved in the regulation of cell growth and differentiation in response to diverse stimuli. Although several downstream target genes of EGR-1, including growth factors, adhesion molecules, cytokines, cell-cycle components, and coagulation system, are identified, the expression of EGR-1 and its role in cancer are complex (Liu et al., 1998). A number of reports indicate that *EGR-1* acts as a tumor suppressor gene. *EGR-1* is down-regulated in several types of neoplasia as well as in an array of tumor cell lines (Huang et al., 1997). *EGR-1* is induced very early in the apoptotic process, in which it mediates the activation of downstream regulators such as p53 (Nair et al., 1997), but EGR-1–induced apoptosis has also been reported in p53−/− cells, indicating the existence of both p53-dependent and -independent pathways. EGR-1 may also activate the phosphatase and tensin homolog tumor suppressor gene during UV irradiation (Virolle et al., 2001). The expression of EGR-1 suppresses the growth of transformed cells both in soft agar and in athymic nude mice (Huang et al., 1995). One of the important downstream targets of EGR-1 seems to be NAG-1, a protein that will suppress the growth of cells on soft agar and inhibit tumor growth in the xenographic nude mouse model. NAG-1 may mediate some of the tumor-suppressor activity of this tumor-suppressor gene, but this transcription factor regulates the expression a number of genes. These genes are linked to the regulation of other biological processes such as angiogenesis, vascular injury, and inflammatory stress (Fahmy et al., 2003). The discovery that some COX inhibitors at physiological concentrations increase the expression of EGR-1 opens a new area of investigation and may provide a better understanding of the chemopreventive, pharmacological, and toxicological activities of this class of drugs.

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