0026-895X/05/6806-1782–1792$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2005 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 68:1782–1792, 2005

Vol. 68, No. 6
17046/3064861
Printed in U.S.A.

1,1-Bis(3′-indolyl)-1-(*p*-substitutedphenyl)methanes Are Peroxisome Proliferator-Activated Receptor γ Agonists but Decrease HCT-116 Colon Cancer Cell Survival through Receptor-Independent Activation of Early Growth Response-1 and Nonsteroidal Anti-Inflammatory Drug-Activated Gene-1

Sudhakar Chintharlapalli, Sabitha Papineni, Seung Joon Baek, Shengxi Liu, and Stephen Safe

Department of Biochemistry and Biophysics, College of Agriculture and Life Sciences, Texas A&M University, College Station, Texas (S.C.); Department of Veterinary Physiology and Pharmacology, College of Veterinary Medicine, Texas A&M University, College Station, Texas (S.P., S.S.); Department of Pathobiology, College of Veterinary Medicine, University of Tennessee, Knoxville, Tennessee (S.J.B.); and Institute of Biosciences and Technology, Texas A&M University System Health Science Center, Houston, Texas (S.L., S.S.)

Received July 19, 2005; accepted September 8, 2005

ABSTRACT

1,1-Bis-(3′-indolyl)-1-(*p*-substitutedphenyl)methanes containing *p*-trifluoromethyl (DIM-C-pPhCF$_3$), *p*-*t*-butyl (DIM-C-pPhtBu), and phenyl (DIM-C-pPhC$_6$H$_5$) substituents decrease survival of HCT-116 colon cancer cells and activate peroxisome proliferator-activated receptor (PPAR) γ in this and other cancer cell lines. These PPARγ-active compounds had minimal effects on expression of cell cycle proteins and did not induce caveolin-1 in HCT-116 cells. However, these compounds induced nonsteroidal anti-inflammatory drug-activated gene-1 (NAG-1) and apoptosis in HCT-116 cells, and in time-course studies, the PPARγ agonists maximally induced early growth response-1 (Egr-1) protein within 2 h, whereas a longer time course was observed for induction of NAG-1 protein. These data, coupled with deletion and mutation analysis of both the Egr-1 and NAG-1 gene promoters, indicate that activation of NAG-1 by these compounds was dependent on prior induction of Egr-1, and induction of these responses was PPARγ-independent. Results of kinase inhibitor studies also demonstrated that activation of Egr-1/NAG-1 by methylene-substituted diindolylmethanes (C-DIMs) was phosphatidylinositol 3-kinase-dependent, and this represents a novel receptor-independent pathway for C-DIM-induced growth inhibition and apoptosis in colon cancer cells.

Nonsteroidal anti-inflammatory drug-activated gene-1 (NAG-1) is a transforming growth factor β-like secreted protein and was initially characterized as a p53-regulated gene (Li et al., 2000; Tan et al., 2000). Overexpression of NAG-1 in breast cancer cells resulted in growth arrest and apoptosis, and similar results were also observed in colon cancer cells (Li et al., 2000; Baek et al., 2001b). Baek, Eling, and their coworkers have extensively investigated the induction of NAG-1 by several different structural classes of drugs and chemoprotective phytochemicals in HCT-116 colon and other cancer cell lines (Baek et al., 2001a,b, 2002, 2004a,b, 2005; Bottone et al., 2002; Kim et al., 2002, 2004, 2005; Newman et al., 2003; Wilson et al., 2003; Yamaguchi et al., 2004). Chem-

This work was supported by National Institutes of Health grants ES09106 and CA11233 and the Texas Agricultural Experiment Station.
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.105.017046.

ABBREVIATIONS: NAG-1, nonsteroidal anti-inflammatory drug-activated gene-1; DIM, diindolylmethane; PPAR, peroxisome proliferator-activated receptor; PGJ2, 15-deoxy-Δ^{12,14}-prostaglandin J2; GW9662, 2-chloro-5-nitrobenzanilide; Egr-1, early growth response-1; C-DIM, methylene-substituted diindolylmethanes; DIM-C-pPhtBu, 1,1-bis(3′-indolyl)-1-(*p*-*t*-butylphenyl)methane; DIM-C-pPhCF$_3$, 1,1-bis(3′-indolyl)-1-(*p*-trifluoromethylphenyl)methane; DIM-C-pPhC$_6$H$_5$, 1,1-bis(3′-indolyl)-1-(*p*-biphenyl)methane; PI3K, phosphatidylinositol 3-kinase; BLOTTO, bovine lacto-transfer optimizer; FBS, fetal bovine serum; β-Gal, β-galactosidase; DMSO, dimethyl sulfoxide; TBST, Tris-buffered saline/Tween 20; ChIP, chromatin immunoprecipitation; bp, base pair(s); PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; T007, 2-chloro-5-nitro-*N*-4-pyridinyl-benzamide; TFIIB, transcription factor IIB; MAPK, mitogen-activated protein kinase; PD98059, 2′-amino-3′-methoxyflavone; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; SRE, serum response element.

icals that induce NAG-1 expression in cancer cell lines generally inhibit growth and/or induce apoptosis in these cells, and these effects are due, in part, to induction of NAG-1 protein. In addition to nonsteroidal anti-inflammatory drugs, other agents that induce NAG-1 include phorbol esters, cyclooxygenase inhibitors, genistein, plant polyphenolics, diallyl disulfide, retinoids, indole-3-carbinol, diindolylmethane (DIM), and peroxisome proliferator-activated receptor (PPAR) γ agonists (Baek et al., 2001a,b, 2002, 2004a,b, 2005; Bottone et al., 2002; Kim et al., 2002, 2004, 2005; Newman et al., 2003; Wilson et al., 2003; Yamaguchi et al., 2004).

Several mechanisms of NAG-1 induction have been described, and these are dependent not only on the structure or class of inducing agents but also on cell context. For example, diallyl disulfide and genistein are antitumorigenic components of garlic and soy, and their induction of NAG-1 in HCT-116 cells is p53-dependent (Bottone et al., 2002; Wilson et al., 2003). In contrast, induction of NAG-1 by indole-3-carbinol and DIM, two anticarcinogenic components in cruciferous vegetables, is p53-independent in the same cell line (Lee et al., 2005). Two PPARγ agonists, 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (PGJ2) and troglitazone, also induce NAG-1 expression in HCT-116 cells, and the PPARγ antagonist GW9662 inhibits the induction response by PGJ2 but not troglitazone (Baek et al., 2004b). It was also shown that the PPARγ-independent activation of NAG-1 by troglitazone is due to induction of early growth response gene (Egr-1), which in turn activates NAG-1 (Baek et al., 2003, 2004b).

Recent studies in this laboratory have identified 1,1-bis-(3′-indolyl)-1-(*p*-substitutedphenyl)methanes [methylene-substituted DIMs (C-DIMs)] as PPARγ agonists, and the most active compounds contain *p*-trifluoromethyl (DIM-C-pPhCF_3), *p*-*t*-butyl (DIM-C-pPhtBu), and phenyl (DIM-C-pPhC$_6$H$_5$) (Chintharlapalli et al., 2004; Hong et al., 2004; Qin et al., 2004; Contractor et al., 2005). These PPARγ agonists decrease survival and induce apoptosis in breast, leukemia, pancreatic, and colon cancer cells. In the latter two cell lines, growth inhibition is associated with receptor-dependent activation of p21 (Hong et al., 2004) and the tumor suppressor gene caveolin-1 (Chintharlapalli et al., 2004). There is also evidence that decreased cancer cell survival induced by these compounds is receptor-independent (Qin et al., 2004; Contractor et al., 2005). The PPARγ-active C-DIMs also decrease cell survival and induce apoptosis in HCT-116 cells but do not induce caveolin-1, as reported previously in HT-29 and HCT-15 colon cancer cells (Chintharlapalli et al., 2004). In contrast, these compounds induce NAG-1 in HCT-116 cells, and PPARγ antagonists do not inhibit this response. Like troglitazone, the PPARγ-active C-DIMs also induce Egr-1, which in turn interacts with proximal (GC-rich) Egr-1 motifs in the NAG-1 gene promoter. However, in contrast to troglitazone, the C-DIM compounds induce Egr-1 through a phosphatidylinositol 3-kinase (PI3K)-dependent pathway, which in turn activates serum response elements in the Egr-1 promoter. This represents a novel pathway for induction of Egr-1 and NAG-1, and these responses contribute to the induction of growth inhibition and apoptosis by the PPARγ-active C-DIMs in colon cancer cells. Moreover, these results also distinguish the mode of action of these C-DIM analogs from that of troglitazone and DIM and identify an important PPARγ-independent mode of action.

Materials and Methods

Cell Lines. Human colon carcinoma cell line HCT-116 and human prostate cancer cell line LNCap were obtained from American Type Culture Collection (Manassas, VA). HCT-116 and LnCap cells were maintained in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 0.22% sodium bicarbonate, 0.011% sodium pyruvate, 0.45% glucose, 0.24% HEPES, 10% FBS, and 10 ml/l of 100× antibiotic antimycotic solution (Sigma-Aldrich). Cells were maintained at 37°C in the presence of 5% CO_2.

Antibodies and Reagents. Antibodies for poly(ADP-ribose) polymerase, cyclin D1, p27, phospho-Akt, Akt, p53, and caveolin 1 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). NAG-1 was from Upstate Biotechnology (Charlottesville, VA), and Egr-1 was from Cell Signaling Technology Inc. (Beverly, MA). Monoclonal β-actin antibody was purchased from Sigma-Aldrich. Reporter lysis buffer and luciferase reagent for luciferase studies were supplied by Promega (Madison, WI). β-Galactosidase (β-Gal) reagent was obtained from Tropix (Bedford, MA), and LipofectAMINE reagent was purchased from Invitrogen (Carlsbad, CA). Western Lightning chemiluminescence reagent was from PerkinElmer Life and Analytical Sciences (Boston, MA). Rosiglitazone was purchased from LKT Laboratories, Inc. (St. Paul, MN). The C-substituted DIMs were prepared in this laboratory as described previously (Chintharlapalli et al., 2004; Qin et al., 2004), and the Egr-1 constructs were kindly provided by C.-C. Chen and W.-R. Lee (Texas A&M University, College Station, TX).

Plasmids. The Gal4 reporter containing 5x Gal4 response elements (pGal4) was kindly provided by Dr. Marty Mayo (University of North Carolina, Chapel Hill, NC). Gal4DBD-PPARγ construct (gPPARγ) was a gift of Dr. Jennifer L. Oberfield (GlaxoSmithKline Research and Development, Research Triangle Park, NC). PPRE-luc construct contains three tandem PPREs with a minimal TATA sequence in pGL2 (Chintharlapalli et al., 2004; Qin et al., 2004). pNAG-1A-pNAG-1D, pNAG-1Dm1, pNAG-1Dm2, and pNAG-1Dm3 were generated previously (Baek et al., 2001a, 2003). pEGR-1A-pEGR-1E constructs containing Egr-1 promoter inserts have also been described previously (Chen et al., 2004).

Transfection and Luciferase Assay. HCT-116 cells (1×10^5 cells/well) were plated in 12-well plates in DMEM/Ham's F-12 media supplemented with 2.5% charcoal-stripped FBS. After 16 h, various amounts of DNA [i.e., Gal4Luc (0.4 μg), β-Gal (0.04 μg), and PPRE-Luc (0.04 μg)] and 0.25 μg of the NAG-1/EGR-1 constructs were transfected by LipofectAMINE (Invitrogen) according to the manufacturer's protocol. Five hours after transfection, the transfection mix was replaced with complete media containing either vehicle (DMSO) or the indicated ligand for 20 to 22 h. Cells were then lysed with 100 μl of 1× reporter lysis buffer, and 30 μl of cell extract was used for luciferase and β-Gal assays. A LumiCount luminometer (PerkinElmer Life and Analytical Sciences) was used to quantitate luciferase and β-Gal activities, and the luciferase activities were normalized to β-Gal activity.

Cell Proliferation Assay. HCT-116 cells (2×10^4/well) were plated in 12-well plates. After cell attachment for 24 h, the medium was changed to DMEM/Ham's F-12 media containing 2.5% charcoal-stripped FBS and either vehicle (DMSO) or the indicated compound. Fresh media and compounds were added every 48 h, and the cells were then trypsinized and counted at the indicated times using a Coulter Z1 cell counter (Beckman Coulter, Fullerton, CA). Each experiment was done in triplicate, and results are expressed as means ± S.E. for each determination.

Western Blot Analysis. HCT-116 cells were seeded in DMEM/Ham's F-12 media containing 2.5% charcoal-stripped FBS for 24 h and then treated with either the vehicle (DMSO) or the compounds for different times as indicated. Cells were collected by scraping in 150 μl of high salt lysis buffer [50 mM HEPES, 0.5 M NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, and 5 μl/ml Protease Inhibitor Cocktail (Sigma-Aldrich)]. The lysates were

incubated on ice for 1 h with intermittent vortexing followed by centrifugation at 40,000*g* for 10 min at 4°C. Before electrophoresis, the samples were boiled for 3 min at 100°C, the amounts of protein were determined, and 60 μg of protein was applied per lane. Samples were subjected to SDS-polyacrylamide gel electrophoresis on 10% gel at 120 V for 3 to 4 h. Proteins were transferred onto polyvinylidene membranes (polyvinylidene difluoride; Bio-Rad, Hercules, CA) by semidry electroblotting in a buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol for 1.5 h at 180 mA. The membranes were blocked for 30 min with 5% TBST-BLOTTO (10 mM Tris-HCl, 150 mM NaCl, pH 8.0, 0.05% Triton X-100, and 5% nonfat dry milk) and incubated in fresh 5% TBST-BLOTTO with 1:1000 (for caveolin 1, p27, and cyclin D1), 1:250 (for PARP), 1:500 (for NAG-1 and Egr-1), and 1:5000 (for β-actin) primary antibody overnight with gentle shaking at 4°C. After washing with TBST for 10 min, the polyvinylidene difluoride membrane was incubated with secondary antibody (1:5000) in 5% TBST-BLOTTO for 90 min. The membrane was washed with TBST for 10 min and incubated with 10 ml of chemiluminescence substrate (PerkinElmer Life and Analytical Sciences) for 1.0 min and exposed to Kodak X-OMAT AR autoradiography film (Eastman Kodak, Rochester, NY).

Chromatin Immunoprecipitation Assay. HCT-116 cells (2×10^7) were treated with Me_2SO (time 0) or DIM-C-pPhC_6H_5 (20 μM) for 1 or 2 h. Cells were then fixed with 1.5% formaldehyde, and the cross-linking reaction was stopped by addition of 0.125 M glycine. After washing twice with phosphate-buffered saline, cells were scraped and pelleted. Collected cells were hypotonically lysed, and nuclei were collected. Nuclei were then sonicated to desired chromatin length (~500 bp). The chromatin was precleared twice by addition of protein A-conjugated beads (Pierce Chemical, Rockford, IL) and then incubated at 4°C for 1 h with gentle agitation. The beads were pelleted, and the precleared chromatin supernatants were immunoprecipitated with antibodies specific to IgG, Sp1, Sp3, Sp4 (Santa Cruz Biotechnology, Inc.), and Egr-1 (Cell Signaling Technology Inc.) at 4°C overnight. Protein-antibody complexes were collected by addition of protein A-conjugated beads at room temperature for 1 h. Beads were extensively washed; the protein-DNA cross-links were eluted and reversed. DNA was purified by phenol extraction/ethanol precipitation followed by PCR amplification. The NAG-1 primers are 5′-TAC TGA GGC CCA GAA ATG TG-3′ (forward) and 5′-GAG CTG GGA CTG ACC AGA TG-3′ (reverse). These primers amplify a 211-bp region of the human NAG-1 promoter containing two Sp1/Egr-1 binding sites. The positive control primers are 5′-TAC TAG CGG TTT TAC GGG CG-3′ (forward) and 5′-TCG AAC AGG AGG AGC AGA GAG CGA-3′ (reverse), which amplify a 167-bp region of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. The negative control primers are 5′-ATG GTT GCC ACT GGG GAT CT-3′ (forward) and 5′-TGC CAA AGC CTA GGG GAA GA-3′ (reverse), which amplify a 174-bp region of human CNAP1 exon. PCR products were resolved on a 2% agarose gel in the presence of 1:10,000 SYBR gold (Invitrogen).

Statistical Analysis. Statistical significance was determined by analysis of variance and Scheffé's test, and the levels of probability are noted. The results are expressed as means ± S.E. for at least three separate (replicate) experiments for each treatment.

Results

Studies in this laboratory have characterized selected C-DIMs as PPARγ agonists that inhibit growth of colon and other cancer cell lines through receptor-dependent and -independent pathways (Chintharlapalli et al., 2004; Hong et al., 2004; Qin et al., 2004; Contractor et al., 2005). Results illustrated in Fig. 1 show that three PPARγ-active C-DIM compounds, namely, DIM-C-pPhCF_3, DIM-C-pPhtBu and DIM-C-pPhC_6H_5, decreased HCT-116 colon cancer cell survival at concentrations of 1 to 10 μM after treatment for 48 or 96 h. Treatment for 96 h resulted in cell death using 10 μM concentrations (i.e., cell numbers were lower than the initial number of seeded cells). In contrast, 10 μM rosiglitazone decreased cell survival, but it did not induce cell death, which is observed only at higher concentrations of this compound (data not shown). Previous studies in colon cancer cells show that PPARγ-active C-DIMs induced transactivation in cells transfected with PPRE-luc or GAL4-PPRγ/pGAL4-luc constructs (Chintharlapalli et al., 2004). In HCT-116 cells, PPARγ-active C-DIMs induced a concentration-dependent increase in transactivation in cells transfected with PPARE-luc, and 10 μM rosiglitazone also increased activity but with lower fold-inducibility (Fig. 2A). The same compounds also induced transactivation in HCT-116 cells transfected with GAL4-PPARγ/GAL4-luc constructs and treated with 5 and 10 μM C-DIMs, and cotreatment with the PPARγ antagonist GW9662 significantly inhibited this response (Fig. 2B). These results confirm that PPARγ-active C-DIMs induce receptor-dependent transactivation in HCT-116 cells, and this complements results of recent studies that show similar response in HT-29 and HCT-15 colon cancer cell lines (Chintharlapalli et al., 2004).

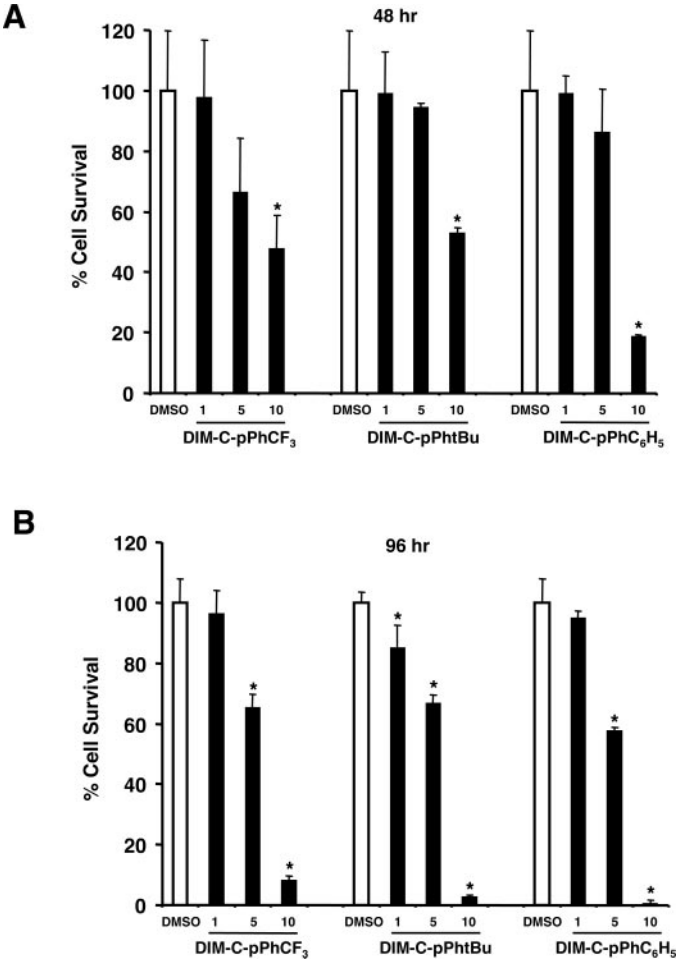

Fig. 1. PPARγ-active C-DIMs decrease HCT-116 cancer cell survival. HCT-116 cells were treated for 48 (A) or 96 (B) h with DMSO or different concentrations of C-DIMs, and cell numbers as a percentage of DMSO-treated cells were determined as described under *Materials and Methods*. Results are expressed as means ± S.E. for three separate determinations for each treatment group, and a significant ($p < 0.05$) decreased in cell survival is indicated with an asterisk.

Treatment of HCT-116 cells for 24 h with 2.5, 5.0, and 7.5 μM DIM-C-pPhCF$_3$, DIM-C-pPhtBu, or DIM-C-pPhC$_6$H$_5$ did not affect expression of cyclin D1 or p27 proteins (Fig. 3A), and p21 levels were barely detectable (data not shown). PPARγ agonists frequently affect expression of these proteins in other cancer cell lines (Clay et al., 1999, 2002; Motomura et al., 2000; Inoue et al., 2001; Palakurthi et al., 2001; Qin et al., 2003; Hong et al., 2004); however, the results (Fig. 3A) are comparable with those observed for the same compounds in HT-29 and HCT-15 cells (Chintharlapalli et al., 2004). The growth inhibitory effects of these compounds in HT-29 and HCT-15 cells are associated with receptor-dependent activation of the tumor suppressor gene caveolin-1 (Chintharlapalli et al., 2004), which inhibits colon cancer cell growth. However, in HCT-116 cells, treatment with 5, 10, or 15 μM DIM-C-pPhCF$_3$ or DIM-C-pPhC$_6$H$_5$ for 72 h did not affect caveolin-1 protein expression (Fig. 3B). Moreover, unlike HT-29 and HCT-15 cells, relatively high levels of caveolin-1 were detected in solvent (DMSO)-treated HCT-116 cells. Other PPARγ agonists such as PGJ2 and troglitazone, induce the transforming growth factor β-like protein NAG-1 in HCT-116 cells (Baek et al., 2003; Wilson et al., 2003); therefore, induction of NAG-1 protein and apoptosis by PPARγ-active C-DIMs was investigated in HCT-116 cells treated with relatively high concentrations (10–20 μM) over 24 h. The results (Fig. 3C) show that NAG-1 protein expression was not detectable in solvent-treated cells; however, treatment with 10 to 20 μM DIM-C-pPhCF$_3$, DIM-C-pPhtBu, or DIM-C-pPhC$_6$H$_5$ induced NAG-1 protein expression. We also observed induction of NAG-1 by C-DIMs in LNCaP cells as described previously for other NAG-1 inducers (Newman et al., 2003); however, NAG-1 was not induced in SW-480 colon cancer cells (data not shown). The induction of NAG-1 in HCT-116 cells was accompanied by activation of apoptosis

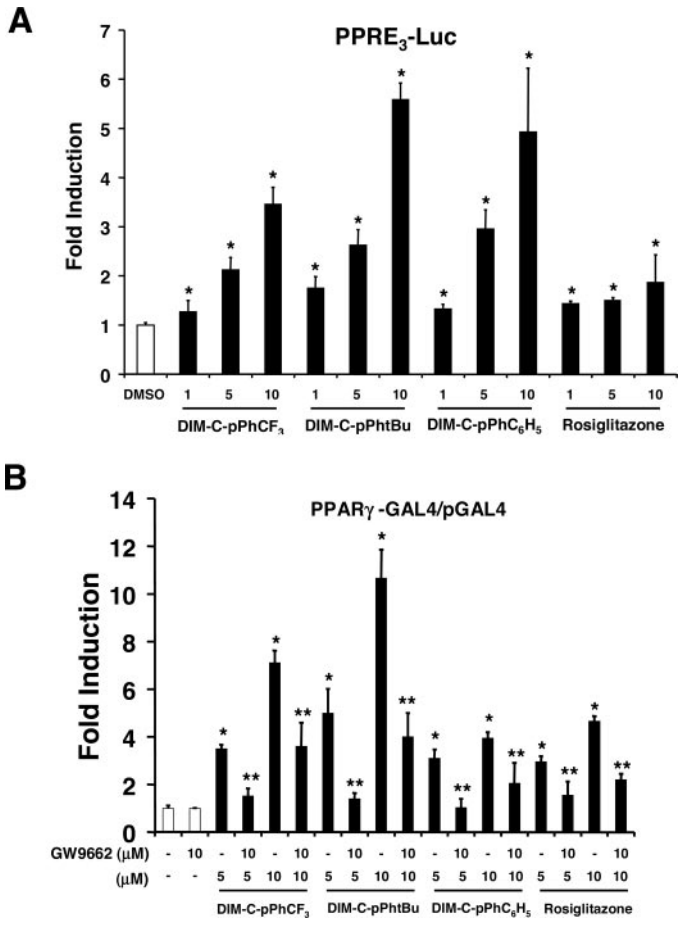

Fig. 2. Activation of PPARγ-dependent transactivation. HCT-116 cells were transfected with PPRE$_3$-luc (A) or PPARγ-GAL4/pGAL4 (B), treated with DMSO, different concentrations of C-DIMs, or rosiglitazone alone or in combination with GW9662, and luciferase activity was determined as described under *Materials and Methods*. Results are expressed as means ± S.E. for replicate determinations for each treatment group, and significant ($p < 0.05$) induction (*) or inhibition after cotreatment with GW9662 (**) is indicated.

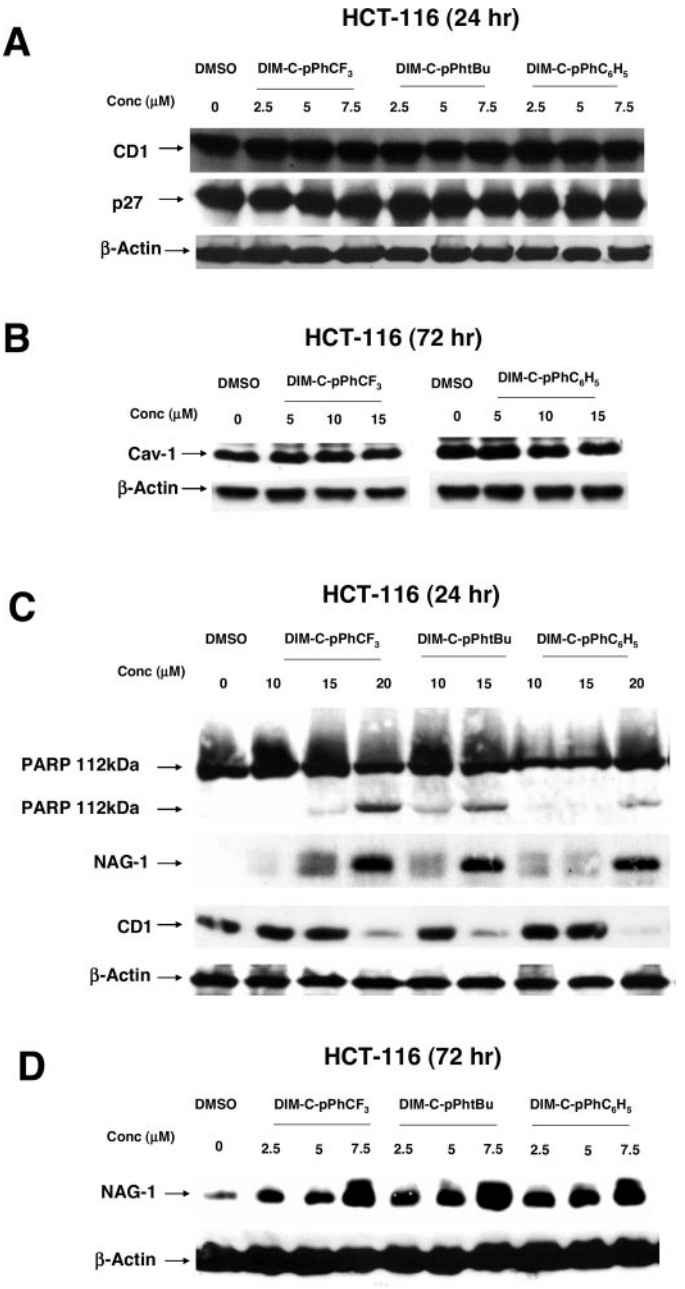

Fig. 3. Modulation of cell cycle proteins, caveolin-1, PARP cleavage, and NAG-1 by PPARγ-active C-DIMs. HCT-116 cells were treated with DMSO or different concentrations of C-DIMs and analyzed for cyclin D1 (CD1)/p27 (A), caveolin-1 (B), PARP cleavage/NAG-1 and CD1 (C), and NAG-1 (D) by Western blot analysis as outlined under *Materials and Methods*. Treatment times were varied and similar results were observed in duplicate experiments for A to D.

as indicated by PARP cleavage. In contrast, p27 expression was unaffected by this treatment (data not shown), and cyclin D1 was down-regulated only at the highest dose level. The coordinate induction of NAG-1 and apoptosis in HCT-116 cells by PPARγ-active C-DIMs after treatment for 24 h is consistent with the effects of these compounds on decreased cell survival (Fig. 1). However, the induction of NAG-1 by PPARγ-active C-DIMs was not strictly a high-dose effect, given that treatment of HCT-116 cells with 2.5, 5.0, and 7.5 μM DIM-C-pPhCF$_3$ and DIM-C-pPhC$_6$H$_5$ for 72 h showed that NAG-1 protein was induced at concentrations as low as 2.5 μM (Fig. 3D).

The concentration-dependent effects of DIM-C-pPhCF$_3$ and DIM-C-pPhC$_6$H$_5$ on induction of NAG-1 and other proteins that are often induced along with NAG-1 were determined in HCT-116 cells after treatment for 24 h (Fig. 4A). NAG-1 protein levels were elevated at concentrations as low as 7.5 μM. After 24 h, maximal induction was observed at concentrations ≤15 μM, and this was accompanied by PARP cleavage. Induction of NAG-1 by some compounds is accompanied by increased expression of p53 protein (Bottone et al., 2002; Wilson et al., 2003) or ATF3 (Baek et al., 2004a; Lee et al., 2005), and the PPARγ-active C-DIMs induced the latter protein but not p53. Although other PPARγ agonists induce NAG-1, the role of the receptor in mediating these responses is structure dependent; studies with PPARγ antagonists showed that induction of NAG-1 by PGJ2 and troglitazone was PPARγ-dependent and -independent, respectively (Baek et al., 2004b). Treatment of HCT-116 cells with DIM-C-pPhCF$_3$ (Fig. 4B) or DIM-C-pPhC$_6$H$_5$ (Fig. 4C) alone or in combination with the PPARγ antagonist GW9662 shows that induction of NAG-1 or apoptosis by the C-DIM compounds is not inhibited by GW9662. We have also repeated the same experiment with another PPARγ antagonist, T007, and ob-

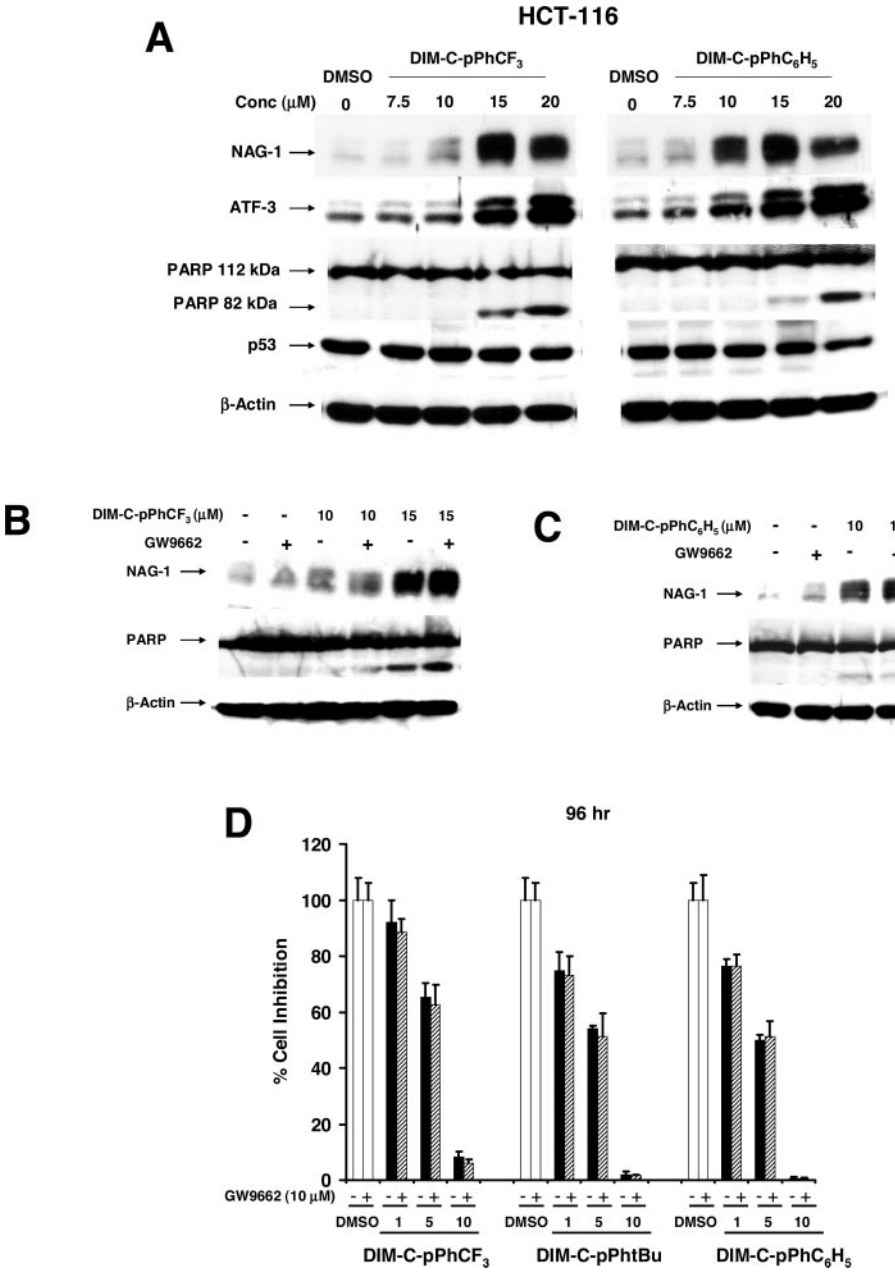

Fig. 4. Induction of NAG-1 and related proteins by C-DIMs and the role of PPARγ. A, induction of NAG-1 and related proteins by C-DIMs. HCT-116 cells were treated with DMSO or 7.5 to 20 μM C-DIMs for 24 h, and proteins were analyzed by Western immunoblot analysis as described under *Materials and Methods*. B and C, role of PPARγ in activation of NAG-1/PARP cleavage by C-DIMs. HCT-116 cells were treated for 24 h with DMSO, 10 or 15 μM C-DIMs alone, or in combination with 10 μM GW9662, and whole cell lysates were analyzed by Western immunoblot analysis as described under *Materials and Methods*. D, role of PPARγ in mediating C-DIM-induced cell survival. Cell survival data (after 96 h) were obtained as described in Fig. 1, and the effects of C-DIMs alone or in combination with GW9662 were determined. No significant ($p < 0.05$) effects on cell survival were observed in cells treated with C-DIMs plus GW9662. Experiments illustrated in B to D were also determined using the PPARγ antagonist T007, and similar results were obtained.

served no inhibition of the C-DIM-mediated induction of NAG-1 or PARP cleavage (data not shown). In addition, GW9662 did not affect decreased HCT-116 cell survival after treatment with the C-DIM compounds for 96 h (Fig. 4D), and similar results were observed after 48 h (data not shown). Apoptosis (PARP cleavage) was induced by PPARγ-active C-DIMs at concentrations as low as 10 μM (after 24 h); this was more pronounced after 48 h (data not shown) and correlated with the cell survival results (Fig. 1). Thus, like troglitazone, the PPARγ-active C-DIMs induce NAG-1 and apoptosis in HCT-116 cells via a PPARγ-independent pathway.

Previous reports have linked the anticarcinogenic activity of troglitazone to induction of both NAG-1 and Egr-1, and enhanced Egr-1 expression has been linked to up-regulation of NAG-1 in HCT-116 cells (Baek et al., 2003, 2004b). The results in Fig. 5A show that both DIM-C-pPhCF$_3$ and DIM-C-pPhC$_6$H$_5$ induce Egr-1 and NAG-1 proteins in HCT-116 cells; however, the temporal expression of both proteins is different. Egr-1 is maximally induced within 2 h after treatment and levels then decline 4 to 24 h after treatment. In contrast, induction of NAG-1 protein increases over the 24-h treatment period, and the highest levels are observed after 24 h. The temporal sequence of C-DIM-induced NAG-1 and -Egr-1 expression is comparable with that observed for troglitazone in HCT-116 cells, except that the time course for induction of both proteins is somewhat delayed (Baek et al., 2003).

We also investigated the effects of the PPARγ-active C-DIMs on transactivation in cells transfected with constructs containing −600 to +12 (pEGR-1A), −460 to +12 (pEGR-1B), and −164 to +12 (pEGR-1C) Egr-1 promoter inserts (Fig. 5, B–D). All three compounds induced transactivation in cells transfected with pEGR-1A and pEGR-1B but not pEGR-1C, suggesting that SRE2-4 motifs within the −460 to −164 region of the promoter were required for activation of Egr-1. Further deletion of the 3′ region of the promoter was investigated in cells transfected with pEGR-1D (−480 to −285) and pEGR-1E (−480 to −376). The C-DIM compound induced transactivation only in cells transfected with pEGR-1D and not pEGR-1E, demonstrating that the minimal region of

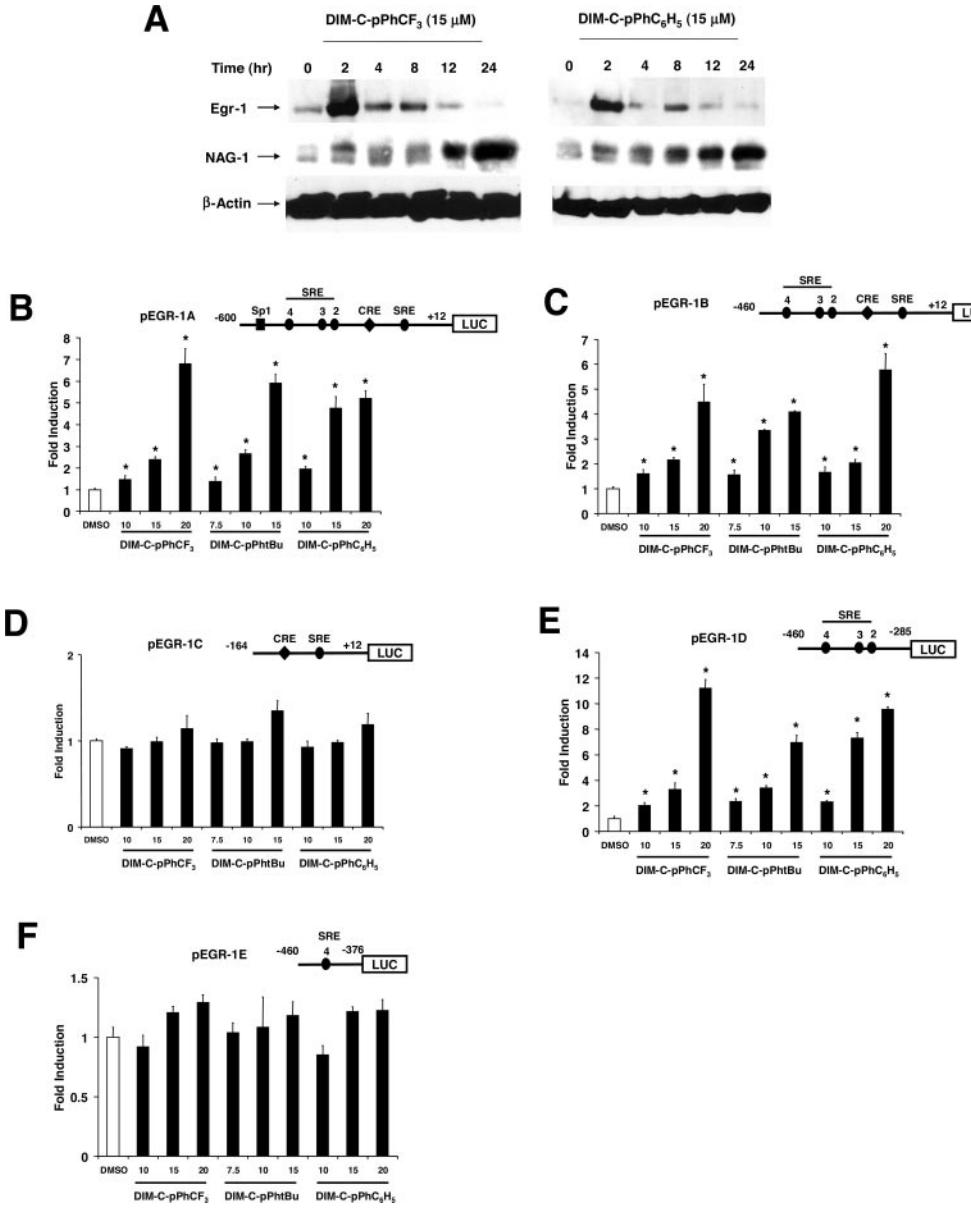

Fig. 5. Induction of Egr-1 protein/reported gene activity by C-DIMs. A, time-course induction of NAG-1 and Egr-1. HCT-116 cells were treated with DMSO or 15 μM C-DIMs for up to 24 h, and whole cell lysates were analyzed for Egr-1, NAG-1, and β-actin (control) by Western immunoblot analysis as described under *Materials and Methods*. Activation of EGR-1A (B), EGR-1B (C), EGR-1C (D), EGR-1D (E), and EGR-1E (F) by C-DIMs. HCT-116 cells were transfected with the various constructs treated with DMSO or C-DIMs, and luciferase activity determined as described under *Materials and Methods*. Results are expressed as means ± S.E. for three replicate determinations for each treatment group, and significant ($p < 0.05$) induction is indicated by an asterisk.

the Egr-1 promoter required for transactivation (−376 to −285) contained SRE3 and SRE2.

The potential role of Egr-1 in mediating induction of NAG-1 was further investigated in HCT-116 cells transfected with a series of constructs containing the −3500 to +41 (pNAG-1A), −1086 to +41 (pNAG-1B), −474 to +41 (pNAG-1C), and −133 to +41 (pNAG1D) NAG-1 promoter inserts (Fig. 6, A–D). The results show that the C-DIM compounds induce transactivation in HCT-116 cells transfected with all four constructs. Previous studies show that the −73 to −44 region of the NAG-1 gene promoter contains two GC-rich Sp1 binding sites that overlap two Egr-1 sites (Baek et al., 2001a,b, 2004b). Therefore, cells were transfected with pNAG-1D or constructs containing a single Egr-1 site mutation (pNAG-1Dm1 and pNAG-1Dm2) or both sites mutated (pNAG-1Dm3). The results (Fig. 6E) show that C-DIM-induced transactivation was decreased in cells transfected with pNAG-1Dm1 and pNAG-1Dm2, and no significant induction was observed in cells transfected with pNAG-1Dm3. Thus, mutation of the Egr-1 sites resulted in loss of inducibility of the NAG-1 constructs. Moreover, in cells transfected with pNAG-1D, cotransfection with Egr-1 expression plasmid also induces transactivation (Fig. 7A). These results are consistent with a mechanism of NAG-1 induction by C-DIMs that involves initial activation of Egr-1, which in turn activates the NAG-1 promoter through proximal Egr-1 motifs. We also investigated interactions of Egr-1 with the NAG-1 promoter in a chromatin immunoprecipitation (ChIP) assay with PCR primers that target the proximal region of the NAG-1 promoter that contain the Egr-1 motifs. HCT-116 cells were treated with DMSO or 20 μM DIM-C-pPhC$_6$H$_5$ for 1 or 2 h; cells were then cross-linked with formaldehyde, and the ChIP assay procedure was used to determine interactions of Sp proteins and Egr-1 with the NAG-1 promoter (Fig. 7B). The results show binding of Sp1, Sp3, and Sp4 proteins to the NAG-1 promoter, and the band intensities are increased after treatment with DIM-C-pPhC$_6$H$_5$. Similar results were observed for Egr-1, suggesting that induction of Egr-1 protein (Fig. 5) facilitates recruitment of Egr-1 and Sp proteins to the NAG-1 promoter. As a control experiment for the ChIP assay, the binding of TFIIB to the GAPDH promoter, but not exon 1 of the CNAP1 gene, is illustrated in Fig. 7C, as described previously (Hong et al., 2004).

Egr-1 is an immediate early gene that is activated by multiple factors in different cell lines, including UV light, endoplasmic reticulum stress, hormones, phorbol esters, and

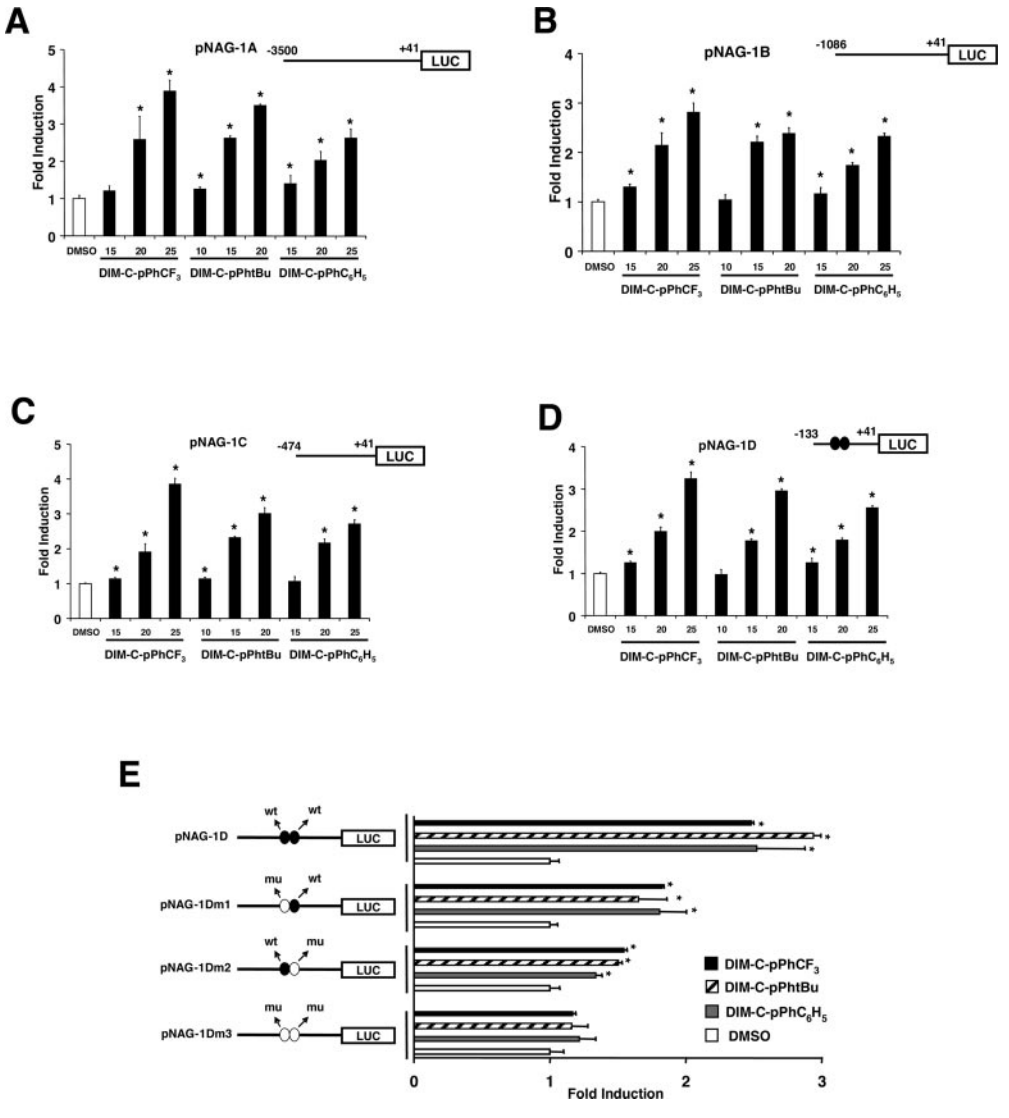

Fig. 6. Activation of NAG-1 promoter constructs by C-DIMs and Egr-1. HCT-116 cells were transfected with pNAG-1A (A), pNAG-1B (B), pNAG-1C (C), pNAG-1D (D), or pNAG-1D mutants (E), treated with DMSO or C-DIMs, and luciferase activity was determined as described under *Materials and Methods*. Results in A to E are expressed as means ± S.E. for three replicate determinations for each treatment group, and significant ($p < 0.05$) induction is indicated by an asterisk.

troglitazone (Sukhatme et al., 1987; Christy and Nathans, 1989; Sukhatme, 1990; Cicatiello et al., 1993; Muthukkumar et al., 1995; Baek et al., 2003, 2004b, 2005; Dziema et al., 2003), and many of the responses involve activation of kinases. Moreover, troglitazone activates Egr-1 and NAG-1 in HCT-116 cells through activation of the mitogen-activated protein kinase (MAPK) pathway (Baek et al., 2003, 2004b). The role of kinases in activation of NAG-1 by C-DIM compounds was therefore investigated in HCT-116 cells treated with 15 μM DIM-C-pPhC$_6$H$_5$ for 24 h in the presence or absence of PD98059 or LY294002, which inhibit MAPK and PI3K-dependent phosphorylation, respectively. The results showed that induction of NAG-1 protein by DIM-C-pPhC$_6$H$_5$ was inhibited by LY294002 but not by PD98059 (Fig. 8A). Similar inhibitory responses were also observed for induction of Egr-1 (Fig. 8B), and DIM-C-pPhCF$_3$-induced responses were also inhibited by LY294002 (data not shown). It is noteworthy that PD98059 alone also induced Egr-1 (Fig. 8B) but not NAG-1 (Fig. 8A), suggesting that Egr-1 alone is not sufficient for activation of NAG-1. The inhibitory effects of LY294002 suggest that the C-DIM compounds induce PI3K. Moreover, in cells transfected with pEGR-1D, induction of luciferase activity by the C-DIM compounds was inhibited by cotreatment with LY294002 (Fig. 8C). The results in Fig. 8D show the time-dependent induction of Akt phosphorylation by DIM-C-pPhC$_6$H$_5$, and a similar induction response was observed for DIM-C-pPhCF$_3$ and DIM-C-pPhtBu. Activation of PI3K was time-dependent, because induction of Akt phosphorylation was not observed after treatment for 2 h (data not shown). These results demonstrate that PPARγ-active C-DIMs coordinately induce Egr-1 and NAG-1 through a novel pathway that involves initial PI3K-dependent activation of Egr-1 through SRE3 and SRE2 motifs on the Egr-1 promoter.

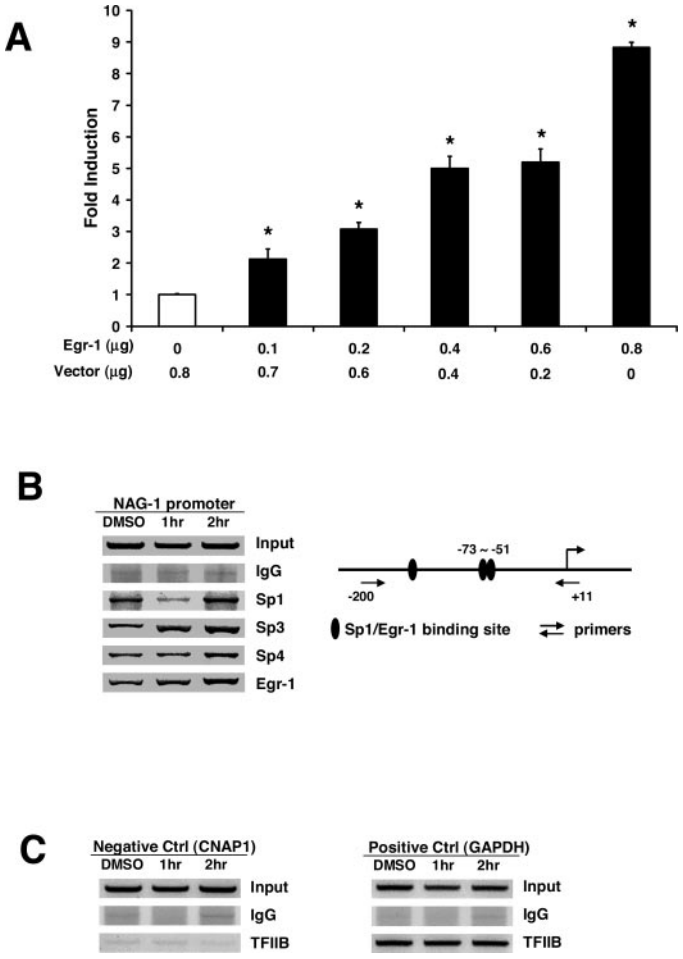

Fig. 7. Activation of NAG-1 by Egr-1. A, activation of NAG-1 promoter constructs by Egr-1 expression plasmid. HCT-116 cells were transfected with pNAG-1D and different amounts of Egr-1 expression plasmid (or empty vector to maintain a constant amount of transfected DNA), and luciferase activity was determined as described under *Materials and Methods*. Results are expressed as means ± S.E. for three replicate determinations for each group, and significant ($p < 0.05$) induction compared with empty vector is indicated by an asterisk. B, ChIP assay. HCT-116 cells were treated with DMSO or 20 μM DIM-C-pPhC$_6$H$_5$, and interactions of various proteins with the NAG-1 promoter were determined in a ChIP assay as described under *Materials and Methods*. C, control binding of TFIIB. The control ChIP assay illustrates binding of TFIIB to the GAPDH promoter but not to exon 1 of the CNAP1 gene (negative control).

Discussion

PPARγ-active C-DIMs and other PPARγ agonists inhibit growth and induce apoptosis in several different cell lines, and these responses are both receptor-dependent and -independent (Sukhatme et al., 1987; Clay et al., 1999, 2002; Takahashi et al., 1999; Motomura et al., 2000; Palakurthi et al., 2001; Qin et al., 2003, 2004; Chintharlapalli et al., 2004; Hong et al., 2004; Contractor et al., 2005). For example, studies in this laboratory have demonstrated that induction of p21 in Panc-28 cells and up-regulation of caveolin-1 in HT-29 and HCT-15 cells is PPARγ-dependent and inhibited by PPARγ antagonists or by PPARγ knockdown with small inhibitory RNAs (Chintharlapalli et al., 2004; Hong et al., 2004). In contrast, induction of apoptosis and down-regulation of cyclin D1 in breast cancer cells was PPARγ-independent (Qin et al., 2004). The interplay between receptor-dependent and -independent pathways has also been reported for the potent triterpenoid-derived PPARγ agonist 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid and related compounds in HCT-15, HT-29, and SW-480 colon cancer cells (Chintharlapalli et al., 2005). At lower growth-inhibitory concentrations, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid induced caveolin-1, and this response was inhibited in cells cotreated with the PPARγ antagonist T007. In contrast, at higher concentrations, caveolin-1 expression was decreased and apoptosis was induced, and this latter response was receptor-independent and not inhibited by T007. The type of differential concentration-dependent induction of PPARγ-dependent and -independent responses has also been observed for PPARγ-active C-DIM compounds in SW-480 cells (S. Chintharlapalli, unpublished results).

In this study, we have investigated the effects of PPARγ-active C-DIMs in HCT-116 cells, and as reported previously in HCT-15 and HT-29 cells (Chintharlapalli et al., 2004), these compounds decrease HCT-116 cell survival (Fig. 1); activate PPARγ-dependent transactivation (Fig. 2); and p21, p27, and cyclin D1 levels were unchanged, except for down-regulation of cyclin D1 at high concentrations (Fig. 3). In contrast to observations in HT-29 and HCT-15 cells (Chintharlapalli et al., 2004), the PPARγ-active compounds did not induce caveolin-1 in HCT-116 cells (Fig. 3), and this may be due, in part, to the relatively high constitutive expression of caveolin-1 in this cell line. Thus, the growth

inhibitory responses of C-DIMs in HCT-116 cells must be related to activation of other pathways. Previous studies in HCT-116 cells have reported that several different structural classes of growth inhibitory/antitumorigenic compounds, including the PPARγ agonists PGJ2 and troglitazone, and DIM induced NAG-1 expression, and this protein exhibits both growth inhibitory and proapoptotic activity (Baek et al., 2001a,b, 2002, 2003, 2004a,b, 2005; Bottone et al., 2002; Kim et al., 2002, 2004; Newman et al., 2003; Wilson et al., 2003; Yamaguchi et al., 2004; Lee et al., 2005). Our results show that PPARγ-active C-DIMs also induce NAG-1 protein expression in HCT-116 cells (Figs. 3 and 4), and this is consistent with their growth-inhibitory (Fig. 1) and apoptotic (Figs. 3 and 4) effects in HCT-116 cells. It was also shown that induction of NAG-1 and apoptosis in HCT-116 cells by DIM-C-pPhCF$_3$ and DIM-C-pPhC$_6$H$_5$ were not inhibited by the PPARγ antagonists GW9662 or T007, and the PPARγ antagonists also did not affect C-DIM-induced growth inhibition (Fig. 4). Thus, induction of NAG-1 by PPARγ-active C-DIMs and troglitazone was receptor-independent, and this was in contrast to induction of NAG-1 by PGJ2 , because this response was inhibited by a PPARγ antagonist (Baek et al., 2004b).

Induction of NAG-1 by troglitazone was linked to activation of the tumor suppressor gene Egr-1, which in turn directly activates NAG-1 promoter constructs. The C-DIM compounds also rapidly activate Egr-1 protein expression in HCT-116 cells and maximal induction was observed within 2 h after treatment, whereas NAG-1 protein expression is increased with time over 24 h (Fig. 5A). In contrast, DIM induced NAG-1 but did not affect Egr-1 expression in HCT-116 cells (Lee et al., 2005), and this clearly differentiated between DIM from the C-DIM compounds. Constructs containing Egr-1 promoter inserts (Fig. 5, B–E) are also activated by the C-DIM compounds, and the minimal promoter region required for transactivation contains SRE3 and SRE2, which have previously been identified as critical *cis*-elements required for activation of Egr-1 (Chen et al., 2004). Moreover, these compounds also activate NAG-1 promoter constructs (Fig. 6), and deletion/mutation analysis of the NAG-1 promoter indicates that mutation of the proximal Egr-1 sites resulted in loss of C-DIM-induced transactivation (Fig. 6E). These results, coupled with the observed interactions of Egr-1 with the NAG-1 promoter in a ChIP assay (Fig. 7), clearly link the induction of NAG-1 by C-DIMs to the rapid induction of Egr-1, which subsequently activates NAG-1 expression in HCT-116 cells.

Thus, the induction of NAG-1 by PPARγ-active C-DIMs and troglitazone in HCT-116 cells is receptor-independent and also involves Egr-1 induction. However, induction of NAG-1 protein and reporter gene activity by troglitazone was inhibited by the MAPK inhibitor PD98059, suggesting that this response may be due, in part, to induction of Egr-1 through activation of kinases by troglitazone (Baek et al., 2004b). Kinase-dependent activation of NAG-1 by C-DIMs was further investigated by determining the effects of PI3K and MAPK inhibitors on induction of NAG-1 and Egr-1 proteins and reporter gene activity (pEGR-1D) in HCT-116 cells (Fig. 8, A–C). The results show that PI3K, and not MAPK inhibitors, block induction of NAG-1 and Egr-1 proteins and induction of transactivation in cells transfected with pEGR-1D, and this was consistent with the identification of kinase-

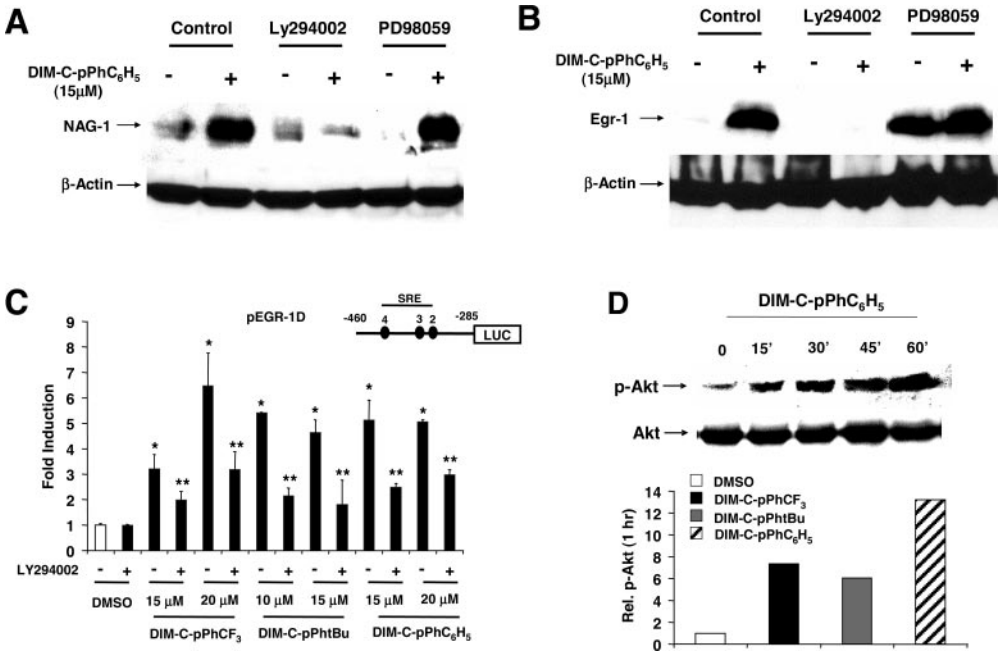

Fig. 8. C-DIM compounds activate PI3K in HCT-116 cells. Effects of kinase inhibitors on induction of NAG-1 (A) or Egr-1 (B) by C-DIMs. HCT-116 cells were treated with DMSO, C-DIMs alone, or in combination with 20 μM PD98059 or 20 μM LY294002 for 24 (A) or 2 (B) h, and whole cell lysates were analyzed by Western immunoblot analysis as described under *Materials and Methods*. Similar results were observed using DIM-C-pPhCF$_3$ or DIM-C-pPhtBu. C, inhibition of pEGR-1D activation by LY294002. HCT-116 cells were transfected with pEGR-1D, treated with 10 or 15 μM C-DIM compounds alone or in combination with 20 μM LY294002, and luciferase activity was determined as described under *Materials and Methods*. Results are expressed as means ± S.E. (triplicate). Significant ($p < 0.05$) induction by C-DIMs and inhibition by LY294002 are indicated by * and **, respectively. D, activation of Akt phosphorylation by C-DIMs. HCT-116 cells were treated with the C-DIM compounds for different periods of time, and whole cell lysates were analyzed by Western blot analysis for phospho-Akt and Akt as described under *Materials and Methods*. Results shown in the bar graph are means of two duplicate determinations.

responsive SRE3 and SRE2 as critical *cis*-elements in the Egr-1 promoter that are required for C-DIM-dependent activation of the Egr-1 constructs (Fig. 5). Kinase-mediated activation of Egr-1 is highly variable and dependent on cell context (Sukhatme et al., 1987; Christy and Nathans, 1989; Sukhatme, 1990; Cicatiello et al., 1993; Muthukkumar et al., 1995; Dziema et al., 2003; Chen et al., 2004; Sarker and Lee, 2004); however, it is apparent from this study that activation of Egr-1 by C-DIMs is PI3K-dependent, whereas troglitazone activation of Egr-1/NAG-1 was linked to a MAPK pathway (Baek et al., 2004b). In this study, 20 μM LY294002 alone did not affect NAG-1 or Egr-1 protein expression, but it inhibited induction of these proteins by PPARγ-active C-DIMs (Fig. 8), and induction of Egr-1-derived promoter constructs by DIM-C-pPhtBu was also inhibited by LY294002 (data not shown). In contrast, a recent study reported that higher concentrations of LY294002 (50 μM) induced NAG-1 protein expression, and this was linked to activation of glycogen synthase kinase-3β through dephosphorylation of this protein (Baek et al., 2005). Thus, PI3K plays a pivotal role in activation of Egr-1 (and NAG-1) by the C-DIM compounds, whereas inactivation of PI3K after treatment with high concentrations of LY294002 results in glycogen synthase kinase-3β-dependent activation of NAG-1 (Baek et al., 2005).

In summary, our results show that PPARγ-active C-DIMs induce both NAG-1 and Egr-1 in HCT-116 cells through receptor-independent pathways. NAG-1 up-regulation is linked to prior PI3K-dependent induction of Egr-1, which directly activates NAG-1 through interaction with Egr-1 *cis*-elements in the NAG-1 promoter. It is also possible that the decreased HCT-116 cell survival and apoptosis observed after treatment with the C-DIM compounds are due, in part, to activation of other Egr-1-dependent genes, which mediate the apoptotic and growth inhibitory effects (Huang et al., 1995). The induction of PI3K by the C-DIM compounds (Fig. 8D) is somewhat paradoxical, because this kinase is linked to cell survival pathways. However, recent studies have demonstrated that activation of PI3K can sensitize caveolin-1-expressing HeLa and 293 cells to the cytotoxicity of arsenite and hydrogen peroxide (Shack et al., 2003). Moreover, caveolin-1 and PI3K also sensitize L929 cells to tumor necrosis factor-α-induced cell death, and it was postulated that this may be caused by Akt-dependent inactivation of forkhead transcription factors (Ono et al., 2004). Although the C-DIM compounds do not induce caveolin-1 in HCT-116 cells, the high endogenous expression of caveolin-1 in these cells (Fig. 3B) coupled with the induction of PI3K may also contribute to the decreased HCT-116 cell survival and apoptosis after treatment with the C-DIMs. Thus, like other PPARγ agonists, the C-DIM compounds activate receptor-independent responses that contribute to their effectiveness as a new class of drugs with potential clinical applications for cancer chemotherapy.

References

Baek SJ, Horowitz JM, and Eling TE (2001a) Molecular cloning and characterization of human nonsteroidal anti-inflammatory drug-activated gene promoter. Basal transcription is mediated by Sp1 and Sp3. *J Biol Chem* **276:**33384–33392.

Baek SJ, Kim JS, Jackson FR, Eling TE, McEntee MF, and Lee SH (2004a) Epicatechin gallate-induced expression of NAG-1 is associated with growth inhibition and apoptosis in colon cancer cells. *Carcinogenesis* **25:**2425–2432.

Baek SJ, Kim JS, Moore SM, Lee SH, Martinez J, and Eling TE (2005) Cyclooxygenase inhibitors induce the expression of the tumor suppressor gene *EGR-1*, which results in the up-regulation of *NAG-1*, an antitumorigenic protein. *Mol Pharmacol* **67:**356–364.

Baek SJ, Kim JS, Nixon JB, DiAugustine RP, and Eling TE (2004b) Expression of NAG-1, a transforming growth factor-b superfamily member, by troglitazone requires the early growth response gene. *EGR-1. J Biol Chem* **279:**6883–6892.

Baek SJ, Kim KS, Nixon JB, Wilson LC, and Eling TE (2001b) Cyclooxygenase inhibitors regulate the expression of a TGF-β superfamily member that has proapoptotic and antitumorigenic activities. *Mol Pharmacol* **59:**901–908.

Baek SJ, Wilson LC, and Eling TE (2002) Resveratrol enhances the expression of non-steroidal anti-inflammatory drug-activated gene (*NAG-1*) by increasing the expression of p53. *Carcinogenesis* **23:**425–434.

Baek SJ, Wilson LC, Hsi LC, and Eling TE (2003) Troglitazone, a peroxisome proliferator-activated receptor γ (PPARγ) ligand, selectively induces the early growth response-1 gene independently of PPARγ. A novel mechanism for its anti-tumorigenic activity. *J Biol Chem* **278:**5845–5853.

Bottone FG Jr, Baek SJ, Nixon JB, and Eling TE (2002) Diallyl disulfide (DADS) induces the antitumorigenic NSAID-activated gene (*NAG-1*) by a p53-dependent mechanism in human colorectal HCT 116 cells. *J Nutr* **132:**773–778.

Chen CC, Lee WR, and Safe S (2004) Egr-1 is activated by 17b-estradiol in MCF-7 cells by mitogen-activated protein kinase-dependent phosphorylation of ELK-1. *J Cell Biochem* **93:**1063–1074.

Chintharlapalli S, Papineni S, Konopleva M, Andreef M, Samudio I, and Safe S (2005) 2-Cyano-3,12-dioxoolean-1,9-dien-28-oic acid and related compounds inhibit growth of colon cancer cells through peroxisome proliferator-activated receptor γ-dependent and -independent pathways. *Mol Pharmacol* **68:**119–128.

Chintharlapalli S, Smith Spaceiiiqq R, Samudio I, Zhang W, and Safe S (2004) 1,1-Bis(3′-indolyl)-1-(*p*-substitutedphenyl)methanes induce peroxisome proliferator-activated receptor g-mediated growth inhibition, transactivation and differentiation markers in colon cancer cells. *Cancer Res* **64:**5994–6001.

Christy B and Nathans D (1989) DNA binding site of the growth factor-inducible protein Zif268. *Proc Natl Acad Sci USA* **86:**8737–8741.

Cicatiello L, Sica V, Bresciani F, and Weisz A (1993) Identification of a specific pattern of "immediate-early" gene activation induced by estrogen during mitogenic stimulation of rat uterine cells. *Receptor* **3:**17–30.

Clay CE, Monjazeb A, Thorburn J, Chilton FH, and High KP (2002) 15-Deoxy-D(12,14)-prostaglandin J2-induced apoptosis does not require PPARg in breast cancer cells. *J Lipid Res* **43:**1818–1828.

Clay CE, Namen AM, Atsumi G, Willingham MC, High KP, Kute TE, Trimboli AJ, Fonteh AN, Dawson PA, and Chilton FH (1999) Influence of J series prostaglandins on apoptosis and tumorigenesis of breast cancer cells. *Carcinogenesis* **20:**1905–1911.

Contractor R, Samudio I, Estrov Z, Harris D, McCubrey JA, Safe S, Andreeff M, and Konopleva M (2005) A novel ring-substituted diindolylmethane 1,1-bis[3′-(5-methoxyindolyl)]-1-(p-t-butylphenyl)methane inhibits extracellular-signal regulated kinase activation and induces apoptosis in acute myeloid leukemia. *Cancer Res* **65:**2890–2898.

Dziema H, Oatis B, Butcher GQ, Yates R, Hoyt KR, and Obrietan K (2003) The ERK/MAP kinase pathway couples light to immediate-early gene expression in the suprachiasmatic nucleus. *Eur J Neurosci* **17:**1617–1627.

Hong J, Samudio I, Liu S, Abdelrahim M, and Safe S (2004) Peroxisome proliferator-activated receptor g-dependent activation of p21 in Panc-28 pancreatic cancer cells involves Sp1 and Sp4 proteins. *Endocrinology* **145:**5774–5785.

Huang RP, Liu C, Fan Y, Mercola D, and Adamson ED (1995) Egr-1 negatively regulates human tumor cell growth via the DNA-binding domain. *Cancer Res* **55:**5054–5062.

Inoue K, Kawahito Y, Tsubouchi Y, Kohno M, Yoshimura R, Yoshikawa T, and Sano H (2001) Expression of peroxisome proliferator-activated receptor g in renal cell carcinoma and growth inhibition by its agonists. *Biochem Biophys Res Commun* **287:**727–732.

Kim JS, Baek SJ, Sali T, and Eling TE (2005) The conventional nonsteroidal anti-inflammatory drug sulindac sulfide arrests ovarian cancer cell growth via the expression of. *NAG-1/MIC-1/GDF-15. Mol Cancer Ther* **4:**487–493.

Kim KS, Baek SJ, Flake GP, Loftin CD, Calvo BF, and Eling TE (2002) Expression and regulation of nonsteroidal anti-inflammatory drug-activated gene (*NAG-1*) in human and mouse tissue. *Gastroenterology* **122:**1388–1398.

Kim KS, Yoon JH, Kim JK, Baek SJ, Eling TE, Lee WJ, Ryu JH, Lee JG, Lee JH, and Yoo JB (2004) Cyclooxygenase inhibitors induce apoptosis in oral cavity cancer cells by increased expression of nonsteroidal anti-inflammatory drug-activated gene. *Biochem Biophys Res Commun* **325:**1298–1303.

Lee SH, Kim JS, Yamaguchi K, Eling TE, and Baek SJ (2005) Indole-3-carbinol and 3,3′-diindolylmethane induce expression of NAG-1 in a p53-independent manner. *Biochem Biophys Res Commun* **328:**63–69.

Li PX, Wong J, Ayed A, Ngo D, Brade AM, Arrowsmith C, Austin RC, and Klamut HJ (2000) Placental transforming growth factor-β is a downstream mediator of the growth arrest and apoptotic response of tumor cells to DNA damage and p53 overexpression. *J Biol Chem* **275:**20127–20135.

Motomura W, Okumura T, Takahashi N, Obara T, and Kohgo Y (2000) Activation of peroxisome proliferator-activated receptor gamma by troglitazone inhibits cell growth through the increase of $p27^{KIP1}$ in human pancreatic carcinoma cells. *Cancer Res* **60:**5558–5564.

Muthukkumar S, Nair P, Sells SF, Maddiwar NG, Jacob RJ, and Rangnekar VM (1995) Role of EGR-1 in thapsigargin-inducible apoptosis in the melanoma cell line A375–C6. *Mol Cell Biol* **15:**6262–6272.

Newman D, Sakaue M, Koo JS, Kim KS, Baek SJ, Eling T, and Jetten AM (2003) Differential regulation of nonsteroidal anti-inflammatory drug-activated gene in normal human tracheobronchial epithelial and lung carcinoma cells by retinoids. *Mol Pharmacol* **63:**557–564.

Ono K, Iwanaga Y, Hirayama M, Kawamura T, Sowa N, and Hasegawa K (2004) Contribution of caveolin-1a and Akt to TNF-a-induced cell death. *Am J Physiol* **287:**L201–L209.

Palakurthi SS, Aktas H, Grubissich LM, Mortensen RM, and Halperin JA (2001) Anticancer effects of thiazolidinediones are independent of peroxisome prolifera-

tor-activated receptor g and mediated by inhibition of translation initiation. *Cancer Res* **61:**6213–6218.
Qin C, Burghardt R, Smith R, Wormke M, Stewart J, and Safe S (2003) Peroxisome proliferator-activated receptor g (PPARg) agonists induce proteasome-dependent degradation of cyclin D1 and estrogen receptor a in MCF-7 breast cancer cells. *Cancer Res* **63:**958–964.
Qin C, Morrow D, Stewart J, Spencer K, Porter W, Smith Spaceiiiqq R, Phillips T, Abdelrahim M, Samudio I, and Safe S (2004) A new class of peroxisome proliferator-activated receptor g (PPARg) agonists that inhibit growth of breast cancer cells: 1,1-bis(3′-indolyl)-1-(*p*-substitutedphenyl)methanes. *Mol Cancer Ther* **3:**247–259.
Sarker KP and Lee KY (2004) L6 myoblast differentiation is modulated by Cdk5 via the PI3K-AKT-p70S6K signaling pathway. *Oncogene* **23:**6064–6070.
Shack S, Wang XT, Kokkonen GC, Gorospe M, Longo DL, and Holbrook NJ (2003) Caveolin-induced activation of the phosphatidylinositol 3-kinase/Akt pathway increases arsenite cytotoxicity. *Mol Cell Biol* **23:**2407–2414.
Sukhatme VP (1990) Early transcriptional events in cell growth: the Egr family. *J Am Soc Nephrol* **1:**859–866.
Sukhatme VP, Kartha S, Toback FG, Taub R, Hoover RG, and Tsai-Morris CH (1987) A novel early growth response gene rapidly induced by fibroblast, epithelial cell and lymphocyte mitogens. *Oncogene Res* **1:**343–355.
Takahashi N, Okumura T, Motomura W, Fujimoto Y, Kawabata I, and Kohgo Y (1999) Activation of PPARg inhibits cell growth and induces apoptosis in human gastric cancer cells. *FEBS Lett* **455:**135–139.
Tan M, Wang Y, Guan K, and Sun Y (2000) PTGF-β, a type β transforming growth factor (TGF-β) superfamily member, is a p53 target gene that inhibits tumor cell growth via TGF-β signaling pathway. *Proc Natl Acad Sci USA* **97:**109–114.
Wilson LC, Baek SJ, Call A, and Eling TE (2003) Nonsteroidal anti-inflammatory drug-activated gene (*NAG-1*) is induced by genistein through the expression of p53 in colorectal cancer cells. *Int J Cancer* **105:**747–753.
Yamaguchi K, Lee SH, Eling TE, and Baek SJ (2004) Identification of nonsteroidal anti-inflammatory drug-activated gene (*NAG-1*) as a novel downstream target of phosphatidylinositol 3-kinase/AKT/GSK-3b pathway. *J Biol Chem* **279:**49617–49623.

Address correspondence to: Dr. Stephen Safe, Department of Veterinary Physiology and Pharmacology, Texas A&M University, College Station, TX 77843-4466. E-mail: ssafe@cvm.tamu.edu