

Aldose Reductase Regulates Growth Factor-Induced Cyclooxygenase-2 Expression and Prostaglandin E₂ Production in Human Colon Cancer Cells

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Abstract

Inhibition of prostaglandin E₂ (PGE₂) and cyclooxygenase (COX)-2 by nonsteroidal anti-inflammatory drugs reduces the progression of colon cancer. Inhibition of aldose reductase (AR; EC. 1.1.1.21.) by sorbinil or by antisense ablation prevented fibroblast growth factor-induced and platelet-derived growth factor-induced up-regulation of PGE₂ synthesis in human colon cancer cells, Caco-2. AR besides reducing aldo-sugars efficiently reduces toxic lipid aldehydes and their conjugates with glutathione. Inhibition of AR prevented growth factor-induced COX-2 activity, protein, and mRNA and significantly decreased activation of nuclear factor- κ B and protein kinase C (PKC) and phosphorylation of PKC- β 2 as well as progression of Caco-2 cell growth but had no effect on COX-1 activity. Cell cycle analysis suggests that inhibition of AR prevents growth factor-induced proliferation of Caco-2 cells at S phase. Treatment of Caco-2 cells with the most abundant and toxic lipid aldehyde 4-hydroxy-trans-2-nonenal (HNE) or its glutathione-conjugate [glutathionyl-HNE (GS-HNE)] or AR-catalyzed product of GS-HNE, glutathionyl-1,4-dihydroxynonane (GS-DHN), resulted in increased COX-2 expression and PGE₂ production. Inhibition of AR prevented HNE- or GS-HNE-induced but not GS-DHN-induced up-regulation of COX-2 and PGE₂. More importantly, *in vivo* studies showed that administration of AR-small interfering RNA (siRNA), but not control siRNA, to nude mice bearing SW480 human colon adenocarcinoma cells completely arrested tumor progression. Collectively, these observations suggest that AR is an obligatory mediator of growth factor-induced up-regulation of COX-2, PGE₂, and growth of Caco-2 cells, indicating that inhibition of AR may be a novel therapeutic approach in preventing the progression of colon cancer. (Cancer Res 2006; 66(19): 9705-13)

Introduction

Colon cancer, a complex multistep process involving progressive disruption of homeostatic mechanisms controlling intestinal epithelial proliferation/inflammation, differentiation, and programmed cell death, is the second leading cause of death after lung cancer in

the United States (1–3). The cytotoxicity observed in colon cancer is usually mediated by dietary and environmental factors and is more pronounced in genetically predisposed subjects (4). One of the main features of colon cancer is the overexpression of the inducible cyclooxygenase (COX)-2 (5) that catalyzes the first two steps in the biosynthesis of prostaglandins from arachidonic acid. Prostaglandins are the major cause of inflammation (5). The *de novo* synthesis of COX-2 is triggered by the exposure of cells to certain stimuli, such as cytokines and growth factors, which lead to induction of altered synthesis of prostaglandins followed by uncontrolled colon epithelial cell proliferation (5–8). Indeed, colorectal epithelial cells obtained from colon cancer patients show up-regulation of the growth factors and their receptors, transforming growth factor, insulin-like growth factor (IGF)-II, hepatocyte growth factor (HGF), HGF receptor, epidermal growth factor receptor, IGF receptor, vascular endothelial growth factor, fibroblast growth factor (FGF), and platelet derived growth factor (PDGF; refs. 9–13).

Widely used nonsteroidal anti-inflammatory drugs (NSAID; ref. 5), besides decreasing the local inflammation in colon, ameliorate pain and pyrogenicity. Broad range of classic NSAIDs, such as sulindac, piroxicam, diclofenac, indomethacin, acetaminophen, and ibuprofen, besides preventing inflammation, also inhibit constitutive COX-1 enzyme, which converts arachidonic acid to prostaglandin E₂ (PGE₂) and maintain the integrity of epithelium of certain organs, such as colon and kidney (5, 6). In addition, such drugs could cause gastrointestinal upset, ulcers, and bleeding. The discovery of COX-2 in 1991 led to the design and synthesis of a new class of NSAIDs, such as celecoxib, refecoxib, valdecoxib, meloxicam, lumiracoxib, and nimesulide. These drugs are being used to treat chronic arthritic conditions in addition to colon cancer. However, refecoxib (Vioxx) and valdecoxib (Bextra) cause cardiovascular complications (5, 6, 14–16).

We have shown earlier that cytotoxic effects of cytokines, chemokines, and growth factors, quantified by following growth in vascular smooth muscle cells (VSMC; ref. 17) and apoptosis in vascular endothelial cells (VEC; ref. 18) and human lens epithelial cells (HLEC; ref. 19), are prevented by inhibiting aldose reductase (AR). This enzyme besides reducing glucose to sorbitol with K_m in micromolar range (50–100 mmol/L) also efficiently reduces lipid peroxidation-derived aldehydes, such as 4-hydroxy-trans-2-nonenal (HNE), and their glutathione conjugates (20–22) with K_m in low micromolar range. Lipid aldehydes and their metabolites are generated in larger amounts in oxidative stress caused by increased cytokines, chemokines, growth factors, endotoxins, and hyperglycemia and may be the major mediators of cytotoxic signals (22, 23). The antioxidant role of AR is consistent with the observation that, in a variety of cell types, AR is up-regulated by oxidants, such as

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hydrogen peroxide, lipid peroxidation-derived aldehydes, advanced glycosylation end products, and nitric oxide (22–25). The elevated reactive oxygen species (ROS) levels besides triggering the inflammatory response in tissues by up-regulating several redox-sensitive kinases, such as mitogen-activated protein kinase and protein kinase C (PKC), regulate transcription of several genes, such as tumor necrosis factor- α , interleukin-8, and AR (17–19). A major signaling pathway associated with the oxidative stress and inflammation is the activation of nuclear factor- κ B (NF- κ B). Modulation of NF- κ B may play a central role in the mitogenic process initiated by ROS and related oxidants in colon cancer. Recently, we have shown that growth factor- and cytokine-induced NF- κ B activation is inhibited by preventing AR in VSMC, VEC, and HLEC (17–19). In the present study, we have investigated the role of AR in mediating the growth factor-induced expression of COX-2 and production of PGE₂ in Caco-2 cells.

Materials and Methods

Materials. McCoy's 5A medium, DMEM, PBS, penicillin/streptomycin solution, trypsin, and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA). Antibodies against COX-1, COX-2, phosphorylated PKC- β 2, and inhibitor NF- κ B (SN50) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Sorbinil and tolrestat were a gift from Pfizer (Groton, CT) and American Home Products (Madison, NJ), respectively. Mouse anti-rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were obtained from Research Diagnostics, Inc. (Concord, MA). DUP-697, COX activity assay, and PGE₂ assay kits were obtained from Cayman Chemical Co. (Ann Arbor, MI). Calphostin C was obtained from EMD Biosciences (San Diego, CA). PDGF, basic FGF (bFGF), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and other reagents used in Western blot analysis were obtained from Sigma (St. Louis, MO). AR-small interfering RNA (siRNA; AATCGGTGTCTC-CAACTCAA) or scrambled siRNA (AAAATCTCCCTAAATCATACA; control) were synthesized by Dharmacon Research (Chicago, IL). All other reagents used were of analytical grade. GS-aldehyde esters were synthesized as described by us previously (26). The glutathione monoethyl-ester (GS-ester) obtained from Sigma was purified by high-pressure liquid chromatography (HPLC) using a reverse-phase column and the conjugate of GS-ester and HNE was made by incubating 1 μ mol [4-³H]HNE with 3-fold excess of GS-ester and 0.1 mol/L potassium phosphate (pH 7.0) at 37°C. The reaction was followed by monitoring absorbance at 224 nm. The glutathionyl-HNE (GS-HNE)-ester thus formed was purified by HPLC (26) and its concentration was calculated based on radioactivity. For synthesis of glutathionyl-1,4-dihydroxynonane (GS-DHN)-ester, 1 μ mol GS-HNE-ester was incubated with 1 unit recombinant human AR and 0.1 mmol/L NADPH in 0.1 mol/L potassium phosphate (pH 7.0) at 37°C. The reaction was followed by monitoring the decrease in absorbance at 340 nm. The enzyme was removed by ultrafiltration using an Amicon Centriprep-10 (Millipore Corporation, Billerica, MA), and GS-DHN-ester in the filtrate was purified on HPLC and confirmed by electrospray ionization/mass spectrometry.

Cell culture. Human colon cancer cell lines, HCT-116 and Caco-2, were obtained from American Type Culture Collection (ATCC; Manassas, VA). HCT-116 cells were maintained and grown in McCoy's 5A medium supplemented with 10% FBS and 1% penicillin/streptomycin and Caco-2 cells were grown in DMEM with 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO₂. Human colon adenocarcinoma (SW480) cells were purchased from ATCC and cultured at 37°C in a humidified atmosphere of 5% CO₂ in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FBS, 1% (v/v) penicillin/streptomycin solution, 2 mmol/L L-glutamine, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate.

Measurement of cytotoxicity. Caco-2 cells were grown to confluency in DMEM, and harvested, and plated ~2,500 per well in a 96-well plate. Subconfluent cells were growth arrested in 0.1% FBS. After 24 hours, bFGF

(10 ng/mL) or PDGF (10 ng/mL) with or without AR inhibitors sorbinil or tolrestat were added to the medium and the cells were incubated for another 24 hours. Cells incubated with the AR inhibitors alone served as control. Cell viability was determined by cell count and MTT assay as described earlier (17–19).

Determination of PKC activity. PKC activity was measured using the Promega SignaTECT (Madison, WI) PKC assay system as described earlier (17). Briefly, aliquots of the reaction mixture [25 mmol/L Tris-HCl (pH 7.5), 1.6 mg/mL phosphatidylserine, 0.16 mg/mL diacylglycerol, and 50 mmol/L MgCl₂] were mixed with [γ -³²P]ATP (3,000 Ci/mmol, 10 μ Ci/ μ L) and incubated at 30°C for 10 minutes. To stop the reaction, guanidine hydrochloride (7.5 mol/L) was added and the phosphorylated peptide was separated on binding paper. The extent of phosphorylation was detected by measuring radioactivity retained on the paper.

PGE₂ assay. Caco-2 cells were plated in six-well plates at a density of 2×10^5 per well. After 24 hours, the medium was replaced with fresh medium containing 0.1% serum with or without sorbinil (20 μ mol/L) followed by treatment with either bFGF (10 ng/mL) or PDGF (10 ng/mL) for another 24 hours. The medium was collected from each well and analyzed for PGE₂ by using an enzyme immunoassay kit according to the manufacturer's instructions. Briefly, diluted standard/sample (50 μ L) was pipetted into a precoated goat polyclonal anti-mouse IgG 96-well plate. Aliquots (50 μ L) of a PGE₂ monoclonal antibody and PGE₂ acetylcholine esterase conjugate (PGE₂ tracer) were added to each well and allowed to incubate at 4°C for 24 hours. After incubation, the wells were washed five times with wash buffer containing 0.05% Tween 20 followed by the addition of 200 μ L Ellman's reagent containing acetylthiocholine and 5,5'-dithio-bis-(2-nitrobenzoic acid). Samples were read after 60 minutes at 412 nm with an ELISA reader. In this procedure, the intensity of yellow color is proportional to the amount of PGE₂ tracer bound to the well and inversely proportional to the amount of free PGE₂ present in the well during incubation.

COX activity assay. For determination of COX activity, growth-arrested Caco-2 cells were treated with either bFGF (10 ng/mL) or PDGF (10 ng/mL) in the presence and absence of sorbinil (20 μ mol/L) for 24 hours. The cells were harvested and homogenized in cold (4°C) buffer containing 0.1 mol/L Tris-HCl (pH 7.8) and 1 mmol/L EDTA and the activity was measured in a 96-well plate according to the manufacturer's instructions. Briefly, standard/sample (10 μ L) was incubated in the presence of arachidonic acid and substrate, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) in a total reaction volume of 210 μ L. The COX peroxidase activity was measured colorimetrically by monitoring appearance of oxidized TMPD at 590 nm by using ELISA reader.

NF- κ B-dependent reporter secretory alkaline phosphatase expression assay. Caco-2 cells (1.5×10^5 per well) were plated in six-well plates, serum starved in Opti-MEM for 24 hours with or without AR inhibitor, sorbinil (20 μ mol/L), and transiently transfected with pNF- κ B-secretory alkaline phosphatase (SEAP) construct or control plasmid pTALSEAP DNA (Clontech, Palo Alto, CA) using the LipofectAMINE Plus reagent. After 6 hours of transfection, cells were treated with either bFGF (10 ng/mL) or PDGF (10 ng/mL) for 48 hours in DMEM containing 0.1% FBS. The cell culture medium was then harvested and analyzed for SEAP activity, essentially as described by the manufacturer, using a 96-well chemiluminescence plate reader and Kodak Image Station 2000R (Rochester, NY).

Determination of NF- κ B activation. The cytosolic as well as nuclear extracts were prepared as described earlier (17) and the NF- κ B activity was determined by using the colorimetric nonradioactive NF- κ B p65 transcription factor assay kit (Chemicon International, Temecula, CA) as per the supplier's instructions. Briefly, a double-stranded biotinylated oligonucleotide containing the consensus sequence for NF- κ B binding (5'-GGGACT-TTCC-3') was mixed with nuclear extract and assay buffer. After incubation, the mixture (probe+extract+buffer) was transferred to the streptavidin-coated ELISA kit and read at 450 nm using an ELISA plate reader. For each experiment, triplicate samples were measured for statistical significance.

Reverse transcription-PCR. Total RNA was isolated from Caco-2 cells by using RNeasy micro isolation kit (Qiagen, Valencia, CA). Total RNA (1.5 μ g) sample was reverse transcribed with Omniscript and Sensiscript reverse transcriptase one-step reverse transcription-PCR (RT-PCR) system

with HotStarTaq DNA polymerase (Qiagen) at 55°C for 30 minutes followed by PCR amplification. The oligonucleotide primer sequences were as follows: 5'-TGAAACCCACTCCAAACACAG-3' (sense) and 5'-TCATCAGGCACAGGAGGAAG-3' (antisense) for COX-2 and 5'-TGAGACCTCAACCC-CAG-3' and 5'-TTCATGAGGTAGTCTGTCTCAGGTCC-3' for β -actin. PCR was carried out in a GeneAmp 2700 thermocycler (Applied Biosystems, Foster City, CA) under the following conditions: initial denaturation at 95°C for 15 minutes and 35 cycles of 94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 1 minute and then 72°C for 5 minutes for final extension (16). PCR products were electrophoresed in 2% agarose-1 \times TAE gels containing 0.5 μ g/mL ethidium bromide.

Flow cytometric analysis of cell cycle. Growth-arrested Caco-2 cells (1.5×10^5 per well in a six-well plate) were preincubated with or without sorbinil (20 μ mol/L) or carrier for 24 hours and then stimulated with either bFGF (10 ng/mL) or PDGF (10 ng/mL) for another 24 hours. The cells were then washed with PBS and harvested by trypsinization. Cellular DNA was stained with low and high salt solutions. Briefly, cells were resuspended in 250 μ L solution A, low salt stain containing polyethylene glycol (30 mg/mL), propidium iodide (0.05 mg/mL), Triton X-100 (1 μ L/mL), sodium citrate (4 mmol/L), and RNase A (10 μ g/mL), and incubated at 37°C for 20 minutes followed by the addition of 250 μ L solution B, high salt stain containing NaCl (400 mmol/L) instead of sodium citrate (4 mmol/L) in solution A, and incubated overnight at 4°C. Cell cycle analysis was done with a minimum of 10,000 events per analysis by using FACScan flow cytometer (Becton Dickinson and Co., San Jose, CA).

Measurement of ROS. Caco-2 cells were plated in a 24-well plate at a density of 1.5×10^4 per well in DMEM and then serum starved at 60% to 70% confluence in the presence and absence of sorbinil (20 μ mol/L) or

tolrestat (20 μ mol/L) for overnight in phenol red-free DMEM supplemented with 0.1% FBS. Cells were then preincubated for 30 minutes with the ROS-sensitive fluorophore 2',7'-dichlorofluorescein diacetate (DCFH-DA), which is taken up and oxidized to the fluorescent dichlorofluorescein by intracellular ROS. After incubation with DCFH-DA, the cells were exposed to bFGF (10 ng/mL) or PDGF (10 ng/mL) for 60 minutes and fluorescence was measured with a CytoFluor II fluorescence plate reader (PerSeptive Biosystems, Inc., Framingham, MA) at excitation of 485 nm and emission of 528 nm.

Western blot analysis. To examine COX-1, COX-2, phosphorylated PKC- β 2, and GAPDH, Western blot analyses were carried. Equal amounts of protein from cell extracts were subjected to 12% SDS-PAGE followed by transfer of proteins to nitrocellulose filters, probing with the indicated antibodies, and the antigen-antibody complex was detected by enhanced chemiluminescence (Pierce, Piscataway, NJ).

Antisense ablation of AR. Caco-2 cells were grown to 50% to 60% confluence in DMEM supplemented with 10% FBS and washed four times with Opti-MEM 60 minutes before the transfection with oligonucleotides (17). The cells were incubated with 2 μ mol/L AR antisense or scrambled control oligonucleotides using LipofectAMINE Plus (15 μ g/mL) as the transfection reagent as suggested by the supplier. After 12 hours, the medium was replaced with fresh DMEM (containing 10% FBS) for another 12 hours followed by 24 hours of incubation in serum-free DMEM (0.1% FBS) before growth factor stimulation. Changes in the expression of AR were estimated by Western blot analysis using anti-AR antibodies.

Effect of AR ablation on tumor growth in nude mice. Hsd: athymic nude *nu/nu* mice were obtained from Harlan (Indianapolis, IN). All animal experiments were carried out in accordance with a protocol approved by

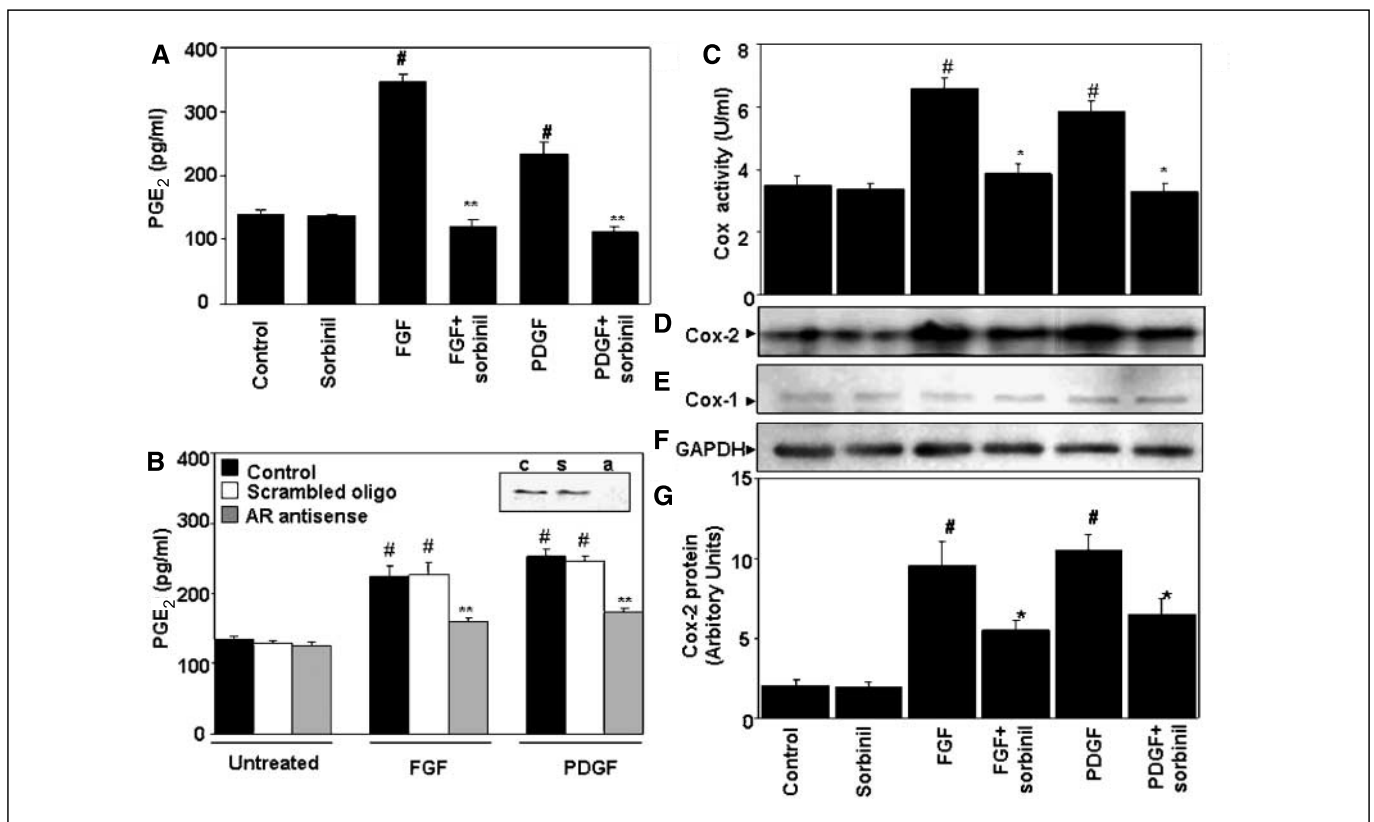


Figure 1. Inhibition or ablation of AR prevents growth factor-induced PGE₂ production and COX-2 expression in colon cancer cells. Growth-arrested Caco-2 cells were preincubated with sorbinil or carrier (A) for 24 hours, with AR antisense or scrambled oligonucleotides (B). B, inset, Western blot analysis for AR protein in untransfected (c), scrambled (s), and AR antisense (a) oligonucleotide-transfected cell extracts. The AR-inhibited and AR-ablated cells were stimulated with bFGF or PDGF. C to G, same as in (A), except that COX activity was measured by COX activity assay kit (C). Western blots were developed using antibodies against COX-2 (D), COX-1 (E), and GAPDH (F). G, densitometric analysis of (D). Columns, mean ($n = 4$); bars, SE. #, $P < 0.001$, compared with treatment without the inhibitor or scrambled oligonucleotide-transfected cells; *, $P < 0.01$; **, $P < 0.001$, compared with cells treated with growth factors.

the Institutional Animal Care and Use Committee. Nine mice (20 weeks old) were divided into three groups of three animals (treated with PBS, scrambled siRNA, and AR-siRNA). All nine animals were injected with 2×10^6 SW480 human colon adenocarcinoma cell suspensions in 100 μ L PBS s.c. Animals were examined daily for signs of tumor growth. Treatment was given when the tumor surface area exceeded 45 mm² (day 25). Treatment consisted of 200 μ g AR-siRNA in 100 μ L PBS. Control groups were treated with 200 μ g/100 μ L scrambled siRNA or diluent (PBS) alone. Tumors were measured in two dimensions using calipers.

Statistical analysis. Data are presented as mean \pm SE and *P*s were determined by unpaired Student's *t* test. *P*s < 0.01 were considered significant.

Results

Inhibition of AR prevents PGE₂ production and COX activity. The growth factors are known to induce PGE₂ production by activating inducible COX-2 in colon cancer (8), but the mechanism is not well understood. Inhibition of AR significantly (>90%) prevented the production of PGE₂ by Caco-2 cells induced by bFGF and PDGF (Fig. 1A). However, sorbinil alone did not inhibit constitutive levels of PGE₂. Because the nonspecificity of AR inhibitors could not be rigorously excluded, we did parallel studies by transfection of Caco-2 cells with antisense AR oligonucleotides that decreased AR protein expression by >95% (Fig. 1B, inset) and the enzyme activity by >90% (data not shown). In contrast to the cells transfected with scrambled oligonucleotides, cells transfected with antisense AR displayed markedly attenuated PGE₂ production on stimulation with bFGF or PDGF (Fig. 1B). PGE₂ generation in COX-2-negative cells (HCT-116) by growth factors was nonsignificant (data not shown).

Because PGE₂ is synthesized from its precursor arachidonic acid catalyzed by COXs, we next examined whether inhibition of AR prevents growth factor-induced expression of COX enzymes. Treatment of Caco-2 cells with bFGF and PDGF significantly (60-80%) increased COX activity (Fig. 1C). Preincubation with sorbinil abolished both bFGF- and PDGF-induced COX activity. Because COX activity is contributed by two isozymes, constitutive COX-1 and inducible COX-2, we next examined the effect of AR inhibition on COX-1 and COX-2 isozymes by Western blot analysis using specific antibodies. The levels of constitutive COX-1 protein were not affected by growth factors or sorbinil (Fig. 1E), whereas COX-2 protein significantly increased and was attenuated by sorbinil (Fig. 1D and G). Further, treatment of Caco-2 cells with bFGF or PDGF significantly (2- to 3-fold) increased the mRNA levels of COX-2 and sorbinil prevented it by 55% to 65% (Fig. 2A and C), suggesting that AR could regulate the transcriptional activation of COX-2 DNA.

Inhibition of AR prevents growth factor-induced NF- κ B activation in colon cancer cells. We next examined the effect of AR inhibitors on growth factor-induced NF- κ B activation because it is known that redox-sensitive transcription factor NF- κ B transcribes COX-2 DNA (27), and our earlier studies show that AR inhibition prevents growth factor- and cytokine-induced NF- κ B activation (17). Both bFGF and PDGF significantly (~3-fold) induced NF- κ B-dependent reporter (SEAP) activation in Caco-2 cells and sorbinil caused >60% inhibition (Fig. 2D). However, sorbinil alone did not affect the NF- κ B-SEAP activity. Stimulation of Caco-2 cells with bFGF or PDGF resulted in a pronounced (~10-fold) activation of NF- κ B DNA binding activity as determined by colorimetric, nonradioactive NF- κ B p65 transcription assay method (Fig. 2E) and sorbinil caused >70% inhibition. These results

validate our measurement of NF- κ B activity and substantiate that the specific activity observed in SEAP and colorimetric methods is due to NF- κ B activation. Based on these observations, we conclude that inhibition of AR prevents growth factor-induced activation of NF- κ B in Caco-2 cells, which transcriptionally may activate COX-2 expression.

Inhibition of AR prevents growth factor-induced PKC activation in colon cancer cells. Because PKC is an upstream kinase for the activation of NF- κ B and activation of PKC- β 2 has been implicated in colon carcinogenesis (28), we next examined the effect of growth factors on total PKC activity in Caco-2 cells in the presence and absence of AR inhibitor. Stimulation with growth factors led to a significant (~3-fold) increase in membrane-bound PKC activity (Fig. 3A) and sorbinil significantly prevented it. However, sorbinil by itself did not alter the total PKC activity in these cells. Both bFGF and PDGF activated PKC- β 2 in Caco-2 cells (Fig. 3B and D). bFGF caused maximal PKC phosphorylation at

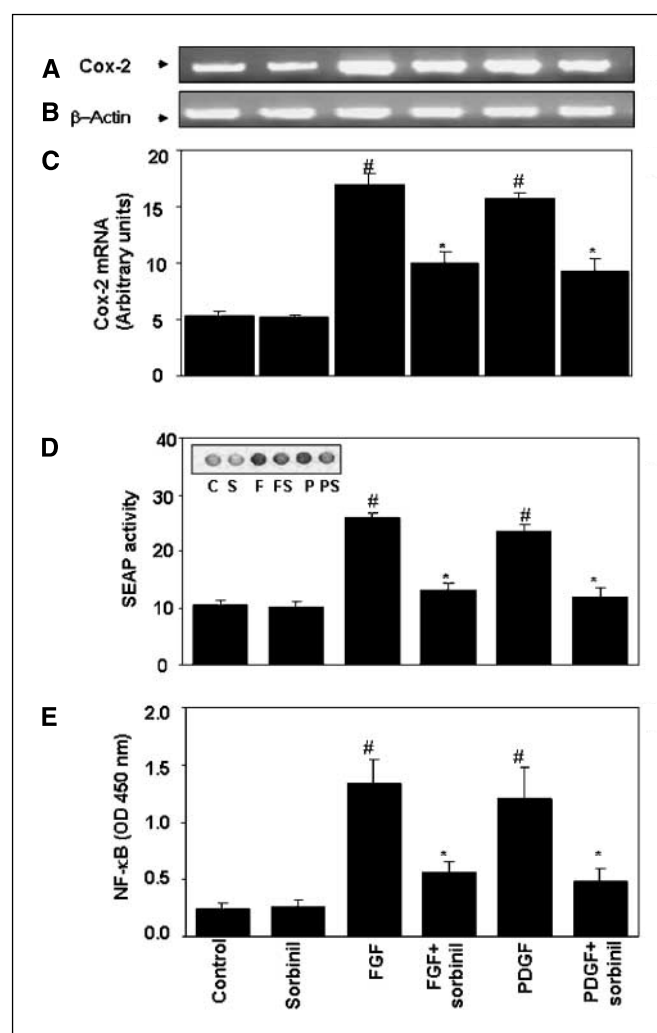


Figure 2. Inhibition of AR prevents growth factor-induced COX-2 mRNA expression and NF- κ B in colon cancer cells. Growth-arrested Caco-2 cells were preincubated with sorbinil or carrier for 24 hours followed by stimulation with bFGF or PDGF for 3 hours. A, COX-2; B, β -actin. C, densitometric analysis of (A). D, NF- κ B-dependent reporter SEAP activity. Inset, chemiluminescence of SEAP. E, NF- κ B activity. Columns, mean (*n* = 4); bars, SE. #, *P* < 0.01, compared with control cells; *, *P* < 0.01, compared with cells treated with growth factors.

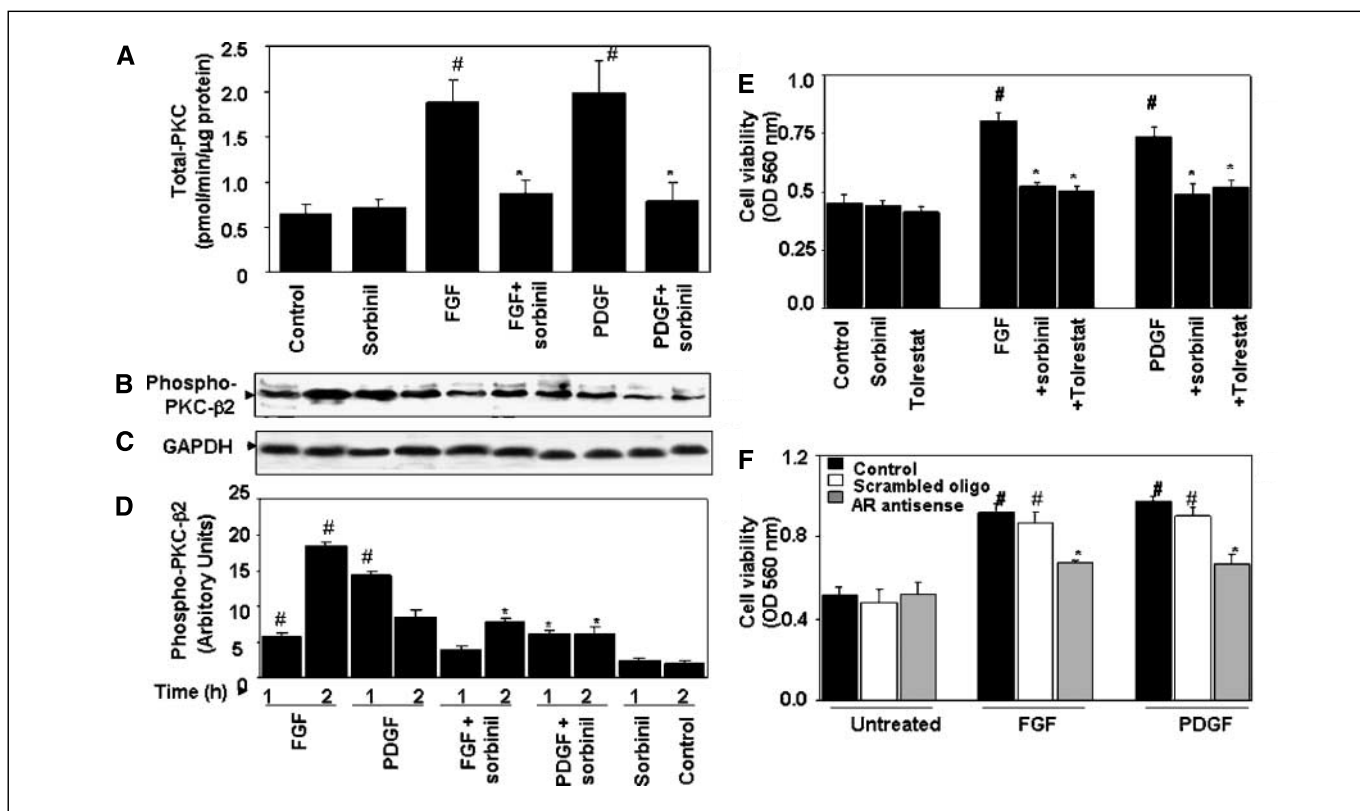


Figure 3. Inhibition of AR abrogates growth factor-induced PKC activation and growth in colon cancer cells. Quiescent Caco-2 cells were preincubated with sorbinil for 24 hours followed by stimulation with bFGF or PDGF for 3 hours. **A**, membrane-bound PKC activity. Western blot analysis using antibodies against phosphorylated-PKC- β 2 (**B**) and GAPDH (**C**). **D**, densitometric analysis of (**B**). **E** and **F**, growth-arrested Caco-2 cells were preincubated with or without sorbinil or tolrestat (**E**) or transfected with AR antisense (**F**) followed by stimulation with bFGF or PDGF for 24 hours, and cell viability was measured by MTT assay. Columns, mean ($n = 4$); bars, SE. #, $P < 0.01$, compared with control cells; *, $P < 0.01$, compared with cells treated with growth factors.

2 hours, whereas PDGF caused maximal phosphorylation at 1 hour and increase in PKC- β 2 phosphorylation was significantly (>70%) attenuated by sorbinil.

Attenuation of growth factor-induced colon cancer cell line proliferation. Because increased COX-2 expression has been shown to facilitate colon cancer progression by stimulating cell proliferation and survival (29), we next examined the role of AR in growth factor-induced Caco-2 cell growth. Treatment of Caco-2 cells with bFGF and PDGF for 24 hours significantly (>40%) stimulated growth (Fig. 3E), which was significantly attenuated (>80%) by sorbinil or by antisense ablation of AR (Fig. 3F), indicating that AR is an obligatory mediator of growth factor-induced colon cancer cell proliferation.

AR inhibition affects S phase of cell cycle. Because inhibition of AR attenuates growth factor-induced Caco-2 cell proliferation, we investigated the stage of cell cycle that is inhibited. Treatment of cells with growth factors significantly induced synthetic (S) phase of cell cycle (Fig. 4), suggesting that the cells were undergoing proliferation. Inhibition of AR prevented growth factor-induced accumulation of cells in S phase and the cells accumulated at G₂-M phase and G₁ phase (Fig. 4), suggesting that AR inhibition prevents synthetic phase of cell cycle, which is an important stage required for cell growth.

Attenuation of growth factor-induced up-regulation of PGE₂ production by inhibitors of signaling cascade for NF- κ B activation. To understand the role of NF- κ B in the growth factor-induced up-regulation of PGE₂, we used inhibitors of PKC

(calphostin c), COX-2 (DUP697), ROS scavenger (*N*-acetyl cysteine), and NF- κ B (SN50). Growth factors caused a pronounced increase in the production of PGE₂ and preincubation of Caco-2 cell with the above inhibitors attenuated, indicating that signaling events that lead to activation of NF- κ B and its dependent COX-2 expression are involved in the production of PGE₂ (Fig. 5A). Further, growth factors caused pronounced increase in ROS, which was inhibited by sorbinil and tolrestat (Fig. 5B).

Effect of AR inhibition on lipid aldehyde-induced signaling in Caco-2 cells. We have shown earlier that AR is an excellent catalyst for the reduction of lipid peroxidation-derived aldehydes, such as HNE, and their conjugates with glutathione to corresponding alcohols (20, 21). Because our results suggest that AR inhibition or ablation prevents growth factor-induced expression of COX-2 and production of PGE₂, we tested whether AR-catalyzed reduction of lipid aldehydes might be involved in this mechanism. Treatment of cells with HNE or cell-permeable esters of GS-HNE or GS-DHN resulted in increased PGE₂ production (Fig. 6A) and COX-2 expression (Fig. 6B and D). Inhibition of AR by sorbinil significantly prevented the HNE- and GS-HNE-induced COX-2 expression and PGE₂ production but had no effect on GS-DHN-induced expression of these inflammatory markers. These results indicate that growth factor-induced mitogenic signaling in colon cancer cells could be mediated by the reduced form of lipid aldehyde-glutathione conjugates catalyzed by AR.

AR-siRNA prevents progression of colon cancer tumor growth in nude mice. The results obtained from *in vitro* studies

were confirmed by *in vivo* nude mice model bearing human colon adenocarcinoma SW480 cells. SW480 (2×10^6) cells were implanted s.c. and allowed to grow in *nu/nu* nude mice to ~ 45 mm² over a period of 25 days followed by i.p. injection of the PBS, scrambled siRNA, or AR-siRNA to different groups of mice on days 1 and 14. Results presented in Fig. 6E clearly shows that the tumor progression was completely arrested in the animals treated with AR-siRNA, whereas uncontrolled growth was observed in the control as well as in scrambled siRNA-treated mice. None of the treatments interfered with the normal weight gain of animals during the experiments. The photographs of animals taken at days 1, 14, and 37 are given in Supplementary Fig. S1. These striking findings indicate that AR inhibition completely halts the colon cancer progression.

Discussion

Colon cancer, characterized by the development of malignant cells in the lining of epithelium of large intestine, is the second leading cause of cancer deaths in the United States (3). Inflammation and pain, caused by proliferation of malignant cells, is the major cause of cachexia (30, 31). The inducible COX-2 enzyme plays an important role in the generation of hyperalgesic and proinflammatory prostaglandins and is up-regulated in numerous pathologic conditions, including inflammation and cancer (7, 32). The *de novo* synthesis of COX-2 is triggered by the

exposure of cancer cells to endotoxin, cytokines, and growth factors (8, 10, 12). In contrast, constitutive COX-1 enzyme is involved in eicosanoid metabolism and homeostasis of tissues (5).

We have shown earlier that cytotoxic effects of cytokines, chemokines, and growth factors, quantified by following proliferation of VSMC and apoptosis of VEC and HLEC, are attenuated by inhibiting AR (17–19). The expression of AR is also enhanced in various forms of cancer, such as hepatocarcinogenesis (33) and colon cancer (30, 31). Therefore, we have analyzed the role of AR in regulating the growth factors, bFGF- and PDGF-induced colon cancer cell growth, and COX-2 expression and PGE₂ production. Inhibition of PGE₂ production in COX-2-positive cells by AR inhibition and no effect of AR inhibition on the PGE₂ production by COX-2-negative cells, HCT-116, indicate that, in Caco-2 cells, COX-2 activation is required for the synthesis of PGE₂. Further, AR regulates the PGE₂ levels by altering the expression of COX-2 protein induced by growth factors. Our results are comparable with other investigations that show Caco-2 cells, but not HCT-116 cells, produce PGE₂ under various stimuli (16, 34, 35). Steady-state increased expression of COX-2 protein and mRNA (Fig. 2) by growth factors and prevention by AR inhibition indicates that AR regulates transcriptional activation of COX-2.

Transcriptional regulation of COX-2 is mediated by a variety of growth factors and cytokines (8, 10, 35) in colon cancer epithelial cells, Caco-2, HT-29 (12, 36), osteoblasts (37), and VSMC (38). Further, we (17) and others (39–41) have shown earlier that growth

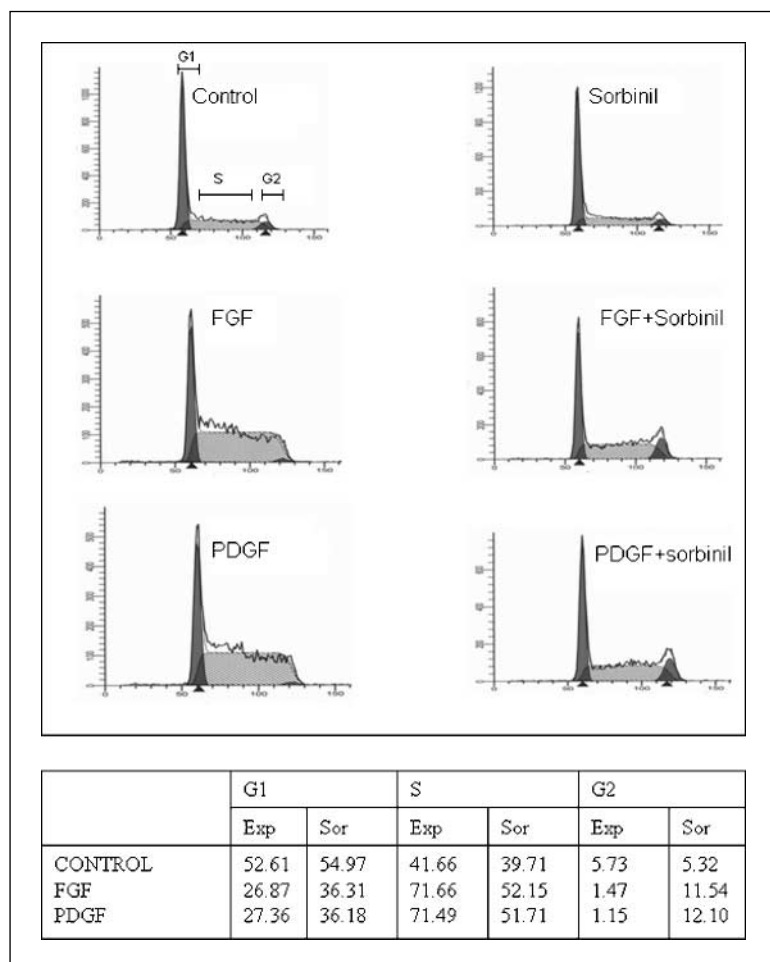
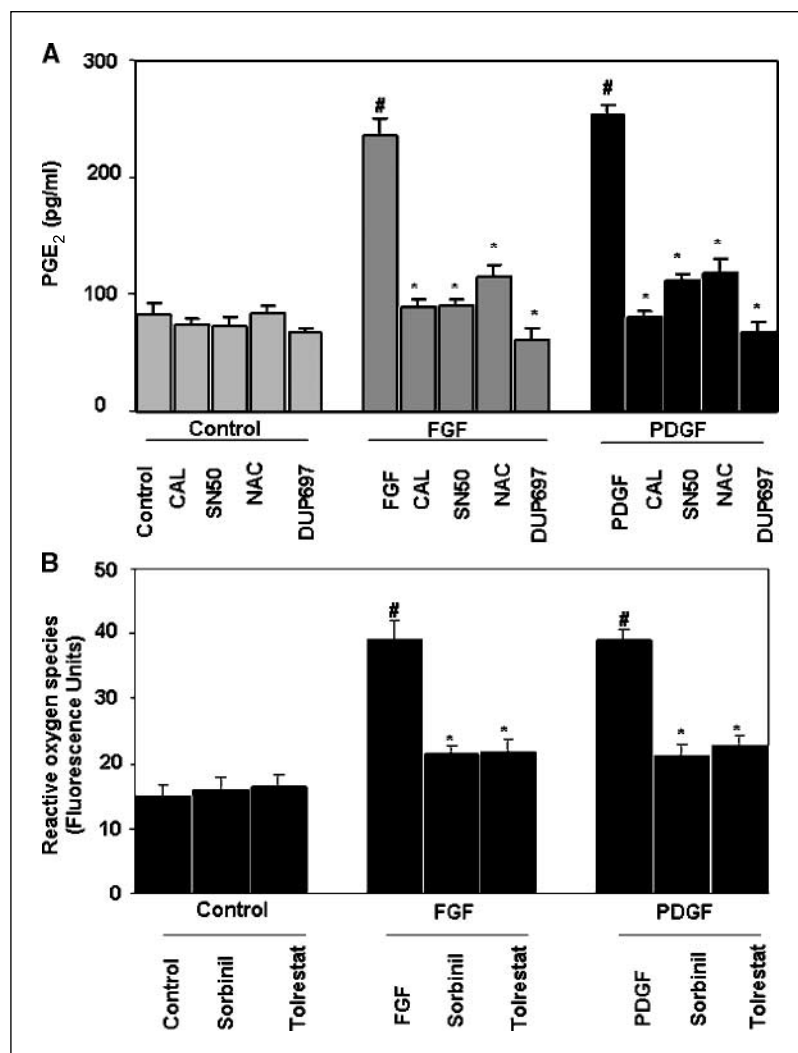


Figure 4. AR inhibition prevents growth factor-induced synthesis phase of cell cycle in colon cancer cells. Growth-arrested Caco-2 cells were preincubated with sorbinil or carrier for 24 hours followed by stimulation with bFGF or PDGF for 24 hours and cell cycle analysis was done by FACS. *Bottom*, percentage of cells in the corresponding phase of cell cycle.

Figure 5. Effect of PKC, NF- κ B, and COX-2 inhibitors and AR inhibitors on growth factor-induced PGE₂ and ROS production, respectively, in colon cancer cells. Growth-arrested Caco-2 cells were preincubated with PKC, NF- κ B, and COX-2 inhibitors or ROS scavenger for 30 minutes (A) and AR inhibitors for 24 hours (B). A and B, further incubated with bFGF or PDGF for 24 hours and 1 hour, respectively. Columns, mean ($n = 4$); bars, SE. #, $P < 0.001$, compared with control cells; *, $P < 0.01$, compared with cells treated with growth factors.



factors and cytokines transcriptionally activate AR, which would facilitate growth factors and cytokine signals that induce COX-2. These observations suggest that AR is a growth-responsive protein, which may be involved in facilitating metabolic changes that accompany growth of colon cancer cells. Consistent with this view, other investigators (23, 40, 42, 43) have shown that the expression of AR is minimal in quiescent cells and is robust under hyperglycemic and growth factor-induced oxidative stress. Prevention of growth factor-induced PGE₂ production and cell growth by pharmacologic inhibition of AR by sorbinil was further confirmed by ablation of AR (Fig. 6E). Involvement of AR with cell growth is consistent with several previous studies on different cell types, such as VSMCs (17), HLECs (18), and human umbilical VEC (HUVEC; ref. 19).

It has been shown that activation of PKC is an obligatory step in promoting colon cancer. In VSMCs (17), HLECs (18), and HUVECs (19), activation of PKC isozymes is linked to AR activity. Oxidative stress in hyperglycemia causes the activation of PKC isozymes, PKC- β 1, PKC- β 2, and PKC- δ , which is prevented by AR inhibition in VSMC (42). Both PKC and AR may be coordinately up-regulated by growth factors but dependence of PKC activation on AR activation is not clear in colon cancer. However, our previous reports show that AR activity is required for the synthesis of

diacylglycerol, which is an obligatory cofactor for the activation of PKC isozymes (42). Significant prevention of growth factor-induced NF- κ B activation by sorbinil suggests that AR is required for the activation of NF- κ B. Similarly, AR is an obligatory mediator in the activation of NF- κ B in VSMC (17), HUVEC (18), and HLEC (19). Our earlier observations that PKC activation by phorbol ester is insensitive to sorbinil (17) suggest that AR is upstream of PKC and indicates that AR plays a pivotal role in redox signaling (17). Indeed, Kawamura et al. (30, 31) have reported that inhibition of AR by ponalrestat significantly reduced the cachexia symptoms in mice induced by colon26 adenocarcinoma. Because inhibition of AR by sorbinil or AR antisense attenuates growth factor-induced Caco-2 cell proliferation, we investigated the cell cycle stage (Fig. 4). Our results showing the arrest of Caco-2 cells at S phase by AR inhibition are similar to human lung adenocarcinoma cells (43).

Several reports suggest that growth factors, such as FGF, PDGF, IGF, HGF, and angiotensin induce intracellular ROS generation by the (a) mitochondrial respiratory chain reaction, (b) arachidonic metabolic reactions of COX-2, and (c) membrane-bound superoxide generating enzyme NADPH oxidase (44–46). The ROS in turn could cause production of toxic lipid peroxidation products, such as HNE, which could readily conjugate

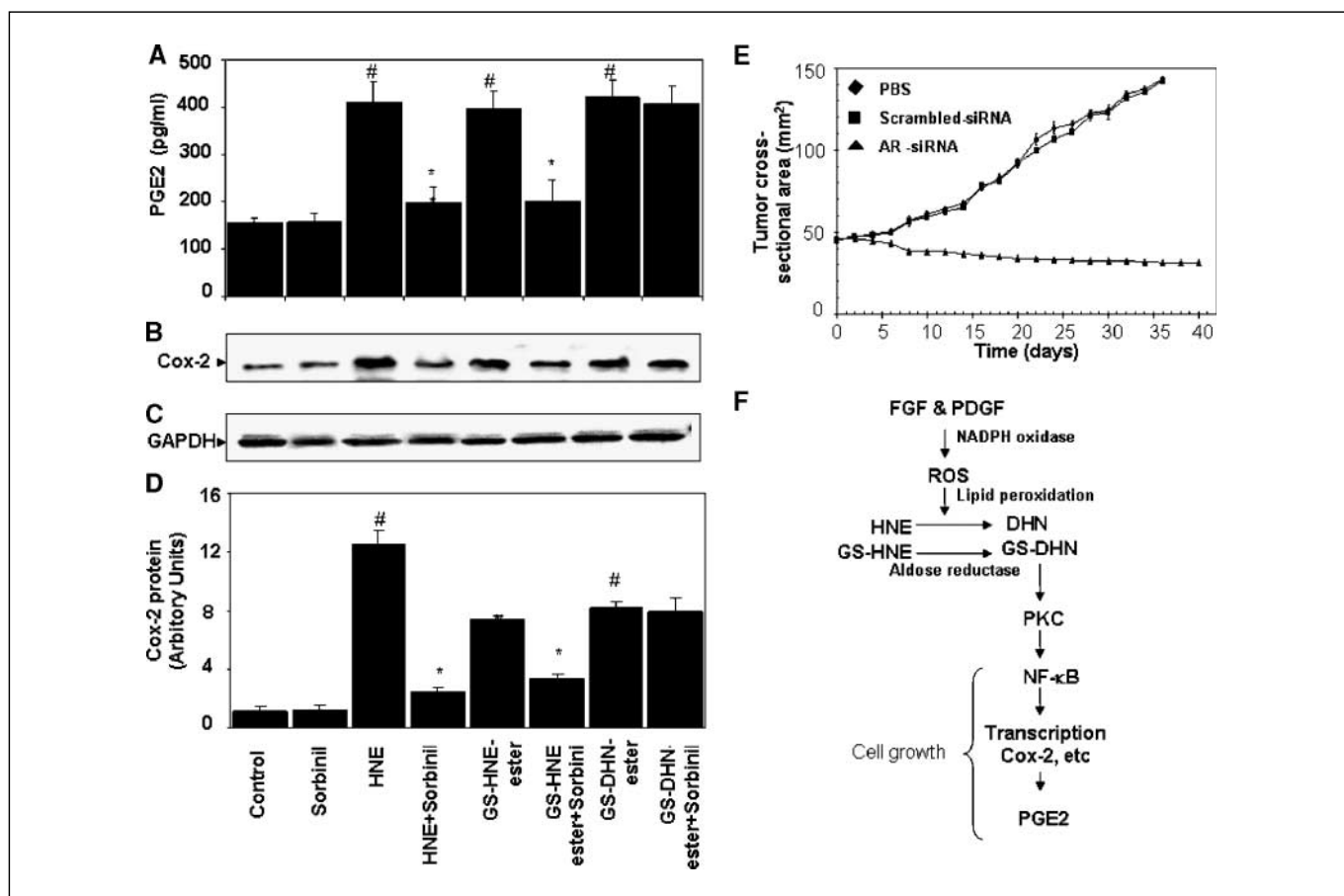


Figure 6. Effect of AR-catalyzed reaction products on PGE₂ and COX-2 in colon cancer cells. The growth-arrested Caco-2 cells preincubated with or without sorbinil for 24 hours were incubated with HNE, GS-HNE-esters, or GS-DHN-esters for 24 hours. *A*, PGE₂. Western blots were developed using antibodies against COX-2 (*B*) and GAPDH (*C*). *D*, densitometric analysis of (*B*). Columns, mean ($n = 4$); bars, SE; #, $P < 0.001$, compared with control cells; *, $P < 0.01$, compared with cells treated with aldehydes. *E*, effect of AR-siRNA on SW480 xenografts. An aliquot of 2×10^6 SW480 cells in 100 μ L PBS was injected s.c. into one flank of each *nu/nu* nude mouse ($n = 3$). When tumors reached a cross-sectional area of ~ 45 mm², animals were treated with PBS, scrambled siRNA, or AR-siRNA as described in Materials and Methods. At different days, tumors were measured in two dimensions using calipers. *F*, modulation of growth factor signaling in Caco-2 cells by AR.

with reduced glutathione, catalyzed by glutathione-*S*-transferase. Both HNE and GS-HNE are efficiently reduced by AR (21, 22, 47). The reduced lipid aldehydes and/or their glutathione conjugates may be the major mediators of ROS-initiated cytotoxic signals, which activate redox-sensitive transcription factors, such as NF- κ B and activator protein-1. A significant inhibition of growth factor-induced PGE₂ production by inhibitors of PKC, COX-2, ROS, and NF- κ B (Fig. 5) indicates the involvement of ROS-mediated signaling cascade. Further, our results suggest that lipid aldehydes especially their glutathione conjugates, catalyzed by AR, could mediate the expression of COX-2 and production of PGE₂.

In conclusion, the present study shows that AR is an obligatory mediator of the inflammatory changes caused by growth factor-induced cytotoxicity in colon cancer and that AR inhibition may represent a novel therapy for colon cancer without affecting constitutive COX-1 expression.

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