Retinoids Suppress Phorbol Ester-mediated Induction of Cyclooxygenase-2

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ABSTRACT

Cyclooxygenase-2 expression is up-regulated in transformed cells and tumors. Because this enzyme catalyzes the synthesis of prostaglandins, strategies aimed at suppressing its expression may prove useful in preventing or treating cancer. We investigated the ability of retinoids to suppress phorbol ester-mediated induction of cyclooxygenase-2 in human oral epithelial cells. Treatment with phorbol myristate acetate (PMA) resulted in a 3-fold increase in the production of prostaglandin E2 (PGE2). Retinoids [all-trans-retinoic acid (RA), 13-cis-RA, and retinyl acetate] markedly suppressed PMA-mediated increases in amounts of cyclooxygenase-2 (Cox-2) and the production of PGE2. Retinoids also suppressed the induction of Cox-2 mRNA by PMA. Nuclear run-offs revealed increased rates of Cox-2 transcription after treatment with PMA; this effect was inhibited by all-trans-RA. Transient transfection experiments showed that PMA caused a 2-fold increase in Cox-2 promoter activity, an effect that was suppressed by all-trans-RA. Our data indicate that treatment of oral epithelial cells with PMA is associated with enhanced transcription of Cox-2 and increased production of PGE2. These effects of PMA were inhibited by retinoids.

INTRODUCTION

In an effort to prevent cancer, considerable effort is being directed toward developing agents that inhibit the activity of Cox. This is so because Cox catalyzes the synthesis of prostaglandins and the production of mutagenic electrophiles (1, 2). Prostaglandins in turn are potentially important in the postinitiation phases of tumorigenesis because they modulate immune surveillance (3). Also, overexpression of Cox-2 in epithelial cells inhibits apoptosis (4), which could increase the tumorigenic potential of initiated cells. The theoretical antitumor benefits of inhibiting Cox have been confirmed in experimental models. Thus, there is strong evidence that inhibitors of Cox, including NSAIDs, protect against colon, mammary, and oral cancer in experimental animals (5–7) and humans (8).

Results from recent studies have established the presence of two distinct Cox enzymes, a constitutive enzyme (Cox-1) and an inducible isoform (Cox-2; Refs. 9 and 10), the products of separate but related genes. The Cox-2 gene is an early response gene that, like c-fos and c-jun, is induced rapidly by phorbol esters, serum, cytokines, and growth factors (11–13). The constitutive isoform of Cox (Cox-1) is unaffected by these factors. The different responses of Cox-1 and Cox-2 reflect, in part, differences in the regulatory elements in the 5'-flanking region of the two genes. For example, an ATF/CRE transcription response element is important in regulating the Cox-2 gene (14). Although chemopreventive strategies have focused on inhibitors of Cox enzyme activity, compounds that suppress the expression of Cox-2 may also have anticancer properties.

Retinoids, a group of naturally occurring and synthetic analogues of vitamin A, represent a major class of chemopreventive agents. Retinoids suppress carcinogenesis in various epithelial tissues, including the oral cavity (15–17). A variety of mechanism(s) have been identified, which may account for the chemoprotective properties of retinoids. For example, retinoids stimulate cellular differentiation and apoptosis (18, 19) and enhance cellular and humoral immunity (20).

Retinoids elicit their biological effects, in part, by blocking AP-1-mediated gene expression (21–23). c-Fos-c-Jun (AP-1) heterodimers can play a role in activating the Cox-2 gene (24). In this study, we investigated whether retinoids could down-regulate phorbol ester-induced expression of Cox-2. Our data show that PMA enhances Cox-2 gene expression, an effect which is suppressed by retinoids.

MATERIALS AND METHODS

Materials. DMEM/F-12, Opti-MEM, and FBS were from Life Technologies, Inc. (Grand Island, NY). Retinoids, PMA, 1'-naphthyl-β-D-galactopyranoside, and sodium arachidonate were obtained from Sigma Chemical Co. (St. Louis, MO). Enzyme immunoassay reagents for PGE2 assays were from Cayman Co. (Ann Arbor, MI). [32P]CTP and [32P]UTP were obtained from DuPont-NEN (Boston, MA). pFx-3, the cationic lipid used for transfections, was from Invitrogen (San Diego, CA). Reagents for the luciferase assay were from Promega (Madison, WI). Random-primer kits were from Boehringer Mannheim Biochemicals (Indianapolis, IN). Nitrocellulose membranes were from Schleicher & Schuell (Keene, NH). Plasmid DNA was prepared using a Quiagen DNA purification kit (Chatsworth, CA). Proteinase K, RNase-free DNase, and RNase A for nuclear run-offs and 18S cDNA were from Ambion, Inc. (Austin, TX).

Cell Line. 1483 squamous carcinoma cells have been described previously (25). Cells were maintained in a 1:1 mixture of DMEM/F-12 supplemented with 10% FBS and 50 μg/ml gentamicin. Cells were grown to 70% confluence, trypsinized with 0.05% trypsin-2 mM EDTA, and plated for experimental use (25).

PGE2 Production. Cells were plated in 100-mm dishes at 106 cells per dish and allowed to attach for 24 h. The DMEM/F-12 medium was then replaced with fresh DMEM/F-12 and retinoids (all-trans-RA, 13-cis-RA, and retinyl acetate) or vehicle (0.01% DMSO) to a density of 1 × 106 cells per milliliter. After 4 h of culture, the medium was harvested and assayed for PGE2 production by a specific prostaglandin assay (26). PGE2 concentrations were determined using a specific EIA kit (Cayman Co.).

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3 The abbreviations used are: Cox, cyclooxygenase; NSAID, nonsteroidal anti-inflammatory drug; FBS, fetal bovine serum; PGE2, prostaglandin E2; PMA, phorbol myristate acetate; RA, retinoic acid; CRE, cyclic AMP response element.
described by Laemmli (28). The resolved proteins were transferred onto nitrocellulose sheets as detailed by Towbin et al. (29). The nitrocellulose membrane was then incubated with a rabbit polyclonal anti-Cox-2 antiserum that was raised against the unique 18-amino acid sequence from the COOH-terminal portion of Cox-2 (30). Nitrocellulose membranes were also probed with a polyclonal anti-Cox-1 antiserum that was a generous gift of Dr. Kenneth Wu (University of Texas, Houston, TX). The membrane was subsequently incubated with a goat anti-rabbit antibody conjugated to alkaline phosphatase and developed as described previously (31). A computer densitometer (Molecular Dynamics, Sunnyvale, CA) was used to determine the density of the bands.

**Northern Blotting.** Cells were plated in 100-mm dishes at a density of 2 x 10^6 cells/dish and allowed to attach for 24 h prior to experiments. To prepare total cellular RNA, cell monolayers were washed and then directly lysed in 4 M guanidium isothiocyanate solution. RNA was then isolated by phenol-chloroform extraction according to Chomczynski and Sacchi (32). For Northern blots, 6 μg of total cellular RNA per lane were electrophoresed in formaldehyde-containing 1.2% agarose gels and transferred to nylon-supported membranes. After baking, membranes were prehybridized overnight and then hybridized in a solution containing 50% formamide, 5X saline-sodium phosphate-EDTA buffer (SSPE), 5 x Denhardt's solution, 0.1% SDS, and 100 μg/ml single-stranded salmon sperm DNA. Hybridization was carried out for 24 h at 42°C with a radioabeled human Cox-2 CDNA probe. After hybridization, membranes were washed for 20 min at room temperature in 2X SSPE-0.1% SDS, twice for 20 min in the same solution at 55°C, and twice for 20 min in 0.1X SSPE-0.1% SDS at 55°C. Washed membranes were then subjected to autoradiography. To verify equivalency of RNA loading in the different lanes, the blot was stripped of radioactivity and rehybridized to determine levels of 18S rRNA. Cox-2 and 18S rRNA probes were labeled with [32P]dCTP by random priming. The density of the bands was quantified by densitometry.

**Nuclear Run-Off.** Four x 10^6 cells were plated in 150-mm dishes and grown in DMEM/F-12 and 10% FBS until approximately 60% confluent. The medium was then replaced with DMEM/F-12 for 24 h to allow the cells to quiesce. Cells were then pretreated with fresh DMEM/F-12 containing 1 μM all-trans-RA or vehicle (0.01% DMSO). Twenty-four h later, this medium was replaced with DMEM/F-12 with or without PMA (50 ng/ml) for 3 h. Nuclei were isolated and stored in liquid nitrogen. For the transcription assay, nuclei (2.5 x 10^7) were thawed and incubated in reaction buffer (10 mM Tris (pH 8), 5 mM MgCl₂, and 0.3 M KCl) containing 100 μCi of uridine 5'-[α32P]triphosphate and 1 mM unlabeled nucleotides. After 30 min, labeled nascent RNA transcripts were isolated. The Cox-2 and β-actin CDNA were immobilized onto nitrocellulose and prehybridized overnight in hybridization buffer. Hybridization was carried out at 42°C for 24 h using equal cpm/ml of labeled nascent RNA transcripts for each treatment group. The membranes were washed twice with 2X SSC buffer for 1 h at 55°C and then treated with 10 mg/ml RNase A in 2X SSC at 37°C for 30 min, dried, and autoradiographed.

**Plasmids.** The Cox-2 promoter construct (−1432/+59) contains 1432 bases 5' of the Cox-2 transcription start site ligated to luciferase (33). pCMV.SPORTβgal was obtained from Life Technologies, Inc. (Grand Island, NY).

**Transient Transfection Assays.** 1483 cells were seeded at a density of 8 x 10^5 cells/well in six-well dishes and grown to 30–40% confluence in DMEM/F-12 containing 10% FBS. For each well, 1.8 μg of Cox-2 luciferase construct and 0.2 μg of pCMV.SPORTβgal were cotransfected into 1483 cells using pFx-3 at a 1:12 ratio of DNA to lipid as per the manufacturer's instructions. After transfection, cells were treated with DMEM/F-12 containing 1 μM all-trans-RA or vehicle (0.01% DMSO). Twenty-four h later, this medium was replaced with DMEM/F-12 with or without PMA (50 ng/ml). The activities of luciferase and β-galactosidase were measured in cellular extract 24 h later.

Each well was washed twice with PBS. Two hundred μl of 1× lysis buffer (Analytical Luminescence Laboratories, San Diego, CA) were added to each well for 30 min. Lysate was centrifuged for 5 min at 4°C. The supernatant was used to assay the activities of luciferase and β-galactosidase. Luciferase activity was measured using a Monolight 2010 luminometer (Analytical Luminescence Laboratories) according to the manufacturer's instructions. To measure the activity of β-galactosidase, 40-μl aliquots of the supernatant were incubated with assay buffer (50 mM Na₃HPO₄, 1 mM MgSO₄, 40 mM 2-mercaptoethanol, 10 mM KCl, and 4 mg/ml o-nitrophenyl-β-D-galactopyranoside) in a total volume of 400 μl for 30 min at 37°C. The reaction was terminated by the addition of 500 μl of 1 M Na₂CO₃, and the absorbance at 420 nm was determined. To adjust for differences in transfection efficiencies, the luciferase values were normalized using β-galactosidase activity.

**Statistics.** Comparisons between groups were made by the Student's t test. A difference between groups of P < 0.05 was considered significant.

**RESULTS**

**Retinoids Suppress Phorbol Ester-mediated Increases in the Production of PGE₂.** We investigated the possibility that phorbol esters could affect the production of PGE₂. As shown in Fig. 1, PMA caused about a 3-fold increase in synthesis of PGE₂. This effect was markedly suppressed by treatment with all-trans-RA, 13-cis-RA, and retinyl acetate.

**Phorbol Ester-mediated Induction of Cox-2 Is Inhibited by Retinoids.** To determine if the above differences in production of PGE₂ could be related to differences in levels of Cox, Western blotting of cell lysate protein was carried out. Fig. 2 shows that PMA caused about a 2-fold increase in amounts of Cox-2. Pretreatment with retinoids partially suppressed the inducing effects of PMA consistent with the results in Fig. 1. In separate experiments, we showed that retinoids inhibited PMA-mediated induction of Cox-2, even when the two agents were administered simultaneously (data not shown). We also attempted to determine if higher doses of retinoid would completely suppress PMA-mediated induction of Cox-2. As shown in Fig. 3, concentrations of all-trans-RA ranging from 0.5–10 μM all-trans-RA led to a similar degree of suppression of Cox-2. Neither PMA nor retinoids affected amounts of Cox-1 (data not shown).

Changes in amounts of Cox-2 enzyme could reflect altered protein synthesis or degradation. To examine the possibility that PMA acti-
Fig. 2. Retinoids inhibit PMA induction of Cox-2. 1483 cells were treated with vehicle (0.01% DMSO; Lanes 1 and 2) or 1 µM all-trans-RA, 13-cis-RA, and retinyl acetate (Lanes 3–5) for 24 h. The medium was replaced with DMEM/F-12 (Lane 1) or DMEM/F-12 and PMA (50 ng/ml; Lanes 2–5) for 5 h. Cellular lysate protein (25 µg/lane) was loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto nitrocellulose. Immunoblots were probed with antibody specific for Cox-2. Densitometry was performed, and results were expressed in arbitrary units (au.). Lane 1, 147 au.; Lane 2, 431 au.; Lane 3, 284 au.; Lane 4, 306 au.; Lane 5, 302 au.

Fig. 3. All-trans-RA suppresses PMA induction of Cox-2. 1483 cells were treated with vehicle (0.01% DMSO; Lanes 1 and 2) or a range of concentrations of all-trans-RA (0.5, 1, 5, and 10 µM; Lanes 3–6) for 24 h. The cellular lysate protein (25 µg/lane) was loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto nitrocellulose. Immunoblots were probed with antibody specific for Cox-2. Densitometry was performed, and results were expressed in arbitrary units (au.). Lane 1, 36 au.; Lane 2, 151 au.; Lane 3, 85 au.; Lane 4, 80 au.; Lane 5, 84 au.; Lane 6, 97 au.

Fig. 4. Retinoids partially inhibit PMA-mediated induction of Cox-2 mRNA. 1483 cells were treated with vehicle (0.01% DMSO; Lanes 1 and 2) or 1 µM all-trans-RA (treatment 1) or 13-cis-RA, and retinyl acetate (treatment 2) for 24 h. The cellular RNA was isolated. Each lane contained 6 µg of RNA. The Northern blot was probed sequentially with probes that recognized Cox-2 mRNA and 18S rRNA. Densitometry was performed, and the results were expressed in arbitrary units (au.). Lane 1, 27 au.; Lane 2, 231 au.; Lane 3, 116 au.; Lane 4, 121 au.; Lane 5, 105 au.

Fig. 5. All-trans-RA inhibits PMA-mediated induction of Cox-2. 1483 cells were treated with vehicle (0.01% DMSO; Lanes 1 and 2) or a range of concentrations of all-trans-RA (0.5, 1, 5, and 10 µM; Lanes 3–6) for 24 h. The cellular RNA was isolated. Each lane contained 6 µg of RNA. The Northern blot was probed sequentially with probes that recognized Cox-2 mRNA and 18S rRNA. Densitometry was performed, and the results were expressed in arbitrary units (au.). Lane 1, 0 au.; Lane 2, 380 au.; Lane 3, 94 au.; Lane 4, 90 au.; Lane 5, 73 au.; Lane 6, 59 au.

DISCUSSION

In this study, we showed that PMA induced the transcription of Cox-2, an effect that was suppressed by retinoids. This observation is important for a variety of reasons. To begin with, the findings are relevant because Cox-2 transcription is enhanced in transformed cells (34, 35), and Cox-2 is overexpressed in tumors (36, 37). Cox has both cyclooxygenase and peroxidase activities. Aside from being important for prostaglandin synthesis, the peroxidase function contributes to the activation of procarcinogens. Drugs such as NSAIDs inhibit the cyclooxygenase but not the peroxidase function of Cox, which potentially limits their effectiveness. Our results demonstrate the feasibility of using retinoids to down-regulate Cox-2 by suppressing transcription. Chemopreventive agents that down-regulate levels of Cox-2 will inhibit all functions of the enzyme and thereby should inhibit carcinogenesis at least as effectively as drugs that inhibit the cyclooxygenase activity of Cox. This is an important consideration for designing future chemoprevention trials. Thus, combining an agent, e.g., retinoid, that suppresses the transcription of Cox-2 with a compound, e.g., NSAID, which inhibits the cyclooxygenase activity of Cox may prove more effective than either agent alone.

Retinoids are active anticancer agents and appear to be an effective treatment for oral leukoplasia (16). The results of this study are likely to be important for understanding the anticancer properties of retinoids. Several classes of chemical carcinogens, e.g., heterocyclic amines, aromatic amines, and dihydrodiol derivatives of polycyclic aromatic hydrocarbons, are activated to mutagenic derivatives by Cox. Retinoid-dependent inhibition of Cox-2 synthesis could, therefore, protect against carcinogenesis by reducing chemical mutagenesis and genomic instability. In fact, retinoids have been reported to inhibit...
the binding of benzo(a)pyrene to DNA in epithelial cells (38). Retinoids also could inhibit carcinogenesis by inducing apoptosis. Retinoids were reported recently to induce apoptosis in the 1483 cell line used in this study (19). Cyclooxygenases and their end products inhibit apoptosis (4, 39). It is possible, therefore, that retinoid-mediated down-regulation of Cox-2 is one of the mechanisms by which retinoids enhance programmed cell death. Additionally, chronic inflammation is associated with an increased risk of epithelial malignancy (40). Although confirmation is needed in an animal model, our data suggest that retinoids may be anti-inflammatory, at least in part, by suppressing the expression of Cox-2. It is possible, therefore, that the anti-inflammatory properties of retinoids (41) may contribute to their chemoprotective properties.

Retinoids elicit their biological response by binding to receptors of the thyroid/steroid superfamily. There are two classes of retinoid nuclear receptors, retinoic acid receptors and retinoid X receptors, both of which are expressed in 1483 cells (42). Upon ligand binding, these nuclear receptors regulate transcription by activating the expression of genes containing retinoic acid response elements or by inhibiting the expression of genes containing AP-1 sites (21–23). The Cox-2 promoter does not contain nucleotide sequences resembling retinoic acid-responsive elements. PMA and AP-1 factors appear to be important for activating Cox-2 gene expression via a CRE within 80 bases of the Cox-2 transcription start site (24, 33, 43). Retinoids have been reported to suppress PMA-induced expression of other genes (44). It is reasonable to postulate, therefore, that retinoids suppress PMA-mediated induction of Cox-2 via a CRE-dependent mechanism. Future experiments are needed to confirm the molecular mechanism(s) by which retinoids suppress PMA-mediated induction of Cox-2.

Aside from needing to elucidate the precise mechanism by which retinoids inhibit PMA induction of Cox-2, the results of this study raise other interesting questions. Cox-2 can be induced by a range of mitogens and cytokines; therefore, it will be important to determine if retinoids can suppress the inducing effects of some of these factors. Finally, one of the current limitations of retinoid treatment is toxicity. Thus, it will be important to determine if receptor-specific retinoids (18) or retinoids that selectively inhibit the transcription factor AP-1 (44, 45) suppress PMA-mediated induction of Cox-2.

REFERENCES

RETINOIDS INhibit CYCLOOXYGENASE-2


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