Peroxisome Proliferators Enhance Cyclooxygenase-2 Expression in Epithelial Cells

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The formation of prostaglandins requires the catalytic activity of cyclooxygenase (COX) which converts arachidonic acid to the prostaglandin endoperoxide PGH₂ from which all other prostaglandins are formed. COX-2 is the highly inducible isozyme of COX which is responsible for much of the prostaglandin production in inflammation and is a key factor in colon carcinogenesis. Because COX-2 activity can be rate-limiting in prostaglandin formation, COX-2 expression must be regulated tightly. Numerous factors, including mitogens, tumor promoters, and cytokines have been found to stimulate the transcription of COX-2. We show that fatty acids, prostaglandins, and non-steroidal anti-inflammatory drugs, compounds that are substrates, products, and inhibitors, respectively, of COX enzymatic activity, also increase its expression. These compounds are members of a heterogeneous group of compounds known as peroxisome proliferators, and the prototypical peroxisome proliferator, WY-14,643, also enhanced COX-2 expression. We demonstrate that these compounds increase COX-2 transcription, and we identify a region of the COX-2 promoter containing a peroxisome proliferator response element that is responsible for the enhancement of COX-2 expression seen with these compounds.

Arachidonic acid and its oxidized derivatives, the prostaglandins (PGs), are important mediators of many physiological and pathophysiological processes. Physiologically, prostaglandins regulate vascular homeostasis, kidney function, ovulation, and parturition. These compounds are equally important as mediators of inflammation, thrombosis, and pain (1). Prostaglandins are formed by the action of cyclooxygenase, a bifunctional enzyme catalyzing both the oxidation of arachidonic acid to the prostaglandin endoperoxide PGG₂ (hence the name cyclooxygenase) and its subsequent reduction to PGH₂. This prostaglandin intermediate is the precursor to all biologically active prostaglandins and thromboxanes, the ultimate prostaglandin product being formed in a cell-specific manner. There are two isoforms of cyclooxygenase, COX-1 and COX-2. COX-1 expression is consistent throughout the cell cycle (2, 3) and is relatively unaffected by treatment of cells with mitogenic or inflammatory compounds, but it has been demonstrated to be developmentally regulated (4). COX-1 is thought to be responsible for “housekeeping” prostaglandin production. COX-2, on the other hand, is highly inducible; its expression is elevated by a variety of stimuli, including mitogens, tumor promoters, and cytokines (1), and, as described below, it is involved in carcinogenesis.

Dietary fat is an important risk factor for certain epithelial cancers, particularly colon cancer (5), but the molecular basis for this risk is unknown. The initiation and progression of other forms of cancer, including breast cancer, may also be regulated by dietary fat. Studies by Bandyopadhyay et al. (6) demonstrated that linoleic acid (LA), the major fatty acid in a normal Western diet, enhances the growth of murine mammary epithelial cells (6). Carter et al. (7) demonstrated that a diet high in fat increased the incidence of tumor formation in rats treated with the carcinogen dimethylbenz(a)anthracene. Treatment with a COX inhibitor attenuated the effects of the high fat diet, suggesting that a COX was essential to the carcinogenic process. More recent studies in colon cancer have identified induction of the COX-2 isoform as a rate-limiting step (8–10).

Work in the last few years has shown that fatty acids and prostaglandins are able to regulate gene expression through peroxisome proliferator-activated receptors (PPARs) (11–15). PPARs are members of the steroid hormone receptor superfamily which act by altering the transcription of genes with which they associate by means of a recognition sequence known as a peroxisome proliferator response element (PPRE) (16). Although nuclear localization of the receptors is independent of ligand, PPARs modulate gene expression only when ligand is bound (17). Compounds that activate PPARs are known as peroxisome proliferators and comprise a heterogeneous group that includes fatty acids and prostaglandins, plasticizers, and anti-diabetic drugs (18 and references therein).

Numerous clinical studies have demonstrated that non-steroidal anti-inflammatory drugs (NSAIDs) prevent colon cancer (19–23). In patients with familial adenomatous polyposis, sulindac reduces both the size and number of polyps, which are precursors of colon cancer (22). As shown by Thun et al. (23), aspirin consumption decreases the risk of fatal colon cancer by as much as 50%. Work by Oshima et al. (10) further supported the involvement of COX-2 in colon carcinogenesis. This group examined polyp formation in mice with a mutation in the APC gene, the gene responsible for familial adenomatous polyposis.

8328 This paper is available on line at http://www.jbc.org
When these mice were crossed with COX-2 knockout mice, the offspring had only a small fraction of the intestinal polyps found in their COX-2-expressing peers. Mice that were heterozygous for expression of COX-2 had intermediate levels of colon polyps. Given the correlation between increased COX-2 expression and colon cancer, the effect of NSAIDs is most certainly mediated, at least in part, by COX inhibition. Interestingly, some NSAIDs have recently been demonstrated to act as peroxisome proliferators (24), suggesting that they may also regulate gene expression as part of their chemopreventative mechanism.

In the experiments described below, we examined the ability of fatty acids, prostaglandins, and NSAIDs to regulate COX-2 expression. We demonstrate that these compounds enhance COX-2 expression and that the regulation occurs transcriptionally. Further, we define a region of the promoter responsible for at least part of the regulation of COX-2 expression by these compounds.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fatty acids, prostaglandins, and NSAIDs were tested from 50 to 1,000 m. Prostaglandins, HETEs, and HODEs were used at the following concentrations: PGD$_2$, PGE$_2$, and PGF$_2\alpha$ at 0.4–7 μM; PGA$_2$, 15-deoxy-Δ12,14-PGJ$_2$, PGI$_2$, 8(R)-HETE, 15-HETE, 9-HODE, and 13-HODE at 0.3–18 μM. The following NSAIDs were tested from 10 to 250 μM: meclofenamate, mefenamic acid, NS-398, indomethacin, sulindac sulfone, and sulindac sulfide, and sulindac sulfide were purchased from BioMol. Other NSAIDs and common reagents were purchased from Sigma. Fatty acids and prostaglandins were diluted in ethanol, 90, 14,643, and NSAIDs in dimethyl sulfoxide. Medium for the culture of human mammary epithelial cells was prepared in house, media for culture of colon cells were obtained from Life Technologies, Inc. Mouse anti-human monoclonal antibodies to COX-1 and COX-2 were used according to the procedures of Habib et al. (25), (27) were obtained by screening with 30-mer oligonucleotides corresponding to the region surrounding the transcriptional start site, according to established procedures. PPARγ (28) was obtained by screening with a random primer-labeled cDNA probe (Cayman). Full-length cDNA clones were obtained for each. The clones were sequenced to ensure correctness and subcloned into pcDNA/Amp (Invitrogen) for mammalian expression.

**Clones for Human PPARs and RXRs**—We screened a human skeletal muscle 5′-STRETCH PLUS cDNA library (CLONTECH) to isolate clones for the known human isoforms of PPAR and for RXRs. PPARα (25), δ (28), and RXRs (27) were obtained by screening with 30-mer oligonucleotides corresponding to the region surrounding the transcriptional start site, according to established procedures. PPARγ (28) was obtained by screening with a random primer-labeled cDNA probe (Cayman). Full-length cDNA clones were obtained for each. The clones were sequenced to ensure correctness and subcloned into pcDNA/Amp (Invitrogen) for mammalian expression.

**Isolation and Subcloning the 7-Kilobase COX-2 Promoter**—Previous work in this laboratory isolated a 1.8-kb human COX-2 promoter clone (9). Because many PPREs are located further upstream from the transcriptional start site (29), we felt it was necessary to obtain a larger promoter clone. We designed polymerase chain reaction primers corresponding to the 5′-most end of the COX-2 promoter, which were used to isolate a P1 clone (Genome Systems). We performed a series of restrictions to the Southern blot, which allowed us to identify the region corresponding to further 5′-regions of the COX-2 promoter. We chose pieces from two restriction digestions, KpnI (5,710 bases) and SacI (9,271 bases), which were subcloned into pBluescript (Stratagene) for sequencing and then into pGL3-basic (see below) attached to our existing COX-2 promoter to allow us to search for potential PPREs in this larger promoter. The result of the subclonings gave us a total of approximately 7,270 bases of promoter for the KpnI construct and 9,290 bases of promoter for the SacI construct. The 7,270-base pair COX-2 promoter has been deposited with GenBank, Accession Number AF044206.

**Reporter Constructs**—Regions of the 7-kb COX-2 promoter were cloned into pGL3-basic (Promega). The putative PPRE in the COX-2 promoter (located at −3900) as well as a consensus PPRE for the rat acyl-CoA oxidase gene (ACO) (30, 31) were cloned into pGL3 promoter. Oligonucleotide sequences for ACO PPRE were: F, 5′-CCCTTCTCCGAACGTGACCTTTGTCCTGGTCCCCTTTTGCT-3′; ACO PPRE R, 5′-GATCTAGAACTGTCAGAGAAGTGAGGCGACAGGTCATAACCCTA-3′; ACO PPRE R, 5′-GATCTAGAACTGTCAGAGAAGTGAGGCGACAGGTCATAACCCTA-3′; and for the COX-2 −3900 PPRE: COX-2 −3900 PPRE F, 5′-CCCGTGGTCTGCCTTCAATTTTAAATAGGAGTTATGACCTGTTGAAGGTTCTGCCCTACTCTCCTCAGGAGTTAATATACTATAATG-3′; COX-2 −3900 PPRE R, 5′-GATCTAGAACTGTCAGAGAAGTGAGGCGACAGGTCATAACCCTA-3′. A pGL3 promoter vector with no insert was used as a negative control in transfection experiments. PPARα cloned into pcDNA/Amp was used for cotransfection to ensure that the cells' native PPAR would not be limiting in detection of transcriptional effects. β-Galactosidase cloned into pHOOK-2 (Invitrogen) was used as a control for normalization of transfections. (33). HCT-116, DLD-1, CaCo-2, and HT-29 colonic epithelial cells were obtained from ATCC and grown according to recommended procedures. HCT-116 and HT-29 were cultured in McCoy's 5A with 10% fetal bovine serum, and CaCo-2 were grown in minimal essential medium with Earle's salts, supplemented with 10% fetal bovine serum. For 24 h before treatment with NSAIDs, the colonocytes were serum starved by replacing the normal growth medium with serum-free medium. To examine COX-2 expression in both mammary epithelial cells and colonocytes, the medium was changed to fresh serum-free medium containing fatty acid, prostaglandin, WY-14,643, or NSAID. For 184B5 cells, the medium also contained the EGF receptor antibody. Cells were incubated with agonist for 4–8 h for mRNA expression or for 10–12 h for protein expression before harvest. For time-course experiments, samples were harvested at the times indicated in the figures. For experiments utilizing actinomycin D, it was added at 5 μg/ml simultaneously with agonist. For each fatty acid, prostaglandin, and NSAID used, a dose-response curve was performed. Only the maximally effective concentration of each NSAID was shown in the figures. Fatty acids including arachidonic acid, eicosapentaenoic acid, eicosatetraenoic acid, linoleic acid, docosahexaenoic acid, and linolenic acid were used at 0.3–18 μM. Prostaglandins, HETEs, and HODEs were used at the following concentrations: PGE$_2$, PGF$_2\alpha$, and PGI$_2$ at 0.4–7 μM; PGA$_2$, 15-deoxy-Δ12,14-PGJ$_2$, PGI$_2$, 8(R)-HETE, 15-HETE, 9-HODE, and 13-HODE at 0.3–18 μM. The following NSAIDs were tested from 10 to 250 μM: meclofenamate, mefenamic acid, NS-398, indomethacin, sulindac sulfone, piroxicam, resveratrol, naproxen, flurbiprofen, and ketorolac. Sulindac sulfide was tested from 1 to 250 μM. Ibuprofen and aspirin were tested from 50 to 1,000 μM.

**Transfection Experiments**—Quiescent 50% confluent 184B5 cells were transfected using 2.5 μg PLD disl LipofectAMINE (Life Technologies, Inc.) at 0.25 μg luciferase reporter plasmid, PPARα (or δ, γ, or vector only), and β-galactosidase and were performed in the presence of monoclonal antibody 225. At 24 h after transfection, the medium was changed to fresh MCDB 170 containing monoclonal antibody 225 with or without 100 μM WY-14,643. The cells were harvested 72 h later, and luciferase and β-galactosidase assays were performed. Luciferase activity was measured using the Promega kit, and β-galactosidase was measured using Galactolight (Tropix). Triplicate wells were assayed in duplicate for each sample.

**Western Blot Analysis**—Cells were washed with ice-cold phosphate-buffered saline and then lysed with buffer consisting of 20 mM Tris, pH 7.5, 1.6 mM CHAPS, 0.5 mM dithiothreitol, 1 mM EDTA, 1 mM benzamidine hydrochloride, 1 μg/ml leupeptin, and 10 μg/ml soybean trypsin inhibitor. Cells were scraped and placed on ice for 30 min. Unsilyzed cells and debris were removed by centrifugation, and protein assays were performed (BCA protein assay, Pierce) to ensure equal loading on SDS-polyacrylamide gel electrophoresis. Proteins were separated through 10% denaturing polyacrylamide gels and transferred to polyvinylidene difluoride membranes. COX-2 and COX-1 monoclonal antibodies were used according to the procedures of Habib et al. (34). Monoclonal anti-human β-actin was used in some experiments as a control for protein integrity. The secondary antibody was horseradish peroxidase-labeled goat anti-mouse (BIOSOURCE), and ECL (Amerasham) was used as a detection method. Quantitation of Western blots was performed by scanning the blots into Photoshop and performing densitometry with NIH Image.

**RNase Protection Assays**—RNA was isolated using TRIzol reagent (Life Technologies, Inc.) and processed according to the manufacturer's instructions. Samples were resuspended in RNase-free water and concentrations determined spectrophotometrically. 10 μg of each sample was used for RNase protection assay with probes for COX-2 and GAPDH. The COX-2 probe corresponds to the region spanning −90 to +332 relative to the COX-2 translational start site. The GAPDH probe is commercially available (Ambion). Radiolabeled fragments were prepared using [α-32P]UTP and the Ambion MAXiScribe in vitro transcription kit. 80,000 cpm/sample of the COX-2 probe and 8,000 cpm/sample of the GAPDH probe were used in the RNase protection assay (RPA II, Ambion). RNA and probes were co-precipitated, resuspended in hybrid.

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ization buffer, and incubated overnight at 43 °C. The following day, unbound RNA was digested with RNase, and the remaining radiolabeled RNA was precipitated, resuspended in loading buffer, and separated on a 6% polyacrylamide gel. Gels were dried and exposed to BioMax MS film at –80 °C for up to 48 h. After autoradiography, the protein assay was quantitated by densitometry as above.

**Nuclear Run-on Assays**—Nuclear run-on assays were used to assess absolute levels of transcription of COX-2 after treatment with EGF, phorbol 12-myristate 13-acetate, or LA. Assays were performed essentially as described by DeWitt and Meade (3). For nuclear isolation, 1 × 10⁷ cells were treated with vehicle, EGF (100 ng/ml), phorbol 12-myristate 13-acetate (60 ng/ml), or LA (18 μM) until the time when the maximal change in transcription was expected based on changes in mRNA levels (30 min for EGF, 1 h for phorbol 12-myristate 13-acetate, and 2 h for LA and vehicle). Cells were scrapped into cold phosphate-buffered saline, centrifuged and rinsed, and placed in buffer (50 mM Tris, pH 8.3, 40% glycerol, 5 mM MgCl₂, and 0.1 mM EDTA) for storage in liquid nitrogen until assayed. mRNA and protein were harvested from identical plates at later time points to ensure that both COX-2 protein and mRNA expression were altered as expected by these treatments. For nuclear run-on assays, the following plasmids were used as probes: a cDNA fragment for COX-2 which corresponded to the entire open reading frame but did not contain the 5'-untranslated region and a full-length cDNA for COX-1. (Previous work demonstrated that COX-1 levels are unaltered by the treatments used in these experiments and that it could serve as a control in these experiments.) Both plasmids were in the same base vector, eliminating the need for a vector control. Plasmids were linearized, denatured, and applied to Hybond N membrane using a Minifold II Slot Blender (Schleicher & Schuell). To begin run-on assays, nuclei were thawed and combined with a buffered nucleotide mixture containing [α-³²P]UTP to label transcripts radioactively. Transcription was allowed to proceed for 30 min at 30 °C, and then RNase-free DNAse (200 units) was added and the incubation continued for an additional 10 min. Next, proteins were digested by the addition of proteinase K (400 μg/ml) for 45 min at 37 °C. The samples were extracted with phenol-chloroform and precipitated with NAOAc and EtOH at –80 °C. After repeating the DNAse and proteinase K digestions, the samples were precipitated overnight at –80 °C. The following day, the samples were centrifuged, and the RNA was resuspended in TE and denatured with NAOH for 10 min on ice. The samples were precipitated a third time and resuspended in TE. Incorporation of radiolabel into the nuclei was ascertained by scintillation counting, and an equal amount of radioactivity was added to individual slot blots for each sample. The slot blots that contained COX-1 and COX-2 probes had been prehybridized for 2 h before the addition of radiolabeled nucleotide. After the addition of the nuclei, the blots were hybridized at 42 °C for 48 h. The blots were then washed as described and exposed to BioMax MS at –80 °C for up to 48 h. After autoradiography, blots were quantitated as described above.

**Gel Shift Assay**—Nuclear extracts were prepared from COS-7 cells transfected with PPARs (or δ, or γ, or vector only) and with RXRα (35). Oligonucleotide cassettes corresponding to the ACO PPRE (30, 31) and the COX-2 –3900 PPRE were either radiolabeled using [γ-³²P]ATP and T4 kinase or used unlabeled for competition experiments. The following oligonucleotides were used: ACO PPRE F, ACO PPRE R, COX-2 –3900 PPRE F, COX-2 –3900 PPRE R, whose sequences were defined earlier, and AP2 F, 5’-GATCGAATCCGAGCGTGATCGTCGATC-3’; and AP2 R, 5’-ACGGCGGCGGCGGCGCTGATCG-3’. Binding reactions were performed by preincubating 20 μg of nuclear lysate in binding buffer with or without competitor oligonucleotide (50-fold excess compared with radiolabeled oligonucleotide) in a volume of 18 μl for 10 min at room temperature. Binding buffer consisted of 10% glycerol, 20 mM Tris, pH 8.0, 80 mM KCl, 10 mM MgCl₂, 2 mM dithiothreitol, and 5 μg of poly(dI-dC): 2 μl of radiolabeled oligonucleotide cassette was then added, and the reactions were incubated 20 min at room temperature. The products were separated by loading on a prerun 4% non-denaturing polyacrylamide gel containing 2.5% glycerol and separated in 1 × TBE buffer at 150 volts at room temperature until the bromphenol blue marker dye was approximately two-thirds of the way down the gel. Controls, including AP2 oligonucleotides, were from the Promega gel shift assay system. Gels were dried and exposed to BioMax MR autoradiography film at –80 °C for up to 48 h.

**RESULTS**

Polyunsaturated fatty acids, including LA, have diverse effects on cells, and we asked whether one component of the response was to alter COX-2 expression. We exposed mammary epithelial cells to LA and found that it strongly enhanced COX-2 expression (Fig. 1A). Many fatty acids are able to stimulate cell responses, so we next tested other fatty acids for their effect on COX-2 expression. We found that all of the unsaturated fatty acids that we tested enhanced COX-2 expression, including arachidonic acid, the normal substrate for COX-2, as well as fatty acids that are abundant in fish oils, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). This result was obtained in two independent experiments.

![Fig. 1](image.png)

**Fig. 1.** Fatty acids enhance the expression of COX-2 in 184B5 mammary epithelial cells. 184B5 cells were grown until they were approximately 70% confluent and then made quiescent as described under “Experimental Procedures.” The cells then were treated with various fatty acids for 10 h, at which time extracts were prepared and examined by immunoblotting using a monoclonal antibody specific for COX-2 (see “Experimental Procedures”). Panel A, LA enhances the expression of COX-2. Cells were exposed to the indicated concentrations of LA. The experiment shown is representative of five. Panel B, a panel of fatty acids was tested for their ability to enhance COX-2 expression. A dose-response curve was performed for each fatty acid; the concentration shown is that which was maximally effective: 18 μM LA, linoleic acid (LEA), arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA).
Peroxisome proliferators act by increasing the transcription of the genes that they regulate. If fatty acids, prostaglandins, and NSAIDs alter COX-2 expression through a PPAR, changes in COX-2 mRNA levels should correspond with changes in COX-2 protein levels. We examined the COX-2 mRNA over time in 184B5 cells treated with LA (Fig. 4A) and found that the levels rose markedly, reaching a maximum by 4 h after treatment. We also measured the change in COX-2 mRNA in CaCo-2 cells that had been treated with the panel of NSAIDs used for Western blot analysis (Fig. 4B). The same pattern of NSAIDs was found to enhance COX-2 mRNA expression as had increased protein levels, suggesting that the mechanism responsible for the increased expression was transcriptional. Actinomycin D inhibited COX-2 expression when used in combination with fatty acids, prostaglandins, and NSAIDs (Fig. 5, A and B), providing further evidence that their effects are transcriptional. Nuclear run-on assays validated this conclusion; in two separate experiments 18 μM LA enhanced COX-2 transcription in 184B5 cells 2.1- and 1.5-fold (COX-2/COX-1), whereas phorbol 12-myristate 13-acetate, which strongly stimulates COX-2 expression in these cells, enhanced transcription 4.9- and 2.1-fold.

We took two approaches to identify the portion of the COX-2 promoter responsible to peroxisome proliferators. First, we isolated new, longer regions of the COX-2 promoter and examined all four colonial epithelial cell lines that we examined. COX-1 expression was unaffected by NSAID treatment. Treatment with NSAIDs at these concentrations did not alter cell viability (n = 2, data not shown).
mately 3900 bases upstream of the translational start site (−3900). We made reporter constructs that contained between 7 kb and 200 base pairs of the COX-2 promoter, the −3900 PPRE, or with a reporter that contained the rat fatty ACO PPRE sequence (30, 31). Cells were co-transfected with an expression plasmid for one of the PPARs. The transfected cells then were stimulated with WY-14,643 or vehicle for 72 h. Luciferase activity was measured and normalized to co-transfected β-galactosidase. In cells containing the −3900 PPRE construct, luciferase activity increased 1.4-fold on treatment with WY-14,643 (n = 6) (Fig. 6A). This response depended on co-transfection with a PPAR, and, although modest, was highly consistent between experiments. The experiments shown used an expression plasmid for PPARα, but essentially equivalent results were obtained with either δ or γ (data not shown). In the second approach, we performed gel shift experiments using nuclear lysate obtained from COS-7 cells transfected with a PPAR and RXRα (Fig. 6B). As a positive control we used an oligonucleotide cassette for the ACO PPRE. Both the COX-2 −3900 PPRE and the ACO PPRE produced bands on gel shift. The ACO PPRE could be competed with cold ACO PPRE but not cold AP2, supporting the hypothesis that this region of the COX-2 promoter is responsible for binding PPAR/RXR heterodimers and therefore is responsible for peroxisome proliferator-stimulated transcription of COX-2. To provide further support that the shifted band was truly a PPAR-COX-2 PPRE complex, we performed supershift experiments using both commercially available antibodies as well as an anti-PPARα antibody that we prepared in house especially for this purpose. Although these antibodies recognized the corresponding PPAR when used for immunoblotting, none would supershift the PPAR-ACO PPRE complex used as a positive control, and none was therefore useful for characterizing the interaction between PPARs and the COX-2 PPRE.

DISCUSSION

Much debate has surrounded the role of dietary fat as a risk factor for the development of cancer. Although diet is a rela-
Peroxisome Proliferators Regulate COX-2

Fatty acids stimulate transcription of COX-2 through a PPAR. Panel A, peroxisome proliferators stimulate the transcription of COX-2. 184B5 cells were transfected with reporter constructs from the COX-2 promoter, the ACO PPRE, or a control promoter, driving luciferase expression. Cells were co-transfected with both PPARs and β-galactosidase. Luciferase and β-galactosidase activities were measured 72 h after the addition of fresh medium with or without 100 μM WY-14,643. The results are expressed as the fold increase in luciferase activity on treatment with WY-14,643 (n = 6). Panel B, gel shift analysis. Nuclear lysates were prepared from COS-7 cells transfected with PPARα and RXRα. After the binding of oligonucleotide cassettes corresponding to the ACO PPRE or the COX-2 −3900 PPRE to these lysates, DNA-protein complexes were detected by separation on non-denaturing polyacrylamide gels. For competition experiments, cold oligonucleotide cassette for ACO, the COX-2 −3900 PPRE, or AP2 was incubated with the lysates before the addition of radiolabeled ACO or COX-2 PPRE. This result was obtained in two independent experiments.

The regulation of COX-2 expression not only by fatty acids, but also by its enzymatic products, demonstrates a rare feed-forward mechanism that may be key in the pathogenesis of cancer. Dietary fat may act through a PPAR to enhance COX-2 expression in one cell type, resulting in the release of prostaglandins to the intercellular milieu. There, the prostaglandins affect neighboring cells, resulting in enhanced COX-2 expression in these cells. Support for such a feed-forward mechanism is found in the work of Oshima et al. (10) who examined expression of COX-2 in the interstitial cells of mice harboring a mutant APC (APC(−/−)). These mice develop large numbers of intestinal polyps. They found COX-2, surprisingly, to be localized in the interstitial cells of the murine intestine rather than in the polyp epithelium where it is expressed in human colon. Thus, expression measured in the polyp epithelium may actually represent a second wave of COX-2 expression due to the paracrine effect of prostaglandins released by the interstitial cells.

NSAIDs are among the most prescribed drugs in the United States. Although these drugs have many well known, long-standing uses, one of the more recent uses is as a colon cancer prophylactic. As demonstrated in this manuscript, NSAIDs have the dual role of inhibiting COX-2 activity while increasing its expression. No in vivo role has currently been defined for this increased COX-2 expression stimulated by NSAIDs. It is likely that NSAIDs would block the activity of the induced COX-2 enzyme as long as the treatment continued, and in such a case, there presumably would be no net effect. However, if NSAID treatment were discontinued, or were intermittent, the effect might be deleterious. For example, if the level of the COX-2 enzyme had been increased markedly by the drug and then it was discontinued, the result might be the exact opposite of the intended therapeutic effect. However, estimating the likelihood of this unfavorable outcome is complex because it depends on the rate at which the NSAID dissociates from COX-2, the t_{1/2} of the protein, and probably other variables. In vivo experiments will be necessary to assess whether the induction of COX-2 by NSAIDs can result in increased prostaglandin synthetic capacity under some circumstances.

It is interesting that COX-1 levels are not altered by treatment with NSAIDs, suggesting that the increase in COX-2 expression seen on treatment with NSAIDs is not a feedback mechanism designed to return prostaglandin production to a base-line level. Further, the different NSAIDs did not enhance COX-2 expression equally, although all were tested at concentrations sufficient to abolish prostaglandin formation. This result supports the hypothesis that enhanced COX-2 expression on treatment with NSAIDs is a direct effect mediated through a PPAR.

While this manuscript was in preparation, Staels et al. (38) reported that activators of PPARα can inhibit the expression of a variety of genes involved in the inflammatory response, including COX-2, in smooth muscle cells. These experiments used a protocol in which the cells were treated with PPARα activators and then with interleukin-1; the PPAR activators had no effects by themselves. One likely explanation for some of the differences between our results and theirs is that our studies used epithelial cells, mammary and colon, whereas theirs were on vascular smooth muscle cells. Moreover, their studies used our previously described 1.8-kb COX-2 promoter/reporter construct (9), which does not have a PPRE and which we found to be unresponsive to the agonists described here. Staels et al. (38) concluded that their results reflected a novel mechanism in lipids and NSAIDs.
which PPARα activation interfered with transcription mediated by nuclear factor-κB and not through a typical PPRE; our work shows that the fatty acid, prostaglandin, and NSAID effects are through a traditional PPAR/PPRE mechanism. It is feasible that both mechanisms are involved in the regulation of COX-2 expression in response to peroxisome proliferators, and the predominant response may be tissue-specific. Several studies examining the role of PPARγ in colon cancer were published at the time that this manuscript was submitted (39–41). In two of them, it was shown that selective activators of PPARγ increased the number and size of colon, but not small intestinal, polyps in Min mice, which have a mutation in one allele of the APC gene (39, 40). The other study reported the opposite effect of PPARγ on the growth of human colon cancer cells transplanted into mice (41). In addition to measuring the size and number of tumors, Lefebvre et al. (39) examined molecular events including whether COX-2 was induced in the mouse adenomas. They also tested the effect of a PPARγ agonist on COX-2 expression in HT-29 cells, a human colon carcinoma cell line that we also examined. The activation of PPARγ did not increase COX-2 expression in their experiments, either in mouse adenomas in vivo or the human cells in culture. In contrast, we found that COX-2 was induced in HT-29 and other colon carcinoma cells in response to NSAIDS, which can activate PPARα. Taken together, we interpret these studies as showing two results: activators of PPARγ can alter colon epithelial cell function in a COX-2-independent manner, and activators of PPARα (or δ) induce transcription of COX-2 in epithelial cells, which may be relevant to carcinogenesis pathways in several organs. We found that the promoter constructs of COX-2 which contained a presumed PPRE were stimulated when a plasmid encoding any of the three human isoforms was cotransfected. Thus, our studies do not establish whether all of them mediate this action in vivo or whether it is a more restricted response. The work of others (as above) suggests that PPARγ is unlikely to serve as a trans-activator of the COX-2 gene in the colon epithelium.

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REFERENCES

1. Meade, E. A., Jones, D. A., Zimmerman, G. A., McIntyre, T. M., and Prescott, S. M. (1992) in Handbook of Lipid Research, vol. 8 (Bell, R. M., Exton, J. H., and Prescott, S. M., eds) pp. 285–305, Plenum Press, New York
2. Smith, W. L., Garavito, R. M., and DeWitt, D. L. (1996) J. Biol. Chem. 271, 33157–33160
3. DeWitt, D. L., and Meade, E. A. (1993) Arch. Biochem. Biophys. 306, 94–102
4. Brannon, T. S., North, A. J., Wells, L. B., and Shaull, P. W. (1994) J. Clin. Invest. 93, 2230–2235
5. Poter, J. D. (1992) JAMA 268, 1573–1577
6. Bandopadhyay, G. K., Imagawa, W., Wallace, D., and Nandi, S. (1987) J. Biol. Chem. 262, 2750–2756
7. Carter, C. A., Milholland, R. J., Shea, W., and Ip, M. M. (1983) Cancer Res. 43, 3559–3562
8. Eberhart, C. E., Coffey, R. J., Radhika, A., Giardiello, F. M., Ferrenbach, S., and DuBois, R. N. (1994) Gastroenterology 107, 1183–1188
9. Kutchera, W., Jones, D. A., Matsunami, N., Groden, J., McIntyre, T. M., Zimmerman, G. A., White, R. L., and Prescott, S. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4816–4820
10. Oshima, M., Dinchuk, J. E., Kargman, S. L., Oshima, H., Hancock, B., Kwong, E., Trzaskos, J. M., Evans, J. F., and Taketo, M. M. (1996) Cell 87, 803–809
11. Gottlicher, M., Widmark, E., Li, Q., and Gustafsson, J.-A. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 4653–4657
12. Keller, H., Dreyer, C., Medin, J., Mahfoudi, A., Ozato, K., and Wahl, W. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2160–2164
13. Kliewer, S. A., Sundeseth, S. S., Jones, S. A., Brown, P. J., Wisely, G. B., Kohle, C. S., Devchand, P., Wahl, W., Wilson, T. M., Lenhard, J. M., and Lehmann, J. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 4318–4323
14. Forman, B. M., Chen, J., and Evans, R. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 4312–4317
15. Yu, K., Bayona, W., Kallen, C. B., Harding, H. P., Raveru, C. P., McMahon, G., Brown, M., and Lazar, M. A. (1995) J. Biol. Chem. 270, 23975–23983
16. Lemberger, T., Desvergne, B., and Wahli, W. (1996) Annu. Rev. Cell Dev. Biol. 12, 335–383
17. Dreyer, C., Keller, H., Mahfoudi, A., Lauledet, V., Krey, G., and Wahl, W. (1995) Biol. Cell 77, 67–76
18. Isselmann, I., and Green, S. (1990) Nature 347, 645–650
19. Giovannucci, E., Egan, K. M., Hunter, D. J., Stampfer, M. J., Colditz, G. A., Willett, W. C., and Speizer, F. E. (1995) N. Engl. J. Med. 333, 609–614
20. Logan, R. F. A., Little, J., Hawtin, P. G., and Hardcastle, J. D. (1995) Br. Med. J. 307, 285–289
21. Williams, C. S., Smalley, W., and DuBois, R. N. (1997) J. Clin. Invest. 100, 1325–1329
22. Giardiello, F. M., Hamilton, S. R., Krush, A. J., Piantadosi, S., Hyland, L. M., Celano, P., Booker, S. V., Robinson, C. R., andOfferhaus, G. J. A. (1993) N. Engl. J. Med. 328, 1313–1316
23. Thun, M. J., Namboodiri, M., and Heath, C. W., Jr. (1991) N. Engl. J. Med. 325, 1595–1596
24. Lehmann, J. M., Lenhard, J. M., Oliver, B. B., Ringold, G. M., and Kliewer, S. A. (1997) J. Biol. Chem. 272, 3406–3410
25. Mukherjee, R., Jow, L., Noonan, D., and McDonnell, D. P. (1994) J. Steroid Biochem. Mol. Biol. 51, 157–166
26. Schmidt, A., Krey, G., and Wahli, W. (1995) J. Biol. Chem. 270, 8071–8076
27. Yaswen, P. (1993) EMBO J. 12, 2394–2398
28. Forman, B. M., and Lazar, M. A. (1995) J. Biol. Chem. 270, 3406–3410
29. Stampfer, M. R., and Bartley, J. C. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 2394–2398
30. Stampfer, M. R., Pan, C. H., Hosoda, J., Bartholomew, J., Mendelsohn, J., and Yaswen, P. (1993) Exp. Cell Res. 208, 175–188
31. Habib, A., Cresmin, C., Frobert, Y., Grassi, J., Pradelles, P., and Maclouf, J. (1993) J. Biol. Chem. 268, 23448–23454
32. Andrews, N. C., and Faller, D. V. (1991) Nucleic Acids Res. 19, 2499
33. Forman, B. M., Tontonoz, P., Chen, J., Brum, R. P., Spiegelman, B. M., and Evans, R. M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 803–812
34. Kliewer, S. A., Wilkson, T. M., Patel, I., McPhail, K. K., and McGuffin, L. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5723–5727
35. Kramer, M. R., Metheny, M. S., and Ayus, J. C. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 1593–1596
36. Kriebel, D., and DuBois, R. N. (1994) Cancer Res. 54, 1046–1052