Peroxisome Proliferator-activated Receptor γ Ligands Suppress the Transcriptional Activation of Cyclooxygenase-2

EVIDENCE FOR INVOLVEMENT OF ACTIVATOR PROTEIN-1 AND CREB-BINDING PROTEIN/p300*

We investigated whether peroxisome proliferator-activated receptor γ (PPARγ) ligands (ciglitazone, troglitazone, and 15-deoxy-Δ12,14 prostaglandin J2) inhibited cyclooxygenase-2 (COX-2) induction in human epithelial cells. Ligands of PPARγ inhibited phorbol ester (phorbol 12-myristate 13-acetate, PMA)-mediated induction of COX-2 and prostaglandin E2 synthesis. Nuclear run-offs revealed increased rates of COX-2 transcription after treatment with PMA, an effect that was inhibited by PPARγ ligands. PMA-mediated induction of COX-2 promoter activity was inhibited by PPARγ ligands in a dose-dependent manner. When CREB-binding protein and c-Jun were overexpressed together, the ability of PPARγ ligands to suppress PMA-mediated induction of COX-2 promoter activity was essentially abrogated. Bisphenol A diglycidyl ether, a compound that binds to PPARγ but lacks the ability to activate transcription, also inhibited PMA-mediated induction of AP-1 activity and COX-2. Taken together, these findings are likely to be important for understanding the anti-inflammatory and anti-cancer properties of PPARγ ligands.

COX1 catalyzes the synthesis of prostaglandins from arachidonic acid. There are two isoforms of COX. COX-1 is constitutively expressed in most tissues and appears to be responsible for various physiologic functions (1, 2). COX-2 is an immediate, early response gene that is rapidly induced by phorbol esters, growth factors, cytokines, and oncogenes (3–9).

COX-2 is an important therapeutic target for preventing or treating arthritis and cancer (10–12). Selective COX-2 inhibitors decrease inflammation and are widely used to treat arthritis (13). COX-2 is overexpressed in transformed cells (8, 14, 15) and in malignant tumors (16–20). COX-2 knockout mice are protected against both intestinal (21) and skin tumors (22). Moreover, selective COX-2 inhibitors suppress the formation of tumors induced by growth factors, cytokines, and oncogenes (3–9). COX-2 knockdown in colorectal cancer cells prevents cancer cell invasion and growth (23–25).

Materials—Minimal essential medium, Opti-MEM, and LipofectAMINE were from Life Technologies, Inc. Keratinocyte basal and growth media were from Clonetics Corp. (San Diego, CA). Phorbol 12-myristate 13-acetate, taxol, sphingomyelinase, sodium arachidate, PMA, PPAR ligands, and PPARγ agonists were from Enzo Life Sciences, Inc. (Cayman Chemical Co.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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This article has been withdrawn by Kotha Subbaramaiah, Derrick T. Lin, and Andrew J. Dannenberg. Janice C. Hart could not be reached. Lanes 1-3 of Fig. 1A were reused in lanes 3-5 of Fig. 1B.

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nate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (thiazolyl blue), lactate dehydrogenase diagnostic kits, epinephrine, epidermal growth factor, hydrocortisone, poly(dI-dC), and o-nitrophenyl-β-D-galactopyranoside were from Sigma. Cigitazone and 15-deoxy-D 12,14-prostaglandin J 2 (15d-PGJ 2 ) were from Biomol Research Labs Inc. (Plymouth Meeting, PA). Troglitazone and its M metabolite were generously provided by Dr. A. Saltiel (Parke-Davis). Biaphenol A diglycidyl ether (BADGE) was obtained from Fluka (Milwaukee, WI). Enzyme immunoassay reagents for PGE 2 assays were from Cayman Co. (Ann Arbor, MI). Western blotting detection reagents, [ 32P]ATP, [ 32P]CTP, and [ 32P]UTP were from PerkinElmer Life Sciences. Random primed kits were from Roche Molecular Biochemicals. Nitrocellulose membranes were from Schleicher & Schuell. Reagents for the luciferase assay were from PharMingen (San Diego, CA). The 18 S rRNA cDNA was from Ambion, Inc. (Austin, TX). T4 polynucleotide kinase was from New England Biolabs (Beverly, MA). Anti sera to PPARγ, COX-2, c-Jun, c-Fos, and ATF-2 were purchased from Santa Cruz Biotechnology, Inc. (San Diego). Plasmid DNA was prepared using a kit from Promega Corp. (Madison, WI). Oligonucleotides were synthesized by Genosys (The Woodlands, TX).

**Tissue Culture**—The 184B5/HER and 184B5 cell lines have been described previously (47). Cells were maintained in minimum essential medium/keratinocyte basal medium mixed in a ratio of 1:1 (basal medium) containing epidermal growth factor (10 ng/ml), hydrocortisone (0.5 μg/ml), transferrin (10 μg/ml), gentamicin (5 μg/ml), and insulin (10 μM) in growth medium. Cells were grown to 60% confluence, trypsinized with 0.05% trypsin, 2 mM EDTA, and plated for experimental use. MSK Leuk1 cells have been described previously (48). Cells were routinely maintained in keratinocyte growth medium and passaged using 0.125% trypsin, 2 mM EDTA. In all experiments, 184B5/HER and MSK Leuk1 cells were grown in basal medium for 24 h prior to treatment. Treatment with vehicle (0.2% Me2SO), PPARγ ligands, or PMA was always carried out in basal medium. Cellular cytotoxicity was assessed by measurements of cell number, release of lactate dehydrogenase, and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, which was performed according to the manufacturer's instructions. The nitrocellulose membranes were prehybridized overnight in a solution containing 10% dextran sulfate, 0.1% SDS, and 0.1% diethyldithiocarbamate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 100 μM leupeptin. Lysates were sonicated for 20 s on ice and centrifuged at 10,000 × g for 10 min to sediment the particulate material. The protein concentration of the supernatant was determined by the method of Lowry et al. (50). SDS-polyacrylamide gel electrophoresis was performed under reducing conditions on 10% polyacrylamide gels as described by Laemmli (51). The resolved proteins were transferred onto nitrocellulose sheets as detailed by Towbin et al. (52). The nitrocellulose membrane was then incubated with primary antisera. Secondary antibody to horseradish peroxidase was used. The blots were incubated with primary antisera. Secondary antibody to horseradish peroxidase was used. The blots were then incubated with horseradish peroxidase-conjugated secondary antibody. Immunoblots were probed with an antibody specific for PPARγ (lane 1) or for the transcription assay, nuclei (1.0 g/ml), transferrin (10 μg/ml), gentamicin (5 μg/ml), and insulin (10 μM) in growth medium. Cells were grown to 60% confluence, trypsinized with 0.05% trypsin, 2 mM EDTA, and plated for experimental use. MSK Leuk1 cells have been described previously (48). Cells were routinely maintained in keratinocyte growth medium and passaged using 0.125% trypsin, 2 mM EDTA. In all experiments, 184B5/HER and MSK Leuk1 cells were grown in basal medium for 24 h prior to treatment. Treatment with vehicle (0.2% Me2SO), PPARγ ligands, or PMA was always carried out in basal medium. Cellular cytotoxicity was assessed by measurements of cell number, release of lactate dehydrogenase, and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, which was performed according to the manufacturer's instructions.

**Western Blotting**—Cells were lysed in buffer (150 mM NaCl, 5 mM EDTA, 10 mM Tris, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Lysates were sonicated for 20 s on ice and centrifuged at 10,000 × g for 10 min to sediment the particulate material. The protein concentration of the supernatant was determined by the method of Lowry et al. (50). SDS-polyacrylamide gel electrophoresis was performed under reducing conditions on 10% polyacrylamide gels as described by Laemmli (51). The resolved proteins were transferred onto nitrocellulose sheets as detailed by Towbin et al. (52). The nitrocellulose membrane was then incubated with primary antisera. Secondary antibody to IgG conjugated to horseradish peroxidase was used. The blots were probed with Renaissance Western blot detection reagents, [ 32P]ATP, [ 32P]CTP, and [ 32P]UTP were from PerkinElmer Life Sciences. Random primed kits were from Roche Molecular Biochemicals. Nitrocellulose membranes were from Schleicher & Schuell. Reagents for the luciferase assay were from PharMingen (San Diego, CA). The 18 S rRNA cDNA was from Ambion, Inc. (Austin, TX). T4 polynucleotide kinase was from New England Biolabs (Beverly, MA). Anti sera to PPARγ, COX-2, c-Jun, c-Fos, and ATF-2 were purchased from Santa Cruz Biotechnology, Inc. (San Diego). Plasmid DNA was prepared using a kit from Promega Corp. (Madison, WI). Oligonucleotides were synthesized by Genosys (The Woodlands, TX).

**Northern Blotting**—Total cellular RNA was isolated from cell monolayers using a RNA isolation kit from Qiagen Inc. 10 μg of total cellular RNA per lane were electrophoresed in a formaldehyde-containing 1.2% agarose gel and transferred to nylon-supported membranes. After baking, membranes were prehybridized overnight in a solution containing 50% formamide, 5× sodium chloride/sodium phosphate/EDTA buffer (SSPE), 5× Denhardt's solution, 0.1% SDS, and 100 μM single-stranded salmon sperm DNA and then hybridized for 12 h at 42 °C with radiolabeled cDNA probes for human COX-2 and 18 S rRNA. COX-2 and 18 S rRNA probes were labeled with [ 32P]CTP by random priming. After hybridization, membranes were washed twice for 20 min at room temperature in 2× SSPE, 0.1% SDS, twice for 20 min in the same solution at 55 °C, and twice for 20 min in 0.1× SSPE, 0.1% SDS at 55 °C. Washed membranes were then subjected to autoradiography.

**Nuclear Run-off Assay**—2.5 × 10 6 cells were plated in four T150 dishes for each condition. Cells were grown in growth medium until ~60% confluent. Nuclei were isolated and stored in liquid nitrogen. For the transcription assay, nuclei (1.0 × 10 6) were thawed and incubated in reaction buffer (10 mM Tris (pH 8), 5 mM MgCl 2 , and 0.3 mM KCl) containing 100 μM of uridine 5′-[ 32P]triphosphate and 1 μM unlabeled nucleotides. After 30 min, labeled nascent mRNA transcripts were isolated. The human COX-2 and 18 S rRNA cDNAs 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 mRNA transcripts for each treatment group. The membranes were washed twice with 2× SSC buffer for 1 h at 55 °C and then treated with 10 mg/ml RNase A in 2× SSC at 37 °C for 30 min, dried, and autoradiographed.

**Plasmids**—The PPRE3-tk-luciferase construct was provided by Dr. Mitchell Lazar (University of Pennsylvania, Philadelphia). The dominant negative PPARγ expression vector was kindly provided by Dr. V. K. K. Chatterjee (University of Cambridge, Cambridge, UK) (53). The COX-2 promoter constructs (−1432/+59, −327/+59, −220/+59, 124/+59, −52/+59, KMB, ILM, CRM, and CRM-ILM) were a gift from Dr. Tadashi Tanabe (National Cardiovascular Center Research Institute, Osaka, Japan) (6). The human COX-2 cDNA was generously provided by Dr. Stephen M. Prescott (University of Utah, Salt Lake City, UT). RSV-c-Jun was a gift from Dr. Tom Curran (Roche Molecular Biochemicals). The AP-1 reporter plasmid (2xTRE-luciferase), composed of two copies of the consensus TRE ligated to luciferase, was kindly provided by Dr. Joan Heller Brown (University of California, La Jolla). P300/CBP expression vector was obtained from Dr. Robert Weinberg (Massachusetts Institute of Technology, Cambridge). The expression vector for CREB was kindly provided by Dr. James Leonard (Strang Cancer Prevention Center, New York). The expression vector for CEbpα was a gift from Dr. Steven McKnight (University of Texas Southwestern Medical Center, Dallas). pSV-β-Galactosidase was obtained from Promega.
**FIG. 2.** PPARγ ligands suppress PMA-mediated induction of PGE2 synthesis. 184B5/HER cells were treated with vehicle, PMA (50 ng/ml), or PMA (50 ng/ml) plus ciglitazone (0–40 μM), A) or 15d-PGJ2 (0–40 μM), B) for 4.5 h. The medium was then replaced with basal medium and 10 μM sodium arachidonate. 30 min later, the medium was collected to determine the synthesis of PGE2. Production of PGE2 was determined by enzyme immunoassay. **Columns, means; bars, S.D.; n = 6.**, *p < 0.001 compared with PMA.

**FIG. 3.** COX-2 induction is blocked by PPARγ ligands. 184B5/HER cells (A–F) and MSK Leuk1 cells (G) were treated for 4.5 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. A, lysate protein was from cells treated with vehicle (lane 2), PMA (50 ng/ml, lane 3), or PMA and ciglitazone (10, 15, 20, 25, 30 μM; lanes 4–8, respectively). B, lysate protein was from cells treated with vehicle (lane 2), PMA (50 ng/ml, lane 3), or PMA and ciglitazone (10, 15, 20, 25, and 30 μM; lanes 4–8, respectively). C, lysate protein was from cells treated with vehicle (lane 2), PMA (50 ng/ml, lane 3), or PMA and troglitazone (25, 50 μM; lanes 4 and 5, respectively). D, lysate protein was from cells treated with vehicle (lane 2), PMA (50 ng/ml, lane 3), or PMA and the M metabolite of troglitazone (25, 50 μM; lanes 4 and 5, respectively). E, lysate protein was from cells treated with vehicle (lane 2), sphingomyelinase (10 milliunits/ml, lane 3), or sphingomyelinase and ciglitazone (10, 20, 30 μM; lanes 4–6, respectively). F, lysate protein was from cells treated with vehicle (lane 2), PMA (50 ng/ml, lane 3), or PMA and the M metabolite of troglitazone (25, 50 μM; lanes 4 and 5, respectively). G, lysate protein was from cells treated with vehicle (lane 2), PMA (50 ng/ml, lane 3), or PMA and troglitazone (25, 50 μM; lanes 4 and 5, respectively). In A–G, lane 1, represents a COX-2 standard.

**FIG. 4.** PPARγ ligands inhibit PMA-mediated induction of COX-2 transcription. A, 184B5/HER cells were treated with vehicle (lane 1), PMA (50 ng/ml, lane 2), or PMA (50 ng/ml) and ciglitazone (10, 15, 20, 25 and 35 μM; lanes 3–7, respectively) for 3 h. B, MSK Leuk1 cells were treated with vehicle (lane 1), PMA (50 ng/ml, lane 2), or PMA (50 ng/ml) and troglitazone (1, 10, and 25 μM; lanes 3–5, respectively) for 3 h. Total cellular RNA was isolated; 10 μg of RNA was added to each lane. The Northern blot was probed for COX-2 mRNA and 18 S rRNA. C, 184B5/HER cells were treated with vehicle (lane 1), PMA (50 ng/ml, lane 2), or PMA (50 ng/ml) and ciglitazone (10, 25, and 30 μM, lane 3–5) for 30 min. Nuclear run-offs were performed as described under "Experimental Procedures." The COX-2 and 18S rRNA cDNAs were immobilized onto nitrocellulose membranes and hybridized with labeled nascent RNA transcripts.

**Oligonucleotides**—The PPRE decoy, scrambled and missense oligonucleotide sequences were as follows: PPRE decoy (ACTTGATC-CCTTGTCAACTC), scrambled (TTAGGGAATCAGCAAGAGGT), and missense (ACTTTGCGCCGTACTACCTC) (38). In addition, the following oligonucleotides containing the CRE of the COX-2 promoter were synthesized: 5′-AAACGATTTTGGTCATGTCATGGGCTTG-3′ (sense) and 5′-CAAGCCCATGTGACGAAATGACTGTTT-3′ (antisense).

**Electrophoretic Mobility Shift Assay**—Cells were harvested, and nuclear extracts were prepared. For binding studies, an oligonucleotide containing the CRE of the COX-2 promoter was used. The complementary oligonucleotides were annealed in 20 mM Tris (pH 7.6), 50 mM NaCl, 10 mM MgCl2, and 1 mM dithiothreitol. The annealed oligonucleotide was phosphorylated at the 5′-end with γ-32P]ATP and T4 polynucleotide kinase. The binding reaction was performed by incubating 5 μg of nuclear protein in 20 mM HEPES (pH 7.9), 10% glycerol, 300 μg of bovine serum albumin, and 1 μg of poly(dIdC) in a final volume of 10 μl for 10 min at 25 °C. The labeled oligonucleotide was added to the reaction mixture and allowed to incubate for an additional 20 min.

**Polymerase Chain Reaction**—Total cellular RNA was isolated; 10 μg of RNA was added to each lane. The Northern blot was probed for COX-2 mRNA and 18 S rRNA. C, 184B5/HER cells were treated with vehicle (lane 1), PMA (50 ng/ml, lane 2), or PMA (50 ng/ml) and ciglitazone (10, 25, and 30 μM, lane 3–5) for 30 min. Nuclear run-offs were performed as described under "Experimental Procedures." The COX-2 and 18S rRNA cDNAs were immobilized onto nitrocellulose membranes and hybridized with labeled nascent RNA transcripts.
at 25 °C. The samples were electrophoresed on a 4% nondenaturing polyacrylamide gel. The gel was then dried and subjected to autoradiography at 280 °C.

Statistics—Comparisons between groups were made with the Student’s t test. A difference between groups of \( p < 0.05 \) was considered significant.

RESULTS

PPARγ Ligands Inhibit the Induction of COX-2 in Human Epithelial Cells—We determined the expression of PPARγ in human breast and oral epithelial cells. Western blotting analysis revealed that PPARγ was expressed in 184B5, 184B5/HER (Fig. 1A), and premalignant oral epithelial cells (data not shown). The receptor was also detected in human breast cancer (Fig. 1B). To investigate if the PPARγ receptor expressed in cell lines was transcriptionally active, 184B5/HER and MSK Leuk1 cells were transfected with a PPAR response element cloned upstream of luciferase (PPRE3-tk-luciferase). Treatment of 184B5/HER (Fig. 1C) or MSK Leuk1 cells (data not shown) with PPARγ ligands (ciglitazone, 15d-PGJ2) caused a dose-dependent increase in promoter activity. Similar effects were observed with troglitazone.

The possibility that PPARγ ligands inhibited PMA-mediated induction of PGE2 synthesis was investigated. Treatment of 184B5/HER cells with PMA led to a severalfold increase in PGE2 production. This effect was suppressed by PPARγ ligands in a dose-dependent manner (Fig. 2). PPARγ ligands also inhibited PMA-mediated induction of PGE2 synthesis in MSK Leuk1 cells (data not shown). To determine whether the above effects on production of PGE2 could be related to differences in amounts of COX-2, Western blotting of cell lysate protein was carried out. PMA induced COX-2 protein (Fig. 3, A–D and G). Treatment with PPARγ ligands (ciglitazone, Fig. 3A; 15d-PGJ2, Fig. 3B; troglitazone, Fig. 3, C and G) caused a dose-dependent decrease in PMA-mediated induction of COX-2. In contrast, the M metabolite of troglitazone, a compound that cannot transactivate PPARγ, did not block the induction of COX-2 by PMA (Fig. 3D). In addition to PMA, sphingomyelinase and taxol are known to induce COX-2 (56, 57). Hence, we also determined whether PPARγ ligands could suppress sphingomyelinase- and taxol-mediated induction of COX-2. Ciglitazone caused dose-dependent suppression of the induction of COX-2 by sphingomyelinase (Fig. 3E) and taxol (Fig. 3F).

Transcriptional Activation of COX-2 Is Inhibited by PPARγ Ligands—To elucidate further the mechanism responsible for the changes in amounts of COX-2 protein, we determined steady state levels of COX-2 mRNA by Northern blotting. As shown in Fig. 4, A and B, treatment with PMA enhanced levels of COX-2 mRNA, an effect that was suppressed by ciglitazone and troglitazone in a concentration-dependent manner. Comparable effects were observed with 15d-PGJ2 (data not shown). Nuclear run-off assays were performed to determine whether differences in amounts of COX-2 mRNA reflected altered rates of transcription. We detected a marked increase in rates of synthesis of nascent COX-2 mRNA after treatment with PMA.
PPARγ Ligands Inhibit the Induction of COX-2

Fig. 6. Decoy PPRE relieves the suppressive effects of PPARγ ligands on COX-2. 184B5/HER cells were transfected with 0.9 µg of a human COX-2 promoter construct ligated to luciferase (-327/+59) or COX-2 promoter plus decoy PPRE (0.4 µg) or COX-2 promoter plus scrambled PPRE (0.4 µg) or COX-2 promoter plus missense PPRE (0.4 µg). All cells received 0.2 µg of pSVβgal. The total amount of DNA in each reaction was kept constant at 2.0 µg by using empty vector. Cells were treated with vehicle (control), PMA (50 ng/ml), or PMA (50 ng/ml) plus either 20 µM ciglitazone (A) or 20 µM 15d-PGJ2 (B). Luciferase activity represents data that have been normalized to β-galactosidase activity. Columns, means; bars, S.D.; n = 6.

Consistent with the differences seen by Northern blotting (Fig. 4C). This effect was reversed by overexpression of the dominant negative form of PPARγ (g, fig. 4C) and 15d-PGJ2 (data not shown).

Transient transfections were performed to elucidate further the effects of PMA and PPARγ ligands on COX-2 transcription. PMA stimulated COX-2 promoter activity, an effect that was blocked by both ciglitazone (Fig. 5A) and 15d-PGJ2 (Fig. 5B). The suppressive effects of ciglitazone and 15d-PGJ2 were blocked by overexpressing a dominant negative form of PPARγ. In addition to blocking transcriptional activation by endogenous PPARγ, the dominant negative form of PPARγ lacks the ability to recruit CBP (55). Additional transient transfections were performed to confirm the role of PPARγ in mediating the inhibitory effects of ciglitazone and 15d-PGJ2. We examined the ability of a PPRE decoy oligonucleotide to prevent the inhibitory effects of ciglitazone and 15d-PGJ2 on PMA-mediated stimulation of COX-2 promoter activity. As shown in Fig. 6, the PPRE decoy oligonucleotide relieved the suppressive effects of both ciglitazone (Fig. 6A) and 15d-PGJ2 (Fig. 6B). In contrast, neither scrambled nor missense PPRE decoy oligonucleotides had any effect.

To define the region of the COX-2 promoter (Fig. 7A) that responded to PMA and PPARγ ligands, transient transfections were performed with a series of human COX-2 promoter deletion constructs. As shown in Fig. 7B, PMA treatment caused nearly a 4-fold increase in COX-2 promoter (-1432/+59) activity, an effect that was suppressed by ciglitazone. Both the inductive effect of PMA and the suppressive effect of ciglitazone were detected with all COX-2 promoter deletion constructs except the -52/+59 construct. A CRE is present between nucleotides -59 and -53, suggesting that this element may be responsible for mediating the effects of PMA. To test this notion, transient transfections were performed using COX-2 promoter constructs in which specific enhancer elements including the CRE were mutagenized. As shown in Fig. 7C, mutagenizing the CRE site caused a decrease in basal promoter activity and a loss of responsiveness to PMA. By contrast, mutagenizing the NF-IL6 or NFκB sites had little effect on COX-2 promoter function.

PPARγ Ligands Inhibit COX-2 Expression via an Anti-AP-1 Mechanism—Electrophoretic mobility shift assays were performed to identify the transcription factor that mediated the induction of COX-2 by PMA. PMA caused increased binding to the CRE site of the COX-2 promoter; this effect was blocked by treatment with ciglitazone (Fig. 8A) or 15d-PGJ2 (Fig. 8B). By contrast, PMA did not increase binding when the CRE site was mutagenized (data not shown). Supershift analyses identified c-Jun, c-Fos, and ATF-2 in the binding complex (Fig. 8C). Taken together, these results indicate that PMA stimulates the binding of the AP-1 transcription factor complex to the CRE of the COX-2 promoter; this effect was blocked by treatment with PPARγ ligands.

Additional experiments were done to define further the mechanism(s) by which PPARγ ligands inhibit PMA-mediated induction of AP-1 activity. As shown in Fig. 9A, ciglitazone caused dose-dependent suppression of PMA-mediated activation of an AP-1 reporter plasmid (2xTRE-luciferase). Similar results were obtained with 15d-PGJ2 (data not shown). More-
PPARγ Ligands Inhibit the Induction of COX-2

Fig. 7. Localization of region of COX-2 promoter that mediates the effects of PMA and PPARγ ligands. A, 184B5/HER cells were cotransfected with 1.8 μg of a series of human COX-2 promoter-luciferase constructs (−1432/+59, −327/+59, KBM, ILM, CRM, and CRM-ILM) and 2 μg of pSVβgal. KBM represents the −327/+59 COX-2 promoter construct in which both the NF-IL6 element and CRE were mutagenized; CRM refers to the −327/+59 COX-2 promoter construct in which the CRE was mutagenized; CRM-ILM represents the −327/+59 COX-2 promoter construct in which both the NF-IL6 element and CRE were mutagenized. After transfection, cells were treated with vehicle (open columns), PMA (50 ng/ml, black columns), or PMA plus ciglitazone (25 μM) for 6 h. Reporter activities were measured in cellular extract 6 h later. Luciferase activity represents data that have been normalized with β-galactosidase activity. Columns, means; bars, S.D.; n = 6.

over, PMA induced c-Jun, a component of the AP-1 transcription factor complex; this effect was also inhibited by ciglitazone (Fig. 9B) or 15d-PGJ2 (data not shown). To determine whether PPARγ ligands blocked PMA-mediated induction of COX-2 solely via effects on c-Jun, transient transfections were performed. As shown in Fig. 10A, ciglitazone blocked PMA-mediated stimulation of COX-2 promoter activity, an effect that was partially reversed by overexpressing c-Jun. In addition to suppressing the expression of c-Jun, ligands of nuclear receptors can potentially inhibit AP-1 activity by other mechanisms. There is growing evidence, for example, that CREB-binding protein (CBP/p300) is important for optimal AP-1-dependent transcription (58). Addition of a PPARγ ligand stimulates the interaction between PPARγ and CBP/p300 (59, 60). Hence, PPARγ ligand-mediated inhibition of AP-1 activity could also be a consequence of competition for limiting amounts of CBP/p300. To evaluate this possibility, transfection experiments...
FIG. 10. Ligands of PPARγ suppress PMA-mediated induction of COX-2 via effects on c-Jun and CBP. 184B5/HER cells were transfected with 0.9 μg of a human COX-2 promoter construct ligated to luciferase (-327/+59) and 0.2 μg of pSVgal. A, c-Jun bar represents cells that received 0.45 μg of expression vector for c-Jun; the CBP bar received 0.45 μg of expression vector for CBP; the c-Jun + CBP bar received 0.45 μg each of expression vectors for c-Jun and CBP. B, the NF-κB, CREB, and CEBP-α bars received 0.9 μg each of expression vectors for NFκB, CREB, and CEBP-α, respectively. The total amount of DNA in each reaction was kept constant at 2 μg by using the corresponding empty expression vectors. Following transfection, cells were treated with vehicle (control), PMA (50 ng/ml), or PMA plus 25 μM ciglitazone for 7 h. Luciferase activity represents data that have been normalized with β-galactosidase activity. Columns, means; bars, S.D.; n = 6.

DISCUSSION

PPARγ ligands, like ligands of other nuclear receptors, modulate gene expression by multiple mechanisms. In the current study, we showed that PPARγ ligands suppressed the induction of COX-2 by an anti-AP-1 mechanism. The AP-1 transcription factor complex consists of a collection of dimers of members of the Jun, Fox, and ATF cAMP-response element-binding protein bZip families. Little is known about the potential of PPARγ ligands to interfere with AP-1-mediated gene expression. Transient transfection analyses indicated that the CRE site of the COX-2 promoter was important for the inductive effects of PMA. Electrophoretic mobility gel shift analyses showed that treatment with PMA augmented binding to the CRE of the COX-2 promoter; c-Jun, c-Fos, and ATF-2 were identified in the DNA binding complex. These findings are consistent with previous reports in which both AP-1 and the CRE were found to be important for the induction of COX-2 in human epithelial cells (57, 62, 63). The results are also consistent with the observations of Xie and Herschman (64, 65) who were the first to demonstrate the importance of c-Jun and the CRE site for mediating the induction of COX-2. Importantly, several different results support the idea that ligands of PPARγ block the induction of COX-2 by antagonizing AP-1. First, PPARγ ligands blocked PMA-, taxol-, and sphingomyelinase-mediated induction of COX-2 (Fig. 2); each of these inducers has been reported to stimulate AP-1-mediated induction of COX-2 transcription (56, 57, 62, 63). Second, PPARγ ligands inhibited PMA-mediated increases in AP-1 binding to the CRE of the COX-2 promoter. Finally, ligands of PPARγ suppressed PMA-mediated activation of an AP-1 reporter plasmid.

Ligands of nuclear receptors, e.g., retinoids, have been reported to antagonize AP-1-mediated transcription by a variety of mechanisms (66, 67). Hence, additional experiments were performed to elucidate the mechanism(s) by which PPARγ ligands inhibited AP-1-mediated induction of COX-2. We found that ligands of PPARγ blocked PMA-mediated induction of c-Jun, a component of the AP-1 transcription factor complex. The functional significance of this effect was confirmed by the finding that overexpressing c-Jun partially relieved the suppressive effects of ciglitazone on PMA-mediated induction of
PPARγ Ligands Inhibit the Induction of COX-2

**Fig. 11.** BADGE, a synthetic antagonist for PPARγ, inhibits PMA-mediated induction of COX-2. A. 184B5/HER cells were treated with vehicle (lane 2), PMA (50 ng/ml, lane 3), or PMA plus BADGE (100, 200, 300, 400, and 500 μM BADGE, lanes 4–8) for 4.5 h. Lane 1 represents a COX-2 standard. Cellular lysate protein (25 μg/lane) was loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto nitrocellulose. The immunoblot was probed with antibody for COX-2. B, 184B5/HER cells infected with 1.8 μg of 2xTRE-luciferase and 0.2 μg of pCP6 (68). Ligands in the transfection medium included vehicle, PMA, or PMA plus BADGE (0–500 μM) for 7 h. Luciferase data that have been normalized with β-galactosidase activity. Columns: means; bars, S.D.; n = 6.

**Fig. 12.** Schematic of proposed mechanism by which PPARγ ligands inhibit AP-1-mediated activation of COX-2 transcription. CBP/p300 links AP-1 with components of the basal transcription machinery: TBP, TATA-box-binding protein; TFIIB, transcription factor IIB; RNA Pol II, RNA polymerase II. Treatment with PMA increases the binding of AP-1 to the CRE site of the COX-2 promoter thereby enhancing transcription. This stimulatory effect of PMA is blocked by cotreatment with a PPARγ ligand. PPARγ ligands inhibit PMA-mediated induction of COX-2 by two mechanisms as follows: 1) induction of c-Jun, a component of the AP-1 transcription factor complex, is blocked; 2) binding of a PPARγ ligand to its receptor enhances the interaction between CBP/p300 and PPARγ. This results in less CBP/p300 being available for AP-1-mediated activation of COX-2, a process known as squelching.

COX-2 promoter activity and transcriptional activation by AP-1 ligands were essentially abrogated. To our knowledge, PPARγ can induce transcriptional activation through specific DNA sites or inhibit the transcription factor AP-1. A pharmacological approach was used to determine whether these two types of receptor actions were mechanistically distinct. As noted above, BADGE is a synthetic ligand that binds to the receptor but is unable to transactivate genes via PPARγ (61). We investigated whether this functionally restricted PPARγ ligand blocked the induction of COX-2 or AP-1 activity like other PPARγ ligands. Importantly, although BADGE did not activate PPARγ, it suppressed PMA-mediated induction of AP-1 activity and COX-2 expression. This finding suggests that it may be feasible to develop a class of PPARγ ligands that selectively inhibit AP-1 activity without stimulating transcription. There is precedent for this idea. AP-1-selective retinoids have been developed (71); these retinoids inhibit AP-1 activity but are unable to stimulate transcription (71). AP-1-selective PPARγ ligands would be anticipated to have different therapeutic properties and toxicity than traditional PPARγ ligands.

Selective COX-2 inhibitors possess both chemopreventive and anti-inflammatory properties. Compounds that interfere with the signaling mechanisms that stimulate COX-2 transcription should also inhibit carcinogenesis and decrease inflammation. In support of this idea, PPARγ ligands can inhibit carcinogenesis (44–46) and reduce inflammation (32, 41). Several of the known anti-neoplastic properties of PPARγ ligands may be explained, in part, by their ability to inhibit COX-2 expression and PG biosynthesis. For example, overexpression of COX-2 promotes angiogenesis (72) and inhibits apoptosis (73), whereas PPARγ ligands inhibit both of these effects (38–40, 42). Both selective COX-2 inhibitors and PPARγ ligands protect against breast cancer in experimental animals (27, 46).
Local production of estrogen in breast adipose tissue, a reaction catalyzed by aromatase, has been implicated in the development of breast cancer. Interestingly, the synthesis of aromatase is stimulated by PGE$_2$ (74) and inhibited by PPAR ligands (75). Our finding that PPAR ligands block the induction of COX-2 and PGE$_2$ synthesis may be important, therefore, for understanding how PPAR ligands inhibit mammary carcinogenesis (46).

Finally, the results of this study may provide additional insights into the mechanisms underlying the anti-diabetic effects of PPAR ligands. COX-2 is constitutively expressed in pancreatic islet cells (76). Prostaglandin E$_2$ negatively modulates glucose-induced insulin secretion, an effect that can be blocked by inhibitors of COX (54). The discovery that PPAR ligands inhibit the production of COX-2-derived PGE$_2$ may help to explain the hypoglycemic effects of this class of agents.

REFERENCES

1. Smith, W. L., Garavito, R. M., and DeWitt, D. L. (1996) J. Biol. Chem. 271, 38157–38160
2. Smith, W. L., and DeWitt, D. L. (1995) Semin. Nephrol. 15, 179–194
3. Kajiyama, H., Akiyama, M., and Nakamura, T. (1995) J. Biol. Chem. 270, 11393–11399
4. Jones, D. A., Carlson, D. P., Mclntyre, T. M., Zimmerman, G. A., and Prescott, S. M. (1993) J. Biol. Chem. 268, 9049–9054
5. DuBois, R. N., Awd, J., Morrow, J. L., and Bishop, P. R. (1994) J. Clin. Invest. 93, 1839–1847
6. Inoue, H., Yokooyama, C., Haro, T., Sone, T., and Yanabe, T. (1995) J. Biol. Chem. 270, 24965–24971
7. Simmons, D. L., Levy, D. B., Yannoni, Y., and Erikson, R. L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 1178–1183
8. Subharamaih, K., Telang, N., Ramonetti, J. T., Araki, R., Devito, B., Weidman, B. B., and Dannenberg, A. J. (1996) Cancer Res. 56, 4424–4429
9. Mastre, J. R., Subharamaih, K., Sacks, P. G., Schantz, S. P., Tanabe, T., Inoue, H., and Dannenberg, A. J. (1997) Cancer Res. 57, 1081–1085
10. Anderson, G. D., Hauser, S. D., McGarity, K. L., Bremer, M. E., Isakson, P. C., and Lipsky, P. E. (1997) Cancer Res. 57, 1081–1085
11. Lipsky, P. E., and Isakson, P. C. (1997) J. Rheumatol. 24, 939–943
12. Dannenberg, A. J., and Zakim, D. (1999) Science 286, 371–373
13. Simon, L. S., Weaver, A. L., Graham, M. H., Hubbard, R. C., Isakson, P. C., and Geis, G. S. (1999) J. Am. Coll. Med. 41, 154–155
14. Kuchler, W., Jones, D., and Zimmerman, G. A. (1999) Semin. Oncol. 26, 195–202
15. Sheng, G. G., Shao, J., Shi, J., and Li, X. (1999) FEBS Lett. 450, 121–127
16. Kargman, S. L., O'Neil, G. P., Vickers, P. J., Evans, J. F., Mancini, J. A., and Robertson, R. P. (1998) Cancer Res. 58, 3344–3352
17. Demetri, G. D., Fletcher, C. D. M., Mueller, E., Sarron, P., Najjoks, R., Campbell, N., Spiegelman, B. M., and Singer, S. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1951–1956
18. Suh, N., Wang, Y., Wang, L., Zheng, R., Gilner, T., Willson, T., and Sporn, M. B. (1997) J. Biol. Chem. 272, 4571–4573
19. Zhai, Y.-F., Pritchard, J. B., Holdin, M., Oakley, C., Esselman, P. W., and Novick, R. J. (1997) Methods 29, 27–51
20. Subharamaih, K., Hart, J. C., Norton, L., and Dannenberg, A. J. (2000) J. Biol. Chem. 275, 14838–14845
21. Kanknecht, R., and Hunter, T. (1996) Nature 383, 22–23
22. Mochiki, S., and Taniguchi, T. (1997) Biochem. Biophys. Res. Commun. 230, 61–64
23. Gelman, L., Zhou, G., Fajas, L., Raspe, E., Fruchart, J.-C., and Auwerx, J. (1997) J. Biol. Chem. 272, 7681–7688
24. Wright, M. H., Clib, C. R., Maki, M., Hauser, S. Y., Yanagi, K., Hiramatsu, R., Serhan, C. N., and Spiegelman, B. M. (2000) J. Biol. Chem. 275, 1873–1877
25. Subharamaih, K., Chung, W. J., Michalpriet, P., Telang, N., Tanabe, T., Inoue, H., Jiang, M., Pizzuto, J. M., and Dannenberg, A. J. (1998) J. Biol. Chem. 273, 21875–21882
26. Subharamaih, K., Michalpriet, P., Sporn, M. B., and Dannenberg, A. J. (2000) Cancer Res. 60, 2399–2404
27. Xie, W., and Hershman, H. R. (1995) J. Biol. Chem. 270, 27622–27628
28. Xie, W., and Hershman, H. R. (1996) J. Biol. Chem. 271, 31742–31748
29. Lee, H. Y., Walsh, G. L., Dawson, M. I., Hong, W. K., and Kurie, J. M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7066–7071
30. Zhou, X. F., Shen, X. Q., and Shemshedini, L. (1999) Mol. Endocrinol. 13, 276–285
31. Kamat, Y., Xu, L., Heinzl, T., Torchi, J., Kurokawa, R., Glass, B., Lin, S. C., Heyman, R. A., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1996) Cell 85, 403–414
32. Newton, R., Seybold, J., Kuiert, L. M. E., Bergmann, M., and Barnes, P. J. (2001) J. Biol. Chem. 276, 32312–32321
33. Chawla, A., Barak, Y., Nagy, L., Liao, D., Tontonoz, P., and Evans, R. M. (2001) Nat. Med. 7, 48–52
34. Kadowaki, T., Hosoda, K., and Moller, M. (2000) FEBS Lett. 477, 137–138
35. Muhi, H., Kadowaki, T., Sacks, P. G., Schantz, S. P., Tanabe, T., and Dannenberg, A. J. (1997) Cancer Res. 57, 1081–1085