Retinoids and Carnosol Suppress Cyclooxygenase-2 Transcription by CREB-binding Protein/p300-dependent and -independent Mechanisms

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ABSTRACT

Treatment with retinoic acid (RA) or carnosol, two structurally unrelated compounds with anticancer properties, inhibited phorbol ester (PMA)-mediated induction of activator protein-1 (AP-1) activity and cyclooxygenase-2 (COX-2) expression in human mammary epithelial cells. The induction of COX-2 transcription by PMA was mediated by increased binding of AP-1 to the cyclic AMP response element (CRE) of the COX-2 promoter. Inhibition of the histone acetyltransferase activity of CREB-binding protein (CBP)/p300 blocked the induction of COX-2 by PMA. Treatment with carnosol but not RA blocked increased binding of AP-1 to the COX-2 promoter. Because AP-1 binding was unaffected by RA, we investigated whether RA inhibited COX-2 transcription via effects on the coactivator CBP/p300. Treatment with RA stimulated an interaction between RA receptor-α and CBP/p300; a corresponding decrease in the interaction between CBP/p300 and c-Jun was observed. Importantly, overexpressing CBP/p300 or dominant-negative RA receptor-α relieved the suppressive effect of RA on PMA-mediated stimulation of the COX-2 promoter. To elucidate the mechanism by which carnosol inhibited COX-2 transcription, its effects on protein kinase C (PKC) signaling were determined. Carnosol but not RA inhibited the activation of PKC, ERK1/2, p38, and c-Jun NH₂-terminal kinase mitogen-activated protein kinase. Overexpressing c-Jun but not CBP/p300 reversed the suppressive effect of carnosol on PMA-mediated stimulation of COX-2 promoter activity. Thus, RA acted by a receptor-dependent mechanism to limit the amount of CBP/p300 that was available for AP-1-mediated induction of COX-2. By contrast, carnosol inhibited the induction of COX-2 by blocking PKC signaling and thereby the binding of AP-1 to the CRE of the COX-2 promoter. Taken together, these results show that small molecules can block the activation of COX-2 transcription by distinct mechanisms.

INTRODUCTION

There are two isoforms of COX,³ designated COX-1 and COX-2. These enzymes catalyze the synthesis of PGs from arachidonic acid. COX-1 is a housekeeping gene that is expressed constitutively in most tissues (1, 2). By contrast, COX-2 is an immediate-early response gene that is induced by a variety of mitogenic and inflammatory stimuli (3–9).

Several lines of evidence suggest that COX-2 is a promising pharmacological target for the prevention and treatment of cancer. COX-2 is commonly overexpressed in transformed cells (8, 10, 11) and in human malignancies (12–18), whereas levels of COX-1 remain essentially unchanged. Overexpression of COX-2 in the mammary glands of multiparous mice was sufficient to induce mammary cancer (19). In a related study, overexpression of COX-2 in basal keratinocytes caused epidermal hyperplasia and dysplasia in transgenic mice, suggesting a mechanistic link between COX-2 expression and the development of skin cancer (20). Mice engineered to be null for COX-2 were protected against developing both intestinal (21) and skin (22) tumors. In addition to the genetic evidence implicating COX-2 in carcinogenesis, selective inhibitors of COX-2 reduce the formation and growth of tumors in experimental animals (23–27) and decrease the number of intestinal tumors in familial adenomatous polyposis patients (28). Several different mechanisms can potentially explain the link between COX-2 and cancer. Enhanced synthesis of COX-2-derived PGs favors tumor growth by stimulating cell proliferation (29), promoting angiogenesis (30, 31), increasing invasiveness (32, 33), and inhibiting apoptosis (34, 35). Because targeted inhibition of COX-2 is a promising approach to preventing or treating cancer, therapeutic strategies have focused on selective inhibitors of COX-2 enzyme activity. An equally important strategy may be to identify compounds that suppress the expression of the COX-2 gene.

Retinoids, a group of naturally occurring and synthetic analogues of vitamin A, and phenolic antioxidants are structurally distinct compounds (Fig. 1) that inhibit carcinogenesis and inflammation (36–41). In experimental models of cancer, both RA and carnosol, a phenolic antioxidant isolated from rosemary, block the tumor-promoting effects of phorbol esters (42, 43). Previously, we identified a series of small molecules including RA that inhibited the activation of COX-2 transcription (9, 44–47). Retinoids elicit their biological effects, in part, by antagonizing AP-1-mediated gene expression (48). The precise mechanisms underlying the antineoplastic effects of carnosol are unknown.

In the present study, we show that both RA and carnosol inhibited AP-1-mediated transcriptional activation of COX-2 in human epithelial cells. However, the detailed mechanisms underlying the anti-AP-1 effects of these compounds were very different. RA acted by a receptor-dependent mechanism to limit the amount of CBP/p300 that was available for AP-1-mediated induction of COX-2. By contrast, carnosol inhibited the activation of COX-2 transcription by blocking the binding of AP-1 to the CRE of the COX-2 promoter. These results provide new insights into the mechanisms controlling COX-2 gene expression and help to explain the anticancer properties of these chemopreventive agents.

MATERIALS AND METHODS

Materials. Minimal essential medium, Opti-MEM, PKC assay kits, and LipofectAMINE were from Life Technologies, Inc. (Grand Island, NY). Keratinocyte basal and growth media were from Clonetics Corp. (San Diego, CA). PMA, sodium arachidonate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (thiazolyl blue), lactate dehydrogenase diagnostic kits, retinoids, epidermal growth factor, hydrocortisone, poly(deoxyinosinic-deoxycytidylic acid) and o-nitrophenyl-β-D-galactopyranoside were from Sigma Chemical Co. (St. Louis, MO). Carnosol was a generous gift from Dr. Allan Conney (Rutgers University, Piscataway, NJ). Lys-CoA was prepared as reported previously (49). Enzyme immunoassay reagents for PGE2 assays were from Cayman Co. (Ann Arbor, MI). Western blotting detection reagents, [32P]ATP, [32P]CTP, and [32P]UTP, were
Tissue Culture. The 184B5/HER cell line has been described previously (50). Cells were maintained in minimum essential medium-keratinocyte basal medium mixed in a ratio of 1:1 (basal medium) containing 10 ng/ml EGF, 0.5 μg/ml hydrocortisone, 10 μg/ml transferrin, 5 μg/ml gentamicin, and 10 μg/ml insulin (growth medium). Cells were grown to 60% confluence, trypsinized with 0.05% trypsin, 2 mM EDTA, and plated for experimental use. Cells were routinely maintained in keratinocyte growth medium and passaged using 0.125% trypsin, 2 mM EDTA. In all experiments, cells were grown in basal medium for 24 h before treatment. Treatment with vehicle (0.2% Me2SO), retinoids, carnosol, 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 method of Denizot and Lang (51). Lactate dehydrogenase assays were performed according to the manufacturer’s instructions. There was no evidence of toxicity in any of our experiments.

PGE2 Production by Cells. Five × 10^4 cells/well were plated in six-well dishes and grown to 60% confluence before treatment. Amounts of PGE2 released by the cells were measured by enzyme immunoassay. Production of PGE2 was normalized to protein concentrations.

from NEN Life Sciences Products (Boston, MA). Random-priming kits were from Boehringer Mannheim Biochemicals (Indianapolis, IN). Nitrocellulose membranes were from Schleicher & Schuell (Keene, NH). Reagents for the luciferase assay were from Pharmingen (San Diego, CA). The 18S rRNA cDNA was from Ambion, Inc. (Austin, TX). T4 polynucleotide kinase was from New England Biolabs, Inc. (Beverly, MA). Antibodies to phosphorylated and unphosphorylated forms of ERK1/2 (p44/p42), p38, and c-Jun were from Cell Signaling Technology, Inc. (Beverly, MA). Plasmid DNA was prepared using a kit from Promega Corp. (Madison, WI). Oligonucleotides were synthesized by Genosys (The Woodlands, TX).
Western Blotting. Cell lysates were prepared by treating cells with lysis buffer [150 mM NaCl, 100 mM Tris (pH 8), 1% Tween 20, 50 mM diethyldithiocarbamate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml trypsin inhibitor, and 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 measured by the method of Lowry et al. (52). SDS-PAGE was performed under reducing conditions on 10% polyacrylamide gels as described by Laemmli (53). The resolved proteins were transferred onto nitrocellulose sheets as detailed by Towbin et al. (54). The nitrocellulose membrane was then incubated with primary antisera. Secondary antibody to IgG conjugated to horse-radish peroxidase was used. The blots were probed with Renaissance Western blot detection system according to the manufacturer’s instructions.

Northern Blotting. Total cellular RNA was isolated from cell monolayers using an RNA isolation kit from Qiagen, Inc. Ten μg of the total cellular RNA per lane was 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 μg/ml single-stranded salmon sperm DNA and then hybridized for 12 h at 42°C with radiolabeled cDNA probes for human COX-2 and 18S rRNA. COX-2 and 18S rRNA cDNA 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. Cells (2 × 10⁷) were plated in four T150 dishes for each condition. Cells were maintained in growth medium until ~60% confluent. Nuclei were isolated and stored in liquid nitrogen. For the transcription assay, nuclei (1.0 × 10⁶) were thawed and incubated in reaction buffer [10 mM Tris (pH 8), 5 mM MgCl₂, and 0.3 M KCl containing 100 μM RNase A in 2× SSC at 37°C], washed twice with 2× SSC, 0.1% SDS at 55°C and then treated with 10 mg/ml trypsin inhibitor for 10 min to sediment the nuclei. After hybridization, membranes were washed twice for 20 min in 0.1× SSC, 0.1% SDS at 55°C. Washed membranes were then subjected to autoradiography.

Luciferase Activity

Fig. 5. ATRA and carnosol inhibit PMA-mediated induction of COX-2 transcription. A, cells were treated with vehicle (Lane 1), PMA (50 ng/ml, Lane 2), or PMA plus ATRA (1.0 μM, Lane 3) for 30 min. B, cells were treated with vehicle (Lane 1), PMA (50 ng/ml, Lane 2), or PMA plus carnosol (60 μM, Lane 3) for 30 min. Nuclear run-offs were performed as described in “Materials and Methods.” The COX-2 and 18S rRNA cDNAs were immobilized onto nitrocellulose membranes and hybridized with labeled nascent RNA transcripts.

Plasmids and Oligonucleotides. The COX-2 promoter constructs (−1432/+59 , −327/+59 , −220/+59 , −124/+59 , −52/+59 , KBM, ILM, CRM, and CRM-ILM) were a gift from Dr. Tadashi Tanabe (National Cardiovascular Center Research Institute, Osaka, Japan; Refs. 6, 55). The human COX-2 cDNA was generously provided by Dr. Stephen M. Prescott (University of California, Los Angeles, CA). p300/CBP expression vectors were a generous gift of Dr. Tso-Pang Yao (Duke University, Durham, NC; Ref. 56). The expression vector for dominant-negative RA receptor-α was provided by Dr. Pierre Chambon (INSERM, Strasbourg, France). pSVβgal was obtained from Promega. The following oligonucleotides containing the CRE of the COX-2 promoter were synthesized: 5′-AAACAGTCATTACGTCATGGGCTTG-3′ (sense), and 5′-CAAGC-CCATGTGACGAAATGACTGTTT-3′ (antisense). CRE oligonucleotides used in the competition assay were: 5′-AGAGATTGCCGTAGCTCAGAG-AGCTAG-3′ (consensus), 5′-AGAGATTGCCGTAGCTCAGAG-AGCTAG-3′ (antisense), and 5′-AGAGATTGCCGTAGCTCAGAG-AGCTAG-3′ (mutant).

Transient Transfection Assays. Cells were seeded at a density of 5 × 10⁴ cells/well in six-well dishes and grown to 50–60% confluence. For each well, 2 μg of plasmid DNA were introduced into cells using 8 μg of LipofectAMINE as per the manufacturer’s instructions. After 7 h of incubation, the

Fig. 6. Localization of region of COX-2 promoter that mediates the effects of PMA. A, shown is a schematic of the human COX-2 promoter. B, cells were transfected with 1.8 μg of a series of human COX-2 promoter deletion constructs ligated to luciferase (−1432/+59 , −327/+59 , −220/+59 , −124/+59 , −52/+59 ) and 0.2 μg of pSVβgal. C, cells were transfected with 1.8 μg of a series of human COX-2 promoter-luciferase constructs (−327/+59 , KBM, ILM, CRM, and CRM-ILM) and 0.2 μg of pSVβgal. KBM represents the −327/+59 COX-2 promoter construct in which the NFκB site was mutated; ILM represents the −327/+59 COX-2 promoter construct in which the NFκB site was mutated; and CRM represents the −327/+59 CRE promoter construct in which the NFκB site was mutated. 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, SD; n = 6.
CHEMOPREVENTIVE AGENTS INHIBIT THE INDUCTION OF COX-2

RESULTS

Retinoids and Carnosol Inhibit the Induction of COX-2. We investigated the possibility that retinoids inhibit PMA-mediated induction of PGE₂ synthesis. Treatment of 184B5/HER cells with PMA caused an increase in PGE₂ production. This stimulatory effect of PMA was suppressed by ATRA, 13-cis-RA, and 9-cis-RA (Fig. 2A). Similarly, carnosol inhibited PMA-mediated induction of PGE₂ synthesis (Fig. 2B). Western blotting of cell lysate protein was performed to determine whether the observed effects on production of PGE₂ could be related to differences in amounts of COX-2. Treatment with PMA induced COX-2 protein, an effect that was suppressed by RA and carnosol in a dose-dependent manner (Fig. 3). In addition, both RA and carnosol inhibited the induction of COX-2 by EGF (Fig. 3C).

To further elucidate 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, treatment with PMA increased levels of COX-2 mRNA, an effect that was blocked by ATRA and carnosol in a concentration-dependent fashion.

Retinoids and Carnosol Inhibit the Activation of COX-2 Transcription. Nuclear run-off assays and transient transfections were performed to determine whether differences in amounts of COX-2

medium was replaced with basal medium. The activities of luciferase and β-galactosidase were measured in cellular extract as described previously (44).

EMSA. 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 DTT. 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 BSA, and 1 μg of poly(dexyinosinic-deoxyctydylacid) 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 at 25°C. For supershift assays, 10 μg of polyclonal antibody were added after incubation of the probe, nuclear extract, and binding buffer. The reaction mixture was kept on ice for 60 min before electrophoresis. For competition assays, cold competitor oligonucleotides were preincubated with nuclear extract and binding buffer for 15 min on ice before the addition of labeled probe. The binding reactions were then incubated on ice for an additional 15 min before electrophoresis. The samples were electrophoresed on a 4% nondenaturing polyacrylamide gel. The gel was then dried and subjected to autoradiography at ~80°C.

PKC Assay. The activity of PKC was measured according to directions from Life Technologies, Inc. Briefly, cells were plated in 10-cm dishes at 10⁶ cells/dish and grown to 60% confluence. Total PKC activity was measured in cell lysates. To determine cytosolic and membrane-bound PKC activity, cell lysates were centrifuged at 100,000 × g for 30 min. The resulting supernatant contains cytosolic PKC; membrane-bound PKC activity is present in the pellet.

Subsequently, DEAE cellulose columns were used to partially purify PKC enzymes. PKC activity was then measured by incubating partially purified PKC with [γ-³²P]ATP (3000–6000 Ci/mmol) and the substrate myelin basic protein for 20 min at room temperature. The activity of PKC is expressed as cpm incorporated/μg protein.

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

Figure 7. Carnosol but not ATRA inhibits PMA-induced binding of AP-1 to the CRE of the COX-2 promoter. A, cells were cotransfected with 1.8 μg of 2X TRE-luciferase and 0.2 μg of pSVβgal. Forty-two h after transfection, cells were treated with vehicle, PMA (50 ng/ml), PMA plus 1.0 μM ATRA, or PMA plus 60 μM carnosol for 8 h. Luciferase activity represents data that have been normalized with β-galactosidase activity. Columns, means; bars, SD; n = 6. B, cells were treated with vehicle (Lane 1), PMA (50 ng/ml, Lane 2), or PMA plus ATRA (1.0 μM, Lane 3) for 4.5 h. Lanes 4, 6, and 9 represent nuclear extract from PMA-treated cells incubated with antibodies to c-Jun (Lane 4), ATF-2 (Lane 6), or c-Fos (Lane 9). Lanes 5, 7, and 8 represent nuclear protein incubated with a 50-fold excess of unlabeled oligonucleotide containing missense (Lane 5), mutant (Lane 7), or consensus CRE (Lane 8). C, cells were treated with vehicle (Lane 1), PMA (50 ng/ml, Lane 2), or PMA plus carnosol (20, 40, 60 μM, Lanes 3–5) for 4.5 h. In B and C, 5 μg of nuclear protein from 184B5/HER cells were incubated with a 32P-labeled oligonucleotide containing the CRE of COX-2. The protein-DNA complex that formed was separated on a 4% polyacrylamide gel.

Figure 8. HAT activity of CBP/p300 is important for PMA-mediated induction of COX-2. A, cells were transfected with 0.9 μg of a human COX-2 promoter construct ligated to luciferase (~327/+59) and 0.2 μg of pSVβgal. The columns labeled CBP/p300 and Mutant CBP/p300 received 0.9 μg of wild-type and HAT-deficient CBP/p300 expression vectors, respectively. The total amount of DNA in each reaction was kept constant at 2.0 μg by using the corresponding empty expression vectors. After transfection, cells were treated with vehicle (Control) or PMA (50 ng/ml) for 7 h. Luciferase activity represents data that have been normalized with β-galactosidase activity. Columns, means; bars, SD; n = 6. B, cells were treated for 4.5 h with vehicle (Lane 1), PMA (50 ng/ml, Lane 2), or PMA plus Lys-CoA (0.1, 0.5, 1.0, 2.5, and 10 μM, Lanes 3–7, respectively), an inhibitor of the HAT activity of CBP/p300. Lane 8, a COX-2 standard. Cellular lysate protein (25 μg/lane) was loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto nitrocellulose. Immunoblots were probed with an antibody specific for COX-2.
mRNA reflect altered rates of transcription. A marked increase in rates of synthesis of nascent COX-2 mRNA was detected after treatment with PMA, consistent with the differences observed by Northern blotting (Fig. 5). This effect was suppressed by ATRA (Fig. 5A) and carnosol (Fig. 5B).

We carried out transient transfections to further elucidate the effects of retinoids and carnosol on PMA-mediated induction of COX-2 transcription. PMA stimulated COX-2 promoter activity, an effect that was blocked by ATRA (Fig. 6B). Other retinoids (13-cis-RA and 9-cis-RA) and carnosol had comparable effects (data not shown). The inductive effect of PMA and the suppressive effects of RA and carnosol were detected with all COX-2 promoter deletion constructs except the −52/+59 construct, suggesting that the CRE site is important for mediating these effects. 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. 6C, mutating the CRE site caused a decrease in basal promoter activity and a loss of responsiveness to PMA and ATRA. Similar effects were observed with carnosol (data not shown). By contrast, mutagenizing the NF-IL6 or nuclear factor-κB sites had little effect on COX-2 promoter function.

Retinoids and Carnosol Inhibit the Transcriptional Activation of COX-2 by Antagonizing AP-1. Both RA and carnosol inhibited PMA-mediated induction of AP-1 activity (Fig. 7A). EMSAs were performed to investigate whether AP-1 was potentially important for the induction of COX-2 by PMA. PMA caused increased binding of c-Jun, c-Fos, and ATF-2, components of the AP-1 transcription factor complex, to the CRE of the COX-2 promoter (Fig. 7B). To further define the mechanism by which PMA stimulated COX-2 gene expression, we also investigated the role of CBP/p300, a coactivator of AP-1-mediated transcription. Overexpressing a mutant form of CBP/p300 lacking HAT activity (56) blocked PMA-mediated induction of COX-2 promoter activity (Fig. 8A). This result strongly suggests that functional CBP/p300 is crucial for AP-1-mediated induction of COX-2. To further investigate this idea, we used the selective CBP/p300 inhibitor Lys-CoA (49). When added to the cell culture medium, Lys-CoA reproducibly decreased PMA-mediated induction of COX-2 (Fig. 8B). Because a number of CoA analogues have low bioavailability, future studies will be needed to evaluate whether cell type or treatment with PMA affects the permeability of Lys-CoA.

To determine whether RA and carnosol blocked PMA-mediated increases in AP-1 binding to the CRE of the COX-2 promoter, EMSAs...
were performed. Treatment with carnosol (Fig. 7B) but not RA inhibits AP-1 binding to the CRE. Because PMA-mediated induction of AP-1 binding was unaffected by RA, we evaluated whether inhibition of COX-2 transcription could be explained by its effects on the coactivator CBP/p300. This possibility was evaluated because inhibition of AP-1 activity can be a consequence of competition for limiting amounts of CBP/p300 in cells (57). In support of this idea, treatment with RA stimulated the interaction between RA receptor-α/H9251 and CBP/p300 (Fig. 9A). A corresponding decrease in the interaction between CBP/p300 and c-Jun was observed (Fig. 9B). Moreover, as shown in Fig. 9C, overexpressing CBP/p300 completely relieved the suppressive effect of RA. By contrast, the inhibitory effects of carnosol on PMA-mediated activation of the COX-2 promoter were unaffected by overexpressing CBP/p300 (Fig. 9C). To confirm the involvement of RA receptors in mediating the inhibitory effects of RA, transient transfections were performed. Overexpressing a dominant-negative form of RA receptor-α abrogated the suppressive effect of RA on PMA-mediated stimulation of the COX-2 promoter (Fig. 9D).

One of the ways that PMA regulates gene expression is by activating the PKC signal transduction pathway. This leads, in turn, to increased AP-1 activity (Fig. 7A). A key feature of this mechanism is the redistribution of PKC activity from cytosol to membrane. We therefore investigated whether carnosol or RA inhibited the redistribution of PKC activity induced by PMA. As shown in Fig. 10A, carnosol but not RA inhibited the translocation of PKC activity from cytosol to membrane. To further investigate the effects of carnosol on the PKC signal transduction pathway, we assessed its effects on the activation of MAPKs. Carnosol caused dose-dependent inhibition of PMA-mediated activation of ERK1/2, p38, and JNK MAPK (Fig. 10B). Blocking PKC signaling can inhibit AP-1 binding activity and thereby suppress gene expression. In support of this mechanism, overexpressing c-Jun relieved the inhibitory effect of carnlosol on PMA-mediated stimulation of COX-2 promoter activity (Fig. 10C).
Carnosol

All-trans-retinoic acid

PKC Activation

Retinoic Receptor

MAPK

CBP/p300

CRE

TATA

RNA POL II

Fig. 11. Schematic of proposed mechanism by which RA and carnosol inhibit AP-1-mediated activation of COX-2 transcription. CBP/p300, a coactivator for AP-1-mediated gene expression, links AP-1 with components of the basal transcription machinery: TATA-box-binding protein (TBP), transcription factor IID (TFIID), and RNA polymerase II (RNA POL II). Treatment with PMA activates PKC signaling which results, in turn, in increased binding of the AP-1 transcription factor complex to the CRE site of the COX-2 promoter, thereby enhancing transcription. This stimulatory effect of PMA is blocked by cotreatment with either RA or carnosol. RA and carnosol inhibit PMA-mediated induction of COX-2 by distinct mechanisms. Carnosol blocks the activation of PKC signaling and thus inhibits the binding of AP-1 to the CRE. By contrast, RA suppresses AP-1-mediated activation of COX-2 transcription without decreasing the binding of AP-1 to the CRE. Treatment with RA stimulates the interaction between the RA receptor and the coactivator CBP/p300. This results, in turn, in decreased interaction between CBP/p300 and AP-1. Thus, RA-induced competition for limiting amounts of CBP/p300 prevents AP-1-mediated activation of COX-2 gene expression.

By contrast, overexpressing c-Jun did not reverse the inhibitory effect of RA on PMA-induced stimulation of COX-2 promoter activity (data not shown).

DISCUSSION

In the present experiments, we showed that RA and carnosol, a phenolic antioxidant, suppressed AP-1-mediated activation of COX-2 transcription, albeit by very different mechanisms. To more completely understand these inhibitory effects, it was first necessary to define the mechanism by which PMA stimulated COX-2 transcription. The AP-1 transcription factor complex consists of a collection of dimers of members of the Jun, Fos, and ATF CREB protein bZip families. EMSAs showed that treatment with PMA augmented the binding of c-Jun, c-Fos, and ATF-2 to the CRE of the COX-2 promoter. This result is consistent with previous findings implicating AP-1 in the activation of COX-2 transcription by a variety of stimuli including PMA (45–47, 58–61). Importantly, there is growing evidence that the coactivator CBP/p300 can be very important for the activation of gene expression by AP-1 (57, 62–64). In this study, we show that the HAT activity of CBP/p300 is required for AP-1-mediated activation of COX-2. This conclusion is supported by two separate findings: (a) overexpressing a HAT-deficient form of CBP/p300 inhibited the induction of COX-2 promoter activity by PMA; and (b) Lys-CoA, a known inhibitor of CBP/p300 HAT activity, blocked PMA-mediated induction of COX-2.

Both RA and carnosol suppressed the activation of COX-2 transcription by PMA. Interestingly, treatment with carnosol but not RA blocked PMA-mediated induction of AP-1 binding to the CRE of the COX-2 promoter. This finding suggested that the two types of agents inhibited the activation of COX-2 transcription by different mechanisms. Ligands of nuclear receptors can antagonize AP-1-mediated transcription by several mechanisms including inhibition of c-Fos expression, suppression of JNK activity, and inhibition of heterodimerization of c-Jun and c-Fos (65–68). Retinoic acid can also inhibit AP-1-mediated transcription by limiting the availability of relatively low intracellular levels of CBP/p300 (57, 62). Thus, competition for limiting amounts of these proteins represents a mechanism for transrepression by nuclear receptors. On the basis of our finding that the HAT activity of CBP/p300 was important for COX-2 gene expression, it was logical to evaluate whether treatment with RA decreased the availability of CBP/p300 for transcription. In fact, treatment with RA stimulated an interaction between CBP/p300 and RA receptor. We also observed less interaction between CBP/p300 and c-Jun after treatment with RA. Transient transfections were performed to further evaluate the functional significance of these effects. Overexpression CBP/p300 completely reversed the suppressive effect of RA on PMA-mediated stimulation of COX-2 promoter activity. Taken together, these results suggest that RA antagonized AP-1-mediated activation of COX-2 transcription by limiting the availability of CBP/p300 for transcription, i.e., squelching of CBP/p300, rather than altering the binding of AP-1 to the CRE of the COX-2 promoter (Fig. 11). In this context, it is noteworthy that ligands of peroxisome proliferator-activated receptor γ, a nuclear receptor that heterodimerizes with retinoid X receptor, inhibit the transcriptional activation of COX-2 via effects on both the binding of AP-1 to the CRE and squelching of CBP/p300 (47). This difference between RA and ligands of peroxisome proliferator-activated receptor γ implies that ligands of different classes of nuclear receptors block the activation of COX-2 transcription by distinct mechanisms.

Carnosol blocked the transcriptional activation of COX-2 by inhibiting the PKC signal transduction pathway (Fig. 11). In contrast to RA, it blocked the translocation of PKC activity from cytosol to membrane. It also inhibited the activation of ERK1/2, p38, and JNK MAPKs by PMA. The latter inhibitory effects help to explain how carnosol blocked PMA-mediated induction of AP-1 binding to the CRE. Thus, ERK1/2 MAPK stimulates AP-1 activity by inducing c-Fos, which heterodimerizes with c-Jun (69). p38 MAPK induces AP-1 activity by phosphorylating ATF-2. A heterodimer comprised of phospho-ATF-2 and c-Jun can induce c-Jun expression (70). JNK induces the expression and phosphorylation of c-Jun (69). In contrast to RA, overexpressing c-Jun but not CBP/p300 reversed the inhibitory effect of carnosol on PMA-mediated induction of COX-2. In all likelihood, these inhibitory effects of carnosol reflect its antioxidant properties because other phenolic antioxidants such as curcumin inhibit both PMA-mediated activation of PKC and AP-1 binding activity (71, 72).

In summary, both RA and carnosol inhibited PMA-mediated activation of COX-2 transcription by antagonizing AP-1 (Fig. 11). Because both AP-1 and COX-2 have been linked to carcinogenesis, these findings help to explain the anticancer properties of RA and carnosol. Remarkably, the suppressive effect of RA was a consequence of squelching CBP/p300. By contrast, carnosol blocked the induction of COX-2 by inhibiting the activation of PKC signaling. Newly developed selective COX-2 inhibitors possess anticancer properties. This study illustrates the potential to reduce amounts of COX-2 by targeting molecules such as CBP/p300 and PKC that control COX-2 gene expression.

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