ABSTRACT

We investigated the effects of ursolic acid, a chemopreventive agent, on the expression of cyclooxygenase-2 (COX-2) in phorbol 12-myristate 13-acetate (PMA)-treated human mammary and oral epithelial cells. Treatment with ursolic acid suppressed PMA-mediated induction of COX-2 protein and synthesis of prostaglandin E2. Ursolic acid also suppressed the induction of COX-2 mRNA by PMA. Nuclear run-offs revealed increased rates of COX-2 transcription after treatment with PMA, an effect that was inhibited by ursolic acid. Transient transfections indicated that the effects of PMA were mediated by a cyclic AMP response element in the COX-2 promoter. Ursolic acid inhibited PMA-mediated activation of protein kinase C, extracellular signal-regulated kinase 1/2, c-Jun N-terminal kinase, and p38 mitogen-activated protein kinases. Treatment with PMA increased activator protein-1 activity and the binding of c-Jun to the cyclic AMP response element of the COX-2 promoter, effects that were blocked by ursolic acid. These data are important for understanding the anticancer and anti-inflammatory properties of ursolic acid.

INTRODUCTION

COX1 catalyzes the synthesis of PGs from arachidonic acid. There are two isoforms of COX. COX-1 is a housekeeping gene that is expressed constitutively in most tissues (1). COX-2 is an immediate, early-response gene that is highly inducible by mitogenic and inflammatory stimuli (2–4).

Multiple lines of evidence suggest that COX-2 is important in carcinogenesis. COX-2 is overexpressed in transformed cells (5–7) and in various forms of cancer (8–12), whereas levels of COX-1 remain essentially unchanged. Moreover, Oshima et al. (13) showed that a null mutation for COX-2 markedly reduced the number and size of intestinal tumors in APCmin/− knockout mice, a murine model of familial adenomatous polyposis. COX-2 deficiency also protected against chemically induced skin papillomas (14). In addition to the genetic evidence implicating COX-2 in tumorigenesis, selective inhibitors of COX-2 decrease tumor formation in experimental animals (13, 15, 16). Because targeted inhibition of COX-2 is a promising approach to prevent cancer, chemopreventive strategies have focused on inhibitors of COX-2 enzyme activity. An equally important strategy may be to identify compounds that suppress the signaling pathways that regulate COX-2 expression (17–20).

Triterpenoids exist widely in nature and are used for medicinal purposes in many Asian countries. Ursolic acid, a pentacyclic triterpenoid found in rosemary, possesses anticancer and anti-inflammatory effects (Refs. 21–24; Fig. 1). It inhibits PMA-induced inflammation and tumor promotion in mouse skin (21). These effects have been attributed, in part, to inhibition of PG synthesis (21, 23), although the underlying mechanisms are incompletely understood. In the current work, we have extended prior observations concerning the effects of ursolic acid on PG synthesis by determining whether ursolic acid inhibits the induction of COX-2 by PMA. Our data show that ursolic acid suppresses the activation of COX-2 gene expression by inhibiting the PKC signal transduction pathway. These data provide a mechanistic basis for the chemopreventive and anti-inflammatory properties of ursolic acid.

MATERIALS AND METHODS

MATERIALS. MEM, PKC assay kits, and LipofectAMINE were from Life Technologies, Inc. (Grand Island, NY). KBM and keratinocyte growth medium were from Clonetics Corp. (San Diego, CA). Ursolic acid, PMA, sodium arachidonate, MTT (Thiazolyl blue), LDH diagnostic kits, epidermal growth factor, hydrocortisone, and o-nitropheno/-galactopyranoside were from Sigma Chemical Co. (St. Louis, MO). Enzyme immunoassay reagents for PGE2, assays were from Cayman Co. (Ann Arbor, MI). [32P]CTP was from New Life Science Products (Boston, MA). Random priming kits were from Roche Molecular Biochemicals (Indianapolis, IN). Nitrocellulose membranes were from Schleicher & Schuell (Keene, NH). Reagents for the luciferase assay were from Analytical Luminescence (San Diego, CA). The 18 S rRNA cDNA was from Ambion, Inc. (Austin, TX). Antibodies to COX-2, COX-1, and c-Jun were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies to phospho-ERK1/2, phospho-c-Jun, and phospho-p38 were from New England Biolabs Inc. (Beverly, MA). Western blotting detection reagents (ECL) were from Amersham Pharmacia Biotech. Plasmid DNA was prepared using a kit from Promega Corp. (Madison, WI).

Tissue Culture. The 184B5/HER cell line has been described previously (25). Cells were maintained in MEM-KBM 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 μg/ml). Cells were grown to 60% confluence, trypsinized with 0.05% trypsin-EDTA, and plated for experimental use. MSK Leuk1 was established from a dysplastic leukoplakia lesion adjacent to a squamous cell carcinoma of the tongue in a 46-year-old nonsmoking female (26). Cells were routinely maintained in keratinocyte growth medium and passaged using 0.125% trypsin-2 mM EDTA, and plated for experimental use. LDH and MTT assay. The MTT assay was performed according to the method of Denizot and Lang (27). LDH assays were performed according to the manufacturer’s instructions. There was no evidence of toxicity in any of our experiments.

PGE2 Production by Cells. Cells (5 × 104 per well) were plated in six-well dishes and grown to 60% confluence in growth medium. The cells were then treated as described below. Levels of PGE2 released by the cells were measured by enzyme immunoassay. Amounts of PGE2 production were normalized to protein concentration.

Western Blotting. Cell lysates were prepared by treating cells with lysis buffer (150 mM NaCl, 100 mM Tris, pH 8.0, 1% Tween 20, 50 mM diethyl-
Using an RNA isolation kit from QIAGEN Inc. Ten m
system according to the manufacturer's instructions.
oxidase was used. The blots were probed with the ECL Western blot detection
COX-1 antiserum. Secondary antibody to IgG conjugated to horseradish per-
bated with a rabbit polyclonal anti-COX-2 antiserum or a polyclonal anti-
detailed by Towbin et al. (30). The nitrocellulose membrane was then incu-
bated with a rabbit polyclonal anti-COX-2 antisera or a polyclonal anti-
COX-1 antiserum. Secondary antibody to IgG conjugated to horseradish per-
obsidase was used. The blots were probed with the ECL 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 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× 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 18 S rRNA. 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. COX-2 and 18 S rRNA probes were labeled with [32P]CTP by random priming.

Nuclear Run-off Assay. Cells (2.5 × 10^5) were plated in four T150 dishes for each condition. Cells were grown in growth medium until they were approximately 60% confluent. Nuclei were isolated and stored in liquid nitrogen. For the transcription assay, nuclei (1.0 × 10^7) were harvested and nuclear extracts were prepared as described previously (35). For binding studies, oligonucleotides containing the CRE of the COX-2 promoter were used: 5′-AACAGCTATTTTGACATGGCGCTG-3′ (sense) and 5′-CAAGG-CATGGCAGAAAGTACGGTCTTC-3′ (antisense; Genosys Biotechnologies, Inc., The Woodlands, TX). The complementary oligonucleotides were annealed in 20 mM Tris, pH 7.6, 50 mM NaCl, 10 mM MgCl_2 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(dI-dC) in a final volume of 10 μl and β-galactosidase were measured in cellular extract as described previously (34).

Electrophoretic Mobility Shift Assay. Cells were harvested and nuclear extracts were prepared as described previously (35). For binding studies, oligonucleotides containing the CRE of the COX-2 promoter were used: 5′-AACAGCTATTTTGACATGGCGCTG-3′ (sense) and 5′-CAAGG-CATGGCAGAAAGTACGGTCTTC-3′ (antisense; Genosys Biotechnologies, Inc., The Woodlands, TX). The complementary oligonucleotides were annealed in 20 mM Tris, pH 7.6, 50 mM NaCl, 10 mM MgCl_2 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(dI-dC) in a final volume of 10 μl.

For each condition. Cells were grown in growth medium until they were

LipofectAMINE as per the manufacturer’s instructions. After 7 h of incubation, the medium was replaced with basal medium. The activities of luciferase

Fig. 1. Structure of ursolic acid.

Fig. 2. Ursoic acid suppresses PMA-mediated induction of PGE_2 synthesis. 184B5/HER cells were treated with vehicle (○), PMA (50 ng/ml), or PMA (50 ng/ml) and ursolic acid (0–30 μM) for 4.5 h. The medium was then replaced with basal medium and 10 μM sodium arachidonate. Thirty min later, the medium was collected to determine the amount of PGE_2 synthesized. Production of PGE_2 was determined by enzyme immunoassay. Columns, means; bars, SD; n = 6. *, P < 0.001 compared with PMA.

Fig. 3. PMA-mediated induction of COX-2 protein is inhibited by ursolic acid. 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, lysates were from 184B5/HER cells treated with vehicle (Lane 1), PMA (50 ng/ml, Lane 2), or PMA (50 ng/ml) and ursolic acid (5, 15, and 30 μM, Lanes 3–6), respectively) for 4.5 h. Lane 1 represents an ovine COX-2 standard. B, lysates were from premalignant oral epithelial (MSK Leuk1) cells treated with vehicle (Lane 1), PMA (50 ng/ml, Lane 2), or PMA (50 ng/ml) and ursolic acid (10, 20, 30, and 40 μM, Lanes 3–6, respectively) for 4.5 h.
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. 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. Cells were then treated with fresh basal medium containing vehicle (0.2% DMSO), PMA (50 ng/ml), or PMA (50 ng/ml) plus ursolic acid (15 μM) for 30 min. Total PKC activity was measured in cell lysates. To determine cytosolic and membrane-bound PKC activity, cell lysates were centrifuged at 100,000 x 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 of protein.

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

**RESULTS**

**Ursolic Acid Inhibits the Induction of COX-2 Transcription in Phorbol Ester-treated Cells.** The possibility that ursolic acid inhibited PMA-mediated induction of PGE₂ synthesis was investigated. PMA caused about a 2-fold increase in synthesis of PGE₂. This effect was suppressed by ursolic acid in a dose-dependent manner (Fig. 2). To determine whether the above effects on production of PGE₂ could be related to differences in levels of COX, Western blotting of cell lysates was carried out. As shown in Fig. 3, ursolic acid caused a dose-dependent decrease in PMA-mediated induction of COX-2. Neither PMA nor ursolic acid altered amounts of COX-1 (data not shown). To confirm that these effects of ursolic acid were not unique to mammary epithelial cells, we determined whether ursolic acid inhibited PMA-mediated induction of COX-2 in a pre-malignant, oral leukoplakia cell line. As shown in Fig. 3B, ursolic acid also suppressed the induction of COX-2 by PMA in this cell line. The maximal effect was observed when 30 μM ursolic acid was used.

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. Treatment with PMA enhanced levels of COX-2 mRNA, an effect that was suppressed by ursolic acid in a concentration dependent manner (Fig. 4A). Nuclear run-ons were performed to determine whether differences in amounts of COX-2 mRNA reflected altered rates of transcription. As shown in Fig. 4B,
membrane PKC activity were measured. Columns, means; bars, SD; n = 6.

we detected 3-fold higher rates of synthesis of nascent COX-2 mRNA after treatment with PMA, consistent with the differences observed by Northern blotting. This effect was suppressed by ursoic acid.

To define the region of the COX-2 promoter (Fig. 5A) that responded to PMA and ursoic acid, transient transfections were performed with a series of human COX-2 promoter deletion constructs. As shown in Fig. 5B, PMA increased COX-2 promoter activity (~1432/+59) about 3-fold, an effect that was suppressed by ursoic acid. Both the inductive effect of PMA and the suppressive effect of ursoic acid 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. 5C, 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 (Fig. 5C).

Defining the Signaling Mechanism by Which Ursoic Acid Suppresses PMA-mediated Induction of COX-2. COX-2 gene expression by activating the PKC signal transduction pathway. A key feature of this mechanism is the translocation of PKC activity from cytosol to membrane. We therefore investigated the possibility that ursoic acid inhibited the redistribution of PKC activity that was mediated by PMA. As shown in Fig. 6, ursoic acid completely inhibited PMA-induced translocation of PKC activity from cytosol to membrane. PKC signaling increases MAPK activity, which, in turn, regulates COX-2 expression (32). Treatment with PMA induced the activities of ERK1/2, JNK, and p38 MAPK; these steps were blocked by ursoic acid in a dose-dependent manner (Fig. 7).

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, an effect that was markedly suppressed by ursoic acid (Fig. 8A). By contrast, treatment with PMA did not increase binding when the CRE site was mutagenized (data not shown). The DNA binding complex induced by PMA was removed by treatment with a blocking antibody to c-Jun, a component of the AP-1 transcription factor complex. This antibody prevents c-Jun from binding to DNA rather than causing a supershift (36). Transient transfections were performed to further investigate the anti-AP-1 activity of ursoic acid. Ursolic acid suppressed PMA-mediated activation of an AP-1 reporter plasmid (2X TRE-luciferase; Fig. 8B).

DISCUSSION

Selective COX-2 inhibitors possess both anti-inflammatory and chemopreventive properties (13, 15, 16, 37). Compounds that interfere with the signaling mechanisms that up-regulate COX-2 should also be useful in this regard because they too decrease COX-2 activity (17–20, 34). We have shown in the present experiments that ursoic acid suppressed PMA-mediated induction of PG synthesis by inhibiting COX-2 gene expression.

Several of the known properties of ursoic acid can be explained, in part, by its ability to inhibit COX-2 and PG synthesis. For example, overexpression of COX-2 promotes angiogenesis (38) and suppresses apoptosis (39), whereas ursoic acid inhibits both of these effects (40, 41). Moreover, because PGs are proinflammatory and chronic inflammation predisposes to malignancy (42), the inhibition of COX-2 by ursoic acid helps to explain both its anti-inflammatory (21–23) and chemopreventive activities (21, 24).

With regard to the mechanism by which ursoic acid modulates gene expression, it suppressed PMA-mediated activation of COX-2 transcription by inhibiting the PKC signal transduction pathway. Ursolic acid blocked PMA-induced translocation of PKC activity from cytosol to membrane and the activation of ERK1/2, JNK and p38 MAPKs. These results are significant because PKC activity is increased in some cancers (43) and is considered a potential target for cancer therapy. Moreover, there is recent evidence that compounds that block the activation of ERK1/2 MAPK inhibit tumor growth (44).

The inductive effects of PMA were mediated by the CRE of the COX-2 promoter. Interestingly, PMA increased the binding of c-Jun to the CRE, an effect that was suppressed by ursoic acid. Previously, Xie and Herschman (45, 46) showed that c-Jun was important for activation of the murine COX-2 promoter via the CRE. To the best of our knowledge, this is the first time a similar observation has been made with the human COX-2 promoter. JNK induces the expression and phosphorylation of c-Jun (47). It is possible, therefore, that ursoic acid blocks PMA-mediated induction of c-Jun binding to the CRE by

![Image](cancerres.aacrjournals.org)
The suppressive effects of ursolic acid on PMA-mediated activation of COX-2 transcription are explained by inhibition of AP-1 activity. Because AP-1 activity has been linked to carcinogenesis (49, 50), the results of this study help to explain the anticancer properties of triterpenoids. Previously, we reported that retinoids blocked PMA-mediated induction of COX-2 by antagonizing AP-1-mediated transcription (34). Ursolic acid and retinoids appear to inhibit AP-1 activity by different mechanisms. In contrast to ursolic acid, retinoids did not block the redistribution of PKC activity from cytosol to membrane mediated by PMA (data not shown). Additionally, retinoids block the activation of COX-2 expression without altering the activity of ERK1/2 MAPK (17). Thus, chemopreventive agents can inhibit AP-1-mediated induction of COX-2 by disrupting PKC signal transduction at different levels in the pathway.

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Ursolic Acid Inhibits Cyclooxygenase-2 Transcription in Human Mammary Epithelial Cells

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