[CANCER RESEARCH 60, 2399–2404, May 1, 2000]

Ursolic Acid Inhibits Cyclooxygenase-2 Transcription in Human Mammary Epithelial Cells[1]

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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 E_2. 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

COX[3] 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 $APC^{\Delta 716}$ 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*-nitrophenyl-β-D-galactopyranoside were from Sigma Chemical Co. (St. Louis, MO). Enzyme immunoassay reagents for PGE_2 assays were from Cayman Co. (Ann Arbor, MI). [^{32}P]CTP was from NEN 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-2 mM 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. 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% DMSO), ursolic acid, or PMA was always carried out in basal medium. Cellular cytotoxicity was assessed by measurements of cell number, release of 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.

PGE_2 Production by Cells. Cells (5×10^4 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 PGE_2 released by the cells were measured by enzyme immunoassay. Amounts of PGE_2 production were normalized to protein concentrations.

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-

Received 10/20/99; accepted 2/29/00.

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[1] This work was supported in part by NIH Grant P01 CA29502 (to K. S.), the Cancer Research Foundation of America (to K. S.), the American Institute for Cancer Research (to A. J. D.), and the Lawrence A. Miller Memorial Foundation.

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[3] The abbreviations used are: COX, cyclooxygenase; CRE, cyclic AMP response element; PG, prostaglandin; PGE_2, prostaglandin E_2; PKC, protein kinase C; AP-1, activator protein-1; PMA, phorbol 12-myristate 13-acetate; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; KBM, keratinocyte basal medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LDH, lactate dehydrogenase; SSPE, sodium chloride-sodium phosphate-EDTA buffer.

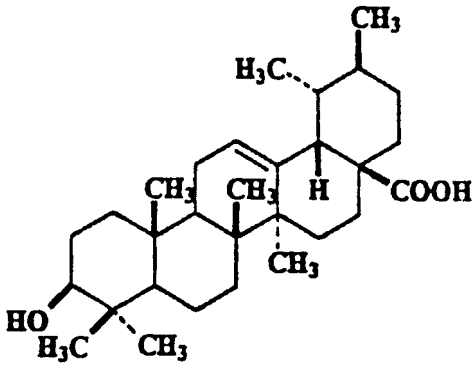

Fig. 1. Structure of ursolic acid.

dithiocarbamate, 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 $\times$ g for 10 min to sediment the particulate material. The protein concentration of the supernatant was measured by the method of Lowry *et al.* (28). SDS-PAGE was performed under reducing conditions on 10% polyacrylamide gels as described by Laemmli (29). The resolved proteins were transferred onto nitrocellulose sheets as detailed by Towbin *et al.* (30). The nitrocellulose membrane was then incubated with a rabbit polyclonal anti-COX-2 antiserum or a polyclonal anti-COX-1 antiserum. Secondary antibody to IgG conjugated to horseradish peroxidase 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$\times$ SSPE, 5$\times$ 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$\times$ SSPE-0.1% SDS, twice for 20 min in the same solution at 55°C, and twice for 20 min in 0.1$\times$ SSPE-0.1% SDS at 55°C. Washed membranes were then subjected to autoradiography. COX-2 and 18 S rRNA probes were labeled with [^{32}P]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 thawed and incubated in reaction buffer (10 mM Tris, pH 8, 5 mM $MgCl_2$, and 0.3 M KCl) containing 100 μCi of uridine 5′[α-^{32}P]triphosphate and 1 mM unlabeled nucleotides. After 30 min, labeled nascent RNA 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 RNA transcripts for each treatment group. The membranes were washed twice with 2$\times$ SSC buffer for 1 h at 55°C and then treated with 10 mg/ml RNase A in 2$\times$ SSC at 37°C for 30 min, dried, and autoradiographed.

Plasmids. The *COX-2* promoter constructs (–1432/+59, –327/+59, –220/+59, –124/+59, –52/+59, KBM, ILM, CRM, and CRM plus ILM) were a generous gift of Dr. Tadashi Tanabe (National Cardiovascular Center Research Institute, Osaka, Japan; Refs. 31 and 32). The human COX-2 cDNA was generously provided by Dr. Stephen M. Prescott (University of Utah, Salt Lake City, UT). 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, CA; Ref. 33). pSV-βgal was obtained from Promega.

Transient Transfection Assays. 184B5/HER cells were seeded at a density of 5×10^4 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 medium was replaced with basal medium. The activities of luciferase 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′-AAACAGTCATTTCGTCACATGGGCTTG-3′ (sense) and 5′-CAAGCCCATGTGACGAAATGACTGTTT-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-^{32}P]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

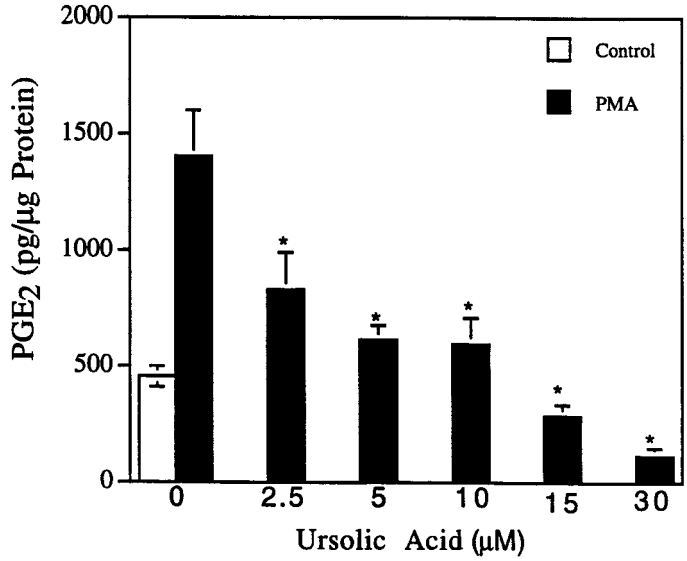

Fig. 2. Ursolic acid suppresses PMA-mediated induction of PGE_2 synthesis. 184B5/HER cells were treated with vehicle (□), PMA (50 ng/ml, ■), or PMA (50 ng/ml) and ursolic acid (0–30 μM) for 4.5 h. The medium was then replaced with basal medium and 10 μM sodium arachidonate. Thirty min later, the medium 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.

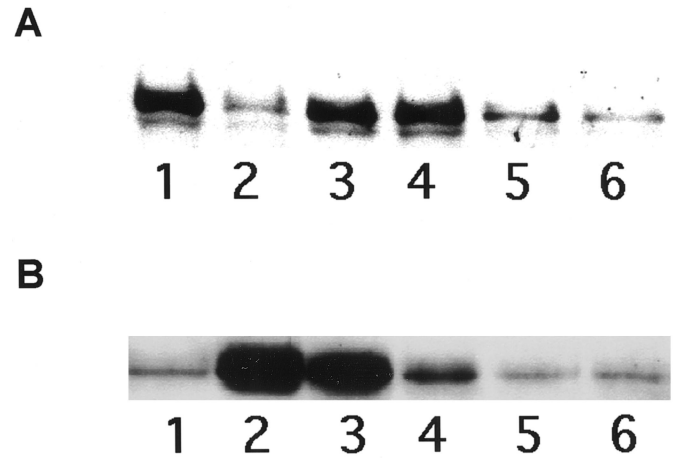

Fig. 3. PMA-mediated induction of COX-2 protein is inhibited by ursolic acid. Cellular lysate protein (25 μg/lane) was loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto nitrocellulose. Immunoblots were probed with antibody specific for COX-2. *A*, lysates were from 184B5/HER cells treated with vehicle (*Lane 2*), PMA (50 ng/ml, *Lane 3*), or PMA (50 ng/ml) and ursolic acid (5, 15, and 30 μM, *Lanes 4–6*, 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 10^6 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 × *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-^{32}P]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 PGE_2 synthesis was investigated. PMA caused about a 2-fold increase in synthesis of PGE_2. This effect was suppressed by ursolic acid in a dose-dependent manner (Fig. 2). To determine whether the above effects on production of PGE_2 could be related to differences in levels of COX, Western blotting of cell lysate protein was carried out. Fig. 3*A* shows that PMA induced COX-2 in human mammary epithelial cells. Treatment with 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 premalignant, oral leukoplakia cell line. As shown in Fig. 3*B*, 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.

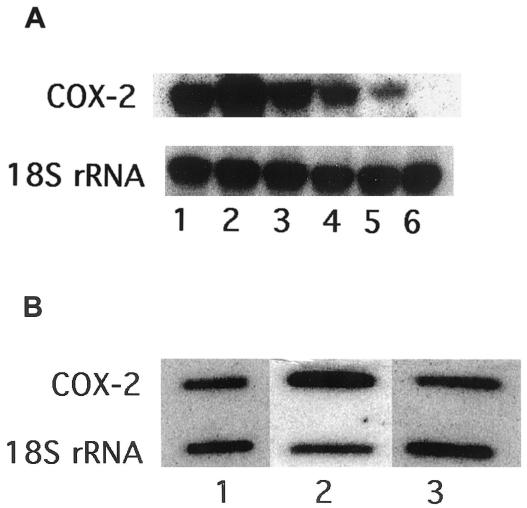

Fig. 4. Ursolic acid inhibits PMA-mediated induction of COX-2 transcription. *A*, 184B5/HER cells were treated with vehicle (*Lane 1*), PMA (50 ng/ml, *Lane 2*), or PMA (50 ng/ml) and ursolic acid (10, 15, 20, and 30 μM, *Lanes 3–6*, respectively) for 3 h. Total cellular RNA was isolated; 10 μg of RNA were added to each lane. The Northern blot was probed with probes that recognized COX-2 mRNA and 18 S rRNA. *B*, 184B5/HER cells were treated with vehicle (*Lane 1*), PMA (50 ng/ml, *Lane 2*), or PMA (50 ng/ml) and ursolic acid (20 μM, *Lane 3*) for 3 h. Nuclear run-offs were performed as described in "Materials and Methods." The COX-2 and 18 S rRNA cDNAs were immobilized onto nitrocellulose membranes and hybridized with labeled nascent RNA transcripts. Results of densitometry in arbitrary units were as follows: *Lane 1*, 20; *Lane 2*, 80; *Lane 3*, 20.

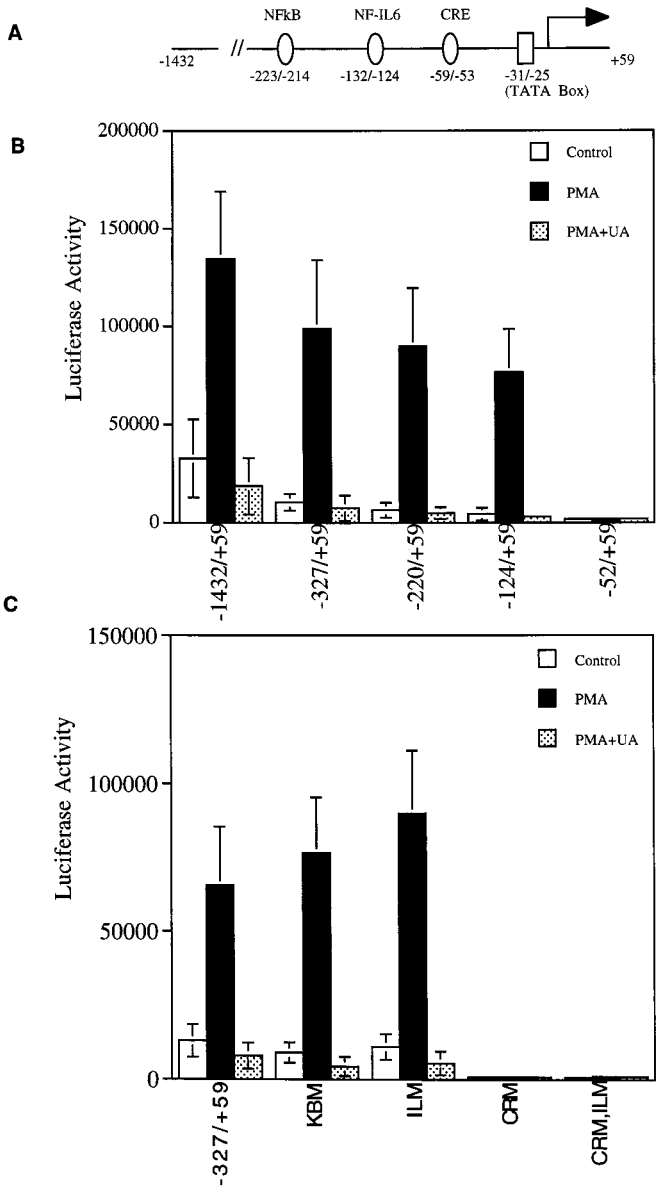

Fig. 5. Localization of region of COX-2 promoter that mediates the effects of PMA and ursolic acid. *A*, shown is a schematic of the human *COX-2* promoter. *B*, 184B5/HER cells were transfected with 1.8 μg of a series of human *COX-2* promoter deletion constructs ligated to luciferase (–1432/+59, –327/+59, –220/+59, –124/+59, and –52/+59) and 0.2 μg of pSVβgal. *C*, 184B5/HER cells were transfected with 1.8 μg of a series of human *COX-2* promoter-luciferase constructs (–327/+59, KBM, ILM, CRM, and CRM plus ILM) and 0.2 μg of pSVβgal. *KBM* represents the –327/+59 *COX-2* promoter construct in which the NFκB site was mutagenized; *ILM* represents the –327/+59 *COX-2* promoter construct in which the NF-IL6 site was 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 (□), PMA (50 ng/ml, ■), or PMA (50 ng/ml) and ursolic acid (15 μM, ▨). Reporter activities were measured in cellular extract 6 h later. Luciferase activity represents data that have been normalized with β-galactosidase. *Columns*, means; *bars*, SD; $n = 6$.

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. 4*A*). Nuclear run-offs were performed to determine whether differences in amounts of COX-2 mRNA reflected altered rates of transcription. As shown in Fig. 4*B*,

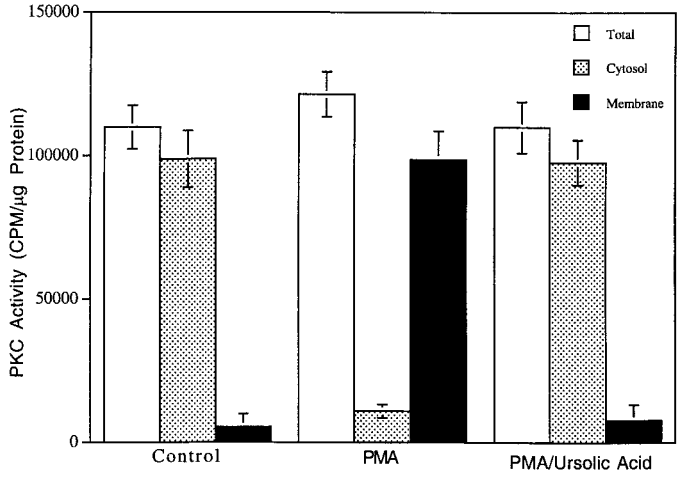

Fig. 6. Ursolic acid inhibits the redistribution of PKC activity induced by PMA. 184B5/HER cells were treated with vehicle, PMA (50 ng/ml), or PMA (50 ng/ml) plus ursolic acid (15 μM) for 30 min. Total PKC activity (□), cytosolic PKC activity (▨), and membrane PKC activity (■) were measured. *Columns*, 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 ursolic acid.

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

Defining the Signaling Mechanism by Which Ursolic Acid Suppresses PMA-mediated Induction of COX-2. PMA regulates *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 ursolic acid inhibited the redistribution of PKC activity that was mediated by PMA. As shown in Fig. 6, ursolic 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 ursolic 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 ursolic acid (Fig. 8*A*). 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 ursolic acid. Ursolic acid suppressed PMA-mediated activation of an AP-1 reporter plasmid (2xTRE-luciferase; Fig. 8*B*).

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 ursolic acid suppressed PMA-mediated induction of PG synthesis by inhibiting *COX-2* gene expression.

Several of the known properties of ursolic 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 ursolic 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 ursolic acid helps to explain both its anti-inflammatory (21–23) and chemopreventive activities (21, 24).

With regard to the mechanism by which ursolic 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 ursolic 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 ursolic acid blocks PMA-mediated induction of c-Jun binding to the CRE by

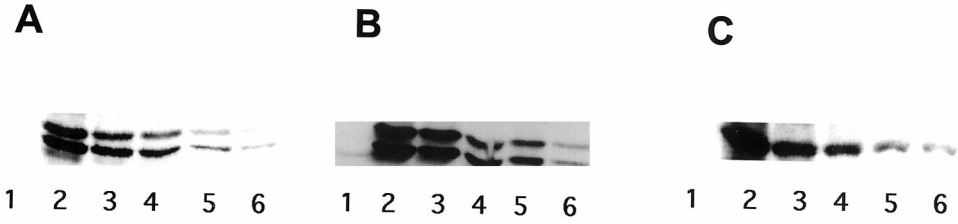

Fig. 7. Ursolic acid inhibits PMA-mediated activation of ERK1/2, JNK, and p38 MAPKs. Cells were treated with vehicle (*Lane 1*), PMA (*Lane 2*), or PMA plus ursolic acid (5, 10, 15, and 20 μM, *Lanes 3–6*, respectively) for 5 min. Cellular protein (50 μg) was loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto nitrocellulose. The blots were subsequently probed with antibodies to the phosphorylated forms of ERK1/2 (*A*), c-Jun (*B*), and p38 (*C*).

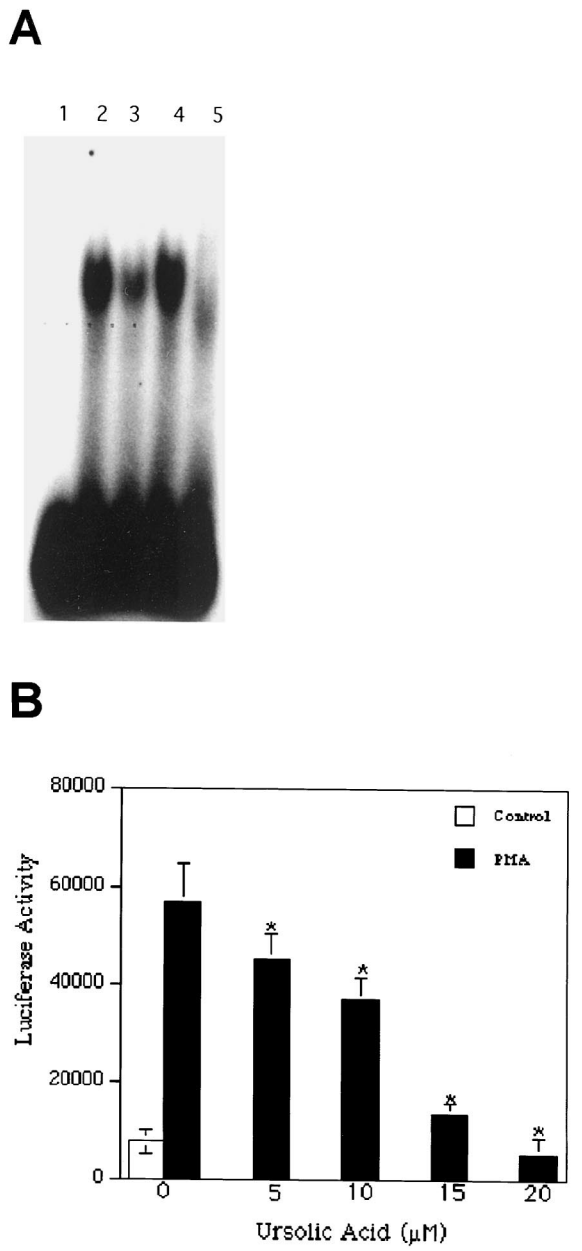

Fig. 8. Phorbol ester-mediated induction of c-Jun binding to the CRE is suppressed by ursolic acid. *A*, 184B5/HER cells were treated with vehicle (control, *Lane 1*), PMA (50 ng/ml, *Lane 2*), or PMA and ursolic acid (15 μM, *Lane 3*) for 4 h. In lanes 1–3, 5 μg of nuclear protein were incubated with a ^{32}P-labeled oligonucleotide containing the CRE of COX-2. In addition, 5 μg of nuclear protein from cells treated with PMA were incubated with IgG (*Lane 4*) or antibody to c-Jun (*Lane 5*) for 30 min. Subsequently, the reaction mixture was incubated with the ^{32}P-labeled oligonucleotide containing the CRE of COX-2 as in *Lanes 1–3*. The protein DNA complex that formed was separated on a 4% polyacrylamide gel. *B*, cells were co-transfected with 1.8 μg of 2xTRE-luciferase and 0.2 μg of pSVβgal. The AP-1 reporter plasmid (2x-TRE-luciferase) is composed of two copies of the consensus TRE (TPA/PMA-responsive element) ligated to *luciferase* (33). After transfection, cells were treated with vehicle, PMA (50 ng/ml), or PMA (50 ng/ml) and ursolic acid (15 μM) for 7 h. Luciferase activity represents data that have been normalized with β-galactosidase activity. *Columns*, means; *bars*, SD; $n = 6$. *, $P < 0.01$ compared with PMA alone.

inhibiting the stimulation of JNK activity. Ursolic acid also inhibited PMA-mediated activation of ERK1/2 and p38 MAPKs. ERK1/2 stimulates AP-1 activity by inducing c-Fos, which heterodimerizes with c-Jun (47). p38 MAPK induces AP-1 by phosphorylating ATF-2. A heterodimer composed of phospho-ATF-2 and c-Jun can induce c-Jun expression (48). Future studies are warranted to determine whether c-Jun heterodimerizes with c-Fos or ATF-2 after treatment with PMA.

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.

REFERENCES

1. Smith, W. L., Garavito, R. M., and DeWitt, D. L. Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. J. Biol. Chem., *271:* 33157–33160, 1996.
2. Kujubu, D. A., Fletcher, B. S., Varnum, B. C., Lim, R. W., and Herschman, H. R. TIS10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. J. Biol. Chem., *266:* 12866–12872, 1991.
3. Jones, D. A., Carlton, D. P., McIntyre, T. M., Zimmerman, G. A., and Prescott, S. M. Molecular cloning of human prostaglandin endoperoxide synthase type II and demonstration of expression in response to cytokines. J. Biol. Chem., *268:* 9049–9054, 1993.
4. DuBois, R. N., Awad, J., Morrow, J., Roberts, L. J., and Bishop, P. R. Regulation of eicosanoid production and mitogenesis in rat intestinal epithelial cells by transforming growth factor-alpha and phorbol ester. J. Clin. Invest., *93:* 493–498, 1994.
5. Subbaramaiah, K., Telang, N., Ramonetti, J. T., Araki, R., DeVito, B., Weksler, B. B., and Dannenberg, A. J. Transcription of cyclooxygenase-2 is enhanced in transformed mammary epithelial cells. Cancer Res., *56:* 4424–4429, 1996.
6. Kutchera, W., Jones, D. A., Matsunami, N., Groden, J., McIntyre, T. M., Zimmerman, G. A., White, R. L., and Prescott, S. M. Prostaglandin H synthase 2 is expressed abnormally in human colon cancer: evidence for a transcriptional effects. Proc. Natl. Acad. Sci. USA, *93:* 4816–4820, 1996.
7. Sheng, G. G., Shao, J., Sheng, H., Hooton, E. B., Isakson, P. C., Morrow, J. D., Coffey, R. J., DuBois, R. N., and Beauchamp, R. D. A selective cyclooxygenase-2 inhibitor suppresses the growth of H-ras-transformed rat intestinal epithelial cells. Gastroenterology, *113:* 1883–1891, 1997.
8. Eberhart, C. E., Coffey, R. J., Radhika, A., Giardiello, F. M., Ferrenbach, S., and DuBois, R. N. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology, *107:* 1183–1188, 1994.
9. Ristimaki, A., Honkanen, N., Jankala, H., Sipponen, P., and Harkonen, M. Expression of cyclooxygenase-2 in human gastric carcinoma. Cancer Res., *57:* 1276–1280, 1997.
10. Parett, M. L., Harris, R. E., Joarder, F. S., Ross, M. S., Clausen, K. P., and Robertson, F. M. Cyclooxygenase-2 gene expression in human breast cancer. Int. J. Oncol., *10:* 503–507, 1997.
11. Chan, G., Boyle, J. O., Yang, E. K., Zhang, F., Sacks, P. G., Shah, J. P., Edelstein, D., Soslow, R. A., Koki, A. T., Woerner, B. M., Masferrer, J. L., and Dannenberg, A. J. Cyclooxygenase-2 expression is up-regulated in squamous cell carcinoma of the head and neck. Cancer Res., *59:* 991–994, 1999.
12. Tucker, O. N., Dannenberg, A. J., Yang, E. K., Zhang, F., Teng, L., Daly, J. M., Soslow, R. A., Masferrer, J. L., Woerner, B. M., Koki, A. T., and Fahey, T. J. Cyclooxygenase-2 expression is up-regulated in human pancreatic cancer. Cancer Res., *59:* 987–990, 1999.
13. Oshima, M., Dinchuk, J. E., Kargman, S. L., Oshima, H., Hancock, B., Kwong, E., Trzaskos, J. M., Evans, J. F., and Taketo, M. M. Suppression of intestinal polyposis in Apc$^{\Delta 716}$ knockout mice by inhibition of cyclooxygenase 2 (COX-2). Cell, *87:* 803–809, 1996.
14. Tiano, H., Chulada, P., Spalding, J., Lee, C., Loftin, C., Mahler, J., Morham, S., and Langenbach, R. Effects of cyclooxygenase deficiency on inflammation and papilloma development in mouse skin. Proc. Am. Assoc. Cancer Res., *38:* 1727, 1997.
15. Kawamori, T., Rao, C. V., Seibert, K., and Reddy, B. S. Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis. Cancer Res., *58:* 409–412, 1998.
16. Fischer, S. M., Lo, H. H., Gordon, G. B., Seibert, K., Kelloff, G., Lubet, R. A., and Conti, C. J. Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, and indomethacin against ultraviolet-light induced skin carcinogenesis. Mol. Carcinog., *25:* 231–240, 1999.
17. Mestre, J. R., Subbaramaiah, K., Sacks, P. G., Schantz, S. P., Tanabe, T., Inoue, H., and Dannenberg, A. J. Retinoids suppress epidermal growth factor-induced transcription of cyclooxygenase-2 in human oral squamous carcinoma cells. Cancer Res., *57:* 2890–2895, 1997.
18. Suh, N., Honda, T., Finlay, H. J., Barchowsky, A., Williams, C., Benoit, N. E., Xie, Q-w., Nathan, C., Gribble, G. W., and Sporn, M. B. Novel triterpenoids suppress inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2) in mouse macrophages. Cancer Res., *58:* 717–723, 1998.
19. Subbaramaiah, K., Chung, W. J., Michaluart, P., Telang, N., Tanabe, T., Inoue, H., Jang, M., Pezzuto, J. M., and Dannenberg, A. J. Resveratrol inhibits cyclooxygen-

ase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells. J. Biol. Chem., *273:* 21875–21882, 1998.
20. Suh, N., Wang, Y., Honda, T., Gribble, G. W., Dmitrovsky, E., Hickey, W. F., Maue, R. A., Place, A. E., Porter, D. M., Spinella, M. J., Williams, C. R., Wu, G., Dannenberg, A. J., Flanders, K. C., Letterio, J. J., Mangelsdorf, D. J., Nathan, C. F., Nguyen, L., Porter, W. W., Ren, R. F., Roberts, A. B., Roche, N. S., Subbaramaiah, K., and Sporn, M. B. 1999. A novel synthetic oleanane triterpenoid, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO), with potent differentiating, anti-proliferative and anti-inflammatory activity. Cancer Res., *59:* 336–341.
21. Huang, M-T., Ho, C-T., Wang, Z. Y., Ferraro, T., Lou, Y-R., Stauber, K., Ma, W., Georgiadis, C., Laskin, J. D., and Conney, A. H. Inhibition of skin tumorigenesis by rosemary and its constituents carnosol and ursolic acid. Cancer Res., *54:* 701–708, 1994.
22. Manez, S., Recio, M. C., Giner, R. M., and Rios, J-L. Effect of selected triterpenoids on chronic dermal inflammation. Eur. J. Pharmacol., *334:* 103–105, 1997.
23. Najid, A., Simon, A., Cook, J., Chable-Rabinovitch, H., Delage, C., Chulia, A. J., and Rigaud, M. Characterization of ursolic acid as a lipoxygenase and cyclooxygenase inhibitor using macrophages, platelets and differentiated HL60 leukemic cells. FEBS Lett., *16:* 213–217, 1992.
24. Nishino, H., Nishino, A., Takayasu, J., Hasegawa, T., Iwashima, A., Hirabayashi, K., Iwata, S., and Shibata, S. Inhibition of the tumor-promoting action of 12-*O*-tetradecanoylphorbol-13-acetate by some oleanane-type triterpenoids compounds. Cancer Res., *48:* 5210–5215, 1988.
25. Zhai, Y-F., Beittenmiller, H., Wang, B., Gould, M. N., Oakley, C., Esselman, W. J., and Welsch, C. W. Increased expression of specific protein tyrosine phosphatases in human breast epithelial cells neoplastically transformed by the neu oncogene. Cancer Res., *53:* 2272–2278, 1993.
26. Sacks, P. G. Cell, tissue and organ culture as *in vitro* models to study the biology of squamous cell carcinomas of the head and neck. Cancer (Phila.) Met. Rev., *15:* 27–51, 1996.
27. Denizot, F., and Lang, R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J. Immunol. Methods, *89:* 271–277, 1986.
28. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randell, R. J. Protein measurement with the Folin phenol reagent. J. Biol. Chem., *193:* 265–275, 1951.
29. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.), *227:* 680–685, 1970.
30. Towbin, H., Staehelin, T., and Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA, *76:* 4350–4354, 1979.
31. Inoue, H., Yokoyama, C., Hara, S., Tone, Y., and Tanabe, T. Transcriptional regulation of human prostaglandin-endoperoxide synthase-2 gene by lipopolysaccharide and phorbol ester in vascular endothelial cells. Involvement of both nuclear factor for interleukin-6 expression site and cAMP response element. J. Biol. Chem., *270:* 24965–24971, 1995.
32. Subbaramaiah, K., Chung, W. J., and Dannenberg, A. J. Ceramide regulates the transcription of cyclooxygenase-2. Evidence for involvement of extracellular signal-regulated kinase/c-Jun N-terminal kinase and p38 mitogen-activated protein kinase pathways. J. Biol. Chem., *273:* 32943–32949, 1998.
33. Trejo, J., Massamiri, T., Deng, T., Dewji, N. N., Bayny, R. M., and Heller Brown, J. A direct role for protein kinase C and the transcription factor Jun/AP-1 in the regulation of the Alzheimer's beta-amyloid precursor protein gene. J. Biol. Chem., *269:* 21682–21690, 1994.
34. Mestre, J. R., Subbaramaiah, K., Sacks, P. G., Schantz, S. P., Tanabe, T., Inoue, H., and Dannenberg, A. J. Retinoids suppress phorbol ester-mediated induction of cyclooxygenase-2. Cancer Res., *57:* 1081–1085, 1997.
35. Zhang, F., Subbaramaiah, K., Altorki, N., and Dannenberg, A. J. Dihydroxy bile acids activate the transcription of cyclooxygenase-2. J. Biol. Chem., *273:* 2424–2428, 1998.
36. Hutchinson, L. E., and McCloskey, M. A. FcεR1-mediated induction of nuclear factor of activated T-cells. J. Biol. Chem., *270:* 16333–16338, 1995.
37. Masferrer, J. L., Zweifel, B. S., Manning, P. T., Hauser, S. D., Leahy, K. M., Smith, W. G., Isakson, P. C., and Seibert, K. Selective inhibition of inducible cyclooxygenase 2 *in vivo* is antiinflammatory and nonulcerogenic. Proc. Natl. Acad. Sci. USA, *91:* 3228–3232, 1994.
38. Tsujii, M., Kawano, S., Tsuji, S., Sawaoka, H., Hori, M., and DuBois, R. N. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. Cell, *93:* 705–716, 1998.
39. Tsujii, M., and DuBois, R. N. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. Cell, *83:* 493–501, 1995.
40. Sohn, K-H., Lee, H-Y., Chung, H-Y., Young, H-S., Yi, S-Y., and Kim, K-W. Anti-angiogenic activity of triterpene acids. Cancer Lett., *94:* 213–218, 1995.
41. Baek, J. H., Lee, Y. S., Kang, C. M., Kim, J-A., Kwon, K. S., Son, H. C., and Kim, K-W. Intracellular Ca^{2+} release mediates ursolic acid-induced apoptosis in human leukemic HL-60 cells. Int. J. Cancer, *73:* 725–728, 1997.
42. Weitzman, S. A., and Gordon, L. I. Inflammation and cancer: role of phagocyte-generated oxidants in carcinogenesis. Blood, *76:* 655–663, 1990.
43. O'Brian, C. A., Vogel, V. G., Singletary, S. E., and Ward, N. E. Elevated protein kinase C expression in human breast tumour biopsies relative to normal breast tissue. Cancer Res., *49:* 3215–3217, 1989.
44. Sebolt-Leopold, J. S., Dudley, D. T., Herrera, R., Van Becelaere, K., Wiland, A., Gowan, R. C., Tecle, H., Barrett, S. D., Bridges, A., Przybranowski, S., Leopold, W. R., and Saltiel, A. R. Blockade of the MAP kinase pathway suppresses growth of colon tumors *in vivo*. Nat. Med., *5:* 810–816, 1999.
45. Xie, W., and Herschman, H. R. v-src induces prostaglandin synthase 2 gene expression by activation of the c-Jun N-terminal kinase and the c-Jun transcription factor. J. Biol. Chem., *270:* 27622–27628, 1995.
46. Xie, W., and Herschman, H. R. Transcriptional regulation of prostaglandin synthase 2 gene expression by platelet-derived growth factor and serum. J. Biol. Chem., *271:* 31742–31748, 1996.
47. Karin, M. The regulation of AP-1 activity by mitogen-activated protein kinases. J. Biol. Chem., *270:* 16483–16486, 1995.
48. Whitmarsh, A. J., and Davis, R. J. Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. J. Mol. Med., *74:* 589–607, 1996.
49. Huang, C. S., Ma, W. Y., Dawson, M. I., Rincon, M., Flavell, R. A., and Dong, Z. G. Blocking activator protein-1 activity, but not activating retinoic acid response element, is required for the antitumor promotion effect of retinoic acid. Proc. Natl. Acad. Sci. USA, *94:* 5826–5830, 1997.
50. Young, M. R., Li, J-J., Rincon, M., Flavell, R. A., Sathyanarayana, B. K., Hunziker, R., and Colburn, N. Transgenic mice demonstrate AP-1 (activator protein-1) transactivation is required for tumor promotion. Proc. Natl. Acad. Sci. USA, *96:* 9827–9832, 1999.