ANTIOXIDANTS & REDOX SIGNALING
Volume 7, Numbers 11 & 12, 2005
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Forum Original Research Communication

Curcumin Inhibits Phorbol Ester-Induced Up-Regulation of Cyclooxygenase-2 and Matrix Metalloproteinase-9 by Blocking ERK1/2 Phosphorylation and NF-κB Transcriptional Activity in MCF10A Human Breast Epithelial Cells

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ABSTRACT

Elevated levels of cyclooxygenase-2 (COX-2) and matrix metalloproteinases (MMPs) are often observed in various types of cancerous and transformed cells, and hence recognized as potential molecular targets for the chemoprevention. In the present study, we investigated the possible inhibitory effects of curcumin on the expression of COX-2 and MMP-9 induced by the tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate (TPA) in MCF10A human breast epithelial (MCF10A) cells and the underlying mechanisms. Curcumin inhibited the TPA-induced COX-2 expression at both transcriptional and post-transcriptional levels, and reduced the synthesis of prostaglandin E_2, one of the major products of COX-2. Likewise, curcumin attenuated invasiveness and motility of MCF10A cells stimulated with TPA through suppression of MMP expression. Curcumin blocked TPA-induced activation of extracellular signal-regulated protein kinase (ERK1/2) and nuclear factor κB (NF-κB) transcriptional activity. Overexpression of the dominant negative forms of ERK2 abrogated the TPA-induced NF-κB transcriptional activity. Treatment of MCF10A cells with U0126, which is a pharmacological inhibitor of ERK1/2, reduced TPA-induced up-regulation of COX-2 and MMP-9. Taken together, these findings suggest that curcumin inhibits the TPA-induced up-regulation of COX-2 and MMP-9 by suppressing ERK1/2 phosphorylation and NF-κB trans-activation in human breast epithelial cells, which may contribute to its chemopreventive potential. *Antioxid. Redox Signal.* 7, 1612–1620.

INTRODUCTION

PROSTAGLANDIN BIOSYNTHESIS is not only essential for the process of inflammation but also implicated in the pathogenesis of malignancy (16). Cyclooxygenase (COX) catalyzes a critical step in the conversion of arachidonic acid to prostaglandins, which have numerous physiologic functions. While the constitutively expressed housekeeping enzyme COX-1 plays a pivotal role in maintaining homeostatic functions of the cell, the inducible isoform COX-2 mediates primarily the inflammatory response. COX-2 is induced by cytokines and tumor promoters. Aberrant COX-2 expression was initially observed in colorectal cancer and has now been detected in many other human malignancies including breast and pancreatic cancers (11, 23). Therefore, the suppression of prostaglandin synthesis through selective inhibition of COX-2 has been suggested as a promising strategy applicable to the identification and development of chemopreventive substances (6).

The extracellular matrix is a framework of proteins and proteoglycans that provides tissues with structural integrity, and plays critical roles in cell growth, differentiation,

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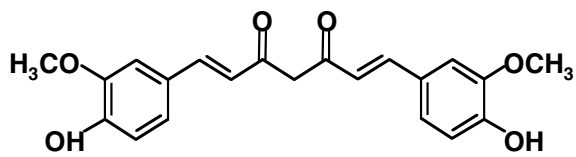
survival, and motility. Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that can degrade the major components of extracellular matrix. Increased activities of the type IV collagenases MMP-2 (72 kDa) and MMP-9 (92 kDa) have been associated with tumor cell invasion and migration (29). MMPs have been implicated in primary and metastatic tumor growth and angiogenesis, and may even contribute to tumor promotion (4, 19). Recent studies have suggested that MMP inhibitors exert substantial protective effects on tumor promotion by blocking, in part, the expression of proinflammatory enzymes, such as COX-2 (26). Based on these findings, it is conceivable that targeted inhibition of MMP is another promising chemoprevention strategy (4, 27).

One of the prime components of the intracellular signaling pathways responsible for the induction of COX-2 and MMP expression is the eukaryotic transcription factor nuclear factor κB (NF-κB) (26). NF-κB activation is primarily regulated via the mitogen-activated protein kinase (MAPK) pathway. There are three distinct groups of well-characterized major MAPK subfamily members: (a) extracellular signal-regulated protein kinase (ERK), (b) p38 MAPKs, which are serine/threonine protein kinases, and (c) c-Jun NH_2-terminal kinase/stress-activated protein kinase. The activated form of each of the above MAPKs then phosphorylates and activates other kinases or transcription factors, thereby altering the expression of the target genes. The induction of COX-2 and MMP is abolished by pharmacological inhibition (8, 20, 21) and dominant-negative knockout (9, 10) of the corresponding MAPKs, lending support to involvement of MAPK cascades in the up-regulation of COX-2 and MMPs.

There has been increasing interest in identifying naturally occurring chemopreventive substances capable of inhibiting, retarding, or reversing multistage carcinogenesis. An example is curcumin (structure shown in Fig. 1), a yellow pigment of turmeric (*Curcuma longa* L., Zingiberaceae), which is used for imparting color and flavor to foods (24). The compound possesses antiproliferative, anti-inflammatory, and antioxidant properties (reviewed in 24, 25). Dietary administration of curcumin significantly reduced the incidence of colon adenocarcinomas in animals (12). Our previous studies demonstrated that curcumin inhibited COX-2 expression by inhibiting ERK activity and NF-κB in 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-stimulated mouse skin (3).

Recent studies suggest that inappropriate up-regulation of COX-2 and MMPs is implicated in the pathogenesis of breast cancer (1, 13, 22). This prompted us to investigate the possible inhibitory effect of curcumin on TPA-induced up-regulation of COX-2 and MMP-9 in MCF10A human breast epithelial (MCF10A) cells and underlying molecular mechanisms. Here we report that curcumin inhibits the TPA-induced up-regulation of COX-2 and MMP-9 through suppression of ERK1/2 phosphorylation and NF-κB transcriptional activity in human mammary epithelial cells.



Curcumin

FIG. 1. Chemical structure of curcumin.

MATERIALS AND METHODS

Materials

Curcumin, cholera toxin, hydrocortisone, anti-actin antibody, and recombinant human epithelial growth factor were obtained from Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle's medium/F-12 nutrient (DMEM/F12), L-glutamine, horse serum, penicillin/streptomycin/Fungizone® (amphotericin B) mixture, NF-κB DNA binding protein detection system, and Trizol® reagent were purchased from Gibco BRL (Grand Island, NY). pELAM-Luc containing the NF-κB site construct was a generous gift from Dr. Youngmi Kim of Asan Hospital, University of Ulsan, Seoul, Korea. Anti-COX-2 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and secondary antibodies were purchased from Zymed Laboratories (San Francisco, CA). BCA protein assay reagents were obtained from Pierce (Rockford, IL), and [α-^{32}P]CTP and [α-^{32}P]ATP were purchased from NEN Life Science (Boston, MA). ExpressHyb hybridization buffer solution was obtained from Clontech (Palo Alto, CA). Transfection reagents and protease inhibitor cocktail were obtained from Boehringer (Mannheim, Germany), and TPA was obtained from Alexis (San Diego, CA). ECL reagent, the prostaglandin E_2 (PGE_2) enzyme immunoassay kit, and the random prime labeling system were obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). Human cox-2 cDNA (1.8 kb) was kindly supplied by Prof. Daniel Hwang (University of California-Davis). Human β-actin cDNA (548 bp) was obtained using polymerase chain reaction. All other chemicals used were of analytical grade or the highest grade available.

Cell culture

MCF10A cells were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air in DMEM/F12 supplemented with 5% heat-inactivated horse serum, 10 μg/ml insulin, 100 ng/ml cholera toxin, 0.5 μg/ml hydrocortisone, 20 ng/ml recombinant human epithelial growth factor, 2 m*M* L-glutamine, and 100 μg/ml penicillin/streptomycin/Fungizone mixture. Cells were grown to 60–80% confluence and trypsinized with 0.05% trypsin containing 2 m*M* EDTA.

Western blot analysis

MCF10A cells (2×10^6) were plated in 100-mm-diameter culture dishes and incubated for 12 h prior to treatment. After treatment, cells were scraped and treated with RIPA lysis buffer (150 m*M* NaCl; 0.5% Triton X-100; 50 m*M* Tris-HCl, pH 7.4; 25 m*M* NaF; 20 m*M* EGTA; 1 m*M* dithiothreitol; 1 m*M* Na_3VO_4; and a protease inhibitor cocktail tablet) for 40 min on ice followed by centrifugation at 14,800 *g* for

30 min. The protein concentration of the supernatant was measured using the BCA reagents. Protein (30 μg) was loaded on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis (PAGE) gel and electrotransferred onto a polyvinylidene difluoride membrane in Tris-glycine buffer (pH 8.4) containing 20% methanol. The membrane was then blocked in 5% fat-free dry milk in phosphate-buffered saline (PBS) with 0.1% Tween-20 for 1 h. The membranes were incubated with 1:5,000 dilutions of actin (Sigma Chemical) and ERK for 2 h or with 1:1,000 dilutions of COX-2 (Cayman Chemical, Ann Arbor, MI) and phosphorylated ERK (Santa Cruz Biotechnology) at room temperature. After three 5-min washes with PBS/0.1% Tween-20, the membranes were incubated with an 1:5,000 diluted horseradish peroxidase-conjugated secondary antibody for 1 h. The transferred proteins were visualized with the ECL western blot detection system according to the manufacturer's instructions (Amersham Pharmacia Biotech).

Northern blotting for measurement of cox-2 mRNA expression

MCF10A cells (1.8×10^6) were plated in six-well plates and stabilized for 4 h, and then total cellular RNA was isolated from cell monolayers using Trizol reagent. Total cellular RNA (6 μg) was electrophoresed on a formaldehyde-containing 1.2% agarose gel and transferred to nylon membranes. After ultraviolet cross-linking, membranes were prehybridized for 30 min in ExpressHyb hybridization solution and hybridized for 1 h at 65°C with radiolabeled DNA probes for human cox-2 cDNA and β-actin cDNA. After hybridization, membranes were washed twice for 30 min at room temperature in SDS solution. Washed membranes were then subjected to autoradiography. Cox-2 and β-actin cDNA probes were labeled with [α-^{32}P]CTP using a random priming kit.

Measurement of PGE_2 production

MCF10A cells (6×10^5) were plated in six-well dishes and grown to 80% confluence in 2 ml of growth medium. The cells were then treated with TPA (60 n*M*) in the absence or presence of curcumin. The amounts of PGE_2 released into medium were measured using the PGE_2 enzyme immunoassay kit.

Determination of COX-2 enzyme activity

MCF10A cells (6×10^5) were plated in six-well dishes and grown to 80% confluence in 2 ml of growth medium. After the cells were treated with TPA (60 n*M*) for 4 h for maximal expression of COX-2 protein, the medium was changed, and curcumin was added. Levels of PGE_2 released into medium were measured 2 h later using the PGE_2 enzyme immunoassay kit.

Gelatin zymographic assay for MMPs

MCF10A cells plated on culture dishes at 90% confluency were maintained in serum-free medium for an additional 24 h. Conditioned medium was collected and concentrated at 10,000 ×*g* for 30 min in a SpeedVac concentrator (Savant, E-C Instruments, Niantic, CT). The protein concentration was measured using BCA protein assay reagents. Equal amounts of conditioned medium were mixed with nonreducing sample buffer, incubated for 15 min at room temperature, and then electrophoresed on 10% SDS-PAGE gels containing 1 mg/ml gelatin. After electrophoresis, the gels were washed with 2.5% Triton X-100 twice for 30 min, rinsed three times for 30 min with a 50 m*M* Tris-HCl buffer (pH 7.6) containing 5 m*M* $CaCl_2$, 0.02% Brij-35, and 0.2% sodium azide, and then incubated overnight at 37°C. The gels were stained with 0.5% Coomassie brilliant blue R-250 solution containing 10% acetic acid and 20% methanol for 30 min, and then destained with 7.5% acetic acid solution containing 10% methanol. Areas of gelatinase activity were detected as clear bands against the blue-stained gelatin background.

Invasion assay

An *in vitro* invasion assay was performed using a 24-well Transwell unit with polycarbonate filters having a diameter of 6.5 mm and a pore size of 8.0 μm (Corning Costar, Cambridge, MA). The lower side of the filter was coated with 10 μl of 0.5 mg/ml type I collagen, and the upper side was coated with 10 μl of 0.5 mg/ml reconstituted basement membrane substance (Matrigel; Collaborative Research, Lexington, KY). The coated filters were air dried for 1 h before the assay. The lower compartment was filled with 600 μl of medium containing 0.1% bovine serum albumin. Fifty thousand cells were resuspended in 100 μl of medium and placed in the upper part of the Transwell plate. Cells were incubated for 24 h in a humidified atmosphere of 5% CO_2 at 37°C. Cells were fixed with methanol and stained with hematoxylin for 10 min followed briefly by eosin. Cells on the upper surface of the filter were removed mechanically by wiping with a cotton swab, and the invasive phenotypes were determined by counting cells that migrated to the lower side of the filter using microscopy at ×400 magnification. Thirteen fields were counted for each assay.

Wound migration assay

Cells plated on culture dishes at 90% confluency were marked the injury line and wounded with a tip 2 mm in width. Plates were rinsed with PBS, and the complete medium was added. Cells were allowed to migrate, and photographs were taken through an inverted microscope (×40 magnification).

Transient transfection and luciferase reporter gene assay

MCF10A cells were plated at a density of 5×10^5 cells per well in six-well dishes and incubated for 4 h for stabilization. The cells were transiently transfected with 2.5 μg of plasmid pELAM-Luc containing the NF-κB site construct ligated to the luciferase gene and 1 μg of pCDNA3.1/hisB/lacZ (coding galactosidase for normalization of luciferase activity) using the transfection reagent DOTAP {*N*-[1(2,3-dioleoyloxy) propyl]-*N,N,N*-trimethylammonium methyl-sulfate} according to the manufacturer's instructions (Boehringer). After overnight transfection, the medium was changed, and TPA was added to each well in the absence or presence of cur-

cumin. Cells were then washed twice with cold PBS and lysed with reporter lysis buffer (Promega). After vortex-mixing and centrifugation at 12,000 *g* for 1 min at 4°C, the supernatant was stored at −70°C until used for the luciferase assay. Twenty milliliters of cell extract and 100 µl of the luciferase assay reagent were mixed at room temperature and placed in a luminometer (AutoLumat LB 953, EG&G Berthold, Bad Wildbad, Germany) for detection of the light produced.

Statistical analysis

Data in all figures are presented as mean ± SD values. Statistical significance was assessed using one-way analysis of variance followed by two-tailed Dunnett's *t* test, with the level of significance set at either $p < 0.01$ or $p < 0.05$.

RESULTS

Curcumin suppressed the TPA-induced expression of COX-2 and its mRNA transcript in MCF10A cells

In a preliminary study, the maximal expression of COX-2 and *cox-2* mRNA occurred at 4 h and 2 h, respectively, in MCF10A cells after the TPA (60 n*M*) treatment (data not shown). We evaluated the ability of curcumin to inhibit TPA-induced COX-2 expression in MCF10A cells. When the cells were treated with curcumin, there was concentration-dependent suppression of COX-2 expression at both protein and mRNA levels (Fig. 2A and B).

Curcumin inhibited TPA-induced PGE_2 synthesis and COX-2 catalytic activity

In parallel with COX-2 up-regulation, PGE_2 production peaked at 6 h following TPA treatment (data not shown). Curcumin at a concentration of 12 µ*M* strongly inhibited TPA-induced PGE_2 production (Fig. 2C). Curcumin was reported previously to directly inhibit the total COX catalytic activity in phorbol ester-treated human gastrointestinal epithelial cells (31), but its effects on COX-2 activity have not been reported in breast cell lines. We investigated the direct effect of curcumin on prostaglandin synthesis in MCF10A cells previously stimulated with TPA to induce COX-2. As shown in Fig. 2D, curcumin at a concentration of 12 µ*M* strongly suppressed COX-2 catalytic activity as well.

Curcumin inhibited TPA-induced up-regulation of MMP-9

Recent studies have suggested that MMPs play a key role in both the promotion and progression processes in breast cancer (1, 13, 22). A previous report found that TPA activated MMP-9 in MCF10A cells, but the underlying mechanisms were not clarified (28). As shown in Fig. 3A, the gelatinolytic activity of MMP-9—but not of MMP-2—was markedly increased in TPA-treated MCF10A cells, indicating a preferential up-regulation of MMP-9 by TPA in MCF10A cells. Invasive and migrative phenotypes of

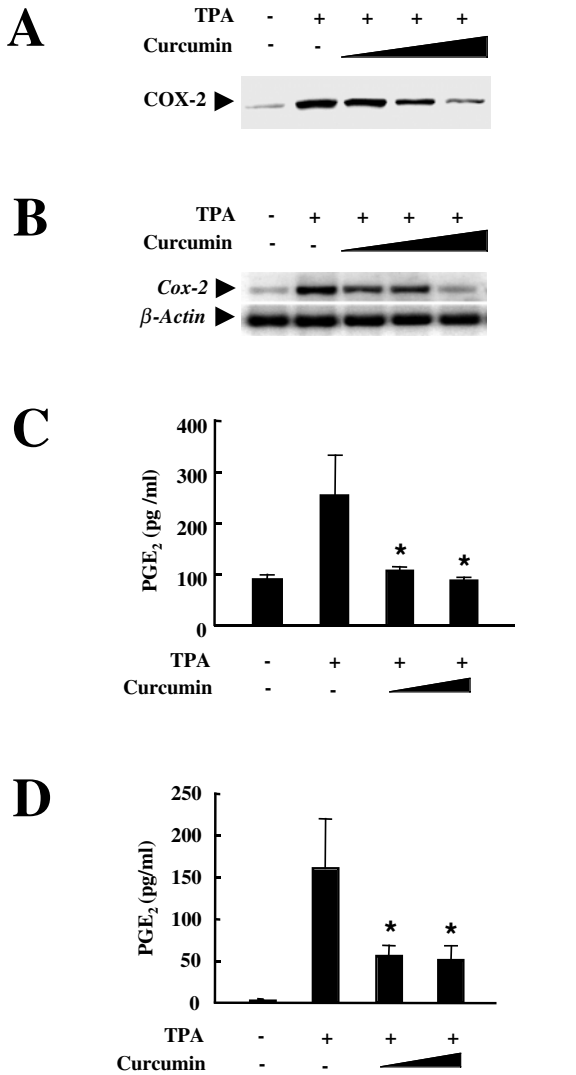


FIG. 2. Effects of curcumin on TPA-induced up-regulation of COX-2 in MCF10A cells. (**A**) Curcumin down-regulates TPA-induced COX-2 expression. MCF10A cells were treated for 4 h with TPA (60 n*M*) alone or together with curcumin at various concentrations (12, 25, and 50 µ*M*). COX-2 expression was determined by western blot analysis as described in Materials and Methods. (**B**) Curcumin inhibits the TPA-induced *cox-2* mRNA expression. MCF10A cells were treated for 2 h with TPA (60 n*M*) alone or together with curcumin at various concentrations (12, 25, and 50 µ*M*). Six micrograms of total RNA was analyzed by northern blot analysis using the [α-^{32}P]-labeled cox-2 cDNA probe. As an internal control, the β-actin mRNA level was analyzed on the same replicated blot. (**C**) Curcumin suppresses TPA-induced induction of PGE_2 synthesis. MCF10A cells were treated with vehicle (striped column) or TPA and curcumin (12 and 25 µ*M*) for 6 h. The medium was collected to determine the production of PGE_2 using an enzyme immunoassay. (**D**) Curcumin directly inhibits COX-2 enzyme activity. MCF10A cells were treated with vehicle, TPA alone, or TPA and curcumin (12 and 25 µ*M*) for 4 h. After TPA stimulation of COX-2, fresh medium containing curcumin (12 and 25 µ*M*) was added for 2 h. The medium was then collected to determine the production of PGE_2 using an enzyme immunoassay. Data are mean ± SD values for three independent experiments. Significantly different from the group treated with TPA alone: $*p < 0.05$, $**p < 0.01$.

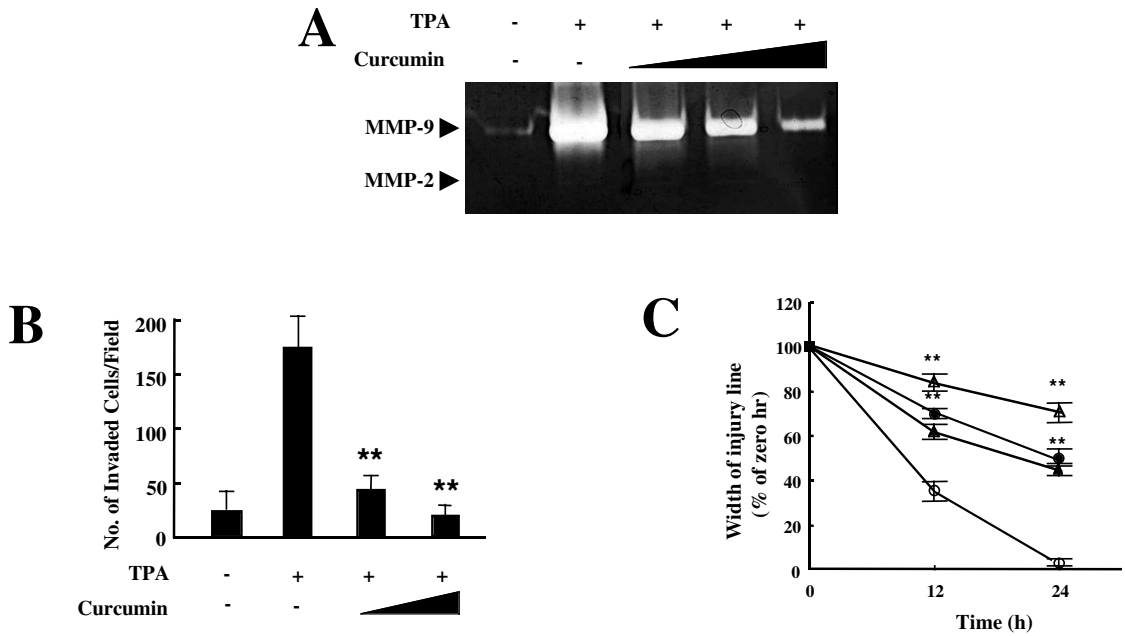


FIG. 3. Effects of curcumin on TPA-induced up-regulation of MMP-9, invasion, and motility in MCF10A cells. (**A**) Curcumin inhibits TPA-induced up-regulation of MMP-9. MCF10A cells were treated for 24 h with TPA (60 n*M*) alone or together with curcumin at various concentrations (12, 25, and 50 μ*M*). The level of secreted MMP-9 (M_r = 92,000) was determined by gelatin zymography as described in Materials and Methods. (**B**) Curcumin inhibits the TPA-induced invasive phenotype. MCF10A cells were treated for 24 h with TPA (60 n*M*) alone or together with curcumin at indicated concentrations (25 and 50 μ*M*). An *in vitro* invasion assay was performed using a 24-well transwell unit with polycarbonate filters having a diameter of 6.5 mm and a pore size of 8.0 μm, and the number of invading cells per field was counted under light microscopy. (**C**) Curcumin inhibits the TPA-induced migrative phenotype. For the wound-migration assay, after pretreatment of cells with mitomycin C (25 μg/ml) for 30 min, a line of damage was made on the confluent monolayer of cells. MCF10A cells were treated with TPA (60 n*M*) alone or together with curcumin at 25 or 50 μ*M*, and cell motility was examined under light microscopy (×40 magnification) at the indicated time points: solid circle, control; open circle, TPA-treated; solid triangle, TPA + 25 μ*M* curcumin; open triangle, TPA + 50 μ*M* curcumin. Data are mean ± SD values for three independent experiments. Significantly different from the group treated with TPA alone: *$p < 0.05$, **$p < 0.01$.

MCF10A cells were also significantly increased by TPA (Fig. 3B and C, respectively), suggesting that the activation of MMP-9 is essential for the TPA-induced invasive/migrative phenotypes in MCF10A cells. Curcumin inhibited TPA-induced up-regulation of MMP-9 in MCF10A cells in a dose-dependent manner (Fig. 3A). Invasive and migrative phenotypes of TPA-treated MCF10A cells were significantly inhibited by curcumin (Fig. 3B and C, respectively). Survival of the treated cells for up to 24 h was comparable to that of cells at 0 h as determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (data not shown), indicating that the inhibition of invasion and motility was not due to cytotoxicity of curcumin.

Curcumin blocked TPA-induced ERK1/2 phosphorylation and NF-κB transcriptional activity in MCF10A cells

There are multiple lines of evidence supporting the proposal that MAPKs play pivotal roles in various cellular responses including up-regulation of COX-2 and MMPs (25, 26). Curcumin inhibited the TPA-induced activation of ERK1/2 but not p38 MAPK (Fig. 4A). When MCF10A cells transfected with a luciferase reporter plasmid containing the NF-κB promoter region ligated to a luciferase reporter gene were incubated with TPA for 6 h, there was a significant increase in the luciferase activity, compared with vehicle-treated control cells (Fig. 4B). The TPA-induced increase in the luciferase activity was blocked when curcumin was added to the cells (Fig. 4B). Ectopic expression of the dominant-negative forms of ERK2 also abrogated TPA-induced NF-κB transcriptional activities as determined by the luciferase reporter gene assay, demonstrating that ERK forms are the upstream molecules responsible for NF-κB activation (Fig. 4C).

TPA-induced up-regulation of COX-2 and MMP-9 was suppressed by an ERK1/2 inhibitor

We next investigated the functional significance of the ERK pathway in TPA-induced up-regulation of COX-2 and MMP-9 in MCF10A cells. As shown in Fig. 5A and B, treatment with 10 μ*M* U0126 (an inhibitor of MAPK kinase [MEK], which is an upstream activator of ERKs) inhibited

TPA-induced up-regulation of COX-2 and MMP-9. Treatment with U0126 almost completely inhibited the invasive and migrative phenotype of TPA-treated MCFA10A cells (Fig. 5C and D). The survival of the treated cells for up to 24 h was comparable to that of cells at 0 h as determined by the MTT assay (data not shown), indicating that the inhibition of invasion and motility was not due to U0126 cytotoxicity. Thus, it is likely that inactivation of the ERK pathway by curcumin could contribute to the suppression of the TPA-induced invasive and migrative phenotype in human mammary epithelial cells. Taken together, the above findings suggest that curcumin inhibits the TPA-induced up-regulation of COX-2 and MMP-9 by suppression of ERK1/2 phosphorylation and NF-κB transcriptional activity in human mammary epithelial cells.

DISCUSSION

Abnormal up-regulation of COX-2 has been frequently observed in various malignancies including those of the esophagus, stomach, breast, pancreas, lung, colon, skin, urinary bladder, and prostate (reviewed in 2). In addition, mice genetically engineered to overexpress COX-2 exhibit increased susceptibility to experimental tumorigenesis, whereas COX-2-knockout mice are less susceptible to tumor promotion. Epidemiological studies have shown that the chronic administration of non-steroidal anti-inflammatory drugs, such as aspirin and sulindac, reduces the risk of colon and breast cancer. COX-2 may be directly involved with mammary carcinogenesis, since expression of COX-2 is suf-

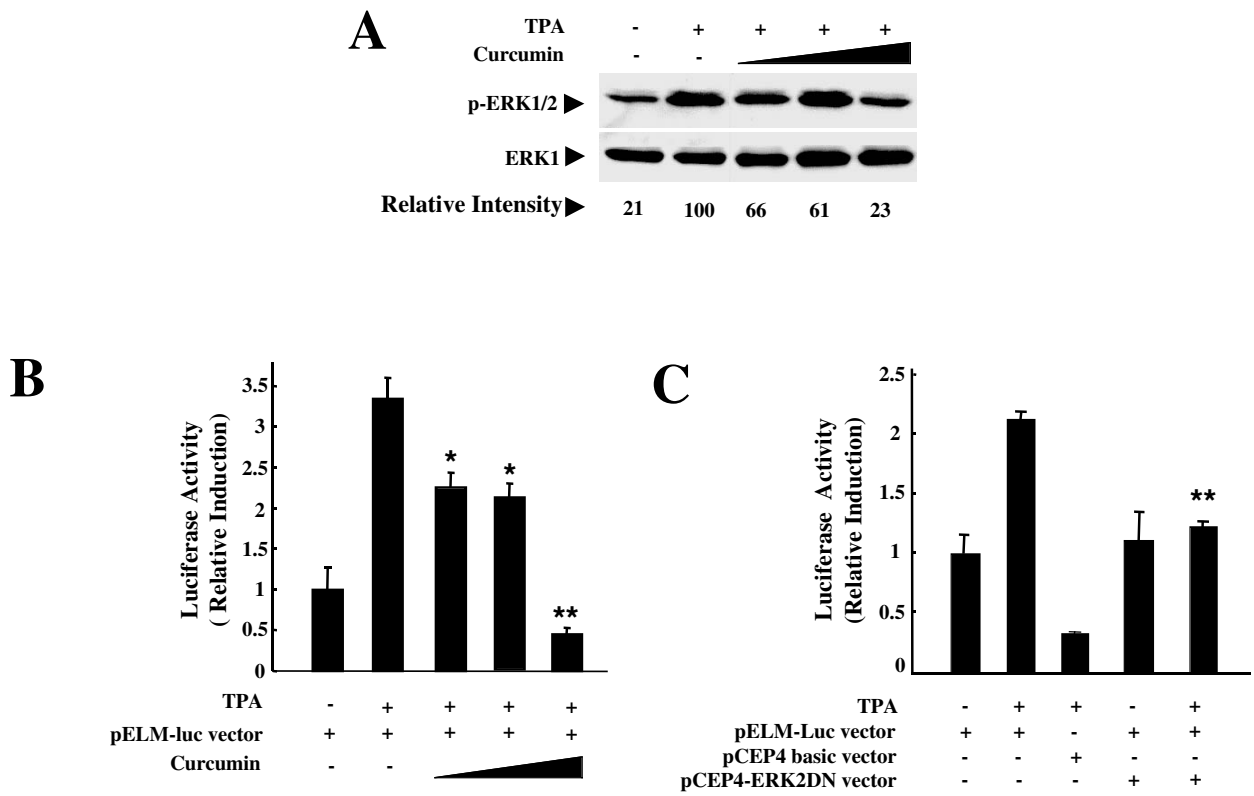


FIG. 4. Curcumin suppresses the TPA-induced ERK1/2 phosphorylation and NF-κB transcriptional activation in MCF10A cells. (**A**) Curcumin suppresses the TPA-induced ERK1/2 phosphorylation. MCF10A cells were treated with TPA (60 n*M*) in the absence or presence of varying concentrations (12, 25, or 50 μ*M)* of curcumin for 15 min. Protein (60 μg) was loaded in each well, and phosphorylated ERK1/2 (p-ERK1/2) was detected by western blot analysis using the appropriate phosphor-specific antibody. Non–phospho-specific ERK1 was used as an internal standard. (**B**) Curcumin suppresses the TPA-induced NF-κB gene transcriptional activity. pELM-Luc (containing the NF-κB site construct ligated to the luciferase gene) vector was transiently transfected into MCF10A cells. After overnight transfection, cells were treated with vehicle, TPA alone, or TPA and curcumin (12, 25, or 50 μ*M*) for 4 h. (**C**) Activation of NF-κB depends on the ERK signal pathway. MCF10A cells were co-transfected with the NF-κB luciferase reporter gene plasmid alone or with pCEP4 vector harboring DN mutant ERK2. After overnight transfection, cells were stimulated with TPA (60 n*M*) for 4h. The indicated luciferase activities are normalized to the ß-galactosidase activity of each protein extract. The luciferase reporter assay is described in Materials and Methods. Data are mean ± SD values for three independent experiments. Significantly different from the group treated with TPA alone: $*p < 0.05$, $**p < 0.01$.

ficient for formation of breast tumors in transgenic mice, and COX-2-selective inhibitors can suppress tumorigenesis in rodent models of breast cancer (reviewed in 1). Thus, COX-2 is an important indicator in the genesis of breast cancer, and COX-2 inhibitors may be used for the treatment and prevention of breast cancer. Therefore, curcumin, which interferes with the signaling mechanism governing the transcription of *cox-2*, is also anticipated to have chemopreventive as well as anti-inflammatory activities.

Numerous studies have shown a correlation between the levels of MMP-2 and/or MMP-9 and the invasive phenotypes of cancer cells (29). However, recent clinical studies suggest that MMP inhibition does not necessarily confer benefits over conventional therapies (4). One plausible reason is that MMP activity is required preferentially for rapidly proliferating and invading tumors rather than for preexisting tumors. Because MMPs are apparently involved in breast cancer initiation and dissemination, inhibition of these proteinases may be of value both in preventing breast cancer and in blocking metastasis of established tumors (reviewed in 7). In the present study, we have found that TPA-induced MMP-9 activation is a critical contributor to the TPA-induced invasive phenotype in human breast epithelial cells and that the inhibition of TPA-induced motility and invasive phenotype in MCF10A cells by curcumin is attributable to the suppression of MMP-9. Thus, the inhibition of MMP activity by curcumin may be effective for preventing the relatively early-stage carcinogenesis, particularly the tumor promotion process before the malignancy manifests (27).

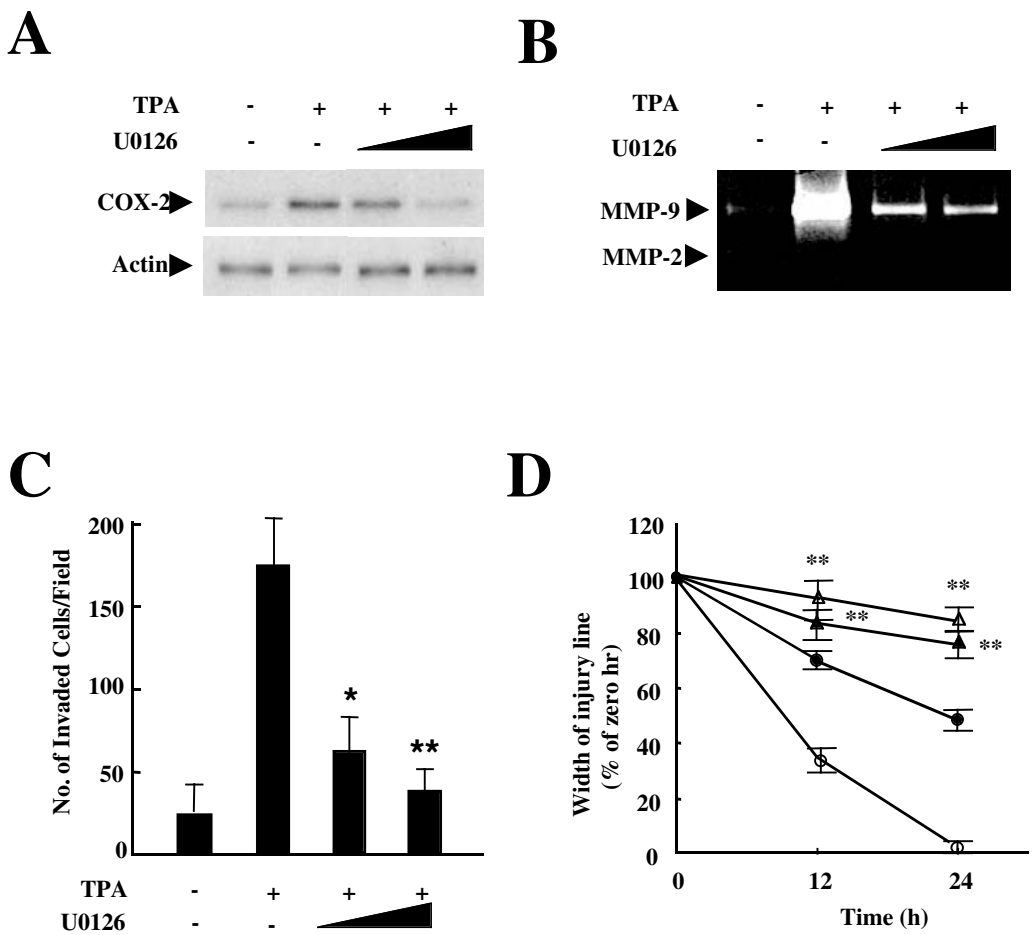


FIG. 5. Role of ERK1/2 in TPA-induced up-regulation of COX-2 and MMP-9, invasion, and motility in MCF10A cells. (**A**) U0126 down-regulates the TPA-induced COX-2 expression. COX-2 expression was determined by western blot analysis as described in Materials and Methods. (**B**) U0126 inhibits the TPA-induced MMP-9 up-regulation. The level of secreted MMP-9 was determined by gelatin zymography. (**C**) U0126 inhibits the TPA-induced invasive phenotype. An *in vitro* invasion assay was performed using a 24-well Transwell unit with polycarbonate filters having a diameter of 6.5 mm and a pore size of 8.0 μm, and the number of invading cells per field was counted under light microscopy. (**D**) U0126 inhibits the TPA-induced migrative phenotype. For the wound migration assay, after pretreatment of cells with mitomycin C (25 μg/ml) for 30 min, a line of damage was made on the confluent monolayer of cells. Cell motility was examined under the light microscope (×40 magnification) at the indicated time points. U0126 was used at concentrations of 10 μ*M* and 20 μ*M* in all assays: solid circle, control; open circle, TPA-treated; solid triangle, TPA + 25 μ*M* curcumin; open triangle, TPA + 50 μ*M* curcumin. Data are mean ± SD values for three independent experiments. Significantly different from the group treated with TPA alone: **p* < 0.05, ***p* < 0.01.

Expression of COX-2 and MMP has been reported to be regulated via distinct MAPK signaling pathways that depend on the type of inducer (2, 5, 26). The induction of COX-2 by platelet-derived growth factor has been demonstrated to require activation of the ERK signaling pathway (30), while interleukin 1β has been shown to induce COX-2 expression by activating both c-Jun NH_2-terminal kinase and p38 MAPK signal pathways in rat renal mesangial cells (9). p38 MAPK has been demonstrated to be an important mediator of cell motility in endothelial and smooth muscle cells. The ERK pathway was shown to be involved in the invasive or migrative behavior of human vascular smooth muscle (18) and glioblastoma cells (14). In contrast, a recent study has shown that H-*ras* up-regulates MMP-2 expression and induces an invasive and migrative phenotype via mainly the p38 pathway in MCF10A human breast epithelial cells (13, 17).

Previous studies showed that TPA induced up-regulation of COX-2 (15) and MMP-9 (28) in MCF10A cells, but the underlying molecular mechanisms remains unclear. In this study, curcumin attenuated the TPA-induced ERK phosphorylation and subsequent NF-κB transcriptional activity. We assume that curcumin can suppress TPA-induced up-regulation of COX-2 and MMP-9 by inhibiting the phosphorylation of ERK in MCF10A cells. Transfection of MCF10A cells with a dominant-negative form of ERK2 abrogated the TPA-induced NF-κB transcriptional activity, verifying that ERK is upstream of NF-κB. The suppression of TPA-induced up-regulation of COX-2 and MMP by the MEK-specific inhibitor (U0126) observed in this study is in agreement with the notion that the ERK signaling pathway is pivotal in regulating the expression of COX-2 and MMP-9 through NF-κB activation. Treatment with U0126 almost completely inhibited the invasive and the migrative phenotype of TPA-treated MCFA10A cells.

Taken together, the above results suggest that curcumin targets the ERK–NF-κB pathway involved in the up-regulation of COX-2 and MMP-9 in human mammary epithelial cells stimulated with TPA, which could contribute to the chemopreventive effects exerted by this chemopreventive phytochemical.

ACKNOWLEDGMENTS

This work was supported by a research grant from the Korea Science and Engineering Foundation (KOSEF), Republic of Korea for Functional Food Research and Development.

ABBREVIATIONS

COX, cyclooxygenase; DMEM/F12, Dulbecco's modified Eagle's medium/F-12 nutrient; ERK, extracellular signal-regulated protein kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; MMP, matrix metalloproteinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor κB; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PGE_2, prostaglandin E_2; SDS, sodium dodecyl sulfate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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Received for publication May 22, 2005; accepted June 27, 2005.

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