Curcumin inhibits phorbol ester-induced expression of cyclooxygenase-2 in mouse skin through suppression of extracellular signal-regulated kinase activity and NF-κB activation

Kyung-Soo Chun1, Young-Sam Keum1, Seong Su Han1, Yong-Sang Song2, Su-Hyeong Kim2 and Young-Joon Surh1,3

1College of Pharmacy and 2Department of Obstetrics and Gynecology, College of Medicine, Seoul National University, Seoul 151-741, South Korea

Introduction

There has been accumulating evidence for the association between inflammatory tissue damage and the process of cancer development (1). Cyclooxygenase (COX), an important enzyme involved in mediating the inflammatory process, catalyzes the rate-limiting step in the synthesis of prostaglandins (PGs) from arachidonic acid. There are two isoforms of COX, designated COX-1 and COX-2 (2). COX-1 is expressed constitutively in most tissues and appears to be responsible for maintaining normal physiological functions. In contrast, COX-2 is detectable in only certain types of tissues and is induced transiently by growth factors, pro-inflammatory cytokines, tumor promoters and bacterial toxins (1,3). Expression of COX-2 has been reported to increase in human colorectal adenocarcinoma (4) and other malignancies such as breast, cervical, prostate and lung tumors (5). Genetic knockout or pharmacological inhibition of COX-2 has been shown to protect against intestinal polyposis in mice (6). Administration of the selective COX-2 inhibitor celecoxib at 400 mg twice a day for 6 months reduced the number of polyps significantly in patients with familial adenomatous polyposis (7). In addition, celecoxib has been found to inhibit experimentally induced colon, breast, bladder and skin carcinogenesis (8–11).

The eukaryotic transcription factor NF-κB plays a central role in general inflammatory as well as immune responses. The 5′-flanking region of the COX-2 promoter contains NF-κB binding sites. In line with this notion, NF-κB has been shown to be a critical regulator of COX-2 expression in many cell lines (12,13). The intracellular signaling cascades controlling NF-κB activation are highly complex and involve the distinct set of kinases. Of the potential protein kinases involved in the activation of NF-κB, mitogen-activated protein (MAP) kinases such as extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase/stress-activated protein kinase signaling pathways have been well characterized (14,15).

Recently, much attention has been devoted to identifying cancer chemopreventive phytochemicals of dietary and medicinal origin (16). One such compound is curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], the major yellow coloring pigment found in turmeric (Curcuma longa Linn, Zingiberaceae), which has been used for centuries in traditional oriental medicine to treat mainly inflammatory disorders. The anti-inflammatory properties of curcumin have also been verified in experimental studies (16). In addition, the compound has been shown to exert anticarcinogenic or antimutagenic effects in many animal models and also in cultured cells (reviewed in 16 and references therein). Thus, curcumin

Abbreviations: AP-1, activator protein 1; COX, cyclooxygenase; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase kinase; PG, prostaglandin; SDS, sodium dodecyl sulfate; TPA, 12-O-tetradecanoylphorbol-13-acetate.
inhibits the development of chemically induced tumors of oral cavity, skin, forestomach, duodenum and colon in rodents (17–20). Curcumin has a variety of biochemical activities that are related to its chemopreventive action. These include antioxidation (21,22), inactivation of activator protein (AP)-1 (23) and NF-κB (24), inhibition of PG biosynthesis (25), and inhibition of activity and expression of ornithine decarboxylase (20,26).

The anti-inflammatory properties of curcumin are considered to contribute to its antitumor promoting activity. In the present study, we examined the effect of curcumin on COX-2 induction by the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) in mouse skin in vivo. To further elucidate the molecular mechanisms by which curcumin regulates cox-2 gene expression, we also investigated its effect on activation of upstream signaling enzymes, such as ERK or p38 MAP kinase, and the transcription factor NF-κB in mouse skin in vivo.

Materials and methods

Chemicals

Curcumin was purchased from Sigma Chemical Co. (St Louis, MO, USA). TPA was obtained from Alexis Biochemicals (San Diego, CA, USA). All other chemicals used were in the purest form available commercially.

Animal treatment

Female ICR mice (6–7 weeks of age) were supplied from the Daen-Han/Bioblink Experimental Animal Center (Daejeon, Korea). The animals were housed in climate-controlled quarters (24 ± 1 °C at 50% humidity) with a 12-h light/12-h dark cycle. The dorsal side of skin was shaved using an electric clipper, and only those animals in the resting phase of the hair cycle were used in all experiments. Curcumin and TPA were dissolved in 200 μl of acetone and applied to the dorsal shaven area.

Western blot analysis

The mice were topically treated on their shaven backs with indicated doses of curcumin 30 min before 10 nmol TPA treatment and were killed by cervical dislocation at the indicated times. For isolation of protein from mouse skin, the dorsal skin was excised, and the fat was removed on ice, immediately placed in ice-cold lysis buffer [150 mM NaCl, 0.5% Triton X-100, 50 mM Tris±HCl (pH 7.4), 20 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM Na3VO4, protease inhibitor cocktail tablet (Roche Molecular Biochemicals, Mannheim, Germany)] for 10 min. Lysates were centrifuged at 12 000 g for 20 min, and supernatant containing 30 μg protein was boiled in sodium dodecyl sulfate (SDS) sample loading buffer for 10 min before electrophoresis on 12% SDS–polyacrylamide gel. After electrophoresis for 2 h, proteins in SDS–polyacrylamide gel were transferred to PVDF membrane (Gelman Laboratories, Ann Arbor, MI), and the blots were blocked with 5% non-fat dry milk-PBST buffer [phosphate-buffered saline (PBS) containing 0.1% Tween-20] for 60 min at room temperature. The membranes were incubated for 2 h at room temperature with 1:1000 dilution of COX-2, p65, IkBα and ERK polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-p65 and phospho-IkBα polyclonal antibodies (Cell Signaling Technology, Beverly, MA) and p38, phospho-p38 and phospho-ERK monoclonal antibodies (Santa Cruz Biotechnology). Equal lane loading was assessed using actin (Sigma Chemical Co.) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) polyclonal antibody (Trevengen, Gaithersburg, MD). The blots were rinsed three times with PBST buffer for 5 min each. Washed blots were incubated with 1:5000 dilution of the horseradish peroxidase conjugated-secondary antibody (Zymed Laboratories, San Francisco, CA) and then washed again three times with PBST buffer. The transferred proteins were visualized with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Northern blot analysis

The pulverized skin was homogenized on ice for 20 s with a Polytron for isolation of total RNA by using TRIzol® reagent (Roche Molecular Biochemicals). For northern blot analysis, 20 μg of total RNA was subjected to electrophoresis on 1.2% agarose–formaldehyde gel and transferred to a Hybond-Nylon membrane (Amersham Pharmacia Biotech). After being fixed by UV irradiation, membranes were pre-hybridized for 30 min at 68 °C in ExpressHyb hybridization solution (Clontech Laboratories, Palo Alto, CA). Hybridization was carried out for 1 h at 68 °C with a cox-2 cDNA probe (Cayman Chemical, Ann Arbor, MI) labeled with [γ-32P]dCTP (NEL Life Science Products, Boston, MA) using a Rediprime II labeling system (Amersham Pharmacia Biotech). After hybridization, the membranes were washed twice for 30 min at room temperature in low-stringency buffer (2× SSC and 0.5% SDS) and twice for 40 min at 50°C in high-stringency buffer (0.1× SSC and 0.1% SDS). Washed membranes were then autoradiographed on the X-ray film using an intensifying screen at 70°C. Each band was quantified using a BAS2000 image analyzer (Fuji Photo Film Co., Tokyo, Japan).

Immunohistochemical staining of COX-2

The dissected skin was prepared for immunohistochemical analysis of COX-2 localization. Four-micrometer sections of formalin-fixed, paraffin-embedded tissue were cut onto silanized glass slides and deparaffinized three times with xylene for 10 min each and rehydrated through graded alcohol bath. The deparaffinized sections were heated and boiled twice for 6 min in 10 mM citrate buffer, pH 6.0, for antigen retrieval. To diminish non-specific staining, each section was treated with 3% hydrogen peroxide in methanol for 15 min. For the detection of COX-2, slides were incubated with 1:50–100 dilutions of the monoclonal mouse anti-COX-2 antibody (Cayman Chemical) at room temperature for 60 min in Tris-buffered saline containing 0.05% Tween-20 and then developed using the HPR EnVision™ System (Dako, Glostrup, Denmark). The peroxidase binding sites were detected by staining with 3,3′-diaminobenzidine tetrahydrochloride (Dako). Finally, counterstaining was performed using Mayer’s hematoxylin.

Preparation of nuclear extracts

The nuclear extract from mouse skin was prepared as described previously (27). Briefly, scraped dorsal skin of mice was homogenized in 1 ml of ice-cold hypotonic buffer A [10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM MgCl2, 1 mM EDTA, 0.1 mM DTT, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonylfluoride (PMSF)]. After 15 min incubation on ice, the nucleoprotein complexes were lysed with 125 μl of 10% Nonidet P-40 (NP-40) solution, followed by centrifugation for 2 min at 14 800 g. The nuclei were washed once with 400 μl of buffer A plus 25 μl of 10% NP-40, centrifuged, resuspended in 150 μl of buffer C [50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF and 10% glycerol], and centrifuged for 5 min at 14 800 g. The supernatant containing nuclear proteins was collected and stored at −70°C after determination of protein concentrations.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed using a DNA–protein binding detection kit (Gibco BRL, Grand Island, NY) according to the manufacturer’s protocol. Briefly, the NF-κB oligonucleotide probe (5′-AGT TGA GGC GAC TTT CCC AGG C-3′) was labeled with [γ-32P]ATP by T4 polynucleotide kinase and purified on a Nick column (Amersham Pharmacia Biotech). The binding reaction was carried out in a total volume of 25 μl containing 10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 4% (v/v) glycerol, 1 mg/ml sonicated salmon sperm DNA, 10 μg of nuclear extracts, and 100 000 c.p.m. of the labeled probe. After 50 min incubation at room temperature, 2 μl of 0.1% bromophenol blue was added, and samples were electrophoresed through a 6% non-denaturing polyacrylamide gel at 150 V in a cold room for 2 h. Finally, the gel was dried and exposed to X-ray film.

MAP kinase assay (non-radioactive)

Kinase assays for determining the catalytic activities of p38 and ERK were carried out by using a non-radioactive MAP kinase assay kit (Cell Signaling Technology) as described in the protocol provided by the manufacturer. Collected tissues were lysed in 200 μl of lysis buffer per sample [20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM glycerolphosphate, 2 mM EDTA, 0.1 mM Na3VO4 and 10 mM MgCl2]. The kinase reactions were carried out in the presence of 100 μM ATP and 2 μg of ATF-2 or Elk-1 at 30°C for 30 min. Phosphorylation of ATF-2 and Elk-1 was selectively measured by immunoblotting with specific antibodies detecting phosphorylation of ATF-2 and Elk-1 at Thr71 and Ser383, respectively.

MAP kinase assay (non-radioactive)
Results

Inhibitory effects of curcumin on TPA-induced COX-2 expression in mouse skin

It has been shown previously that TPA is a potent stimulator of COX-2 expression in various cell lines (28,29). We determined whether this was also the case in mouse skin. When 10 nmol of TPA was applied topically to the shaven backs of female ICR mice, the COX-2 protein level increased in a time-related manner with maximal expression observed at 4 h (Figure 1A). Under the same experimental conditions, \(\text{cox-2}\) mRNA expression peaked 1 h after the TPA application (Figure 1B). TPA application caused dose-dependent increases in both COX-2 protein and mRNA expression (Figure 2). To determine localization of COX-2 in mouse skin, we conducted immunohistochemical analysis. In acetone-treated control skin, specific COX-2 immunostaining was barely detectable in dorsal layer. Upon treatment with TPA for 4 h, the expression of COX-2 increased mainly in the epidermal layer (Figure 3), which was suppressed by curcumin pre-treatment (Figure 4). Curcumin-mediated suppression of COX-2 expression was verified by western blot analysis (Figure 5).

Inhibition of TPA-induced NF-\(\kappa\)B DNA binding activity by curcumin

Because NF-\(\kappa\)B is known to play a critical role in regulating the induction of COX-2, we have determined whether curcumin could suppress activation of this transcription factor in nuclear extracts obtained from the mouse skin stimulated with TPA. Our previous studies demonstrated an apparent increase in epidermal NF-\(\kappa\)B DNA binding as early as 10 min after 10 nmol TPA application, which was abolished by the excess unlabeled probe (27). Effects of curcumin on NF-\(\kappa\)B activation were examined with varying doses of the compound topically applied 30 min before, simultaneously with or 30 min after the TPA treatment. Pre-treatment of curcumin strongly inhibited TPA-induced NF-\(\kappa\)B activation, whereas co- and post-treatment exhibited weaker inhibitory effects on NF-\(\kappa\)B DNA binding in TPA-stimulated mouse skin (Figure 6A).

Effects of curcumin on TPA-induced phosphorylation and degradation of I\(\kappa\)B\(\alpha\) and nuclear translocation of \(\text{p65}\)

One of the most critical steps in NF-\(\kappa\)B activation is the dissociation of I\(\kappa\)B, which is mediated through phosphorylation and subsequent proteolytic degradation of this inhibitory subunit. To determine whether the inhibitory effect of curcumin on NF-\(\kappa\)B DNA binding was due to its suppression of I\(\kappa\)B\(\alpha\) degradation via phosphorylation, the cytoplasmic levels of I\(\kappa\)B\(\alpha\) and phospho-I\(\kappa\)B\(\alpha\) were determined by western blot analysis. Topical application of TPA led to phosphorylation and degradation of I\(\kappa\)B\(\alpha\), which were significantly repressed.

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**Fig. 1.** TPA-induced COX-2 protein (A) and its mRNA (B) expression in mouse skin. (A) Dorsal skins of female ICR mice were treated topically with acetone alone or with 10 nmol TPA in acetone for indicated time periods. Protein extracts (30 \(\mu\)g) were loaded onto a 12% SDS–polyacrylamide gel, electrophoresed, and subsequently transferred onto PVDF membrane. Immunoblots were a probed with a goat polyclonal COX-2 antibody. (B) \(\text{cox-2}\) mRNA expression was determined by northern blot analysis using the \(\text{cox-2}\) cDNA probe labeled with [\(\alpha\)-\(^{32}\)P]dCTP. Quantification of \(\text{cox-2}\) mRNA signal intensities was normalized to those of GAPDH mRNA.

**Fig. 2.** Dose-related expression of COX-2 protein (A) and its mRNA (B) in TPA-treated mouse skin. Dorsal skins of female ICR mice were treated topically with acetone alone or with various doses of TPA in acetone. Mice were killed 4 or 1 h later for immunoblot analysis of COX-2 protein (A) or northern blot analysis of \(\text{cox-2}\) mRNA (B), respectively.
by curcumin pre-treatment (Figure 6B and C). This finding is consistent with NF-κB DNA binding affected by the same treatment (Figure 6A). We also measured the level of p65, the functionally active subunit of NF-κB, in nucleus. Upon TPA treatment, the nuclear translocation of p65 increased, which was blocked by curcumin (Figure 6B). These results indicate that curcumin inhibits TPA-induced translocation of p65 to the nucleus through blockade of IκBa phosphorylation.

Effects of curcumin on TPA-induced activation of MAP kinases

MAP kinases are known to regulate NF-κB activation by multiple mechanisms. Accumulating evidence indicates that NF-κB activation is modulated by ERK as well as p38 MAP kinase. As shown in Figure 7, ERK and p38 in mouse skin were phosphorylated in response to TPA treatment. Phosphorylation of each MAP kinase was evident at 30 min following TPA application and was sustained up to 4 h. To confirm that phosphorylation of ERK and p38 reflected their enhanced catalytic activity, kinase assays were performed. In parallel with elevated phosphorylation, the activities of ERK and p38 increased rapidly after TPA application (Figure 7). However, the JNK activity remained unchanged even after TPA treatment (data not shown). After verifying the ERK and p38 activation in TPA-stimulated mouse skin, we examined whether curcumin could down-regulate the aforementioned MAP kinases, thereby inactivating NF-κB and further suppressing the COX-2 induction. Curcumin inhibited catalytic activities of both p38 MAP kinase and ERK1/2. In addition, curcumin inhibited activation of p38 MAP kinase through phosphorylation while it did not much influence the phosphorylation of ERK1/2 in mouse skin (Figure 8). Under the same experimental conditions, the level of the total form of each kinase remained almost constant.

Inhibition of TPA-induced NF-κB DNA binding activity and COX-2 expression by MAP kinase inhibitors

To determine which of the MAP kinases is involved in activation of NF-κB in mouse skin, we investigated the inhibitory
effect of the pharmacological inhibitors of MAP kinases on NF-κB activation by TPA. SB203580 is known to selectively inhibit p38 MAP kinase and U0126 is an ultrapotent inhibitor of MAP kinase kinase (MEK)1/2 responsible for activation of ERK. We have confirmed that SB203580 and U0126 at 4 μmol each suppressed the activity of p38 MAP kinase and phosphorylative activation of ERK1/2, respectively (data not shown). U0126 blocked the NF-κB DNA binding activity, whereas the p38 MAP kinase inhibitor SB203580 failed to (Figure 9A). This result suggests that the activation of NF-κB occurs via the ERK-dependent pathway in mouse skin. Many studies have revealed a close association between the ERK activity and phosphorylation and degradation of IκB protein, which leads to increased nuclear translocation of NF-κB and subsequent DNA binding in various cell systems (30–33). To investigate the possible role of ERK in IκB phosphorylation and degradation, we measured the levels of phosphorylated IκBα with and without U0126 pre-treatment. As illustrated in Figure 9B, U0126 blocked the phosphorylation of IκBα, thereby suppressing degradation of this inhibitory protein. To further investigate the possible involvement of ERK in the signaling pathway mediating COX-2 induction, we examined the effect of U0126 on the TPA-induced COX-2 expression. As shown in Figure 9C, U0126 at 4 μmol almost completely abrogated COX-2 induction in TPA-treated mouse skin. These data suggest that ERK plays a central role in intracellular signaling cascades mediating TPA-induced COX-2 expression in mouse skin.

Discussion

In recent years considerable efforts have been made to develop chemopreventive agents that could inhibit, retard or reverse the multi-stage carcinogenesis (34). Chemoprevention has become an emerging area of cancer research that, in addition to providing a practical approach to identifying potentially useful inhibitors of malignant transformation, affords opportunities to study the mechanisms of anticarcinogenesis (34). Chemopreventive agents can act in any stages of carcinogenesis, i.e. initiation, promotion or progression. The intervention of cancer in the promotion stage, however, seems to be most appropriate and practical since tumor promotion is a reversible event, which requires repeated and prolonged exposure to promoting agents (35). Tumor promotion is closely linked to inflammation and oxidative stress (35,36), and it is hence
Curcumin is likely to contribute to both anti-inflammatory and antioxidative activities as act as antitumor promoters as well.

Curcumin, a naturally occurring anti-inflammatory and anti-oxidant compound, has been shown to inhibit experimentally induced tumorigenesis in several animal models, including 7,12-dimethylbenz[a]anthracene-initiated and TPA-promoted skin tumors, benzo[a]pyrene-induced forestomach tumors, and azoxymethane-induced intestinal tumors in mice (18,19,37–39). There is a growing body of compelling evidence that targeted inhibition of COX-2 expression or activity is valuable for not only alleviating inflammation, but preventing cancer. Therefore, agents that interfere with the intracellular signaling mechanisms governing the transcription of COX-2 are considered to be potential chemopreventives. Treatment of several human gastrointestinal cell lines with curcumin suppressed expression of COX-2 protein and mRNA as well as PGE2 production induced by TPA (40). Since chronic inflammation predisposes to malignancy (41), the inhibition of COX-2 by curcumin is likely to contribute to both anti-inflammatory and chemopreventive effects this phytochemical exerts. While the majority of non-steroidal anti-inflammatory drugs inhibit the catalytic activity of COX-2, our results indicate that curcumin can inhibit expression of the COX-2 induced by the typical tumor promoter TPA in mouse skin. These data suggest that previously reported chemopreventive properties of curcumin against mouse skin carcinogenesis are attributable in part to its inhibition of expression of COX-2 as well as its catalytic activity. TPA treatment can induce or activate a wide array of genes and their protein products. Like COX-2, elevated expression of inducible nitric oxide synthase (iNOS) and/or its catalytic activity has been observed in several human malignancies as also in chemically induced tumors in experimental animals (42–44). We have found that topically applied curcumin abrogated iNOS expression in TPA-stimulated mouse skin (K.-S.Chun et al., unpublished observation). Besides aforementioned pro-inflammatory enzymes, curcumin may also target other molecules such as NAD(P)H:oxidoreductase (45) and ornithine decarboxylase (20,26) in exerting its chemopreventive activity. Thus, it is unlikely that COX-2 is the only target for chemoprevention by curcumin in mouse skin.

Control of \textit{cox}-2 induction involves a complex array of regulatory factors including NF-\textit{kb} (12,13). Curcumin blocked tumor promoter-mediated NF-\textit{kb} transactivation by inhibiting the NF-\textit{kb}-inducing kinase (NIK)/IkB kinase (IKK) signaling complex, probably at the level of IKK\textalpha/\beta (46). When the effect of curcumin on NF-\textit{kb} activation by TPA was examined in mouse skin, pre-treatment of curcumin was found to be most effective in terms of inhibiting NF-\textit{kb} DNA binding, whereas co- or post-treatment caused a weaker effect. The inhibitory effect of curcumin on NF-\textit{kb} activation by TPA was due to inhibition of IkB degradation and p65 translocation to the nucleus. Our previous study revealed that
curcumin could also suppress NF-κB activation through direct interruption of NF-κB DNA binding in TPA-pre-treated nuclear extract (47). Production of PGE₂ and 6-ketoPGF₁α in LPS-stimulated J774 macrophages was reported to be reduced by the antioxidant pyrrolidine dithiocarbamate (PDTC) and the serine protease inhibitor, N-α-p-tosyl-L-lysine chloromethylketone (TPCK), both of which are inhibitors of NF-κB (12). Suppression of the prostaglandin production by PDTC or TPCK was not mediated through direct inhibition of COX-2 activities since these NF-κB inhibitors did not influence the catalytic activity of the enzyme when added to the new media after bacterial lipopolysaccharide challenge (12). According to our previous study, topical application of PDTC resulted in dose-related suppression of TPA-induced activation of NF-κB and also caused reduced COX-2 protein expression in mouse skin (48). These data also support the notion that the naturally occurring anti-inflammatory agent curcumin inhibits TPA-induced COX-2 expression in mouse skin, possibly by blocking NF-κB activation.

Another transcription factor that plays an important role in controlling cox-2 gene is AP-1. A role of AP-1 in COX-2 induction has been demonstrated in various cell lines (49–51). In our previous data, topical application of curcumin suppressed TPA-stimulated AP-1 activation by directly blocking the binding of the pre-activated nuclear extract to the AP-1 consensus sequence (47). Therefore, it is plausible that curcummin inhibits COX-2 expression through not only inactivation of NF-κB but also other transcription factors including AP-1 in mouse skin.

The molecular signaling mechanisms involved in the induction of COX-2 as well as activation of NF-κB in response to various external stimuli have not been fully clarified. One of the most extensively investigated intracellular signaling cascades involved in pro-inflammatory responses is the MAP kinase pathway. MAP kinases regulate NF-κB activation via multiple mechanisms. A substantial body of data indicates that NF-κB activation is modulated by MAP kinase/ERK kinase-1 (MEKK1), a kinase upstream of MAP kinases (52–54). There is accumulating evidence indicating that enzymes of the MAP kinase family play a role in cox-2 gene expression. The Parke-Davis MEK inhibitor PD98059 partially blocked LPS-induced COX-2 expression in RAW 264.7 cells and also in lysophosphatidic acid-stimulated rat mesangial cells (55), which further supports the association of ERK activation with COX-2-mediated PG production. LPS-induced expression of COX-2 was blunted by SB203580, the p38 MAP kinase inhibitor, which resulted in decreased PGE₂ production in RAW 264.7 cells (56). Similar effects were observed in LPS-stimulated monocytes (57).

Although the MAP kinase signaling pathways have been extensively investigated in cultured cell lines, much less is known about the specificity of MAP kinases and extent to which they are activated during the tumor promotion in mouse skin in vivo. Topical application of TPA on the ears of CD1 mice (58) and skin of SENCAR mice (59) induced a rapid and sustained activation of ERK but not of p38 MAP kinase. Most importantly, we have found that treatment of dorsal skins of female ICR mice with TPA significantly enhanced both catalytic activities and phosphorylation of p38 MAP kinase and ERK1/2. Topically applied curcumin inhibited activities of both p38 and ERK1/2 MAP kinases in mouse skin.

To determine which MAP kinase play an important role in activation of NF-κB in mouse skin, we investigated the inhibitory effects of the ultrapotent MEK1/2 inhibitor U0126 and the p38 inhibitor SB203580 on NF-κB activation. Interestingly, only U0126 abolished the NF-κB binding activity. In another experiment, we examined an inhibitory effect of U0126 on TPA-induced COX-2 expression. U0126 almost completely abrogated TPA-induced COX-2 protein expression, which supports the idea that ERK may play an important role in the signaling pathway of TPA-induced COX-2 expression and NF-κB activation in mouse skin.

Dhawan and Richmond (30) showed that in Hs294T cells, ERK regulation of NF-κB activation involves increased IκB phosphorylation with concomitant elevation in the NF-κB DNA binding activity. An increase in phosphorylation of IκB is responsible for enhanced degradation of this inhibitory
subunit, which leads to increased nuclear localization of NF-κB and subsequent DNA binding. In our present study, U0126 inhibited the TPA-induced phosphorylation of IκBα in mouse skin. Most inhibitors of NF-κB activation, such as silymarin (60) and oleandrin (61), exert their anti-inflammatory effects through suppression of phosphorylation and degradation of IκBα. Thus, we examined whether curcumin could also block NF-κB activation by inhibiting IκBα phosphorylation. Topical application of curcumin suppressed TPA-induced IκBα phosphorylation in a dose-dependent manner. Based on these findings, we suggest that the ERK signaling pathway regulates NF-κB activation through inhibition of IκB phosphorylation. These results may explain the molecular mechanism responsible for the inhibitory effect of curcumin on NF-κB activation in TPA-treated mouse skin (Figure 10).

In conclusion, the present study demonstrates that curcumin inhibits induction of COX-2 in TPA-treated mouse skin in vivo. Since improper and abnormal over-expression of COX-2 is implicated in the pathogenesis of various types of human cancers, assessment of the effects of curcumin on COX-2 gene expression may represent a useful surrogate biomarker for the evaluation of its chemopreventive potential. Our findings that curcumin inhibits TPA-induced COX-2 expression by blocking the ERK and NF-κB signaling cascades may provide molecular basis for suppression of tumor promotion as well as inflammation exerted by this chemopreventive phytochemical in mouse skin. Although chemopreventive and chemoprotective properties of curcumin have been extensively investigated and well documented, the compound, under certain pathophysiologic conditions, may exert detrimental effects. According to a recent study by Frank and colleagues, 0.5% curcumin in the diet failed to protect Long-Evans Cinnamon rats against hepatic and renal carcinogenesis, but rather shortened the median survival time, probably due to enhanced oxidative stress induced by this phenolic in the presence of excess copper (62). Therefore, a caution should be made for intake of curcumin by patients suffering from metal storage disorders, such as Wilson’s disease or hepatitis C viral infection.

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References


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