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Curcumin Suppresses Activation of NF-κB and AP-1 Induced by Phorbol Ester in Cultured Human Promyelocytic Leukemia Cells

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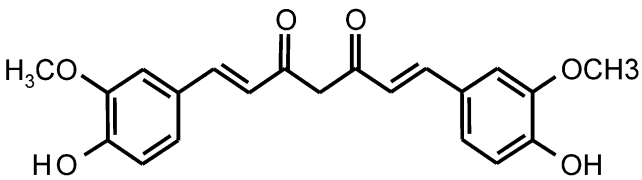
Many components that are derived from medicinal or dietary plants possess potential chemopreventive properties. Curcumin, a yellow coloring agent from turmeric (*Curcuma longa* Linn, Zingiberaceae), possesses strong antimutagenic and anticarcinogenic activities. In this study, we have found that curcumin inhibits the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced nuclear factor κB (NF-κB) activation by preventing the degradation of the inhibitory protein IκBα and the subsequent translocation of the p65 subunit in cultured human promyelocytic leukemia (HL-60) cells. Alternatively, curcumin repressed the TPA-induced activation of NF-κB through direct interruption of the binding of NF-κB to its consensus DNA sequences. Likewise, the TPA-induced DNA binding of the activator protein-1 (AP-1) was inhibited by curcumin pretreatment.

Keywords: AP-1, Curcumin, HL-60 cells, NF-κB, 12-*O*-Tetradecanoylphorbol-13-acetate

Introduction

A wide variety of naturally-occurring substances in edible plants possess substantial chemopreventive or chemoprotective activities. These are often attributed to their antioxidative and anti-inflammatory properties (Surh, 1999; Surh *et al*., 2001). Curcumin (diferuloylmethane; structure shown in Fig. 1), a yellow pigment that is derived from turmeric (*Curcuma longa* L., Zingiberaceae), is protective against a wide range of experimentally-induced tumors. These include mammary, forestomach, duodenal, skin, and colon cancers (Nagabhushan *et al*., 1992; Huang *et al*., 1997; Samaha *et al*., 1997; Verma *et al*., 1997; Huang *et al*., 1998; Dorai *et al*., 2000). The compound alleviates the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced oxidative DNA damage in mouse epidermis (Huang *et al*., 1997) and cultured mouse fibroblast cells (Shih and Lin, 1993), as well as superoxide production in macrophages (Ruby *et al*., 1995). It also suppresses the expression of phospholipase, cyclooxygenase, and inducible nitric oxide synthase that are involved in mediating inflammatory responses (Huang *et al*., 1991; Rao *et al*., 1995; Pan *et al*., 2000). Moreover, curcumin preferentially causes the apoptosis of several types of cancer cells (Jiang *et al*., 1996a,b; Kuo *et al*., 1996; Samaha *et al*., 1997; Shim *et al*., 2001). For instance, curcumin inhibits the proliferation/growth of Jurkat T leukemia cells (Sikora *et al*., 1997) and BKS-2 B lymphoma cells (Han *et al*., 1999) more effectively than the primary thymocytes and normal splenic B cells, respectively.

The nuclear transcription factor kappa-B (NF-κB) is one of the most ubiquitous transcription factors that regulates the expression of distinct sets of genes that encode proteins involved in mediating cellular proliferation, inflammatory responses, cell adhesion, etc. The functionally active NF-κB exists mainly as a hetero-dimer consisting of subunits of the Rel family, which are normally sequestered in the cytosol as an inactive complex by binding to the inhibitory protein IκB. Phosphorylation and subsequent ubiquitination of IκB upon exposure of the cells to various extracellular stimuli causes rapid degradation of this inhibitory subunit by proteosomes. The resulting free NF-κB translocates to the nucleus, where it binds to the specific κB binding sites that are located in the promoter region of the target genes, thereby controlling their



Curcumin (*Curcuma longa* Linn, Zingiberaceae)

Fig. 1. Structure of curcumin.

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expression (Sen and Packer, 1996; Barnes *et al*., 1997). NF-κB has dual functions in terms of regulating cell survival and apoptosis (Shishodia and Aggarwal, 2002). Another transcription factor, activator protein-1 (AP-1), also has a central role in controlling the eukaryotic gene expression. AP-1 is composed of Jun and Fos proteins, which interact via a leucine-zipper domain. Like NF-κB, DNA binding of AP-1 is influenced by the cellular redox state (Abate *et al*., 1990; Sen and Packer, 1996). AP-1 activation is required for TPA-stimulated cellular proliferation and transformation. It is considered to be essential in tumor promotion (Angel and Karin, 1991; Huang *et al*., 1991; Dong *et al*., 1994; Li *et al*., 1997).

Curcumin has multifaceted functions in influencing the expression of proteins that are involved in cellular proliferation, inflammation, adhesion, malignant transformation, etc. As part of our research program to elucidate the molecular mechanisms that underlie the pleiotropic actions of this chemopreventive phytochemical, we investigated its effects on the activation of two prototype eukaryotic transcription factors, NF-κB and AP-1.

Materials and Methods

Chemicals Curcumin and gentamycin were purchased from the Sigma Chemical Co. (St. Louis, USA). TPA was a product of Alexis Biochemicals (San Diego, USA). RPMI 1640 and fetal bovine serum were supplied from Gibco-BRL (Rockville, USA).

Preparation of cytosolic and nuclear extracts from HL-60 cells Unless otherwise specified, the HL-60 cells (1×10^7) were grown in a RPMI 1640 medium that was supplemented with 10% heat-inactivated fetal bovine serum and gentamycin (5 μg). Cells were treated with dimethyl sulfoxide (DMSO) or TPA (10 nM) for 1 h. When necessary, varying concentrations of curcumin were added 30 min before the TPA treatment. Curcumin was dissolved in DMSO. The proportion of DMSO in the culture media did not exceed 0.5%. The control cells were treated with the same volume of solvent. The cells were lysed by incubation at 4°C for 10 min in 400 μl of buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride (PMSF)]. The cell lysate was centrifuged for 6 min. The resulting pellet was resuspended in 100 μl of ice-cold buffer C [20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 20% (v/v) glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF], followed by incubation at 4°C for 20 min. After centrifugation, the supernatant was collected, aliquoted, and stored at −70°C (Dent and Latchman, 1993). The protein content of the final extracts was estimated using the BCA kit that was supplied from Bio-Rad (Richmond, USA), according to the manufacturers protocol.

Electrophoretic mobility shift assay (EMSA) EMSA was performed using a DNA-protein binding detection kit (Gibco-BRL; Rockville, USA) for the measurement of NF-κB binding, according to the manufacturer's protocol with minor modifications. Briefly, the double-strand NF-κB oligonucleotide was labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase and purified on a Nick column (Pharmacia Biotech Inc., Buckinghamshire, UK). The binding reaction was carried out in 25 μl of a mixture that contained 5 μl of an incubation buffer [10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 4% (v/v) glycerol, and 0.1 mg/ml sonicated salmon sperm DNA], 10 μg of nuclear extract, and 100,000 cpm of the labeled probe. To verify the specificity of NF-κB, fifty-fold excess of unlabeled NF-κB oligonucleotide was added to the reaction mixture as a competitor. For the supershift assay, 2 μg of p50 or p65 antibody was added. After a 20-min incubation at room temperature, 2 μl of 0.1% bromophenol blue was added. The samples were then electrophoresed through a 6% nondenaturing polyacrylamide gel at 150 V at room temperature. Finally, the gel was dried and exposed to x-ray film. EMSA for AP-1 was carried out in the same manner as that for NF-κB, except that the AP-1 oligonucleotide (Promega, Madison, USA) was used as a probe (Kwon *et al*., 2001).

Western blot analysis of p65 and IκBα Both the nuclear and cytosolic extracts that were prepared from the HL-60 cells were subjected to 12% SDS-polyacrylamide gel electrophoresis for measuring p65 and IκBα levels. After a 3-h transfer of the gel to the PVDF membrane (Amersham Life Sciences, Arlington Heights, USA), the blots were blocked with 5% fat-free dry milk in phosphate-buffered saline that contained 0.1% Tween-20 for 2 h at room temperature, then washed in the same buffer. The p65 protein was detected with a rabbit p65 polyclonal antibody (Santa Cruz Biotech., Santa Cruz, USA) that was diluted 1:2000. IκBα protein was detected with a rabbit IκBα polyclonal antibody (Santa Cruz Biotech, Santa Cruz, USA) that was diluted 1 : 1000. Goat anti-rabbit immunoglobulin G-conjugated horseradish peroxidase (diluted 1 : 5000) was used as a secondary antibody. The transferred proteins were visualized with an enhanced chemiluminescence (ECL) detection kit (Amersham Life Sciences, Arlington Heights, USA).

Results

TPA induces the activation of NF-κB in HL-60 cells in a dose- and time-related manner In order to investigate the effect of TPA on the activation of NF-κB, the HL-60 cells were incubated with various concentrations of TPA for 1 h, and EMSA was performed. The activation of NF-κB, as assessed in terms of its DNA binding activity, was evident when the cells were incubated with 1 nM TPA. The maximal NF-κB DNA binding was observed with 10 nM TPA (Fig. 2A). We then examined the kinetics of the TPA-induced activation of NF-κB in the same cell line after treatment with 10 nM TPA. As shown in Figure 2B, NF-κB activation peaked at 1 h, and decreased to the baseline level in 6 h.

One of the most predominant forms of NF-κB/Rel proteins is a heterodimer of p50 and p65 (Thanos and Maniatis, 1995). In order to ascertain the specificity, as well as the identity of NF-κB in HL-60 cells, EMSA was performed with excess amounts of unlabeled NF-κB oligonucleotide for the competition assay and with antibodies against typical NF-κB subunits, p50 and p65, for the super-shift assay. As illustrated

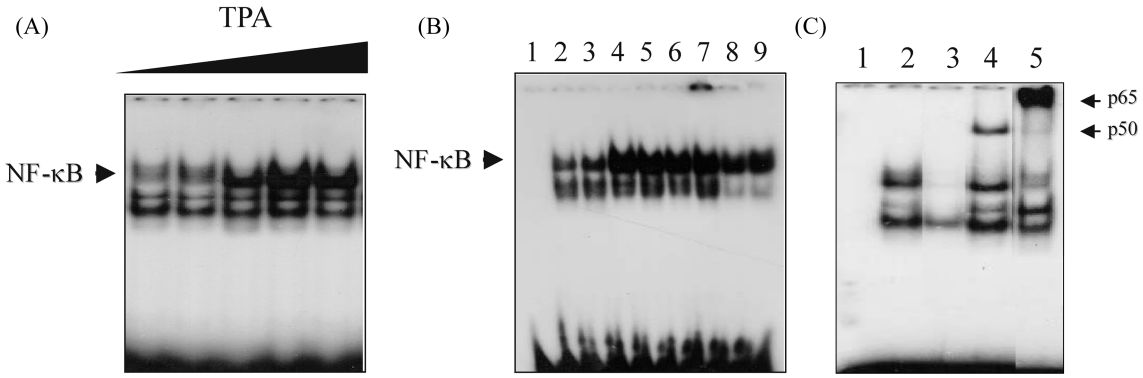

Fig. 2. (A) Concentration-dependent activation of NF-κB in HL-60 cells treated with TPA. HL-60 cells (1×10^6/ml) were treated with 0, 1, 5, 10, or 20 nM of TPA for 1 h. (B) Kinetics of NF-κB DNA binding in HL-60 cells treated with 10 nM TPA. HL-60 cells (1×10^6/ml) were treated with 10 nM TPA for various time periods. Lane 1, probe only; lane 2, DMSO control; lane 3, 0.5 h; lane 4, 1 h; lane 5, 1.5 h; lane 6, 2 h; lane 7, 4 h; lane 8, 6 h; lane 9, 8 h. Nuclear extracts (10 μg) were incubated with radiolabeled NF-κB oligonucleotide at room temperature for 20 min. (C) Competition and super-shift assays for NF-κB DNA binding. Nuclear extracts (10 μg) from TPA (10 nM)-treated HL-60 cells were incubated with 50-fold excess of unlabeled NF-κB oligonucleotide (lane 3), 2 μg of p50 antibody (lane 4), and 2 μg of p65 antibody (lane 5). Lane 1, probe only. Lane 2, nuclear extract from TPA-treated cells alone. EMSA was performed as described in Materials and Methods.

in Figure 2C, incubation of the TPA-stimulated nuclear extract with the 50-fold excess unlabeled NF-κB oligonucleotide before EMSA abolished the NF-κB DNA binding that was induced by TPA. This indicates that the retarded band that was observed in EMSA is indeed NF-κB. Incubation of TPA-stimulated nuclear extracts with an antibody against either p50 or p65 shifted the band with the higher molecular weight (Fig. 2C). These results indicate that the TPA-activated NF-κB complex in HL-60 cells exists as a heterodimer that consists of at least two typical NF-κB subunits, p50 and p65 proteins.

Curcumin inhibits TPA-induced NF-κB activation by blocking the degradation of IκBα and nuclear translocation of p65 subunits To examine whether curcumin could modulate TPA-induced NF-κB activation in the HL-60 cells, the cells were treated with various concentrations of curcumin for 30 min prior to the stimulation with 10 nM TPA for 1 h. TPA-induced NF-κB activation was significantly inhibited when the HL-60 cells were pretreated with 5 μM or 10 μM curcumin, while no significant effect was observed at 1 μM (Fig. 3A). In an attempt to elucidate the mechanism that underlies the inhibitory effects of curcumin on TPA-induced NF-κB activation, we tested whether curcumin could block the TPA-induced degradation of IκBα and nuclear translocation of p65. As shown in Figure 3B, curcumin inhibited both processes in a concentration-dependent manner.

Curcumin suppresses the activation of AP-1 induced by TPA in HL-60 cells Besides NF-κB, AP-1 also plays a crucial role in the regulation of a vast variety of genes that are responsible for cell proliferation and differentiation.

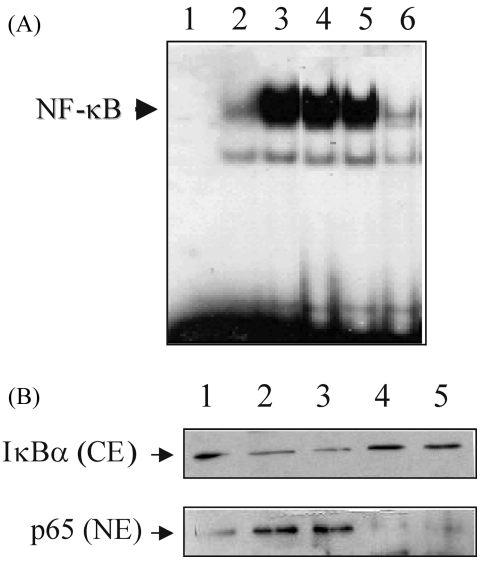

Fig. 3. (A) Effects of curcumin on TPA-induced NF-κB activation in HL-60 cells. HL-60 cells (1×10^6/ml) were treated with DMSO alone (lane 2) or 10 nM TPA for 1 h in the absence (lane 3) or presence of 1 μM (lane 4), 5 μM (lane 5), or 10 μM (lane 6) of curcumin. Experimental details are described in Materials and Methods. Lane 1 represents the probe only. (B) Effects of curcumin on the levels of IκBα in cytosol and p65 in nucleus. HL-60 cells (1×10^6/ml) were treated with DMSO or curcumin (5 or 10 μM) for 30 min prior to stimulation with TPA (10 nM). Nuclear and cytosolic fractions were prepared 1 h later and subjected to an immunoblot analysis to detect p65 and IκBα, respectively. Lane 1, DMSO as a control; lane 2, TPA alone; lane 3, 1 μM curcumin + TPA; lane 4, 5 μM curcumin + TPA; lane 5, 10 μM curcumin + TPA. Abbreviations: NE, nuclear extract; CE, cytosolic extract.

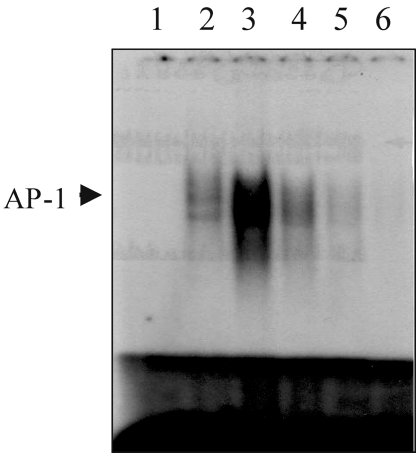

Fig. 4. Effect of curcumin on TPA-induced AP-1 activation in HL-60 cells. HL-60 cells (1×10^6/ml) were treated with 0 μM (lane 3), 1 μM (lane 4), 5 μM (lane 5), or 10 μM (lane 6) of curcumin for 30 min prior to TPA as described in the legend to Fig. 3. The control cells were treated with DMSO alone (lane 2). The nuclear extract (10 μg) was subjected to EMSA, described under Materials and Methods. Lane 1 represents the probe only.

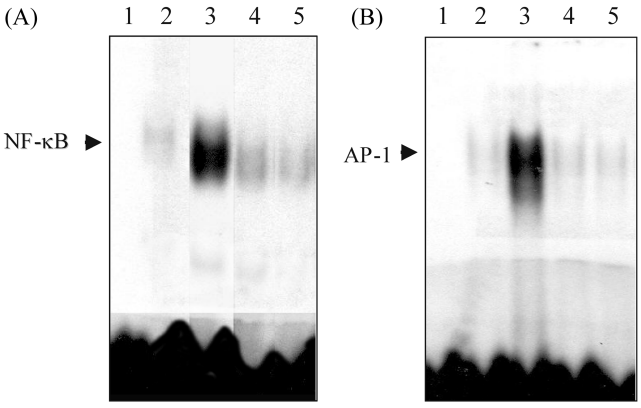

Fig. 5. Effect of curcumin on DNA-binding activity of NF-κB (A) and AP-1 (B) in HL-60 cells. HL-60 cells were treated with DMSO (lane 2) or 10 nM TPA in the absence (lane 3) or presence (lane 4) of 10 μM curcumin. For lane 5, the nuclear extracts from TPA-stimulated HL-60 cells were incubated with 10 μM curcumin for 20 min at room temperature before EMSA with an oligonucleotide probe for NF-κB or AP-1. Lane 1 represents the probe only.

Therefore, we also examined the effect of curcumin on the DNA binding of AP-1. When the HL-60 cells were preincubated with various concentrations of curcumin, TPA-induced activation of AP-1 was inhibited in a concentration-dependent manner (Fig. 4). An almost complete inhibition of AP-1 activation was achieved with curcumin at a concentration as low as 1 μM (Fig. 4).

Curcumin can also directly interrupt DNA binding of NF-κB and AP-1 to their consensus sequences In the previous experiment, curcumin was found to inhibit NF-κB activation by blocking IκBα degradation in the cytoplasm and translocation of p65 to the nucleus. Alternatively, curcumin could suppress NF-κB activation by directly interfering with the DNA binding of the functionally active subunit of NF-κB. To test this possibility, the nuclear extract that was isolated from the TPA-stimulated HL-60 cells was treated with 10 μM curcumin *in vitro*, and EMSA was conducted. Curcumin directly inhibited the ability of NF-κB to bind DNA (Fig. 5A). Likewise, the direct DNA binding capability of AP-1 was repressed by addition of curcumin to the EMSA mixture containing preactivated nuclear extracts (Fig. 5B).

Discussion

Recent advances in our understanding of the biochemical and molecular basis of inflammatory processes reveal that the transcription factors NF-κB and AP-1 are implicated in the inducible expression of a wide array of genes in response to proinflammatory cytokines, reactive oxygen species, and mitogens (Chabot-Fletcher, 1996; Sen and Packer, 1996). Since intracellular signaling pathways that lead to the activation of NF-κB and AP-1 may differ from one cell type to another, and depend on the types and duration of stimuli (Angel and Karin, 1991; Johnson *et al*., 1996), we initially tried to assess whether or not the classical tumor promoter TPA can activate NF-κB and AP-1 in the HL-60 cells, as it does in other cell lines. Our results show that TPA rapidly and transiently induces the activation of the former transcription factor. NF-κB that was activated in the TPA-stimulated HL-60 cells was found to consist of p65 and p50 subunits.

There is accumulating evidence that the activation of NF-κB is pivotal in regulating the expression of proteins that are associated with tumor promotion, which is suppressed by some chemopreventive agents, including curcumin (Singh and Aggarwal, 1995; Natarajan *et al*., 1996; Bierhous *et al*., 1997; Plummer *et al*., 1999; Surh *et al*., 2000). Therefore, we found that curcumin inhibits the activation of this transcription factor in TPA-treated HL-60 cells. Since the translocation of p65 to the nucleus is preceded by the phosphorylation and degradation of IκBα in the cytoplasm (Remacle *et al*., 1995; Thanos and Maniatis, 1995), we also determined whether or not curcumin could inhibit the nuclear translocation of p65 by preventing the degradation of the inhibitory protein IκBα. Our results reveal that curcumin does inhibit the TPA-induced NF-κB activation by preventing the degradation of IκBα and subsequent translocation of the p65 subunit, which agrees with previously reported findings (Singh *et al*., 1996; Jobin *et al*., 1999). Curcumin completely blocks the TPA- and bacterial lipopolysaccharide-induced activation of NF-κB in endothelial cells (Pendurthi *et al*., 1997). Curcumin also prevents the tumor necrosis factor-α (TNF-α)-induced NF-κB activation by inhibiting the phosphorylation and degradation of IκB in human myelomonoblastic leukemia cells (Singh and Aggarwal, 1995). According to Pendurthi *et al*. (1997), the

TNF-α-induced activation of NF-κB was suppressed by curcumin through the blockade of the degradation of IκBα and subsequent activation of p65 in endothelial cells. Likewise, curcumin inhibited the interleukin-1β-mediated phosphorylation and subsequent degradation of IκBα in rat intestinal epithelial cells, which led to the inactivation of NF-κB (Jobin *et al*., 1999). Therefore, it seems likely that curcumin regulates the upstream pathway(s) of IκBα phosphorylation, thereby preventing IκBα degradation, which results in the repression of NF-κB activation (Jobin *et al*., 1999; Pan *et al*., 2000). Additional studies will be necessary in order to determine whether or not curcumin can suppress the activation of mitogen-activated protein kinases, which in turn blocks the phosphorylation of IκBα through the down-regulation of IκB kinase. Curcumin may also affect the ubiquitination and proteosome-mediated degradation of IκBα. Evidence supports the roles of reactive oxygen intermediates as common and critical regulators in the activation of NF-κB (Eicher *et al*., 1994; Koong *et al*., 1994). In consideration of the strong antioxidant activity that curcumin retains, it is conceivable that this compound inhibits NF-κB activation by scavenging reactive oxygen species that are generated in the TPA-stimulated HL-60 cells.

AP-1 is another well-defined transcription factor that is known to be regulated by the intracellular redox state. It is involved in the inducible expression of a wide variety of genes. The functional activation of AP-1 may play a pivotal role in the signal transduction mediating TPA-induced cellular proliferation and malignant formation. The binding site of AP-1 on DNA is recognized as the TPA response element (TRE) that is present in the promoter region of several genes, including the metallothioneine IIA gene, collagenase, interleukin-2, etc. (Abate *et al*., 1990; Sen and Packer, 1996). Besides the NF-κB activation, the activation of AP-1 that is induced by TPA in HL-60 cells was also inhibited by curcumin. Curcumin also inhibits the TPA-induced TRE binding of c-Jun/AP-1 in mouse fibroblast cells (Huang *et al*., 1991). Curcumin down-regulates AP-1 activation in the human breast tumor cell line (Mehta *et al*., 1997) and the TPA-induced AP-1 activation in mouse skin (Han *et al*., 2001). Apoptotic death in dexamethasone-treated rat thymocytes and UV-irradiated Jurkat cells was suppressed by curcumin, which was accompanied by the inhibition of AP-1 DNA binding activity in these cells (Sikora *et al*., 1997). Similarly, bufalin-induced apoptosis in human leukemia U937 cells was attenuated by curcumin, which appeared to be mediated through the inactivation of AP-1 (Watabe *et al*., 1998). Curcumin was recently reported to inhibit the TNF-α-induced binding of AP-1 to DNA in bovine aortic endothelial cells (Bierhaus *et al*., 1997).

In addition to blocking the degradation of the IκBα and p65 translocation to nucleus, curcumin also directly inhibited the binding of NF-κB and AP-1 to their consensus sequences on DNA. Since curcumin has two α,β-unsaturated ketone moieties, the compound may covalently interact with the nucleophilic sites of NF-κB and AP-1 transcription factors through the Michael addition, thereby hampering their DNA binding.

In conclusion, our findings indicate that curcumin suppresses the TPA-stimulated activation of NF-κB and AP-1. This may contribute to the anti-tumor promoting properties this chemopreventive phytochemical retains.

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