Activation of Transcription Factor NF-κB Is Suppressed by Curcumin (Diferulolylmethane)*

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When activated, NF-κB, a ubiquitous transcription factor, binds DNA as a heterodimeric complex composed of members of the Rel/NF-κB family of polypeptides. Because of its intimate involvement in host defense against disease, this transcription factor is an important target for therapeutic intervention. In the present report we demonstrate that curcumin (diferulolylmethane), a known anti-inflammatory and anticarcinogenic agent, is a potent inhibitor of NF-κB activation. Treatment of human myeloid ML-1a cells with tumor necrosis factor (TNF) rapidly activated NF-κB, which consists of p50 and p65 subunits, and this activation was inhibited by curcumin. AP-1 binding factors were also found to be down-modulated by curcumin, whereas the Sp1 binding factor was unaffected.

Besides TNF, curcumin also blocked phorbol ester- and hydrogen peroxide-mediated activation of NF-κB. The TNF-dependent phosphorylation and degradation of IκBα was not observed in curcumin-treated cells; the translocation of p65 subunit to the nucleus was inhibited at the same time. The mechanism of action of curcumin was found to be different from that of protein tyrosine phosphatase inhibitors. Our results indicate that curcumin inhibits NF-κB activation pathway at a step before IκBα phosphorylation but after the convergence of various stimuli.

Members of the transcription factor NF-κB family play a central role in various responses leading to host defense, activating a rapid progression of gene expression. These transcription factors are dimeric complexes composed of different members of the Rel/NF-κB family of polypeptides. This family is distinguished by the presence of a Rel homology domain of about 300 amino acids that displays a 35 to 61% identity between various family members (for review, see Ref. 1). Although NF-κB is a ubiquitous transcription factor, it plays a critical role in the cells of the immune system, where it controls the expression of various cytokines and the major histocompatibility complex genes. The inappropriate regulation of NF-κB and its dependent genes have been associated with various pathological conditions including toxic/septic shock, graft versus host reaction, acute inflammatory conditions, acute-phase response, viral replication, radiation damage, atherosclerosis, and cancer (1, 2). No wonder NF-κB is an important target for therapeutic intervention.

Unlike other transcription factors, the NF-κB proteins and other members of the Rel family reside in the cytoplasm in an inactive state but upon activation, they are translocated to the nucleus. The nuclear translocation of Rel proteins is induced by many agents, including inflammatory cytokines (e.g. tumor necrosis factor (TNF), 1 lymphotoxin, and interleukin-1), mitogens, bacterial products, protein synthesis inhibitors, oxidative stress (H2O2), ultraviolet light, and phorbol esters (3, 4). Upon activation of NF-κB, a large number of genes are induced including various inflammatory cytokines, adhesion molecules, and Rel proteins (for review, see Refs. 3 and 4).

Curcumin (diferulolylmethane) has been shown to block many reactions in which NF-κB plays a major role. This agent is a major active component of turmeric (Curcuma longa) and it gives specific flavor and yellow color to curry. The compound has been shown to display anticarcinogenic properties in animals as indicated by its ability to inhibit both tumor initiation induced by benz(a)pyrene and 7,12-dimethylbenz(a)anthracene (5–8) and tumor promotion induced by phorbol esters (9, 10), which are known to activate NF-κB. Curcumin has also been shown to inhibit type 1 human immunodeficiency virus long terminal repeat (HIV-LTR) directed gene expression and virus replication stimulated by TNF and phorbol ester (11), which likewise require NF-κB activation. The anti-inflammatory and antioxidant properties of curcumin have been well documented (12–14). How these inhibitory responses are modulated by curcumin is not understood.

In the present report we show that curcumin is a potent inhibitor of NF-κB activation induced by various agents. The results also indicate that curcumin inhibits at a step in the signal transduction cascade of NF-κB activation that occurs before IκBα phosphorylation but after the point at which various signals transduced by different stimuli converge. This study shows that curcumin is a potential candidate for modulation of NF-κB-dependent pathological conditions.

EXPERIMENTAL PROCEDURES

Materials—Penicillin, streptomycin, RPMI 1640 medium, and fetal calf serum were obtained from Life Technologies, Inc. Curcumin, glycine, NaCl, and bovine serum albumin were obtained from Sigma, and phenylarsine oxide from Aldrich. Bacteria-derived recombinant human TNF, purified to homogeneity with a specific activity of 5 × 107 units/mg, was kindly provided by Genentech, Inc. (South San Francisco, CA). Antibody against IκBα, cyclin D1, and NF-κB subunits p50 and p65 and double-stranded oligonucleotides having AP-1 and Sp1 consensus sequences were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Lines—The cell line employed in this study was ML-1a, a human myelomonoblastic leukemia cell line kindly provided by Dr. Ken Takeda.

1 The abbreviations used are: TNF, tumor necrosis factor; DTT, dithiothreitol; DMP, 2,3-dimercaptopropanol; HIV-LTR, human immunodeficiency virus-1 long terminal repeat; PMA, phorbol 12-myristate 13-acetate; EMSA, electrophoretic mobility shift assay; TPCK, i-1-to-sylamido-2-phenethyl chloromethyl ketone; ROI, reactive oxygen intermediates.

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Activation of transcription factor NF-κB by curcumin.

**RESULTS**

In this report we examined the effect of curcumin on the activation of transcription factor NF-κB. We used human ML-1a cells for these studies because their response to NF-κB activation by various stimuli has been well characterized (21-23). The time of incubation and the concentration of the drugs used in our studies had no effect on the cell viability (data not shown).

Curcumin Inhibits TNF-dependent NF-κB Activation—ML-1a cells were preincubated for 1 h with different concentrations of curcumin followed by treatment with TNF (0.1 nM) for 30 min at 37°C. They were then examined for NF-κB activation by electrophoretic mobility shift assay. The results in Fig. 1a indicate that 40–60 μM curcumin inhibited most of the TNF response. Curcumin by itself did not activate NF-κB.

We next tested the kinetics of inhibition, incubating the cells with curcumin for 60, 30, and 10 min prior to the addition of TNF. The cells were treated with TNF for 30 min either with or without 0.1 nM TNF. – indicates time curcumin was present before the addition of TNF; 0 indicates co-incubation with TNF; and + indicates time curcumin was added after TNF. For panel c, ML-1a cells (2 × 10^6/ml) were incubated at 37°C with 50 μM curcumin for 60 min followed by treatment with 10 nM TNF for different times. After these treatments nuclear extracts were prepared and then assayed for NF-κB as described under "Experimental Procedures." The arbitrary units represent the relative amounts of the radioactivity present in respective bands.
Inhibition of NF-κB Activation by Curcumin

The concentration of TNF (10 nM) can activate NF-κB and curcumin was not effective. Both treated with curcumin (Fig. 1b). Cotreatment of cells with TNF and curcumin was not effective.

Previous studies from our laboratory have shown that a high concentration of TNF (10 nM) can activate NF-κB within 5 min and this induction is higher in its intensity than that obtained with cells using 10-fold lower concentration of TNF for longer time (23). To determine the effect of curcumin on NF-κB activation at higher TNF concentration and its effect on kinetics of TNF-mediated activation of NF-κB, curcumin-pretreated cells were exposed to 10 nM TNF for various times (Fig. 1c). In agreement with our previous results, the induction of NF-κB by 10 nM TNF was very high and occurred within 5 min. Curcumin could completely inhibit the activation of NF-κB induced by 10 nM as efficiently as it did with 0.1 nM TNF. This suggests that curcumin is a very potent inhibitor of NF-κB activation.

To show that the retarded band observed by EMSA in TNF-treated cells was indeed NF-κB we incubated the nuclear extracts with antibody to either p50 (NF-κB1) or p65 (Rel A) subunits and then carried out EMSA. The results from this experiment (Fig. 2a) show that antibodies to either subunit of NF-κB shifted the band to higher molecular weight, thus suggesting that the TNF-activated complex consisted of p50 and p65 subunits. Neither preimmune serum nor irrelevant antibody against cyclin Di had any effect on the mobility of NF-κB.

Both TPCK and herbimycin A have been shown to interfere with the binding of NF-κB to the DNA (25, 52). To determine the effect of curcumin on the binding of NF-κB to the DNA, the nuclear extracts from TNF-pretreated cells were incubated with curcumin and then EMSA was performed. The results of this experiment (Fig. 2b) show that curcumin did not modify the ability of NF-κB to bind to the DNA.

Curcumin Also Blocks Phorbol Ester- and Hydrogen Peroxide-Mediated Activation of NF-κB—Besides TNF, NF-κB activation is also induced by phorbol ester (PMA), and hydrogen peroxide (49). However, the initial signal transduction pathway induced by these agents that leads to the NF-κB activation differs. Therefore we examined the effect of curcumin on activation of the transcription factor by these various agents. The results shown in Fig. 3 indicate that curcumin completely blocked PMA and hydrogen peroxide-induced activation of NF-κB. Thus these results suggest that curcumin is a general suppressor of NF-κB activation.

Curcumin Down-modulates AP-1 but Not Sp1 Transcription Factors—Whether curcumin specifically blocks the activation of NF-κB or also affects other transcription factors was investigated. Curcumin had no effect on the Sp1 transcription factor (Fig. 4); however, DNA binding of AP-1 transcription factors was found to be down-modulated. This result is in agreement with an earlier report which showed that curcumin not only inhibits the DNA binding activity of c-jun/AP-1 binding factors but also down-modulates the level of these factors (24).

Reducing Agents Do Not Reverse the Effect of Curcumin—It has been shown that agents like TPCK that modify the sulfhydryl group in NF-κB inhibit its activation but this inhibition is prevented in the presence of DTT and DMP (25, 31). DTT and DMP can also reverse the inhibitory effect of phenylarsine oxide (a potent protein tyrosine phosphatase inhibitor) on NF-κB activation (21). To determine if the inhibitory effect of curcumin on NF-κB was reversed by these reducing agents, ML-1a cells were treated with curcumin in the presence and absence of either DTT or DMP and then examined for the activation of NF-κB by TNF. As shown in Fig. 5, DTT and DMP did not reverse the inhibition caused by curcumin but completely reversed the phenylarsine oxide-mediated inhibition. These results thus suggest that the mechanism of action of curcumin is different from that of protein tyrosine phosphatase inhibitors.

Curcumin Inhibits TNF-dependent Phosphorylation and Degradation of IκBα and Hence Translocation of p65 Subunit of NF-κB to the Nucleus—The translocation of NF-κB to the nucleus is preceded by the phosphorylation and proteolytic degradation of IκBα (for review, see Ref. 26). To determine whether the inhibitory action of curcumin was due to its effect on IκBα degradation, the cytoplasmic levels of IκBα protein were examined by Western blot analysis. IκBα was phosphorylated within 5 min of TNF treatment of ML-1a cells and then disappeared within 15 min. However, curcumin abolished both the phosphorylation (as indicated by absence of the slow mi-
We also measured the level of p65 in the cytoplasm and nucleus. As expected upon TNF treatment, the level of p65 declined in the cytoplasm with a concurrent increase in the nucleus (Fig. 6, B and C). The treatment of cells with curcumin abolished the TNF-dependent change in the nuclear and cytoplasmic p65 levels. These results show that curcumin inhibits the TNF-induced translocation of p65 to the nucleus and this is consistent with the inhibition of TNF-dependent degradation of IkBα by curcumin.

**DISCUSSION**

Curcumin is a pharmacologically safe compound with known anti-inflammatory, anticarcinogenic, and free radical scavenger properties (6, 10, 27–30). However, how curcumin carries out these functions is not very clear. We investigated curcumin’s effect on NF-κB activation because NF-κB is involved in so many of the activities that curcumin is known to block. NF-κB plays a pivotal role in cells of the immune system because it is rapidly activated by a wide variety of pathogenic signals and functions as a potent and pleiotropic transcriptional activator. Intervention in NF-κB activation may be beneficial in suppressing toxic/septic shock, graft versus host reactions, acute inflammatory reactions, HIV replication, acute phase response, and radiation damage.

Our results show that curcumin completely blocked the TNF-dependent activation of NF-κB. The activation induced by various other agents including phorbol ester and H2O2 was also inhibited by curcumin. As has been shown with other inhibitors, the effect of curcumin was not due to the chemical modification of NF-κB proteins (25, 31, 52). The inhibition of NF-κB activation was accompanied by the inhibition of p65 translocation to the nucleus and of IkBα degradation. Identifying how curcumin blocks the activation of NF-κB requires an understanding of the mechanism by which various inducers activate this important transcription factor. The role of different TNF-activated signals including acidic and neutral sphingomyelinase-generated ceramides, proteases, serine/threonine protein kinase, protein tyrosine kinase, protein tyrosine phosphatase, and superoxide radicals in the activation of NF-κB have been implicated (1, 21, 22, 32–35). Whether these signals are generated by TNF sequentially or independently of each other, however, is not understood.

All three inducers of NF-κB used in our studies are known to produce reactive oxygen intermediates (ROI). Therefore, it is possible that the effect of curcumin is through quenching of ROI production. The inhibitors of mitochondrial electron transport have been shown to impair the TNF-induced activation of NF-κB (36), thus also suggesting the role of ROI. Several additional, indirect lines of evidence suggest a role for ROI as a...
Curcumin may also block NF-κB activation by inhibiting a protein kinase. In vitro, curcumin has been shown to inhibit both serine/threonine protein kinase and protein tyrosine kinase (44). The protein kinase needed for the activation of NF-κB has not, however, been identified. Although PMA is an activator of protein kinase C, both TNF and H₂O₂ have been shown to activate both protein kinase C and protein tyrosine kinase. NF-κB activation by TNF and H₂O₂ has been shown to be blocked by inhibitors of both protein kinase C and protein tyrosine kinase (50). The role of a protein tyrosine kinase has also been implicated in NF-κB activation by ultraviolet light, lipopolysaccharide, hypoxia, and v-src (37–40, 51). We have shown that TNF-dependent activation of NF-κB is dependent on erbstatin-sensitive protein tyrosine kinase (22). Studies of Schieven et al. (43) showed that protein tyrosine kinase inhibitors block γ-irradiation-induced NF-κB activation, a stimulant thought to work through the immediate generation of ROI, which suggest that protein tyrosine kinase activation may precede ROI generation. Thus there are different early events involved in activation of NF-κB but all of them may converge to phosphorylate the IκBα which precedes its degradation and the subsequent translocation of p65 into the nucleus.

It has been shown that curcumin not only inhibits the DNA binding of c-jun/AP-1 transcription factor but it also down-modulates c-jun level by preventing its transcription (24). Our data are in agreement, but this raises the question of what other transcription factors curcumin inhibits. We found that curcumin did not inhibit the Sp1 transcription factor under the same conditions in which it inhibited NF-κB and AP-1 transcription factors. Curcumin has also been shown to inhibit TNF and phorbol ester-stimulated type 1 HIV-LTR-directed gene expression and virus replication (11), and this may be mediated through the inhibition of NF-κB. Recently it has been reported that curcumin can also inhibit nitric oxide synthase (45–47). These observations can be explained based on our results since the expression of this enzyme is NF-κB dependent. This is consistent with the observation that TPCK, a protease inhibitor that blocks NF-κB activation, also blocks the expression of nitric oxide synthase (48). TPCK, however, may exert its effect by a different mechanism than curcumin does. It has been shown that TPCK chemically modifies NF-κB, thus altering its release from IκBα (25). Curcumin, however, does not chemically modify the DNA binding properties of NF-κB.

Another level of modification that could prevent formation of p50/p65 heterodimer is down-modulation of the cytoplasmic pool of p65 subunit of NF-κB. Our results, however, show that p65 was not down-modulated by curcumin but its translocation to the nucleus was inhibited, most likely through inhibition of degradation of IκBα.

The observation that TNF-induced phosphorylation and degradation of IκBα is abolished by curcumin indicate that the step in the signal transduction pathway of NF-κB activation inhibited by this agent is at or before the phosphorylation step of NF-κB (Fig. 7). That it can inhibit NF-κB activation by diverse agents indicate that this step is after or at the step where the diverse signals converge. Overall we conclude that because of its very low pharmacological toxicity and its ability to modulate activation of NF-κB by various agents, curcumin has a high potential for use in modulating expression of genes regulated by NF-κB.

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