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Inhibition of Nuclear Factor κB by Direct Modification in Whole Cells—Mechanism of Action of Nordihydroguaiaritic Acid, Curcumin and Thiol Modifiers

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ABSTRACT. This study was set up to investigate the mechanism of four inhibitors of interleukin-1(IL-1)-α and tumor necrosis factor-(TNF)α activated nuclear factor κB (NFκB) in whole cells. The compounds fall into two classes: the first comprised two chain-breaking antioxidants, curcumin (diferulolylmethane) and nordihydroguaiaritic acid. The second class were two thiol-modifying agents, *N*-ethylmaleimide (NEM) and 2-chloro-1,3-dinitrobenzene (CDNB). Both sets of compounds were found to inhibit NFκB in tumour necrosis factor-activated Jurkat T lymphoma cells and interleukin 1-activated EL4.NOB-1 thymoma cells as determined by electrophoretic mobility shift assay using a specific NFκB DNA probe. In unstimulated cells the compounds were found to modify NFκB prior to chemical dissociation with sodium deoxycholate. They also inhibited DNA binding by NFκB when added to nuclear extracts from stimulated cells. Both of these effects occurred over a concentration range comparable to that which inhibited cytokine-activated NFκB in intact cells. All four agents were found to react directly with the p50 subunit of NFκB. However, only the antioxidants, curcumin and nordihydroguaiaritic acid (NDGA) were found to inhibit IκBα degradation activated by tumour necrosis factor-α. These results suggest that NFκB itself is susceptible to direct inhibition by a range of pharmacological agents. Furthermore, curcumin and nordihydroguaiaritic acid inhibit NFκB by interfering with IκBα degradation and reacting with p50 in the NFκB complex. These findings are likely to be useful in the attempt to develop agents which inhibit NFκB-dependent gene transcription. BIOCHEM PHARMACOL **55**;7:965–973, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. nuclear factor κB; interleukin-1; tumor necrosis factor; nordihydroguaiaritic acid; curcumin; *N*-ethylmaleimide

NFκB† is a transcription factor implicated in the expression of many genes, particularly those of the inflammatory and immune responses [1]. It is activated by a wide range of agents including lipopolysaccaride, double stranded RNA, viruses and the cytokines IL-1α and TNFα. Several of the genes induced in response to IL-1α and TNFα have NFκB binding motifs in their 5′ flanking regions including those for IL-2, IL-2 receptor α chain, serum amyloid A, the human immunodeficiency virus long terminal repeat and several cell adhesion molecules [2–6]. NFκB activation may therefore be viewed as a key event in the signaling pathway leading to changes in the expression of such genes. For these reasons, NFκB has been implicated in many pathological conditions, including AIDS, rheumatoid arthritis, septic shock and asthma [7]. The selective inhibition of NFκB may offer a target for therapeutic intervention in some of these conditions.

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† *Abbreviations:* CDNB, 2-chloro-1,3-dinitrobenzene; DOC, sodium deoxycholate; EMSA, electrophoretic mobility shift assay; IκB, inhibitor of nuclear factor for immunoglobulin κ chain in B cells; IL-1, interleukin 1; NDGA, nordihydroguaiaritic acid; NEM, *N*-ethylmaleimide; NFκB, nuclear factor κB; TNF, tumour necrosis factor.

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While there are many different forms of NFκB, the predominant form of NFκB in unstimulated cells occurs in the cytoplasm as a heterodimer of two proteins, p50 and p65 complexed to an inhibitory subunit called IκBα (see [1] for review). Upon cell stimulation this inhibitory subunit is released allowing migration of the heterodimer to the nucleus where it can bind to DNA and increase transcription. A mechanism of activation has been proposed in which IκB becomes phosphorylated and dissociates from the NFκB heterodimer [8, 9]. The proteolysis of IκB has been shown to be crucial in the activation process [8, 10]. The sites of IκB phosphorylation have been demonstrated to be serines 32 and 36. A kinase which phosphorylates these sites, entitled IκB kinase, has also been identified [11]. However, other kinases have also been shown to affect these sites either directly or indirectly such as MEKK and p90 ribosomal S6 kinase [12, 13] suggesting that IκB phosphorylation may be a site of integration of different signalling pathways.

Experiments have also been presented suggesting that reactive oxygen intermediates are a common signal which drive the activation process [14–17]. Much of the evidence is based on a pharmacological approach, whereby antioxidants such as *N*-acetylcysteine have been shown to

inhibit NFκB activation [14, 16]. In addition, one study has demonstrated that over-expression of enzymes which affect the level of oxygen radicals in cells modulate NFκB activation [17]. Apart from the oxidative activation of NFκB, other studies have somewhat paradoxically demonstrated that DNA binding by NFκB can be inhibited by the modification of a sensitive thiol at cysteine 62 in the p50 subunit [18–20]. This thiol must, therefore, be maintained in a reduced state for DNA binding to occur. It has been hypothesised that oxidative inhibition of NFκB may occur in the cytosol and that NFκB may be translocated to the nucleus as a reversibly oxidised protein [21].

In this study we investigated the mechanisms of inhibition of two naturally occurring chain-breaking anti-oxidants, curcumin (diferulolylmethane) and NDGA. We have compared their effects to two thiol-modifying agents, NEM and CDNB, in order to assess the importance of oxygen radicals and thiol reactivity in whole cells. Curcumin and NDGA inhibit the activation process but also directly modify NFκB. In contrast, NEM and CDNB appear to inhibit solely by modifying NFκB, indicating that it contains the most important target thiol in the NFκB system for both activation and DNA binding. This data demonstrates the importance of NFκB modification in whole cells suggesting that this may offer an important mechanism of inhibition.

MATERIALS AND METHODS

Materials

The murine thymoma cell line EL4.NOB-1 and the human lymphoblast Jurkat E6.1 were obtained from the European Collection of Animal Cell Cultures. RPMI 1640 and FCS were from Greiner GmbH. Penicillin-streptomycin was purchased from Life Technologies. Human recombinant TNFα was a gift from Dr. Steve Foster, Zeneca Pharmaceuticals. IL-1α was a gift from Dr. J. Saklatvala (Kennedy Institute for Rheumatology-Sunley Division). Poly (dI.dC) was from Pharmacia Biosystems. T4 polynucleotide kinase, the 22-bp oligonucleotide containing the NFκB consensus sequence (underlined) (5′-AGTTGAGGGGACTTTCC CAGGC-3′) and human recombinant p50 were purchased from Promega. Affinity-purified rabbit polyclonal antibody raised against the amino terminal domain of human IκBα/MAD-3 was supplied by Santa Cruz Biotechnology, Inc. Enhanced Chemiluminescence (ECL) reagents and anti-mouse IgG peroxidase conjugate were from Amersham International. Curcumin, NDGA, CDNB, NEM, nuclease-free bovine serum albumin, goat anti-rabbit IgG (whole molecule) peroxidase conjugate, sodium deoxycholate and all other reagents including nuclease free BSA and Nonidet P40 were supplied from Sigma.

Cell Culture

EL4.NOB-1 and Jurkat E6.1 T cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and 100 units/mL of penicillin-streptomycin. Cells were maintained at 37° in a humidified atmosphere of 5% CO_2.

Preparation of Subcellular Fractions

Subcellular fraction were prepared as described previously [22]. Briefly EL4.NOB-1 or Jurkat E6.1 cells (1×10^6/mL–5×10^6/mL) were plated into 24 well plates (16 mm diameter) 20 min before stimulation. Cells were then pretreated with inhibitors as indicated and activated with IL-1α (10 ng/mL) or TNFα (10 ng/mL) for 1 hr. Stimulation of cells was terminated by addition of ice-cold PBS and nuclear and cytosolic extracts were prepared as described previously [18]. Protein concentrations of nuclear extracts were determined by the method of Bradford [23] and the extracts were assayed immediately for NFκB activity or stored at −20°. All of the above steps were performed at 4° unless otherwise stated.

Electrophoretic Mobility Shift Assay

NFκB binding activity was determined using the EMSA [24]. Four micrograms of protein was incubated with 10,000 cpm of a [γ-^{32}P]-labelled 22-bp oligonucleotide containing the NFκB consensus sequence, 2 μg of poly(dI-dC) and binding buffer (4% glycerol, 1 mM of EDTA, 10 mM of Tris, pH 7.5, 100 mM of NaCl, 0.1 mg/mL of nuclease free BSA) at room temperature for 30 min. The oligonucleotide was labelled with [γ-^{32}P]ATP (10 mCi/mL) by T4 polynucleotide kinase. In the case of cytosolic extracts cell free activation of latent NFκB activity was required. This was done by the pretreatment of extracts (4 μg of protein) with 0.8% (w/v) sodium deoxycholate and 1.1% (w/v) Nonidet P40 for 10 min on ice prior to incubation as described above with the labelled oligonucleotide probe. All incubations were subjected to electrophoresis on a 5% native polyacrylamide gel. The gel was dried and autoradiographed at −70° overnight.

In Vitro *Experiments with Nuclear Extracts and p50*

Nuclear extracts (4 μg) from TNFα or IL-1α-stimulated cells (10 ng/mL, 1 hr) were incubated *in vitro* with increasing concentrations of curcumin, NDGA, CDNB, NEM or vehicle control for 10 min at room temperature. Binding buffer and poly dI.dC were then added, followed by a 30 min incubation with radiolabelled NFκB probe. Extracts were analysed for NFκB-DNA binding activity by EMSA.

Experiments were also carried out with recombinant human p50. p50 was in a DTT-free buffer comprising 10 mM of Tris-HCl pH 7.5, 1 M of NaCl, 10 mM of EDTA, 40% glycerol 1 mg/mL of nuclease-free BSA. Eleven ng of p50 were incubated with increasing concentrations of curcumin, NDGA, CDNB or NEM for 15 min at room temperature prior to assessing DNA binding.

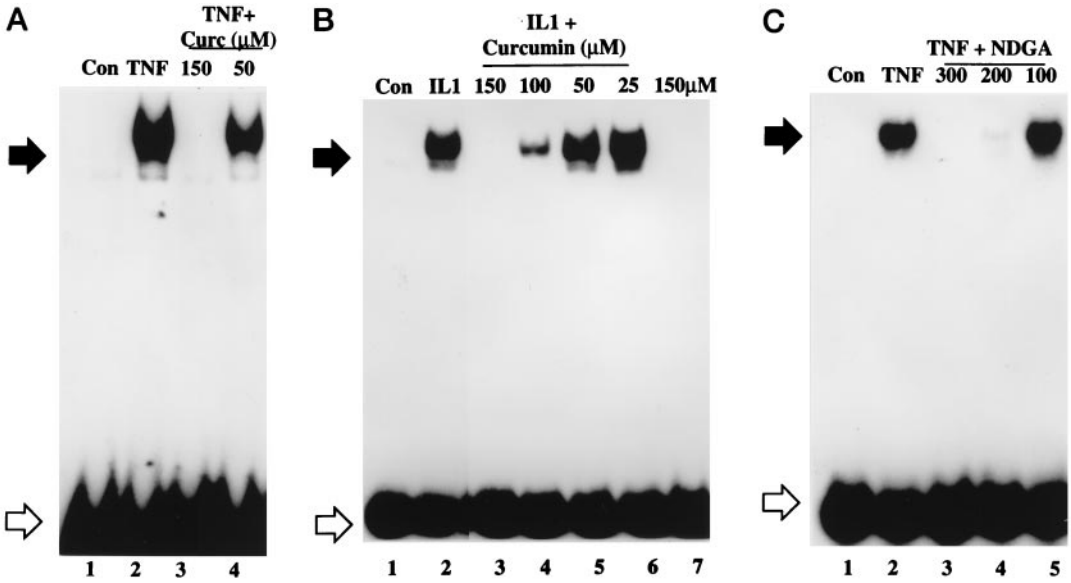

FIG. 1. Curcumin and NDGA inhibit NFκB activation by TNFα and IL-1α in Jurkat E6.1 and EL4.NOB-1 cells. Cells (5×10^6/mL) were pretreated with the indicated concentrations of curcumin (*A, Jurkat E6.1 and B, EL4.NOB-1*) or NDGA (*C, Jurkat E6.1*) for 30 min, followed by stimulation with TNFα (10 ng/mL, *A and C*) or IL-1α (10 ng/mL, *B*) for 1 hr. Nuclear extracts were prepared subsequent to stimulation and analysed for NFκB binding as described under Materials and Methods. Closed arrowheads indicate induced NFκB-DNA complexes and open arrowheads indicate unbound DNA.

Anti-IκBα Immunoblot Analysis

Cells (5×10^6–1×10^7) were preincubated in RPMI for 15 min at 37° prior to treatment with curcumin, NDGA, CDNB or NEM for 30 mins. Samples were then either unstimulated or stimulated with IL-1α (10 ng/mL) or TNFα (10 ng/mL) for 20 min. Stimulation was terminated by the addition of ice-cold PBS and the samples were centrifuged at 150 *g* for 5 min. Cell lysates were prepared and then assessed for IκBα by immunoblotting as described previously [18].

RESULTS

Curcumin and NDGA Inhibit NFκB Activated by IL-1α or TNFα

Two T cell lines, the murine thymoma EL4.NOB-1 and the human lymphoma Jurkat E6.1 were tested for inhibition of NFκB by curcumin and NDGA. We have previously shown EL4 to respond strongly to the cytokine IL-1α [18] while Jurkat are commonly used in TNFα studies. The NFκB complexes have been characterised in both cell types [18, 25]. Both cell types were pre-incubated with the agents for 30 min prior to cytokine stimulation for 1 hr. Cells were harvested and washed once in PBS. Nuclear extracts were generated and NFκB binding activity in the nuclear extracts was assayed by electrophoretic mobility shift assay. NFκB binding was activated strongly by both IL-1α and TNFα. The response to both cytokines was inhibited by curcumin and NDGA (Fig. 1). A concentration of 150 μM curcumin abolished the effect of both TNFα in Jurkat (Fig. 1A, compare lanes 2 and 3) and IL-1α in EL4 (Fig. 1B, compare lanes 2 and 3). Two hundred μM of NDGA was required to fully inhibit TNFα (Fig. 1C, compare lanes 2 and 4) or IL-1α (not shown). No toxicity was apparent at these concentrations, as judged by trypan blue exclusion and lactate dehydrogenase release assays (not shown), and neither agent on their own activated NFκB, as shown for 150 μM of curcumin in Fig. 1B (lane 7).

Curcumin and NDGA Modify NFκB Prior to Chemical Dissociation with Deoxycholate and React with NFκB In Vitro

In a previous study we demonstrated inhibition of NFκB with herbimycin A, a putative tyrosine kinase inhibitor, by chemical modification of the NFκB complex [18]. We therefore next determined whether the effect of the compounds under examination here could also be explained in terms of such modification. Two approaches were taken. The first involved treating Jurkat with either agent, preparing a cytosolic extract, releasing NFκB from IκB with the detergent deoxycholate and carrying out a DNA binding assay. Secondly, the agents were added *in vitro* to nuclear extracts from stimulated cells prior to assaying for DNA binding. In both cases the agents were found to be inhibitory. Lanes 1 and 2 in Fig. 2A and 2B demonstrate how deoxycholate reveals latent NFκB in cytosolic extracts. Prior incubation of cells with curcumin or NDGA markedly decreased the DNA binding revealed by deoxycholate *in vitro*. Cells incubated with 150 μM of curcumin (Fig. 2A lane 4) or 200–300 μM of NDGA (Fig. 2B, lanes 4 and 6) were found have less NFκB DNA binding activity released by deoxycholate in cytosolic extracts. This suggests that NDGA and curcumin are able to modify the NFκB-IκB complex in the cytosol. These concentrations were similar to those previously found to block activation in cells

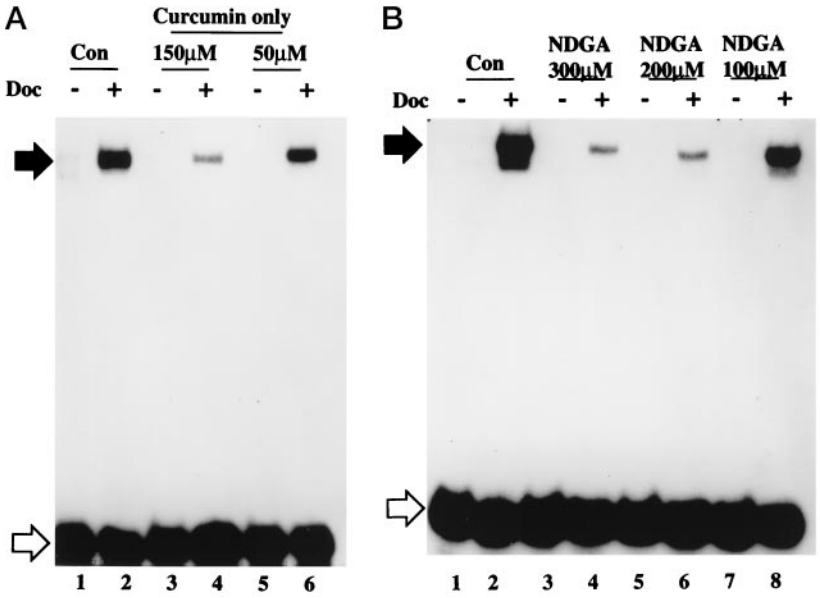

FIG. 2. Curcumin and NDGA modify cytosolic NFκB prior to chemical dissociation with deoxycholate. *A and B*, Jurkat E6.1 (5×10^6/mL) were left untreated (*A and B, lanes 1 and 2*) or were treated with the indicated concentrations of curcumin (*A*) or NDGA (*B*) for 2 hr; cytosolic extracts were prepared and left untreated (*A and B, lanes 1, 3, 5 and 7* (*B* only)) or were treated with deoxycholate and Nonidet P-40 (*A and B, lanes 2, 4, 6 and 8* (*B only*)) as described. Samples were then assayed for NFκB. Closed arrowheads indicate detergent-released NFκB-DNA complexes and open arrowheads indicate unbound DNA.

treated with TNFα or IL-1α. Figure 3A and 3B demonstrate that NDGA or curcumin added to nuclear extracts prepared from TNFα-treated Jurkat inhibited DNA binding. Again, similar concentrations to those in intact cells were effective, with 150 μM of curcumin (Fig. 1C, lane 4) and 300 μM of NDGA (Fig. 1D, lane 4) inhibiting. Vehicle (DMSO) was without effect (Fig. 3A and 3B, lanes 6 and 3 respectively). A similar result was obtained with extracts from IL-1α-treated cells (not shown). These results implied that the anti-oxidant effect of these compounds would not be the only means by which they inhibit NFκB.

The Thiol-Reactive Agents CDNB and NEM Inhibit NFκB by Direct Modification

We next compared the effect of curcumin and NDGA to the thiol-reactive agents CDNB and NEM, which would be expected to modify NFκB directly. NEM has been charac-

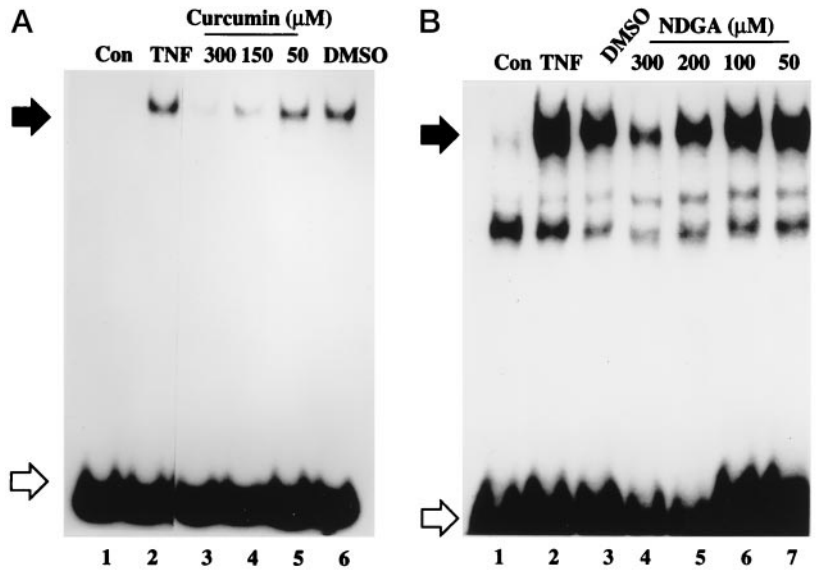

FIG. 3. NDGA and curcumin inhibit NFκB DNA binding *in vitro* *A and B*, nuclear extracts (4 μg) from TNFα-stimulated cells (10 ng/mL) (*A and B, lane 2*) were incubated *in vitro* with the indicated concentrations of curcumin (*A, lanes 3–5*) or NDGA (*B, lanes 4–7*) or vehicle carrier (DMSO) (*A, lane 6; B, lane 3*) for 15 min at room temperature. Binding buffer and poly(dI-dC) were then added, followed by a 30 min incubation with radiolabelled NFκB probe. Samples were then assayed for NFκB. Closed arrowheads indicate NFκB-DNA complexes and open arrowheads indicate unbound DNA.

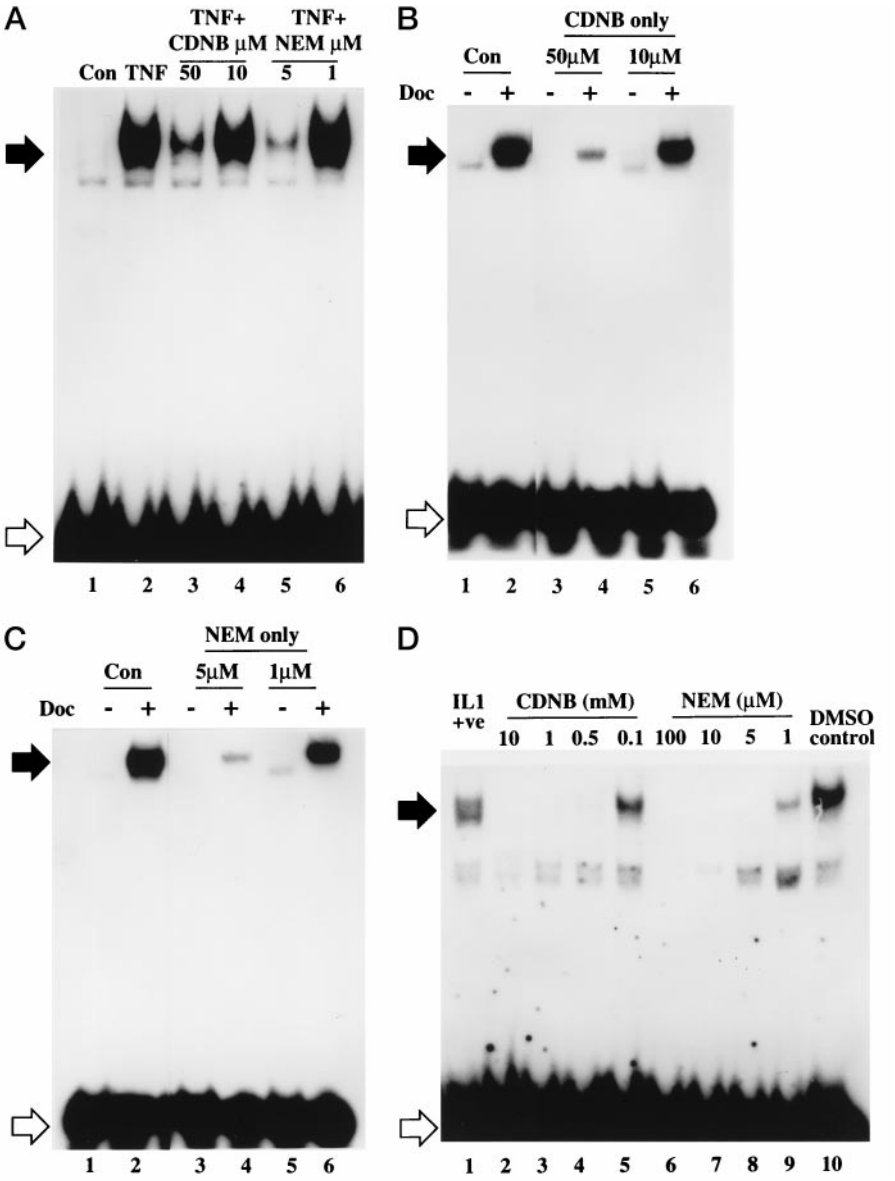

FIG. 4. CDNB and NEM inhibit NFκB activation by TNFα, modify cytosolic NFκB prior to chemical dissociation with deoxycholate and react directly *in vitro* with activated NFκB. A, Jurkat E6.1 (5×10^6/mL) were pretreated with the indicated concentrations of CDNB or NEM for 30 min, followed by stimulation with TNFα (10 ng/mL) for 1 hr. Nuclear extracts were prepared subsequent to stimulation and analysed for NFκB binding as described. Closed arrowhead indicates induced NFκB-DNA complexes and open arrowhead indicates unbound DNA. ***B and C,*** Jurkat E6.1 (5×10^6/mL) were left untreated ('Con') (***B and C, lanes 1 and 2***) or were treated with the indicated concentrations of CDNB (***B***) or NEM (C) for 2 hr; cytosolic extracts were prepared and left untreated (***B and C, lanes 1, 3 and 5***) or were treated with deoxycholate and Nonidet P-40 (***B* and C, *lanes 2, 4* and 6**) as described. Samples were then assayed for NFκB. Closed arrowheads indicate detergent-released NFκB-DNA complexes and open arrowheads indicate unbound DNA. ***D,*** nuclear extracts (4 μg) from IL-1α-stimulated cells (10 ng/mL) ('Con IL-1α + ve') (***lane 1***) were incubated *in vitro* with the indicated concentrations of CDNB (***lanes 2–5***) or NEM (***lanes 6–9***) or vehicle carrier ('DMSO control') (***lane 10***) for 15 min. Binding buffer and poly(dI-dC) were then added, followed by a 30 min incubation with radiolabelled NFκB probe. Samples were then assayed for NFκB. Closed arrowhead indicates NFκB-DNA complexes and open arrowhead indicates unbound DNA.

terised in terms of effects on NFκB *in vitro* [26] and it has been suggested that it can modify a key cysteine in the p50 subunit of NFκB at position 62 [20]. Both compounds were found to block NFκB activation by TNFα in Jurkat cells, with 50 μM of CDNB and 5 μM of NEM proving inhibitory (Fig. 4A, lanes 3 and 5). Similar to curcumin and NDGA, both CDNB and NEM inhibited NFκB as revealed by chemical dissociation with deoxycholate, as shown in Fig. 4B and 4C. Fifty μM of CDNB (Fig. 4B, lane 4) and 5 μM of NEM (Fig. 4C, lane 4) were effective, which agreed with the inhibitory concentrations in TNFα-treated cells.

Both of these compounds were also shown to react with NFκB *in vitro*. Incubation of nuclear extracts from IL-1α-treated cells with 500 μM of CDNB (Fig. 4D, lane 5) or 5 μM of NEM (Fig. 4D, lane 9) inhibited DNA binding

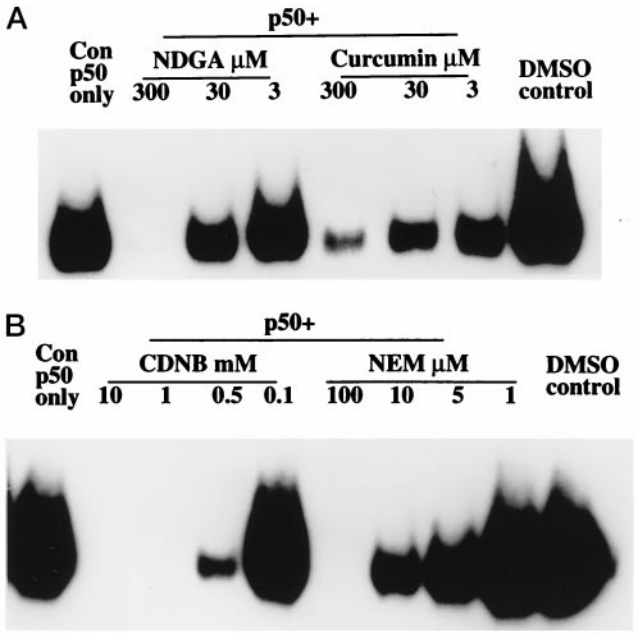

FIG. 5. p50 inhibition by curcumin, NDGA, CDNB and NEM. Eleven ng of human recombinant p50 were incubated with the indicated concentrations of compounds for 15 min at room temperature. Binding buffer and poly(dI-dC) were then added and the samples were assessed for κB binding as described. Protein-DNA complexes are shown.

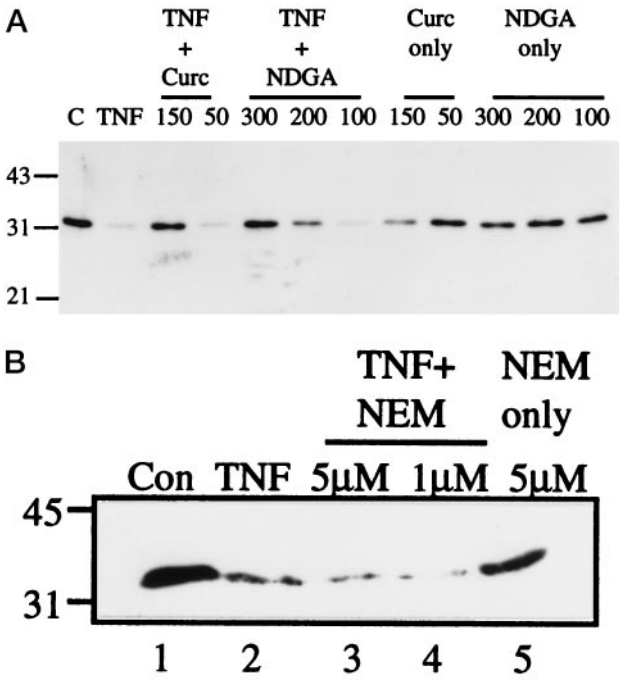

FIG. 6. Curcumin and NDGA but not NEM inhibit TNFα-stimulated IκBα degradation. Jurkat E6.1 (5 × 10^6/mL) were pretreated with either medium or the indicated concentrations of curcumin or NDGA (*A*) or NEM (*B*) for 30 min prior to stimulation with 10 ng/mL TNFα (*A, lanes 2–7; B, lanes 2–4*) or medium alone for 20 min (*A, lanes 8–12; B, lane 5*). Cell lysates were prepared according to the method outlined under Materials and Methods and subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes and probed with anti-IκBα antibody. Blots were developed as recommended for enhanced chemiluminescence. The major protein detected was of M_r 37,000 and corresponded to IκBα as indicated. Molecular weight markers are shown in kilodaltons.

relative to untreated or vehicle-treated extracts (lanes 1 and 10 respectively). A similar result was obtained with extracts from TNFα-treated cells (not shown). Again, these concentrations were comparable to those which inhibited NFκB activation by IL-1α or TNFα in intact cells.

Curcumin, NDGA and Thiol Modifiers React Directly with Human Recombinant p50

The results indicated that all four compounds could modify NFκB directly and that this was likely to be important for their inhibitory effect in intact cells. We felt it was important to determine if this could occur in the presence of only p50 or if other cytoplasmic proteins were required. To test this various doses of all four inhibitors were incubated with recombinant p50. All four compounds inhibited (Fig. 5). Figure 5A demonstrates the inhibition of p50 binding by NDGA and curcumin. Curcumin inhibited substantially at 3 μM, which was lower than the concentration required to inhibit in whole cells (Fig. 1). For NDGA the same concentration, 300 μM, inhibited p50 binding and NFκB binding in whole cells. However, with CDNB and NEM higher concentrations were required to inhibit p50 DNA binding than were required in whole cells (compare Fig. 5B and Fig. 4A). This could indicate that different NFκB complexes were modified in whole cells or may reflect an increase in effective concentration in whole cells.

Curcumin and NDGA but not NEM Inhibit TNFα-Activated IκB Degradation

Given the anti-oxidant properties of curcumin and NDGA, a possible event sensitive to these agents would be IκB degradation, as this is a key step in the NFκB activation process. We therefore tested the effect of the compounds on IκBα degradation induced by TNFα. Treatment of Jurkat for 20 min with 10 ng/mL of TNFα caused a marked degradation of IκBα (Fig. 6A, lane 2). Pretreatment of cells with 150 μM of curcumin (lane 3) or 200 μM of NDGA (lane 6) inhibited this response. Neither drug induced degradation on their own (lanes 8–12). This result was in contrast to NEM, which at 5 μM had no effect on TNFα-induced degradation (Fig. 6B, lane 3). Similarly, no effect was seen with CDNB (not shown). This result highlighted another difference between the 2 sets of compounds, indicating that curcumin and NDGA target IκB degradation and NFκB while CDNB and NEM target NFκB alone.

In summary these data establish the mechanism of action of curcumin, NDGA and the thiol modifiers NEM and CDNB as a direct interaction with NFκB itself. This modification can occur in the presence of IκB in intact cells

suggesting that NFκB is the most sensitive thiol of the activation pathway.

DISCUSSION

This study has shown that the antioxidants NDGA and curcumin inhibit NFκB by direct modification. Modification can occur in the cytosol of intact cells, where NFκB was complexed to IκB, implying that the site of modification was not obscured by IκB. We reached this conclusion from experiments with deoxycholate, in which we found that in cytosolic extracts prepared from drug-treated cells, DNA binding by NFκB as revealed by deoxycholate treatment *in vitro* was inhibited. It is also possible, although less likely, that both agents only reacted with NFκB *in vitro*, following release by deoxycholate. Both NDGA and curcumin also inhibited degradation of IκBα. This could have occurred by either limiting access of the NFκB-IκB complex to the proteosome or blocking proteosome activity directly. This would rule out the need to implicate an anti-oxidant effect by the compounds. Modification of p50 could prevent both degradation of IκB and DNA binding.

For comparison purposes we carried out similar studies with the alkylating agents CDNB and NEM. Important differences were observed. Both agents inhibited NFκB binding but neither CDNB nor NEM inhibited IκB degradation. As they are highly thiol reactive, other thiols would be potential targets for these compounds in intact cells. A recent review has suggested that a number of thiols may be a site of redox control in NFκB activation [27]. As neither compound inhibited IκB degradation however, it is likely that thiols within the activated NFκB complex are the most sensitive thiols in the activation pathway. It has been show that 2 other alkylating agents, L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) and N^a-*p*-tosyl L-lysine chloromethyl ketone (TLCK), which were originally used as serine protease inhibitors to implicate IκB degradation as a key step in NFκB activation, also react directly with NFκB [28]. These agents act by modifying histidine in the active site of serine proteases but are also likely to react with exposed cysteine residues, as in the case of cysteine 62 in p50. This would explain their direct effect on NFκB. However, since neither NEM nor CDNB inhibited IκB degradation it can be assumed that they are acting more selectively than TLCK or TPCK here, and that there are no important thiol groups on proteins leading to IκB degradation.

Another study from this laboratory identified the direct modification of NFκB by herbimycin A in EL4.NOB-1 and Jurkat [18]. Also, the use of pyrolidine dithiocarbamate and diamide causes a change in the cellular redox status that alters the oxidation state of NFκB [25]. This leads to an inhibition of NFκB-DNA binding. Herbimycin A has been shown to inhibit NFκB directly at cysteine 62 [18]. This may be the site at which NEM and CDNB modify NFκB but the site at which NDGA and curcumin inhibit is unknown. Direct modification of NFκB has been shown to inhibit gene expression. Herbimycin A inhibited IL-1α induced interleukin-2 production which requires the activity of NFκB [18]. This demonstrates that the direct modification of NFκB will inhibit cytokine expression. Further, both NDGA and curcumin have been shown to inhibit human immunodeficiency virus long terminal repeat-directed gene expression [29, 30] which is NFκB-dependent. The ability of curcumin to inhibit NFκB may explain some of its identified anti-inflammatory properties [31], as genes such as cyclooxygenase type 2 and the inducible form of nitric oxide synthase are NFκB regulated [32, 33]. NDGA inhibition of NFκB is likely to be the basis for its inhibitory effect on IL2 production, as NFκB is important for IL2 gene regulation [2].

NDGA and curcumin have been shown in previous studies to inhibit NFκB [30, 34] and were identified in this study following a screen of antioxidant compound performed to test the hypothesis that NFκB is activated by a mechanism involving oxygen radicals or oxidative stress. However in our system their inhibitory action seems to correlate strongly with their direct modification of NFκB. This and previous studies [22, 24] suggest that in our system the oxidative stress model of NFκB activation is unlikely to be conserved in all cell types. For example hydrogen peroxide did not induce NFκB in EL4.NOB-1 cells and very high concentrations were required to activate in Jurkat cells [22]. This data suggests that in T-cells, or perhaps in IL-1α signalling, reactive oxygen intermediates are unlikely to be involved. Singh and Aggarwal also investigated whether curcumin could modify NFκB modification *in vitro* [34]. Unlike our study, they found no effect, although they only tried concentrations up to 20 μM. Their experiments were performed in ML-1a leukemic cells. It is therefore possible that the differences are due to cell type, with NFκB in ML-1a cells being less susceptible to modification than in EL4 or Jurkat. Although curcumin and NDGA modified NFκB in our study, their effect on IκBα degradation may have been due to an anti-oxidant effect. In a previous study however, we found that *N*-acetylcysteine did not inhibit NFκB activated by IL-1α in EL4.NOB-1 [22]. This may reflect different anti-oxidant properties of NDGA and curcumin over *N*-acetylcysteine. It is possible that the compounds are more effective than *N*-acetylcysteine at inhibiting lipid peroxidation. Both agents are well known potent inhibitors of lipid peroxidation [35, 36] and it has been suggested that lipid peroxides may be more important than H_2O_2 in the activation of NFκB [37]. We reached a similar conclusion in a recent study carried out in the endothelial cell line ECV304 which compared *N*-acetylcysteine and PDTC [38]. *N*-acetylcysteine could not prevent lipid peroxidation and did not inhibit NFκB activation by TNFα. PDTC was inhibitory however, because of its ability to block iron-dependent lipid peroxidation. Another possible explanation for the differences between *N*-acetylcysteine and curcumin or NDGA is that the inhibitory effect on IκB degradation is a product of the direct interactions between these compounds and NFκB.

Recently a direct link between MEKK1 and the IκBα kinase has been identified [12]. MEKK1 may be controlled in a very complex fashion [39] such as rac, cdc42 and PAK. The small G-protein rac has also been suggested to be involved in NFκB regulation via the production of reactive oxygen intermediates [40]. This could explain how reactive oxygen intermediates can activate NFκB in some cell systems. However if different cytokines activate different small G-proteins leading to MEKK and the IκB kinase activation, some may involve reactive oxygen intermediates whereas others may not. Direct inhibition of NFκB could offer a mechanism for inhibition despite many different signaling pathways.

The concentration of NEM used in this study was lower than any of the other inhibitors suggesting that if another step was also inhibited by curcumin and NDGA it is not as sensitive to inhibition as NFκB itself. Herbimycin A was also shown to inhibit at a similar concentration to NEM (2 μM) [18]. This suggests that inhibition of NFκB by direct modification may offer the most important and selective mechanism of inhibition perhaps by engineering a compound based on herbimycin A. Compounds that alter the redox state of the cell, such as PDTC and diamide have been shown to trigger a change in the thiol status of NFκB which can be reversed in vitro. However this study shows that NFκB can be modified independent of this redox state. The effect of NEM on NFκB occurs in the absence of significant changes in total glutathione (not shown) which suggests that NFκB is more sensitive to thiol modification than the redox system of the whole cell.

In summary, this study indicates that the NFκB system can be interfered with at two points: inhibition of the activation process (as detected by monitoring IκB degradation) and direct modification of NFκB itself. The main target for alkylating agents appears to be NFκB. Dietary chain-breaking anti-oxidants inhibit activation and also appear capable of modifying NFκB. These compounds and the others identified, TPCK and herbimycin suggest that modification of NFκB may be in important mechanism of inhibition. Furthermore, as all of these agents also inhibit NFκB-driven gene expression, they may prove useful in attempts to design specific inhibitors of NFκB which would have potential as anti-inflammatory agents.

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References

1. Baldwin S Jr, The NFκB and the IκB proteins: New discoveries and insights. *Annu Rev Immunol* **14:** 649–681, 1996.
2. Ballard DW, Bohnlein E, Lowenthal JW, Wano Y, Franza BR and Greene WC, HTLV-I tax induces cellular proteins that activate the κB element in the IL-2 receptor α gene. *Science* **241:** 1652–1655, 1988.
3. Cybulsky MI, Fries JW, Williams AJ, Sultan P, Eddy R, Byers M, Shows T, Gimbrone MA Jr. and Collins T, Gene structure, chromosomal location, and basis for alternative mRNA splicing of the human VCAM1 gene. *Proc Natl Acad Sci USA* **88:** 7859–7863, 1991.
4. Edbrooke MR, Burt DW, Cheshire JK and Woo P, Identification of *cis*-acting sequences responsible for phorbol ester induction of human serum amyloid A gene expression via a nuclear factor κB-like transcription factor. *Mol Cell Biol* **9:** 1908–1916, 1989.
5. Hoyos B, Ballard DW, Bohnlein E, Siekevitz M and Greene WC, Kappa B-specific DNA binding proteins: role in the regulation of human interleukin-2 gene expression. *Science* **244:** 457–460, 1989.
6. Nabel G and Baltimore D, An inducible transcription factor activates expression of human immunodeficiency virus in T cells. *Nature* **326:** 711–713, 1987.
7. Baeuerle PA and Henkel T, Function and activation of NFκB in the immune system. *Annu Rev Immunol* **12:** 141–179, 1994.
8. Beg AA, Finco TS, Nantermet PV and Baldwin AS Jr, Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of IκBα: a mechanism for NFκB activation. *Mol Cell Biol* **13:** 3301–3310, 1993.
9. Cordle SR, Donald R, Read MA and Hawiger J, Lipopolysaccharide induces phosphorylation of MAD3 and activation of *c*-Rel and related NFκB proteins in human monocytic THP-1 cells. *J Biol Chem* **268:** 11803–11810, 1993.
10. Henkel T, Machleidt T, Alkalay I, Kronke M, Ben Neriah Y and Baeuerle PA, Rapid proteolysis of IκBα is necessary for activation of transcription factor NFκB. *Nature* **365:** 182–185, 1993.
11. Chen ZJ, Parent L and Maniatis T, Site-specific phosphorylation of IκBα by a novel ubiquitination-dependent protein kinase activity. *Cell* **84:** 853–862, 1996.
12. Lee FS, Hagler J, Chen ZJ and Maniatis T, Activation of the IκBα kinase complex by MEKK1, a kinase of the JNK pathway. *Cell* **88:** 213–222, 1997.
13. Schouten GJ, Vertegaal ACO, Whiteside ST, Israel A, Toebes M, Dorsman JC, van der Eb, AJ and Zantema A, IκBα is a target for the mitogen-activated 90 kDa ribosomal S6 kinase. *EMBO J* **16:** 3133–3144, 1997.
14. Schreck R, Rieber P and Baeuerle PA, Reactive oxygen intermediates as apparently widely used messengers in the activation of the NFκB transcription factor and HIV-1. *EMBO J* **10:** 2247–2258, 1991.
15. Schreck R, Meier B, Mannel DN, Droge W and Baeuerle PA, Dithiocarbamates as potent inhibitors of nuclear factor κB activation in intact cells. *J Exp Med* **175:** 1181–1194, 1992.
16. Suzuki YJ, Aggarwal BB and Packer L, Alpha-lipoic acid is a potent inhibitor of NFκB activation in human T cells. *Biochem Biophys Res Commun* **189:** 1709–1715, 1992.
17. Schmidt KN, Amstad P, Cerutti P and Baeuerle PA, Identification of hydrogen peroxide as the relevant messenger in the activation pathway of transcription factor NFκB. *Adv Exp Med Biol* **387:** 63–68, 1996.
18. Mahon TM and O'Neill LA, Studies into the effect of the tyrosine kinase inhibitor herbimycin A on NFκB activation in T lymphocytes. Evidence for covalent modification of the p50 subunit. *J Biol Chem* **270:** 28557–28564, 1995.
19. Matthews JR, Kaszubska W, Turcatti G, Wells TN and Hay RT, Role of cysteine 62 in DNA recognition by the P50 subunit of NFκB. *Nucleic Acids Res* **21:** 1727–1734, 1993.
20. Matthews JR, Wakasugi N, Virelizier JL, Yodoi J and Hay RT, Thioredoxin regulates the DNA binding activity of NFκB by reduction of a disulphide bond involving cysteine 62. *Nucleic Acids Res* **20:** 3821–3830, 1992.
21. Galter D, Mihm S and Droge W, Distinct effects of glutathione disulphide on the nuclear transcription factor κB and the activator protein-1. *Eur J Biochem* **221:** 639–648, 1994.

22. Brennan P and O'Neill LA, Effects of oxidants and antioxidants on nuclear factor κB activation in three different cell lines: evidence against a universal hypothesis involving oxygen radicals. *Biochim Biophys Acta* **1260:** 167–175, 1995.
23. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
24. Stylianou E, O'Neill LA, Rawlinson L, Edbrooke MR, Woo P and Saklatvala J, Interleukin 1 induces NFκB through its type I but not its type II receptor in lymphocytes. *J Biol Chem* **267:** 15836–15841, 1992.
25. Brennan P and O'Neill LA, 2-mercaptoethanol restores the ability of nuclear factor κB (NFκB) to bind DNA in nuclear extracts from interleukin 1-treated cells incubated with pyrollidine dithiocarbamate (PDTC). Evidence for oxidation of glutathione in the mechanism of inhibition of NFκB by PDTC. *Biochem J* **320:** 975–981, 1996.
26. Toledano MB and Leonard WJ, Modulation of transcription factor NFκB binding activity by oxidation-reduction *in vitro*. *Proc Natl Acad Sci USA* **88:** 4328–4332, 1991.
27. Droge W, Schulze-Osthoff K, Mihm S, Galter D, Schenk H, Eck HP, Roth S and Gmunder H, Functions of glutathione and glutathione disulfide in immunology and immunopathology. *FASEB J* **8:** 1131–1138, 1994.
28. Finco TS, Beg AA and Baldwin AS Jr, Inducible phosphorylation of IκBα is not sufficient for its dissociation from NFκB and is inhibited by protease inhibitors. *Proc Natl Acad Sci USA* **91:** 11884–11888, 1994.
29. Li CJ, Zhang LJ, Dezube BJ, Crumpacker CS and Pardee AB, Three inhibitors of type 1 human immunodeficiency virus long terminal repeat-directed gene expression and virus replication. *Proc Natl Acad Sci USA* **90:** 1839–1842, 1993.
30. Israel N, Gougerot-Pocidalo MA, Aillet F and Virelizier JL, Redox status of cells influences constitutive or induced NFκB translocation and HIV long terminal repeat activity in human T and monocytic cell lines. *J Immunol* **149:** 3386–3393, 1992.
31. Mukhopadhyay A, Basu N, Ghatak N and Gujral PK, Anti-inflammatory and irritant activities of curcumin analogues in rats. *Agents Actions* **12:** 508–515, 1982.
32. Appleby SB, Ristimaki A, Neilson K, Narko K and Hla T, Structure of the human cyclo-oxygenase-2 gene. *Biochem J* **302:** 723–727, 1994.
33. Geller DA, Nussler AK, Di Silvio M, Lowenstein CJ, Shapiro RA, Wang SC, Simmons RL and Billiar TR, Cytokines, endotoxin, and glucocorticoids regulate the expression of inducible nitric oxide synthase in hepatocytes. *Proc Natl Acad Sci USA* **90:** 522–526, 1993.
34. Singh S and Aggarwal BB, Activation of transcription factor NFκB is suppressed by curcumin (diferuloylmethane). *J Biol Chem* **270:** 24995–25000, 1995.
35. Robinson TW, Sevanian A and Forman HJ, Inhibition of arachidonic acid release by NDGA and its antioxidant action in rat alveolar macrophages and Chinese hamster lung fibroblasts. *Toxicol Appl Pharmacol* **105:** 113–122, 1990.
36. Sreejayan RMA, Curcuminoids as potent inhibitors of lipid peroxidation. *J Pharm Pharmacol* **46:** 1013–1016, 1994.
37. Flohe L, Brigelius-Flohe R, Saliou C, Traber M and Packer L, Redox regulation of NFκB activation. *Free Radic Biol Med* **22:** 1115–1126, 1997.
38. Bowie AG, Moynagh PN and O'Neill LAJ, Lipid peroxidation is involved in the activation of NFκB by TNF but not IL1 in the human endothelial cell line ECV304. *J Biol Chem* **272:** 25941–25950, 1997.
39. Xu S, Robbins DJ, Christerson LB, English JM, Vanderbilt CA and Cobb MH, Cloning of rat MEK kinase 1 cDNA reveals an endogenous membrane-associated 195-kDa protein with a large regulatory domain. *Proc Natl Acad Sci USA* **93:** 5291–5295, 1996.
40. Sulciner DJ, Irani K, Yu ZX, Ferrans VJ, Goldschmidt-Clermont P and Finkel T, *rac*1 regulates a cytokine-stimulated, redox-dependent pathway necessary for NFκB activation. *Mol Cell Biol* **16:** 7115–7121, 1996.