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Suppression of NFκB Activation and NFκB-Dependent Gene Expression by Tepoxalin, a Dual Inhibitor of Cyclooxygenase and 5-Lipoxygenase

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Abstract Tepoxalin, a dual inhibitor of cyclooxygenase (CO) and 5-lipoxygenase (5LO) with cytokine modifying activity, is also a potent inhibitor of the transcription factor, nuclear factor κB (NFκB). NFκB is a pleiotropic activator that is involved in the regulation of many genes whose products participate in immune or inflammatory responses. Tepoxalin inhibited in a dose related manner NFκB activation by PMA + ionomycin or H_2O_2 in Jurkat and HeLa cells. TNF-α-induced NFκB was also inhibited by tepoxalin in HeLa cells, while relatively less marked inhibition was observed in Jurkat cells. Activation of NFκB in several monocytic cell lines was also suppressed by tepoxalin. However AP-1 stimulation under the same conditions was not affected by tepoxalin. Other CO, LO inhibitors such as naproxen or zileuton did not inhibit NFκB activities. This inhibitory activity of tepoxalin was further illustrated by its suppression of NFκB regulated genes such as IL-6 in PMA stimulated human PBL and c-myc in IL-2 dependent T cell lines. Tepoxalin also blocked PMA + ionomycin-induced IκB degradation in a time-dependent fashion. The possible mechanism of tepoxalin in NFκB activation and its potential clinical application are discussed.

Key words: tepoxalin, anti-inflammation, immunosuppression, NFκB, DNA binding, transactivation, IκB, PDTC, quantitative PCR

The transcription factor NFκB [Sen and Baltimore, 1986] is involved in the regulation of many genes, especially those whose products participate in the stimulation of immune and inflammatory processes [Baeuerle, 1991; Grilli et al., 1993]. Originally identified as a heterodimer of two subunits p50 and p65 which is constitutively nuclear only in mature B cells and certain monocytic cell lines, NFκB is a posttranslationally inducible transcription factor in most other cell types studied [Baeuerle and Baltimore, 1988; Baeuerle, 1991; Grimm and Baeuerle, 1993]. In resting cells, NFκB exists predominantly as an inactive complex with an inhibitor protein, IκB, in the cytoplasm. Upon cell activation, degradation of IκB results in the release of p50-p65 dimer which can then translocate into the nucleus [Sun et al., 1993; Rice and Ernst, 1993; Beg and Baldwin, 1993] and bind its consensus element (GGGRNNYYCC) to initiate gene transcription. The key features of NFκB transcription control are that it is fast and versatile and is used in many different gene systems. It also has the important ability to carry signals from the cytoplasm into the nucleus and transactivate specific genes. Many cytokine genes and several immunoreceptors contain functional NFκB sites in their promoters [Baeuerle, 1991; Grilli et al., 1993; Grimm and Baeuerle, 1993]. More recently, it has been shown that anti-sense to p65 subunit inhibits the upregulation of Mac1, the β_2 integrin present on neutrophils and also the expression of ELAM1 and VCAM1 on human umbilical vein endothelial cells (HUVEC), thus confirming the important role of NFκB in inflammatory processes [Eck et al., 1993; Sokoloski et al., 1993].

The signal transduction pathway involved in NFκB activation remains unclear. The first enzymatic process identified to be associated with the activation is phosphorylation [Ghosh and Baltimore, 1990]. Treatment with a variety of kinases including protein kinase A or C, or heme-regulated kinase eIF-2, released *NFκB* from its

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inhibition by IκB [Shirakawa and Mizel, 1989]. More recently tyrosine kinases were postulated to be associated with the activation process especially in synergy with H_2O_2 activation [Schieven et al., 1993]. Inhibitors of PKC such as staurosporin block NFκB at very low concentrations. The involvement of serine/threonine kinases was confirmed by recent observations that transfection of an isoform of PKC or activated Raf-1 kinase can spontaneously activate NFκB [Diaz-Meco et al., 1993; Finco and Baldwin, 1993]. Recently, a cytoplasmic chymotrypsin-like protease was shown to cause rapid degradation of IκB, leading to the activation of NFκB in the presence of various inducing agents [Henkel et al., 1993].

Another extensively explored pathway is the involvement of reactive oxygen intermediates (ROI) in the mediation of NFκB activation. ROI generators such as H_2O_2 and irradiation are potent inducers of NFκB, while strong antioxidants such as pyrrolidine dithiocarbamate (PDTC) block NFκB activation by various stimulants including PMA and TNF-α [reviewed in Schreck et al., 1992a]. However, besides their potent NFκB inhibitory activity, chronic treatment with antioxidants is also known to desensitize cells for physiological regulation [Schreck et al., 1992b].

Tepoxalin [5-(4-chlorophenyl)-N-hydroxy-(4-methoxyphenyl)-N-methyl-1H-pyrazole-3-propanamide] is a dual inhibitor of 5-lipoxygenase (5LO) and cyclooxygenase (CO) synthesis with potent anti-inflammatory activity in several animal models [Argentieri et al., 1990; Wallace et al., 1991, 1993]. The inhibition of prostaglandin synthesis by tepoxalin in adjuvant arthritis rats as well as in isolated guinea pig preparations was comparable to naproxen and indomethacin. Tepoxalin shows a favorable in vivo toxicity profile in several acute, subacute, and mutagenicity studies. In acute toxicity studies, the LD_{50} of tepoxalin in mice/rats was determined to be >400 mg/kg, which is several fold higher than the doses required for biological effects. Moreover, unlike NSAIDS, tepoxalin was virtually devoid of ulcerogenic actions in the gastrointestinal tracts of experimental animals [Wallace et al., 1991], possibly because of the differential inhibition of prostaglandin synthesis in different tissues [Wallace et al., 1993].

The anti-inflammatory activity of tepoxalin was further confirmed by its unique ability to inhibit cytokine production (unpublished observations). Recently, tepoxalin was shown to be a potent suppressor of IL-2 transcription in human PBL [Zhou et al., in press]. Tepoxalin's regulatory effect on IL-2 is probably unrelated to its effect on CO or LO because other known LO, CO inhibitors have no effect on IL-2 transcription [Zhou et al., in press]. Upon further investigation, we discovered that tepoxalin is in fact a potent inhibitor of the transcription factor NFκB. In this report we discuss the profile of tepoxalin as a transcription factor inhibitor and the implications for physiological regulation.

MATERIALS AND METHODS

Cell Culture

Jurkat E6.1, HL-60 and U937 cells (American Type Tissue Culture Collection, Rockville, MD) were cultured at 37°C and 95% air:5% CO_2 in RPMI-1640 supplemented with 10% fetal calf serum (FCS) and 1% glutamine (Gibco BRL, Grand Island, NY). β-Mercaptoethanol (5 μM) was included in the media for the propagation of Jurkat cells. HeLa cells (ATCC CC12) were grown in Dulbecco's modified Eagles's medium (DMEM) containing 10% FCS and 1% glutamine. In HeLa experiments using H_2O_2, DMEM was replaced by α-minimum essential medium (α-MEM). Human breast carcinoma T47D (ATCC HTB133) cells were cultured in α-MEM containing 10% FCS, 0.1 mM nonessential amino acids, 6 μg/ml insulin, and 1% glutamine.

Oligonucleotides and Plasmid Constructs

Oligonucleotides were synthesized using an Applied Biosystems (Foster City, CA) automated DNA synthesizer. Complementary sequences were annealed, end-filled with [α-^{32}P] dGTP and [α-^{32}P]dCTP (Dupont-New England Nuclear, Boston, MA) using Klenow enzyme (Gibco BRL), column purified, and used for DNA binding studies. The sequences of the double-stranded oligonucleotides used to determine the binding of NFκB and AP-1 were as follows (binding sites are underlined). NFκB, derived from the mouse immunoglobulin kappa chain: 5'-AGCTTCAGAGGGGACTTTCCGAGAGG-3', AP-1: 5'-AGCTTTCCAAAGAGTCATCAGG-3', derived from human IL-2 promoter. The reporter plasmid p6xNFκBtk-CAT contains three copies of a sequence from the HIV-1 LTR (−105 to −80) encompassing the two κB motifs that were cloned HindIII/BamHI into pBLCAT5. pBLCAT5 [Boshart et al., 1993] contains two

copies of the polyadenylation signal of the SV40 large T encoding gene inserted upstream of the herpes simplex virus thymidine kinase promoter (HSV-tk), which serves to minimize the background transcription initiated from cryptic regulatory elements within the backbone vector. AP-1-CAT was constructed by cloning PCR amplified human collagenase promoter sequences (−527 to +50) into Xho1/HindIII-digested pBLCAT2 reporter plasmid. GRE_2tk-CAT containing two copies of the glucocorticoid/progesterone response element from tyrosine aminotransferase has been described earlier [Kazmi et al., 1993].

Electrophoretic Mobility Shift Assay (EMSA)

Jurkat, HeLa, HL-60, and U937 cells were incubated with PMA, PMA + ionomycin (Sigma Chemical Co. St. Louis, MO), human recombinant TNF-α (R & D Systems, Minneapolis, MN), or H_2O_2 (Sigma) in the presence and absence of test drug(s) as described in the figure legends. The test compounds were added 1 h prior to the stimulation of cells with NFκB-inducers. Nuclear extracts were prepared using the method of Dignam et al. [1983], with modifications. Briefly, cells were pelleted, washed once with PBS, and resuspended in two packed cell volumes of buffer A (15 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% glycerol, 2 mM DTT, 0.5 mM PMSF, 5 μg/ml aprotinin). Resuspended cells were homogenized on ice in a dounce homogenizer (30 strokes with pestle B) and the homogenates were centrifuged (7,500 rpm × 15 min at 4°C) to separate the cytoplasmic from the nuclear fractions. Nuclear fractions were resuspended in high-salt buffer C (20 mM HEPES, pH 7.9, 20% glycerol, 0.55 M NaCl, 1.5 mM $MgCl_2$, 2 mM EDTA, 2 mM DTT, 0.5 mM PMSF, 5 μg/ml benzamidine, 5 μg/ml aprotinin), incubated 30 min at 4°C with occasional mixing, and centrifuged at 17,000 rpm for 30 min at 4°C. The supernatant, representing solubilized nuclear proteins, was used for EMSA. The protein concentration in nuclear extracts was determined by the Bradford assay [Bradford, 1976].

Binding reactions were performed with 4–6 μg of protein in binding buffer (10 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM EDTA, 5% glycerol) containing 2 μg poly dI-dC, 20 μg BSA, 0.05% NP-40, and 0.1 ng [^{32}P]-labeled oligonucleotide probe in a final volume of 20 μl for 25 min at 22°C. Samples were analyzed on 4%, nondenaturing polyacrylamide gels. Dried gels were exposed to Kodak XRP film for 4–16 h at −70°C and the binding was quantified by image analysis. For the competition experiments, [^{32}P]-labeled DNA probe was mixed with a 200-fold excess of the corresponding oligonucleotides prior to the initiation of binding reactions. For supershift experiments, anti-p50 antiserum raised in our laboratory against the recombinant NFκB p50 fusion protein was used, while anti-p65 antiserum was a generous gift of Dr. Nancy Rice.

Transient Transfection and CAT Assay

Jurkat cells and T47D mammary carcinoma cells were transiently transfected with 6xNFκBtk-CAT plasmids using electroporation and DEAE-dextran methods, respectively. In either case, cells were transfected in large batches, which were then separated into different drug treatment groups. For electroporation, Jurkat cells were harvested in the log phase of growth, washed once with serum-free RPMI-1640, and resuspended in the same medium at 6×10^7 cells/ml. Two hundred and fifty microliters of the cell suspension (1.5×10^7 cells) were electroporated with 1 μg 6xNFκBtk-CAT at settings of 250 mv and 960 uFD using the Bio-Rad electroporation system (Bio-Rad, Hercules, CA). Electroporated cells were replated in the complete medium for drug treatment and reporter enzyme assays (see below). T47D cells were incubated with 5 μg 6xNFκBtk-CAT and 200 μg/ml DEAE-dextran in Ca^{++}, Mg^{++}-free PBS for 20 min at room temperature. The DNA mix was removed, cells were washed with PBS and grown in the complete medium for 16 h. Preliminary studies were carried out with AP-1-CAT and GRE_2tk-CAT reporter plasmids, transfected in CV-1 cells using the calcium phosphate method, as described earlier [Kazmi et al., 1993].

The transfected cells were stimulated with PMA (20 ng/ml), or a combination of PMA (20 ng/ml) and ionomycin (1 μM) or recombinant human TNF-α (5 ng/ml) in the presence or absence of test drugs. Details of treatment conditions for each experiment are described in the corresponding figure legend. Twenty-four hours after the stimulation, cells were harvested and extracts were prepared by rapid freeze-thawing. Protein concentration was determined by the Bradford assay [Bradford, 1976] and was used to normalize CAT enzyme activities. CAT assays

were carried out by the radiometric scintillation method [Seed and Sheen, 1988], essentially as described earlier [Kazmi et al., 1993].

Western Analysis

Jurkat cells were treated with PMA + ionomycin in the presence or absence of tepoxalin under conditions similar to EMSA experiments. Cells were subjected to mild detergent lysis after various time periods (indicated in the figure legend) and supernatants containing cytoplasmic fractions were used for protein determination [Bradford, 1976]. Proteins resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were electrophoretically transferred to nitrocellulose membrane using a transblot semidry cell (Bio-Rad). The filters were treated with the blocking buffer containing 10% nonfat dry milk in PBS for 12–16 h at room temperature. Membranes were then incubated with anti-IκB/MAD-3 antiserum (gift of Dr. W. Greene) [Sun et al., 1993], followed by the goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Sigma). Immunoblots were reacted with an enhanced chemiluminescence reagent (ECL; Amersham, Arlington Heights, IL) to detect signals.

Quantitative PCR (QPCR)

Human peripheral blood leukocytes and IL-2-dependent T cell blasts were treated with PMA + ionomycin, with or without tepoxalin or cyclosporin (CsA), and the total RNA was isolated as described earlier [Zhou et al., 1992]. QPCR with IL-6 primers was performed essentially according to our published method [Zhou et al., 1992]. The internal control of IL-6 was obtained from Clontech (Palo Alto, CA). The cDNA of c-myc was cloned by the method of RT-PCR, using the cloning strategies similar to those applied for isolating the gene for human high affinity IgE receptor [Pang et al., 1993]. The internal control cDNA for c-myc was constructed by the deletion of 286 bp from its cDNA (Sac II-Cla I fragment). The sequences for IL-6 and c-myc primers were as follows. IL-6: 5′-ATGAACTGGTTCTCCACAAGCGC and 3′-GTCAGGTCGGACTCCCGAGAAG; C-MYC: 5′-CAGCCTCCCGCGACGATGCCCC and 3′-GGAGGTCGTCTTCCACT AGGTCTGAG. The PCR amplification and quantification of DNA bands from agarose gels were performed as described [Zhou et al., 1992].

RESULTS

Tepoxalin Interferes With Activation of NFκB

We have demonstrated previously [Zhou et al., in press] that tepoxalin potently inhibited the transcription of IL-2 as well as transcription of IL-2-induced genes by a mechanism different from that of CsA. The present study was designed to further investigate the mechanism of transcriptional inhibition by tepoxalin using NFκB, a transcription factor common to the regulation of IL-2 and IL-2-inducible genes. First, the effect of tepoxalin on the stimulation of NFκB binding to its cognate response elements was investigated. κB binding proteins present in nuclear extracts of Jurkat T cells treated with PMA (20 ng/ml) and ionomycin (1 μM) were first detected by EMSA with a synthetic oligonucleotide probe containing the NFκB binding site of the kappa immunoglobulin light chain enhancer [Sen and Baltimore, 1986; Baeuerle, 1991]. As shown in Figure 1, PMA + ionomycin strongly induced a slow migrating complex representing NFκB binding. Both untreated and stimulated cell nuclear extracts also contained an intense faster migrating DNA-protein complex. To ascertain the sequence specificity of these complexes, competition assays were performed in the presence of excess unlabeled NFκB or an unrelated (AP-1) DNA competitor oligonucleotide. The NFκB competitor completely abolished the inducible upper complex, whereas the AP-1 oligonucleotide was ineffective (Fig. 1). The faster migrating complex appears to correspond to nonspecific DNA binding, as it is always present, even in unstimulated cells. However, the nonspecific DNA binding proteins were also partially reduced, perhaps due to the presence of such a high affinity DNA binding element (NFκB) in greater excess. In addition, the antisera for both p50 (NFκB1) and p65 (RelA) caused the characteristic supershift of the upper DNA complex in Jurkat E6.1 T cells, suggesting the heterodimeric (P50/P65) binding to NFκB motif.

Tepoxalin treatment to Jurkat cells blocked PMA + ionomycin-induced activation of NFκB binding in a dose-dependent fashion (Fig. 2). Jurkat cells were pretreated with various concentrations of tepoxalin for 1 h, followed by 2–3 h of coincubation with PMA + ionomycin, and nuclear extracts were subjected to EMSA, as described in Materials and Methods. The IC_{50} of tepoxalin for NFκB inhibition was 5–7 μM, while

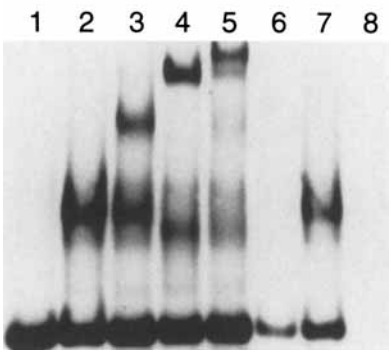

Fig. 1. EMSA analysis of NFκB-DNA binding in activated T cells. Jurkat E6.1 cells were stimulated with 50 ng/ml PMA and 1 μM ionomycin for 3 h. EMSA was performed with nuclear extracts from unstimulated (lane 1) and stimulated (lanes 2–7) cells. The NFκB complex (lane 2) was supershifted with P50 (lane 3), P65 (lane 4), and P50 + P65 (lane 5) antibodies. For supershift assays, nuclear extracts were incubated with 1 μl of specific antiserum for 15 min at room temperature prior to the addition of labeled oligonucleotide. Binding competition assays were performed with a 200-fold excess of cold NFκB (lane 6) or AP-1 (lane 7) oligonucleotides. Lane 8 represents NFκB probe in the absence of any nuclear extract.

the cell viability measured by MTT dye-exclusion assay remained over 85% throughout the course of the treatment. The nonspecific activity showed no striking variation in response to tepoxalin (Fig. 2).

Since tepoxalin is a potent dual inhibitor of CO and 5LO, we next examined the ability of two well-known inhibitors of these enzymes to influence NFκB binding in Jurkat cells. As shown in Figure 2, naproxen (CO inhibitor) and zileuton (5LO inhibitor), either alone or together did not influence the stimulation of NFκB binding in nuclear extracts. These observations provided initial indications of the unique activity of tepoxalin in the suppression of transcription factor(s) for antiinflammatory response, which may be unrelated to the general inhibition of arachidonic acid metabolism. In addition, comparison of the immunosuppressive drug, CsA (100 nM), and tepoxalin in similar EMSA experiments showed less marked effects of CsA on the induction of NFκB DNA binding activity (data not shown).

The specificity of tepoxalin was assessed by measuring DNA binding activity of AP-1, which binds complexes of jun and fos transcription factors upon PMA stimulation. Treatment of Jurkat cells with PMA + ionomycin strongly induced the binding of AP-1, which was unaffected in the presence of 10–30 μM tepoxalin (Fig. 3).

Tepoxalin Abrogates NFκB Activation in Different Cell Types

To determine whether tepoxalin-induced inhibition of NFκB represents physiological regulation of NFκB target genes, the effect of tepoxalin on NFκB induction was examined in various cell types of fibroblast (HeLa) and myeloid (HL-60 and U937) origins. PMA + ionomycin strongly induced NFκB binding in nuclear extracts of all three cell lines and tepoxalin was clearly effective in specifically suppressing the DNA binding at 5–30 μM (Fig. 4). As described earlier for Jurkat cells, the specificity of the NFκB-DNA complex was determined with excess unlabeled NFκB oligonucleotide as the competitor. *PDTC*, an anti-oxidant with NFκB inhibitory activity, caused significant inhibition of NFκB-DNA binding at 50 and 100 μM in these cell lines, as reported earlier [Schreck et al., 1992b].

Tepoxalin Interferes With NFκB Stimulation by Different Agents

A variety of extracellular stimuli activate NFκB and subsequently induce gene expression. These agents include PKC activators, cytokines, free radical generator H_2O_2, UV and γ-irradiation, and LPS [Baeuerle, 1991; Schreck et al., 1992a; Grilli et al., 1993]. Since antioxidants can block NFκB stimulation mediated by many of these activators, it is postulated that ROIs may play a key role in the signalling of NFκB-DNA binding [Schreck et al., 1992b]. However, it has also been demonstrated that ceramide, produced upon TNF-α or IL-1 treatment to Jurkat cells, can activate NFκB in PKC-independent fashion [Krasnow et al., 1991]. To further understand the mechanism of tepoxalin-induced inhibition of NFκB, we selected TNF-α and H_2O_2 as stimulants for NFκB in Jurkat and HeLa cells.

The time course of stimulation of NFκB with PMA, TNF-α, or H_2O_2 was similar, and NFκB binding activity was maximal between 45 and 240 min after activation (data not shown). Tepoxalin (10–30 μM) effectively inhibited NFκB binding induced by 200 μM H_2O_2 in both Jurkat and HeLa cell nuclear extracts (Fig. 5). PDTC treatment, at concentrations known to impair NFκB binding (50–100 μM) [Schreck et al., 1992b] also suppressed DNA binding to activated κB binding proteins in these cells (Fig. 5). Surprisingly, tepoxalin and PDTC only marginally affected NFκB binding induced by TNF-α in

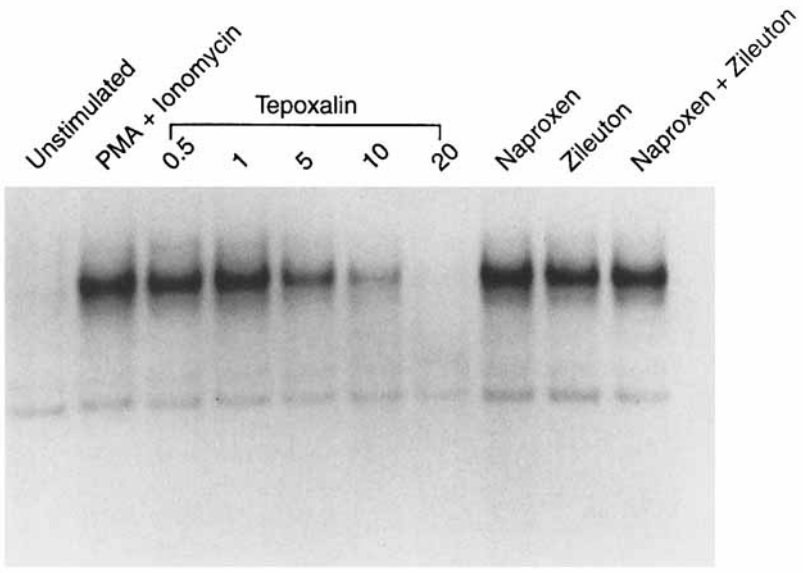

Fig. 2. Dose-dependent inhibition of NFκB-DNA binding by tepoxalin. Jurkat cells were pretreated for 1 h with different concentrations of test drugs, followed by 3 h incubation with PMA + ionomycin. [^{32}P]-labeled NFκB oligonucleotide (0.05 ng) was incubated with nuclear extracts (4 μg) from unstimulated (first lane only) and stimulated cells. Relative to the PMA + ionomycin control, tepoxalin, at increasing concentrations of 0.5, 1, 5, 10, and 20 μM, inhibited NFκB-DNA binding, whereas 20 μM naproxen, 20 μM zileuton, or 20 μM each naproxen + zileuton did not exhibit any marked inhibition of NFκB-DNA binding.

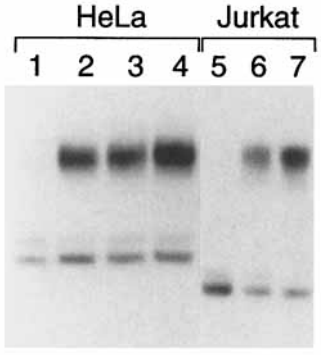

Fig. 3. Hela (lanes 2–4) and Jurkat (lanes 6–7) cells were treated with PMA + ionomycin in the presence and absence of tepoxalin and the nuclear extracts were used for EMSA with [^{32}P]-labeled AP-1 oligonucleotide, as described in Materials and Methods. Lane 1 (HeLa) and lane 5 (Jurkat) represent AP-1 DNA binding in unstimulated cells. In all treatment groups, the cell viability during the incubation period was consistently > 90%, as determined by the trypan blue dye-exclusion assay.

this clone of Jurkat cells (E6.1). However, tepoxalin (10, 20 μM) and PDTC (50, 100 μM) showed marked suppression of TNF-α-induced NFκB binding in HeLa cells (Fig. 5).

Since NFκB proteins are reported to be susceptible to oxidizing agents in cell free systems [Toledano and Leonard, 1991], we investigated the in vitro effects of tepoxalin on NFκB-DNA binding. Tepoxalin (10–30 μM) was incubated with preparations of nuclear extracts from either untreated or PMA + ionomycin-stimulated Jurkat cells prior to EMSA analysis. Tepoxalin was found to be completely ineffective in blocking NFκB binding under these conditions (data not shown), suggesting the involvement of cellular mediators in tepoxalin-induced inhibition of NFκB DNA-binding activities.

In addition, the potential free radical scavenging activity of tepoxalin was evaluated in another in vitro assay based on the spectrophotometric determination of 1,1 dipheny-2-picrylhydrazine (DPPH) absorbance in the presence of antioxidants [Smith et al., 1987]. The absorbance of 100 μM DPPH at 517 nm was reduced by known antioxidants such as PDTC and NDGA by transferring electrons to DPPH to rapidly form the colorless product in 1–2 min (Fig. 6). In contrast, tepoxalin at 20–50 μM caused a slight reduction in DPPH absorbance over the 10-min period (Fig. 6). These observations suggest that the inhibition of NFκB by tepoxalin may not be due to its free radical scavenging properties.

Tepoxalin Inhibits NFκB-Mediated Transactivation

The results described above clearly demonstrate suppression of NFκB binding to its cog-

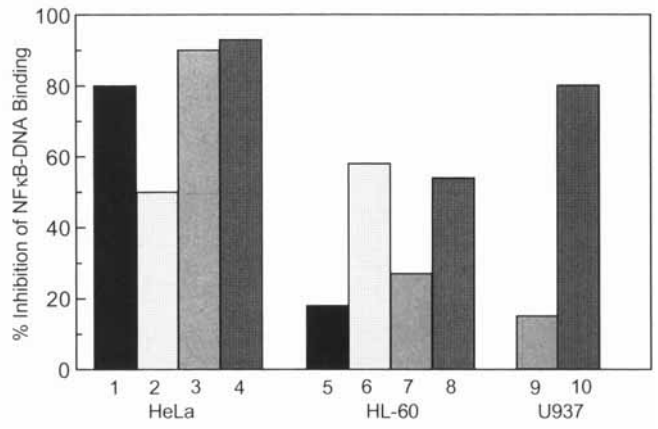

Fig. 4. Inhibition of nuclear factor binding to NFκB site by tepoxalin and PDTC in different cell types. HeLa, HL-60, and U937 cells were pretreated with tepoxalin or PDTC, followed by PMA + ionomycin, as described in Figure 2. Nuclear extracts were incubated with NFκB probe and the DNA-protein complexes retained on the gel were quantified using the Bio-Image analysis system. Data shown are percent inhibition of PMA + ionomycin-induced NFκB protein binding to kB site by tepoxalin (10, 20 μM) or PDTC (50, 100 μM). Bars 1 and 5 represent PDTC 50 μM; bars 2 and 6 represent PDTC 100 μM; bars 3, 7, and 9 represent tepoxalin 10 μM; and bars 4, 8, and 10 represent tepoxalin 20 μM. From densitometric analysis, the ratio of PMA + ionomycin stimulated NFκB DNA binding over background binding (unstimulated cells) was determined to be approximately 16-fold for HeLa cells and ranged from 4–5 fold for both HL-60 and U937 cells.

nate elements. The effect of tepoxalin on NFκB-mediated gene transactivation was next examined. 6xNFκBtk-CAT, a reporter construct consisting of three copies of NFκB binding site repeats from HIV-1 cloned in front of the CAT gene, was transiently transfected into Jurkat cells or human fibroblast (breast cancer) T47D cells.

Jurkat cells, when cultured overnight in 5% serum/RPMI-1640 prior to transfection with 6xNFκB-CAT, showed 10–12 fold induction of CAT activity by PMA + ionomycin (Fig. 7A). The NFκB-mediated transactivation was suppressed 35 and 64% by tepoxalin at 10 and 20 μM, respectively (Fig. 7A). The IC_{50} of tepoxalin for the specific inhibition of NFκB-CAT activity was calculated to be 11.5 μM, which is similar in range for the suppression of NFκB binding to enhancer sequences (Fig. 2). In addition, treatment of transfected fibroblast T47D cells with TNF-α caused 6–8 fold induction of NFκB-dependent promoter activity, which was markedly suppressed in the presence of increasing concentrations of tepoxalin (Fig. 7B). The antioxidant PDTC also inhibited NFκB-mediated transactivation (Fig. 7A), while naproxen and zileuton were ineffective (Fig. 7B).

Tepoxalin Prevents Inducible Decay of IκB

The inhibitory protein, IκB, undergoes rapid degradation upon cellular stimulation, which is coincident with the appearance of NFκB activity [Rice et al., 1992; Beg and Baldwin, 1993]. Treatment of cells with PDTC or protease inhibitors has been shown to completely prevent the inducible decay of IκB, together with the loss of NFκB activity [Schreck et al., 1992b; Rice et al., 1992; Henkel et al., 1993]. To further characterize the inhibition of NFκB activation by tepoxalin and to establish a correlation between NFκB activity and IκB disappearance, we measured the level of PMA + ionomycin-induced IκB in the presence and absence of tepoxalin. As shown in Figure 8, PMA + ionomycin caused the degradation of IκB within 20–40 min, followed by reappearance of the molecule after 1–2 h presumably due to new protein synthesis. Tepoxalin (20 μM) completely prevented the decay of IκB after 30–40 min of stimulation. These observations indicate that tepoxalin may affect the phosphorylation of IκB mediated by specific kinases.

Tepoxalin Inhibits the Transcription of IL-6 and c-myc

In order to evaluate the functional consequences of NFκB inhibition by tepoxalin, we measured the synthesis of a representative inflammatory cytokine, IL-6, and an oncogene, c-myc, whose promoters contain the κB motif [Libermann and Baltimore, 1990; Duyao et al., 1990; Donnelly et al., 1993]. QPCR [Zhou et al., 1992] was employed to determine the levels of mRNA from peripheral blood lymphocytes (PBL), which were treated with PMA + ionomy-

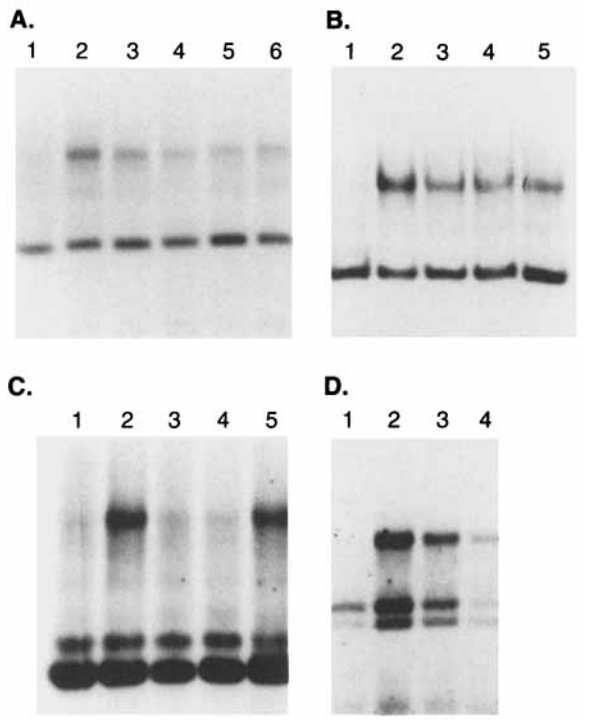

Fig. 5. Effect of tepoxalin on H_2O_2- and TNF-induced NFκB-DNA binding in Jurkat and HeLa cells. NFκB-DNA binding activity in Jurkat T cells, induced by H_2O_2 (**A, lanes 1–6**) or TNF-α (**B, lanes 1–5**), was assessed by EMSA. In both panels, lane 1 represents DNA binding in unstimulated cells. Cells were pretreated with PDTC or tepoxalin, followed by 2 h of stimulation. The treatment conditions were as follows. **A**: lane 2, H_2O_2 200 μM alone; lanes 3–4, H_2O_2 + PDTC at 50 and 100 μM, respectively; lanes 5–6, H_2O_2 + tepoxalin at 10 and 20 μM, respectively. **B**: lane 2, TNF-α (5 ng/ml) alone; lanes 3–5, TNF-α + tepoxalin at 1, 5, and 10 μM, respectively. NFκB binding in HeLa cells induced by H_2O_2 (**C, lanes 1–5**) or TNF-α (**D, lanes 1–4**). Binding conditions were similar to those described for Jurkat cells. **C**: lane 1, unstimulated; lane 2, H_2O_2 200 μM alone; lanes 3–4, H_2O_2 + tepoxalin at 10 and 20 μM, respectively; lanes 5, H_2O_2 + PDTC at 50 μM. **D**: lane 1, unstimulated; lane 2, TNF-α 5 ng/ml alone; lane 3, TNF-α + tepoxalin at 10 μM; lane 4, TNF-α + PDTC at 50 μM.

cin in the presence or absence of tepoxalin (10 μM). As shown in Figure 9A and Table I, PMA + ionomycin strongly induced IL-6 mRNA that was suppressed by 10-fold upon tepoxalin treatment. Tepoxalin (10 μM) also caused a 15.5-fold reduction in c-myc mRNA production (Fig. 9B, Table I). We have previously demonstrated a similar reduction in the transcription of IL-2 and IFN-γ genes by tepoxalin in lymphocytes [Zhou et al., in press]. Taken together, these results strongly suggest the novel antiinflammatory and immunosuppressive activities of tepoxalin could be attributed to its effects on NFκB activation.

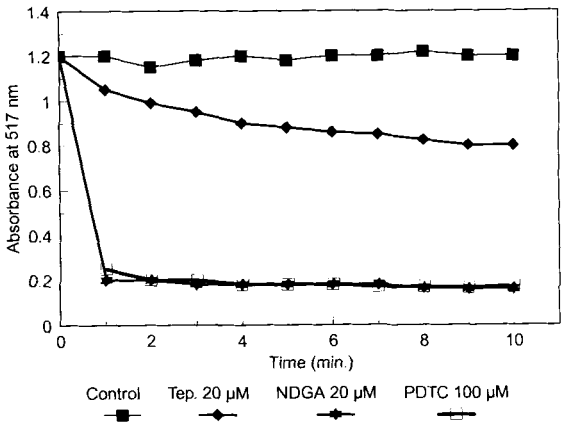

Fig. 6. Free radical scavenging activity of tepoxalin and the known antioxidants. Tepoxalin (20–50 μM) or PDTC (100 μM) or NDGA (20 μM) was mixed with 100 μM 1,1-diphenyl-2-picrylhydrozine (DPPH) and the colorometric change in absorbance at 517 nm was monitored over a 10-min period according to the method of Smith et al. [1987].

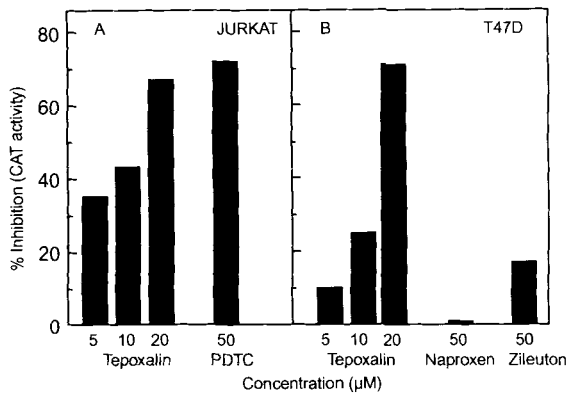

Fig. 7. Regulation of NFκB-mediated transactivation by tepoxalin. **A:** Jurkat E6.1 cells (6×10^7 cells/ml) were electroporated with 6xNFκBtk-CAT and treated with tepoxalin or PDTC at indicated concentrations for 1 h, followed by 18–20 h stimulation with PMA (20 ng/ml) and ionomycin (1 μM). **B:** T47D cells (7×10^6 cells/flask) were transfected with 6xNFκBtk-CAT using the DEAE-dextran method. After 16 h, cells were replated in 24-well plates (1.5×10^5 cells/well) and pretreated with test compounds for 1 h. Thereafter, cells were stimulated with hTNF-α (5 ng/ml) for 18–20 h. The induction of CAT activity, as a marker for the transactivation, was measured as described in Materials and Methods. Data shown are averages of replicate determinations from a representative experiment. Similar results were obtained from 3–4 experiments, each carried out in duplicate.

DISCUSSION

The present study describes an effective inhibition of NFκB activity by tepoxalin, previously identified as a dual inhibitor of CO and 5LO [Argentieri et al., 1990; Wallace et al., 1991, 1993]. The interference of tepoxalin in NFκB

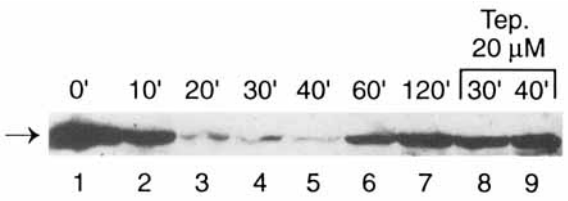

Fig. 8. Inhibition of PMA-induced degradation of IκB by tepoxalin. Jurkat E6.1 T cells were stimulated with PMA (50 ng/ml) + ionomycin (1 μM) in the presence and absence of 20 μM tepoxalin. At the indicated time points, cells were collected and cytosolic fractions were isolated by mild detergent lysis. Proteins were resolved on SDS-PAGE and detected with a peptide antiserum specific for the COOH terminus (amino acids 289 to 317) of IκB [Sun et al., 1993]. Lane 1: unstimulated cells. Lanes 2–7: cells stimulated for 10, 20, 30, 40, 60, and 120 min, respectively. Lanes 8, 9: cells pretreated with tepoxalin for 1 h, followed by PMA + ionomycin stimulation for 30 and 40 min, respectively.

activation was determined in the following ways: (1) EMSA analysis of the inhibition of the κB protein binding to NFκB enhancer sequences by several stimuli in different cell types, (2) inhibition of NFκB-mediated transactivation, (3) suppression of cytosolic IκB degradation, and (4) inhibition of the transcription of genes that have κB motifs in their promoter regions. The specificity of tepoxalin action on NFκB was supported by the lack of inhibition of PMA-induced AP-1 binding to DNA in the same extracts (Fig. 3). Tepoxalin was also ineffective in suppressing the transactivation mediated by unrelated transcription factors such as corticosteroid or progesterone receptors in CV-1 cells (data not shown).

The inhibitory activity of tepoxalin was not related to its effect on prostaglandin or leukotriene production, since potent inhibitors of CO (naproxen) and 5LO (zileuton) did not interfere with NFκB activation in both DNA binding and transactivation assays. Certain LO inhibitors, such as nordihydroguarietic acid (NDGA) and butylated hydroxyanisole (BHA) have been reported to be inhibitors of NFκB-DNA binding, which is most likely due to their potent antioxidant activities [Israel et al., 1992].

It should be pointed out that the effects of tepoxalin on cellular processes were not due to general cytotoxicity. At doses that caused marked inhibition of NFκB (IC_{50} 5–7 μM) and IL2 transcription [Zhou et al., in press], >85% cells were viable in MTT assay. Furthermore, tepoxalin at concentrations required for NFκB inhibition did not inhibit either total Pi incorporation (general phosphorylation) or tyrosine phosphorylation in Jurkat cells (data not shown).

The inhibition of NFκB activity and the related cytokine gene expression by antioxidants, particularly PDTC, has led to the suggestion that ROIs may serve as a common messenger in the activation of NFκB [for review, see Schreck et al., 1992a,b; Ziegler-Heitbrock et al., 1993]. The overlapping functional regulation of NFκB by tepoxalin and PDTC include, (1) inhibition of PMA-, PMA + ionomycin-, and TNF-induced NFκB, (2) inhibition of H_2O_2-stimulated activity, suggesting direct interference with ROI generation, (3) NFκB inhibition in lymphocytes, monocytes, and fibroblasts, and (4) lack of inhibition of AP-1 binding to DNA [Meyer et al., 1993]. Despite these similarities, tepoxalin does not appear to act entirely as an antioxidant. Unlike PDTC and NDGA, tepoxalin (20–50 μM) showed very weak (20–30%) free radical scavenging effects (Fig. 6) in the DPPH conversion assay [Smith et al., 1987].

The differential inhibition of NFκB activation by tepoxalin was evident in different cell types and in the presence of different stimulating agents (Fig. 5). Tepoxalin exhibited maximal inhibition of NFκB activation by PMA, while markedly less effect was observed with TNFα-induced NFκB in Jurkat and HeLa cells. It is conceivable that TNF-α and PMA stimulate separate signal transduction pathways which converge to activate gene expression in T lymphocytes. TNF-α was shown to induce NFκB binding by a mechanism different from PMA-induced PKC [Yang et al., 1993; Krasnow et al., 1991]. Due to the involvement of sphingomyelin and ceramide on TNFα-induced NFκB activity, it may be assumed that tepoxalin does not interfere with the generation of TNFα-induced ceramide in Jurkat T cells. Furthermore, while H_2O_2 strongly induced NFκB-DNA binding activity in HeLa cells, in agreement with previous reports [Schreck et al., 1992b; Meyer et al., 1993], it was not an effective activator of NFκB activity in Jurkat cells. These observations suggest differential redox mechanisms in the two cell types and may also account for differences in the degree of inhibition by tepoxalin in the presence of H_2O_2 (Fig. 5).

The actual mechanism of tepoxalin-induced inhibition of NFκB remains unclear, but the observed suppression of PMA-induced IκB disappearance by tepoxalin and the suggested role of phosphorylation in NFκB activation deserve further investigation. The plausible targets in the regulatory mechanism may include, (1) inhibi-

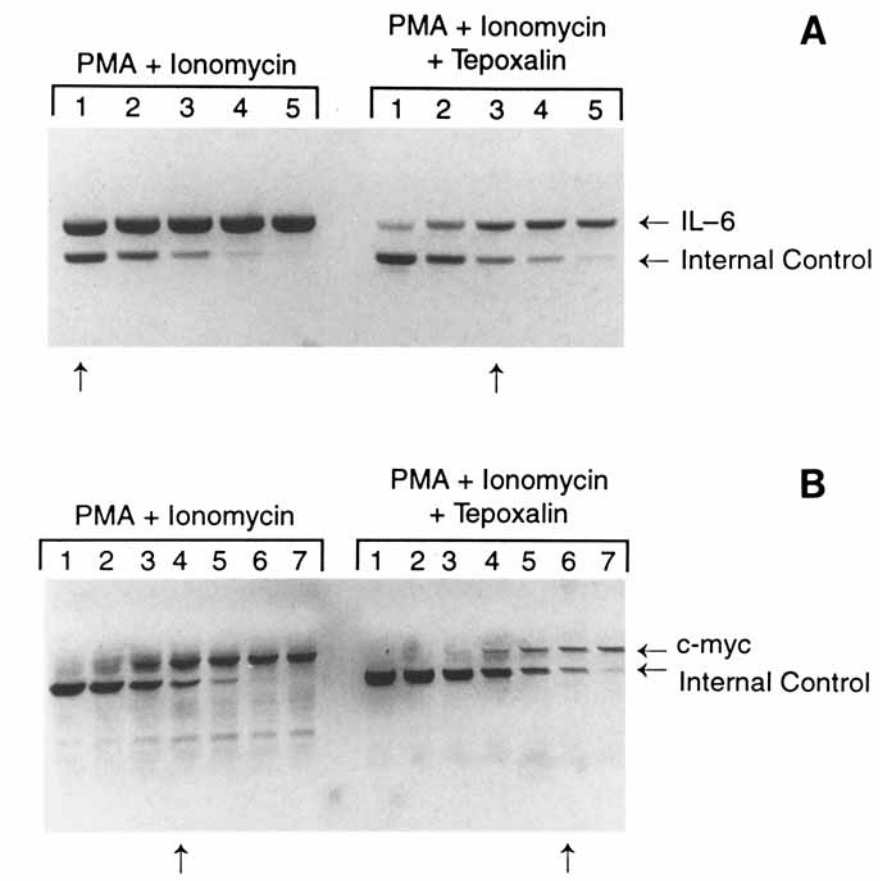

Fig. 9. Effect of tepoxalin on PMA + ionomycin-induced IL-6 and c-myc mRNA production. Human PBL (**A**) and human IL2-dependent T cell blasts (**B**) were treated with tepoxalin (10 μM) or vehicle (DMSO, 0.1%) for 1 h, followed by stimulation by PMA (20 ng/ml) and ionomycin (1 μM) for 4 h. Total RNA was isolated from the treated cells, reverse transcribed using oligo-dT as primer, and QPCR was performed using IL-6- (A) or c-myc- (B) specific primers as described in Materials and Methods. For IL-6, the amplified DNA bands of 500 bp (internal standard control) and 628 bp (IL-6 gene) are marked (A). For c-myc, the amplified DNA bands were 427 bp (internal standard control) and 713 bp (c-myc gene) as indicated. Arrows below the gels refer to the approximate positions at which the densities of both the targeted cDNA and internal standard control bands are equal. The three-fold dilution of the IL-6 internal standard, indicated as attomoles, was as follows. A: lane 1, 1×10^{-2}; lane 2, 3.3×10^{-3}; lane 3, 1.1×10^{-3}; lane 4, 3.7×10^{-4}; and lane 5, 1.2×10^{-4}. The internal standard dilution for c-myc was as follows. B: lane 1, 1×10^{-1}; lane 2, 3.3×10^{-2}; lane 3, 1.1×10^{-2}; lane 4, 3.7×10^{-3}; lane 5, 1.2×10^{-3}; lane 6, 4×10^{-4}; lane 7, 1.3×10^{-4}. Similar experiments performed with CsA (10 nM) treatment to PMA + ionomycin-stimulated PBL showed no marked effect on transcription of IL-6 (data not shown).

tion of PKC or specific tyrosine kinases, (2) activation of IκB-specific phosphatases to increase the stability of IκB, (3) induction of IκB like protein synthesis, and (4) inhibition of certain specific proteases that are required for the degradation of IκB. Since the induction of AP-1 by PMA was not inhibited in the presence of tepoxalin, it is unlikely that tepoxalin impairs PKC activity. Further studies are needed to characterize the signal molecules that mediate the inhibition of NFκB by tepoxalin, particularly for the inhibition of IκB degradation by tepoxalin.

In addition to the interference of NFκB activation in EMSA analysis and transient transfection studies, tepoxalin also inhibited expression of NFκB-dependent genes. For this study, we chose IL-6, a cytokine, and c-myc, an oncogene, both genes with an NFκB motif that is crucial to their expression [Libermann and Baltimore, 1990; Duyao et al., 1990; Donnelly et al., 1993]. IL-6 induces many functional responses, including proliferation of hemopoietic stem cells, differentiation and antibody secretion in B cells, increase in T cell proliferation, and induction of hepatic acute phase protein synthesis [Libermann and Baltimore, 1990; Donnelly et al., 1993]. Furthermore, the potential role of NFκB in carcinogenesis and enhanced cell proliferation is exemplified in the NFκB-dependent acti-

TABLE I. Effect of Tepoxalin on PMA + Ionomycin-Induced IL-6 and c-myc mRNA Production*

Treatment condition	mRNA (Attomole) IL6	C-Myc
Vehicle	1.8×10^{-2}	7.0×10^{-3}
Tepoxalin (10 μM)	1.8×10^{-3} (10-fold)	4.5×10^{-4} (15.5-fold)

*Values in parentheses represent fold reduction of mRNA production.

vation of c-myc [Duyao et al., 1990; Finco and Baldwin, 1993]. The observed 10–15 fold reduction in the transcription of these genes by tepoxalin, as shown by QPCR in this study (Table I), provides crucial evidence for the suppression of NFκB-mediated pathways by tepoxalin. In addition, we have also demonstrated that tepoxalin effectively inhibits the expression of IL-8 and adhesion molecules, ELAM and VCAM-1, coincident with the suppression of NFκB-DNA binding (manuscript in preparation). Taken together, these observations strongly suggest that tepoxalin could serve as an excellent pharmacological tool to investigate mechanisms underlying the activation of NFκB and the associated signal transduction pathways.

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