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Suppression of Tumor Necrosis Factor-Activated Nuclear Transcription Factor-κB, Activator Protein-1, c-Jun N-Terminal Kinase, and Apoptosis by β-Lapachone

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ABSTRACT. β-Lapachone, the product of a tree from South America, is known to exhibit various pharmacologic properties, the mechanisms of which are poorly understood. In the present report, we examined the effect of β-lapachone on the tumor necrosis factor (TNF)-induced activation of the nuclear transcription factors NF-κB and activator protein-1 (AP-1) in human myeloid U937 cells. TNF-induced NF-κB activation, p65 translocation, IκBα degradation, and NF-κB-dependent reporter gene expression were inhibited in cells pretreated with β-lapachone. Direct treatment of the p50-p65 heterodimer of NF-κB with β-lapachone had no effect on its ability to bind to the DNA. Besides myeloid cells, β-lapachone was also inhibitory in T-cells and epithelial cells. β-Lapachone also suppressed the activation of NF-κB by lipopolysaccharide, okadaic acid, and ceramide but had no significant effect on activation by H_2O_2 or phorbol myristate acetate, indicating that its action is selective. β-Lapachone also abolished TNF-induced activation of AP-1, c-Jun N-terminal kinase, and mitogen-activated protein kinase kinase (MAPKK or MEK). TNF-induced cytotoxicity and activation of caspase-3 were also abolished by β-lapachone. Because reducing agents (dithiothreitol and *N*-acetylcysteine) reversed the effect of β-lapachone, it suggests the role of a critical sulfhydryl group. Overall, our results identify NF-κB, AP-1, and apoptosis as novel targets for β-lapachone, and this may explain some of its pharmacologic effects. BIOCHEM PHARMACOL **57**;7:763–774, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. lapachone; TNF; JNK; apoptosis; NF-κB

β-Lapachone (3,4-dihydro-2,2-dimethyl-2*H*-naphthol[1,2-*b*]pyran-5,6-dione) (Fig. 1), a derivative of the naturally occurring substance lapachol, found in the South American lapacho tree (*Tabebuia avellanedae*) [1, 2], shows antibacterial, antifungal, and antitrypanosomal activities, the mechanisms of which are unclear [3, 4]. β-Lapachone also inhibits reverse transcriptase and DNA polymerase-α [2] and blocks DNA repair [5]. Like camptothecin, it is a potent inhibitor of DNA topoisomerase I, but its mode of action differs [6]. β-Lapachone exhibits several biological properties that are similar to those of another plant-derived product, curcumin, but the two products are structurally unrelated. Both inhibit gene expression directed by the HIV-1 LTR† [7], and both induce apoptosis in a wide variety of tumor cells through a similar mechanism [8–10]. Curcumin, however, has been shown to block the activation of the nuclear transcription factors NF-κB [11] and AP-1 [12]. It is not known whether β-lapachone possesses these properties.

NF-κB is a ubiquitous transcription factor whose dysregulation is associated with the development of various diseases and pathologic conditions such as septic shock, graft versus host disease, acute inflammation, the acute-phase response, radiation damage, atherosclerosis, and cancer [13]. When NF-κB is activated, apoptosis is also blocked [14]. Under normal conditions, NF-κB is retained in the cytoplasm as a complex consisting of p50, p65, and IκBα subunits. Once activated, IκBα is then phosphorylated,

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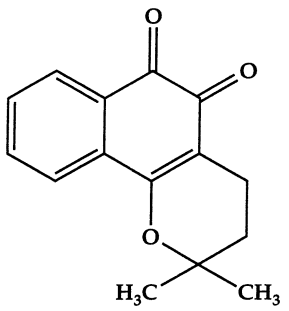
† *Abbreviations*: LTR, long terminal repeat; NF-κB, nuclear transcription factor-κB; TNF, tumor necrosis factor; IκB, inhibitory subunit of NF-κB; EMSA, electrophoretic mobility shift assay; JNK, c-jun N-terminal kinase; PARP, poly (ADP) ribose polymerase; LPS, lipopolysaccharide; MAP, mitogen-activated protein; ECL, enhanced chemiluminescence; MEK, mitogen-activated protein kinase kinase; AP-1, activator protein-1; DTT, dithiothreitol; IL, interleukin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DOC, deoxycholate; SAPK, stress-activated protein kinase; PDTC, pyrrolidine dithiocarbamate; NAC, *N*-acetyl-l-cysteine; CAT, chloramphenicol acetyl transferase; and TPCK, *N*-tosyl-l-lysyl chloromethyl ketone.



β-Lapachone

(3,4-dihydro-2,2-dimethyl-2H-napthol[1,2-b] pyran-5,6-dione; M.W. 242)

FIG. 1. Chemical structure of β-lapachone.

ubiquitinated, and degraded; this causes the p50 and p65 heterodimers to be translocated to the nucleus. Various agents that induce inflammation and apoptosis trigger this chain of events. These agents include TNF, lymphotoxin, IL-1, mitogens, bacterial products, protein synthesis inhibitors, phorbol esters, oxidative stress, and ultraviolet light. After it reaches the nucleus, NF-κB activates a wide range of genes, associated with acute inflammatory responses, arthritis, viral replication, apoptosis, and cancer.

Various agents that activate NF-κB also activate another transcription factor called AP-1. AP-1 consists of a homodimer, and heterodimers of members of the Jun family (c-Jun, JunB, and JunD), and heterodimers of the Jun family and the Fos family (c-Fos, FosB, Fra1, and Fra2) [15]. AP-1 is regulated in part by activation of the JNK/SAPK [15]. JNK is activated by a dual-specificity kinase called MAPKK or MEK. Both AP-1 and JNK play an important role in growth modulation and apoptosis [16].

Because NF-κB, AP-1, and JNK are known to be involved in inflammation, HIV replication, and apoptosis, drugs that negatively affect these activities would be of great therapeutic value. In the study described here, we investigated the inhibitory effects of β-lapachone on TNF-activated NF-κB, AP-1, JNK, and apoptosis. We observed that TNF-induced activation of NF-κB, AP-1, JNK, MEK, and apoptosis was suppressed in cells treated with β-lapachone, thus suggesting that β-lapachone may be able to ameliorate pathological conditions.

MATERIALS AND METHODS

Materials

Highly purified β-lapachone (MW 242) was provided by Dr. A. Sedlacek (Ciba-Geigy). It was dissolved in DMSO to a 10 mM concentration, aliquoted, and kept at −20°. Penicillin, streptomycin, RPMI 1640 medium, and fetal bovine serum were obtained from GIBCO. Glycine, DTT, PDTC, EDTA, and NAC were obtained from the Sigma Chemical Co. Bacteria-derived recombinant human TNF, purified to homogeneity with a specific activity of 5×10^7 U/mg, was provided by Genentech, Inc. Phospho-specific anti-p44/42 MAP kinase (Thr 202/Tyr 204) antibody was obtained from New England Biolabs, Inc. Antibodies against IκBα and p65, double-stranded oligonucleotides for NF-κB and AP-1, and the single-stranded oligonucleotide of NF-κB were obtained from Santa Cruz Biotechnology. The rat MDR1bCAT plasmid -243RMICAT, which contains the CAT gene with either a wild-type or a mutated NF-κB binding site, was supplied by Dr. M. Tien Kuo of The University of Texas M. D. Anderson Cancer Center. The characterization of these plasmids has been described previously [17].

Cell Lines

U937 (human histiocytic lymphoma), Jurkat (human T-cell line), HeLa (human epithelial cell), and H4 (human glioma cell) cell lines were obtained from the American Type Culture Collection. All cells were free of *Mycoplasma* as shown by the Gen-Probe Mycoplasma Rapid Detection Kit (Fisher Scientific).

Assay for NF-κB and AP-1

NF-κB was assayed according to the method of Chaturvedi *et al.* [18]. Briefly, nuclear extracts prepared from 2×10^6 cells were incubated with ^{32}P-end-labeled, 45-mer, double-stranded NF-κB oligonucleotides from the HIV-LTR (5′-TTGTTACAA**GGGACTTTC**CGCTG**GGGACTTTC**CAGGGAGGCGTGG-3′), after which the extracts were subjected to EMSA on native polyacrylamide gels. A double-stranded, mutated oligonucleotide, (5′-TTGTTACAA**CTCACTTTC**CGCTG**CTCACTTTC**CAGGGAGGCGTGG-3′) was used to examine the specificity of the binding of NF-κB to the DNA. The specificity of binding was also examined by competition with the unlabeled oligonucleotide.

The EMSA for AP-1 was performed in the same way as that for NF-κB except that the nuclear extracts were incubated with ^{32}P-end-labeled double-stranded oligonucleotide of AP-1 (5′-CGCTTGA**TGACTCA**GCCGGAA-3′; 3′-GCGAACT**ACTGAGT**CGGCCTT-5′). The nuclear extract containing 6 μg of protein was analyzed in 6.6% native polyacrylamide gel. The specificity of binding was determined routinely by using an excess amount of unlabeled oligonucleotide for competition. A PhosphorImager (Molecular Dynamics) equipped with "Image-quant" software was used to visualize and quantitate the radioactive bands.

Western Blot Analysis of IκBα and p65

After cells were treated with TNF or β-lapachone for different times, postnuclear cytoplasmic extracts were prepared and resolved on 8.5% SDS-polyacrylamide gels to determine the levels of IκBα. Nuclear and cytoplasmic extracts were resolved by 8.5% SDS–PAGE to determine

the p65 levels. After electrophoresis was performed, the proteins were electrotransferred to nitrocellulose filters, probed with a rabbit polyclonal antibody to IκBα and p65, and detected by ECL (Amersham).

MEK Assay

MEK was assayed using the modified method of Cowley *et al*. [19]. Briefly, U937 cells cultured in 2% serum for overnight were stimulated with different concentrations of TNF for 30 min at 37° and washed. Then cell extracts were prepared using lysis buffer containing 20 mM HEPES (pH 7.4), 2 mM EDTA, 250 mM NaCl, 0.1% NP-40, 2 μg/mL of leupeptin, 2 μg/mL of aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 μg/mL of benzamidine, 1 mM DTT, and 1 mM sodium orthovanadate. Fifty micrograms of protein per lane was resolved by 10% SDS–PAGE, electrotransferred onto nitrocellulose filters, and probed with the phospho-specific anti-p44/42 MAP kinase antibody raised in rabbits. After this, the membrane was incubated with peroxidase-conjugated anti-rabbit IgG (1:3000 dilution), and the bands were detected by chemiluminescence using the ECL detection kit.

JNK Assay

The JNK assay was performed using a previously described modified method [20, 21]. Briefly, cells (3×10^6/mL) were treated with TNF for 10 min, then cell extracts were prepared, and samples of 150–250 μg were immunoprecipitated with 0.3 μg of anti-JNK antibody for 60 min at 4°. After incubation with protein A/G Sepharose beads for 45 min at 4°, immune complexes were collected and washed extensively, and the kinase assays were performed for 15 min at 30° using GST-Jun (1–79) as a substrate in the presence of 10 μCi [γ-^{32}P]ATP. Reactions were stopped by exposure to SDS sample buffer, after which samples were boiled and subjected to SDS–PAGE (9%). GST-Jun (1–79) was visualized by staining the gel with Coomassie blue, and the dried gel was analyzed using the PhosphorImager.

Transient Transfection and CAT Assay

U937 cells were transiently transfected with -243RMICAT (wild type) and -243RMICAT-κm (mutant) plasmids for 6 hr using the calcium phosphate method according to the instructions of the manufacturer (Life Technologies). Then the cells were preincubated for 2 hr at 37° with β-lapachone (5 μM) before being stimulated with 1 nM TNF for 1 hr. After this, the cells were washed with PBS, and the CAT activity was examined as described [22].

MTT Assay

U937 cells (5×10^3/0.1 mL) were pretreated with different concentrations of β-lapachone for 2 hr and then exposed to 0.1 nM TNF for 24 hr at 37° in a CO_2 incubator. After this, cell viability was determined by the MTT dye uptake assay, in which the dye is converted into formazan granules in the presence of reactive oxygen. After incubating the cells with the dye for 2 hr at 37°, the granules were lysed with SDS (20%) and dimethylformamide (50%) by overnight incubation at 37°, and the absorbance was detected at 590 nm using a 96-well multiscanner autoreader (Dynatech MR 5000; Dynatech Laboratories).

Immunoblot Analysis of PARP Degradation

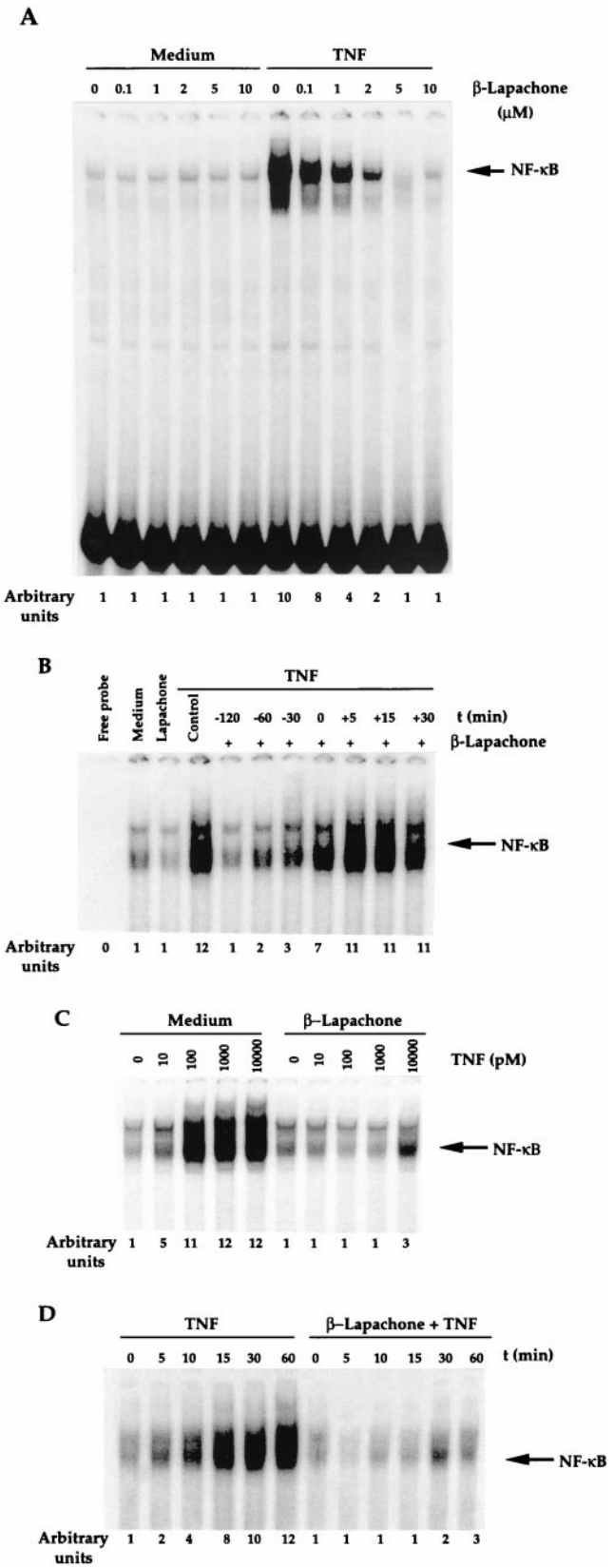
TNF-induced apoptosis was examined by proteolytic cleavage of PARP [20]. Briefly, β-lapachone pretreated (for 2 hr) and untreated cells (2×10^6/mL) were exposed to cycloheximide (10 μg/mL) and TNF (1 nM) for 2 hr at 37°. After treatment, cell extracts were prepared by incubating the cells for 30 min on ice in 0.05 mL buffer containing 20 mM HEPES (pH 7.4), 2 mM EDTA, 250 mM NaCl, 0.1% NP-40, 2 μg/mL of leupeptin, 2 μg/mL of aprotinin, 1 mM PMSF, 0.5 μg/mL of benzamidine, and 1 mM DTT. The lysate was centrifuged, and the supernatant was collected. Cell extract protein (50 μg) was resolved on 7.5% SDS–PAGE, electrotransferred onto a nitrocellulose membrane, blotted with mouse anti-PARP antibody, and then detected by ECL (Amersham). Apoptosis was represented by the cleavage of 116-kDa PARP into a 85-kDa peptide product.

RESULTS

The aim of this study was to investigate the effects of β-lapachone on TNF-induced activation of NF-κB, AP-1, JNK, and apoptosis. Because β-lapachone is known to induce apoptosis in some cells, we first examined the viability of various cell types (U937, Jurkat, HeLa, and H4) for different times after treatment with different concentrations of β-lapachone. We found that more than 97% of the cells remained viable when treated with β-lapachone at the concentration and for the time used in the different experiments.

Effect of β-Lapachone on TNF-Dependent NF-κB Activation

U937 cells were incubated for 2 hr with different concentrations of β-lapachone and then treated with TNF (100 pM) for 30 min at 37°. EMSA was performed to detect NF-κB activation. Figure 2A shows that although β-lapachone by itself did not activate NF-κB, it did inhibit TNF-induced NF-κB activation, with a maximum effect seen at 5 μM. We next tested the time it took for β-lapachone to inhibit TNF-induced NF-κB activation. To determine this, cells were incubated with β-lapachone for 120, 60, or 30 min before the addition of TNF; β-lapachone was added at the same time as the TNF; or cells were incubated with β-lapachone for 5, 15, and 30 min after the addition of TNF. In all instances the cells were treated with



TNF for 30 min. NF-κB activation was almost completely inhibited only in the cells pretreated with β-lapachone for 120 min, with the degree of inhibition decreasing gradually with shorter preincubation times. Treatment with β-lapachone at the same time as or after the addition of TNF did not inhibit NF-κB activation by TNF completely (Fig. 2B).

To examine the effect of β-lapachone on the time course of NF-κB activation by TNF, cells were treated with 100 pM TNF for different times in the absence and presence of β-lapachone, and then assayed for the NF-κB. Figure 2D shows that optimal NF-κB activation occurred within 30–60 min in the absence of β-lapachone but that no significant activation occurred in cells treated with β-lapachone. Previous studies in our laboratory have shown that a high concentration (10 nM) of TNF can activate NF-κB within 5 min and that this induction is more intense than that in cells treated at a 100-fold lower concentration of TNF but for longer [23]. To determine the effect of β-lapachone on NF-κB activation at an even higher concentration, both untreated and β-lapachone-treated cells were incubated with various concentrations of TNF (0–10,000 pM) for 30 min, and then the activation of NF-κB was determined by EMSA. Although NF-κB was activated intensely by 10,000 pM TNF, β-lapachone inhibited the activation as completely as it did at the 0.1 nM concentration. These results show that β-lapachone is a very potent inhibitor of NF-κB activation (Fig. 2C).

Composition of NF-κB Inhibited by β-Lapachone

Various combinations of Rel/NF-κB proteins can serve as active NF-κB heterodimers that bind to specific sequences in DNA. To show that the retarded band visualized by EMSA in TNF-treated cells was indeed NF-κB, we incubated nuclear extracts from TNF-activated cells with antibody to either the p50 (NF-κBI) or p65 (Rel A) subunits and then performed EMSA. Antibodies to either subunit shifted the band to a higher molecular weight (Fig. 3A),

FIG. 2. Effect of β-lapachone on TNF-dependent NF-κB activation. (A) U937 cells (2 × 10^6/mL) were incubated at 37° for 2 hr with different concentrations (0–10 μM) of β-lapachone, followed by a 30-min incubation with 0.1 nM TNF at 37°. After these treatments, nuclear extracts were prepared and then tested for NF-κB activation. (B) Cells were incubated at 37° with 5 μM β-lapachone for either 120, 60, or 30 min before the addition of TNF; or at the same time as the TNF or 5, 15, and 30 min after the addition of TNF. Total incubation time with TNF (0.1 nM) was 30 min; (−) indicates the time β-lapachone was present before the addition of TNF, (0) indicates coincubation with TNF, and (+) indicates the time β-lapachone was added after TNF. Nuclear extracts were prepared after these treatments and then assayed for NF-κB. (C) Cells were incubated at 37° with 5 μM β-lapachone for 2 hr and then tested for NF-κB activation after treatment at 37° for 30 min with different concentrations of TNF as indicated. (D) Cells were incubated at 37° with 5 μM β-lapachone for 2 hr, then treated with 0.1 nM TNF at 37° for different times as indicated and tested for NF-κB activation.

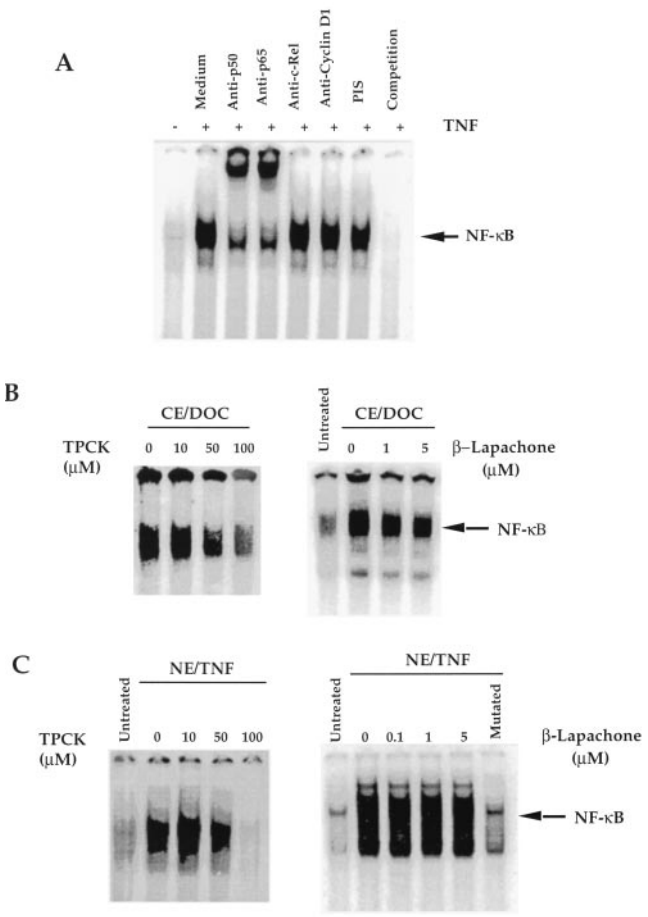


FIG. 3. (A) Supershift and specificity of NF-κB. Nuclear extracts were prepared from untreated or TNF (0.1 nM)-treated U937 cells (2 × 10^6/mL), incubated for 15 min with either the antibodies indicated or with unlabeled NF-κB probe (for competition), and then assayed for the NF-κB activation. (B and C) *In vitro* effect of β-lapachone on DNA binding of NF-κB. In panel B, cytoplasmic extracts (CE) from untreated U937 cells (10 μg sample of protein) were treated with 0.8% DOC for 15 min at room temperature, incubated with different concentrations of TPCK or β-lapachone for 2 hr at room temperature, and then assayed for DNA binding by EMSA. In panel C, nuclear extracts were prepared from 0.1 nM TNF-treated U937 cells; 5 μg of nuclear (NE) protein was treated with the indicated concentrations of TPCK or β-lapachone for 2 hr at room temperature and then assayed for DNA binding by EMSA.

thus suggesting that the TNF-activated complex consists of p50 and p65 subunits. Neither preimmune serum nor irrelevant antibodies such as anti-cRel and anti-cyclin DI had any effect on the mobility of NF-κB. The addition of excess cold NF-κB oligonucleotide probe (100-fold) caused the band to disappear completely, indicating the specificity of NF-κB.

DNA-Binding Ability of NF-κB Proteins after Treatment with β-Lapachone

Both TPCK, a serine protease inhibitor, and herbimycin A, a protein tyrosine kinase inhibitor, down-regulate NF-κB activation by chemically modifying the NF-κB subunits, thus preventing their binding to DNA [24, 25]. To determine whether β-lapachone also directly modifies NF-κB proteins, we prepared a p50-p65 heterodimer by treating cytoplasmic extracts with 0.8% DOC for 15 min or took nuclear extracts from TNF-triggered cells. DOC has been shown to dissociate the IκBα subunit, thus releasing NF-κB for binding to the DNA. After this, we treated the p50-p65 heterodimer with various concentrations of either TPCK or β-lapachone *in vitro*. The DNA-binding activity was then determined by EMSA. Panels B and C of Fig. 3 show that TPCK modified the DNA-binding ability of NF-κB proteins derived from either DOC or TNF treatments, but under these conditions β-lapachone did not. Therefore, it can be concluded that the mechanism by which β-lapachone inhibits NF-κB activation differs from that of TPCK or herbimycin A.

Effect of β-Lapachone on TNF-Dependent Degradation of IκBα and Nuclear Translocation of p65

NF-κB is translocated to the nucleus after the phosphorylation, ubiquitination, and proteolytic degradation of IκBα [13]. To determine whether the inhibitory action of β-lapachone is due to its effect on IκBα degradation, cells were treated with β-lapachone for 2 hr and next with TNF for different times. After this, cytoplasmic extracts were prepared, and the IκBα status was determined by western blot analysis. In β-lapachone-untreated cells, the IκBα was fully degraded within 5 min of TNF treatment but reappeared by 30 min (Fig. 4A). On the other hand, TNF-mediated degradation of IκBα was abolished in cells treated with β-lapachone (Fig. 4A). These results indicate that β-lapachone blocks NF-κB activation by blocking IκBα degradation.

Because the p65 subunit of NF-κB also must be translocated to the nucleus for NF-κB to be activated, we measured the level of p65 in the cytoplasm and nucleus. As expected, the level of p65 in the cytoplasm declined in response to TNF treatments with a concurrent increase in the nucleus (Fig. 4B). In contrast, the TNF-dependent change in the levels of p65 in the nucleus and cytoplasm was also abolished in cells treated with β-lapachone. These results show that β-lapachone inhibits the TNF-induced translocation of p65 to the nucleus, and this is consistent with the inhibition of TNF-dependent NF-κB activation and degradation of IκBα.

Effect of β-Lapachone on TNF-Induced NF-κB Reporter Gene Expression

So far we have shown that β-lapachone blocks NF-κB activation on the basis of the findings from DNA-binding studies. Whether this agent blocks NF-κB-dependent gene expression was also examined. To determine this, cells were transfected with a plasmid consisting of a multidrug resistance gene promoter containing NF-κB binding sites ligated to the *CAT* reporter gene, treated with β-lapachone and then stimulated with TNF, after which the CAT

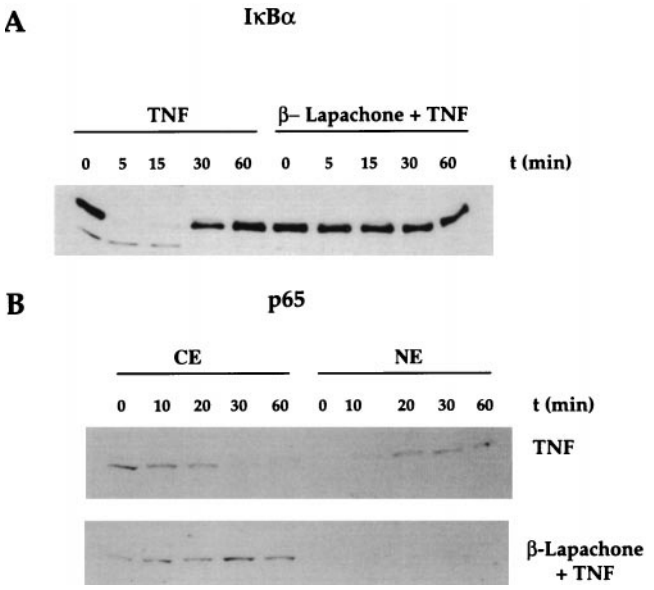


FIG. 4. Effect of β-lapachone on TNF-induced degradation of IκBα and on the level of p65. U937 cells (2×10^6/mL) were either not treated or treated for 2 hr with 5 μM β-lapachone at 37°, next incubated for the indicated times with TNF (0.1 nM), and then assayed for IκBα (A) in cytosolic fractions by western blot analysis, and p65 (B) from cytoplasmic (CE) as well as nuclear (NE) extracts by western blot analysis.

activity was examined. As shown in Fig. 5, there was an almost 5-fold increase in CAT activity in response to stimulation with 1 nM TNF. However, although β-lapachone alone had no effect on CAT activity, TNF-induced CAT activity was inhibited by more than 90% in cells treated with β-lapachone for 2 hr before TNF treatment. CAT activity was not induced by TNF in cells transfected with a plasmid containing a mutated NF-κB binding site. These results show that β-lapachone also suppresses NF-κB-dependent reporter gene expression induced by TNF.

Effect of β-Lapachone on PMA-, LPS-, H_2O_2-, Okadaic Acid-, and Ceramide-Mediated Activation of NF-κB

NF-κB is also activated by various other inflammatory agents, including phorbol ester, H_2O_2, LPS, okadaic acid, and ceramide. Because the mechanism by which these agents activate NF-κB may differ from that of TNF, we studied the effect of β-lapachone on the activation of the transcription factor by these various agents. Figure 6 shows that β-lapachone completely blocked the activation of NF-κB induced by LPS, okadaic acid, and ceramide, indicating that β-lapachone may act at a step prior to the point where all these agents converge in the pathway leading to NF-κB activation. The activation of NF-κB by PMA and H_2O_2, however, was not affected significantly in cells treated with β-lapachone (Fig. 6), suggesting that the mechanism by which these agents activate NF-κB differs from the mechanism of TNF, LPS, okadaic acid, and ceramide.

Cell Type-Specific Inhibition of NF-κB Activation by β-Lapachone

All the effects of β-lapachone that have been described here were observed in human myeloid U937 cells. Recent reports indicate, however, that the NF-κB activation pathway may differ in different cell types [26]. Therefore, we also investigated whether β-lapachone affects other cell types. Specifically, we studied the ability of β-lapachone to block TNF-induced NF-κB activation in T-cells (Jurkat), epithelial (HeLa) cells, and glioma (H4) cells. The results of these experiments (Fig. 7) indicate that β-lapachone inhibited NF-κB activation in all cell types, but the effects were not as pronounced as they were in U937 cells. Under conditions where NF-κB activation was inhibited completely in U937 cells, β-lapachone inhibited it by 55, 72, and 40% in Jurkat, HeLa, and H4 cells, respectively. These results suggest that β-lapachone is somewhat selective in its action in different cell types.

Effect of β-Lapachone on TNF-Induced AP-1 Activation

TNF also activates the transcription factor AP-1 [15], but the mechanism by which AP-1 is activated may differ from

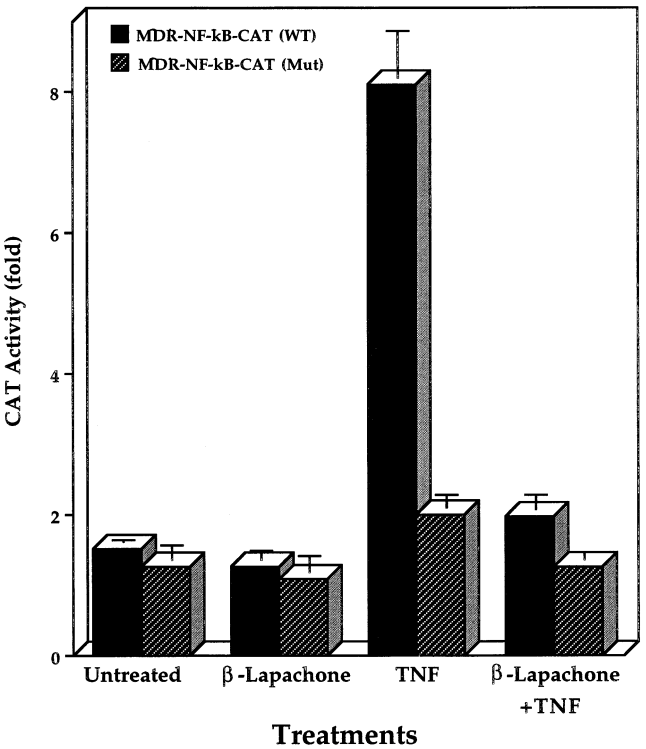


FIG. 5. Effect of β-lapachone on the NF-κB-dependent CAT reporter gene expression. Cells were transiently transfected with plasmids MDR-NF-κB-CAT (-243RMICAT) that had either wild (WT) or mutated (Mut) NF-κB binding sites, treated with 5 μM β-lapachone for 2 hr, exposed to 1 nM TNF for 1 hr, and then assayed for CAT activity as described in Materials and Methods. Results are expressed as the fold increase in activity over that in the nontransfected control. The results shown are the means (± SEM) of triplicate assays.

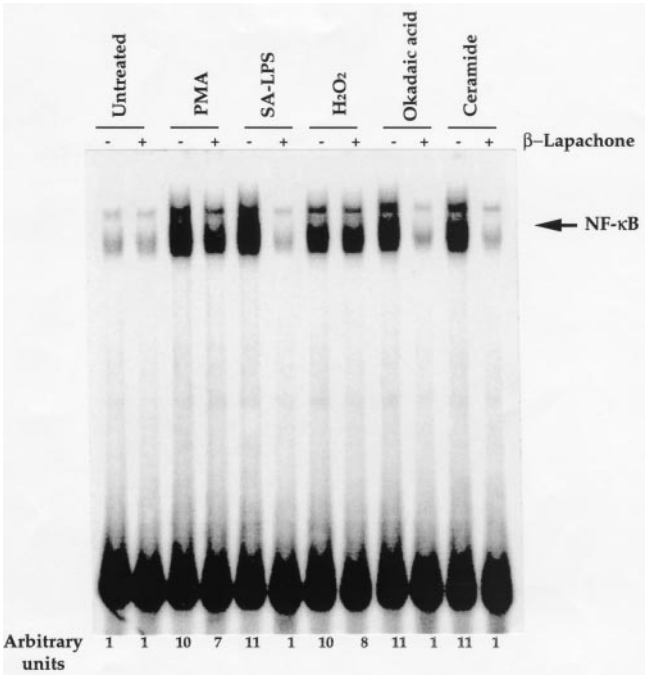


FIG. 6. Effect of β-lapachone on NF-κB activation induced by PMA, serum-activated LPS, H_2O_2, okadaic acid, and ceramide. U937 cells (2 × 10^6/mL) were incubated for 2 hr at 37° with β-lapachone (5 μM) and then with PMA (25 ng/mL), SA-LPS (10 μg/mL), H_2O_2 (250 μM), okadaic acid (500 nM), or ceramide (10 μM) for 30 min, and then assayed for NF-κB activation.

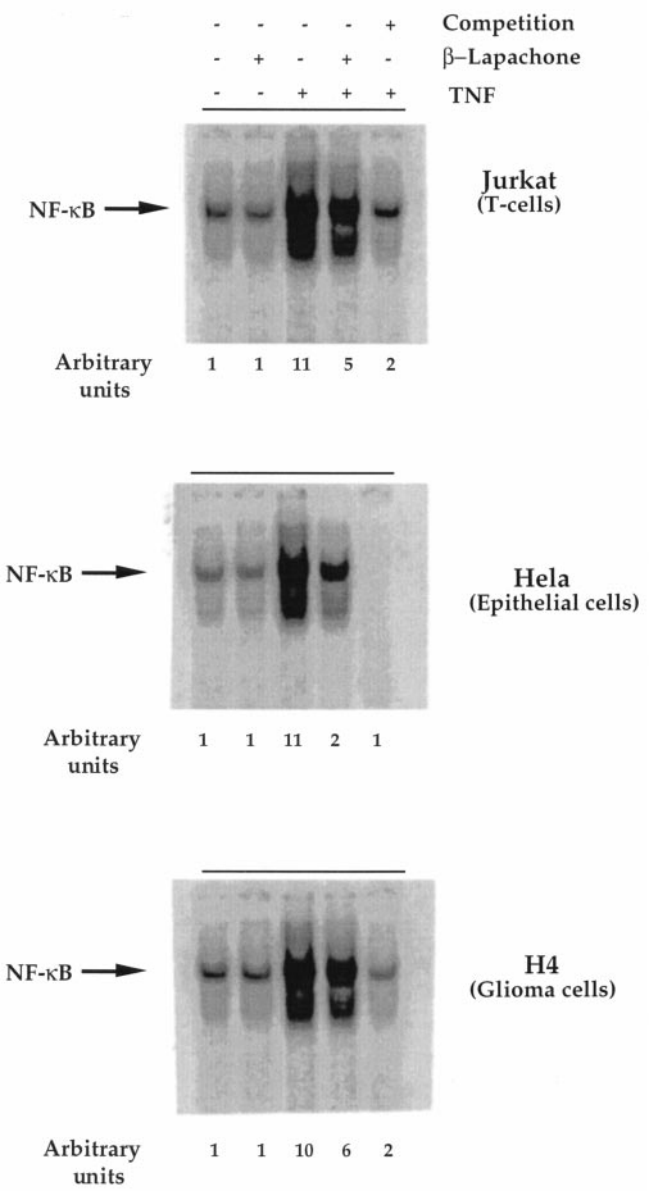


FIG. 7. Effect of β-lapachone on activation of NF-κB induced by TNF in different cell lines. HeLa, H4, and Jurkat cells (2 × 10^6/mL) were incubated at 37° with 5 μM β-lapachone for 2 hr and then NF-κB was activated at 37° for 30 min with 100 pM TNF. After these treatments, nuclear extracts were prepared and assayed for NF-κB.

that of NF-κB [27]. Therefore, we examined the effect of β-lapachone on TNF-induced AP-1 activation. For this study, U937 cells were pretreated with different concentrations of β-lapachone for 2 hr, and then the AP-1 was activated by incubation with 1 nM TNF for 1 hr. After this, nuclear extracts were prepared, and the AP-1 activity was determined by EMSA. As shown in Fig. 8A, TNF induced a 7-fold activation of AP-1 in U937 cells, and this activation was inhibited by β-lapachone in a concentration-dependent manner. The β-lapachone by itself, however, had no effect on AP-1 activation. In addition, this drug blocked not only the AP-1 activation induced by 1 nM TNF but also that induced by higher concentrations of TNF (Fig. 8B).

Effect of β-Lapachone on TNF-Induced JNK Activation

The activation of AP-1 by TNF is regulated in part by JNK [15]. We, therefore, also investigated whether β-lapachone affects the TNF-induced JNK activation. To do this, U937 cells were pretreated with different concentrations of β-lapachone for 2 hr and then stimulated with TNF (1000 pM) for 10 min, after which JNK activation was assayed. As shown in Fig. 9A, JNK activation was increased 16-fold in response to TNF. However, it gradually decreased with increasing concentration of β-lapachone and was reduced to the basal levels by 5 μM β-lapachone. β-Lapachone by itself did not activate JNK.

Effect of β-Lapachone on TNF-Mediated MEK

There are reports that the MAP kinase pathway may be involved in TNF-induced NF-κB activation [28, 29]. Therefore, we examined whether β-lapachone affects TNF-induced MEK. For this study, U937 cells were treated with variable concentrations of β-lapachone for 2 hr and then stimulated with TNF (100 pM) for 30 min, after which the phosphorylated form of MAP kinase was detected by western blot analysis (Fig. 9B). TNF activated MEK. In addition, although β-lapachone by itself had no effect on MEK, it completely blocked the TNF-induced activation in a concentration-dependent manner, with a maximum ef-

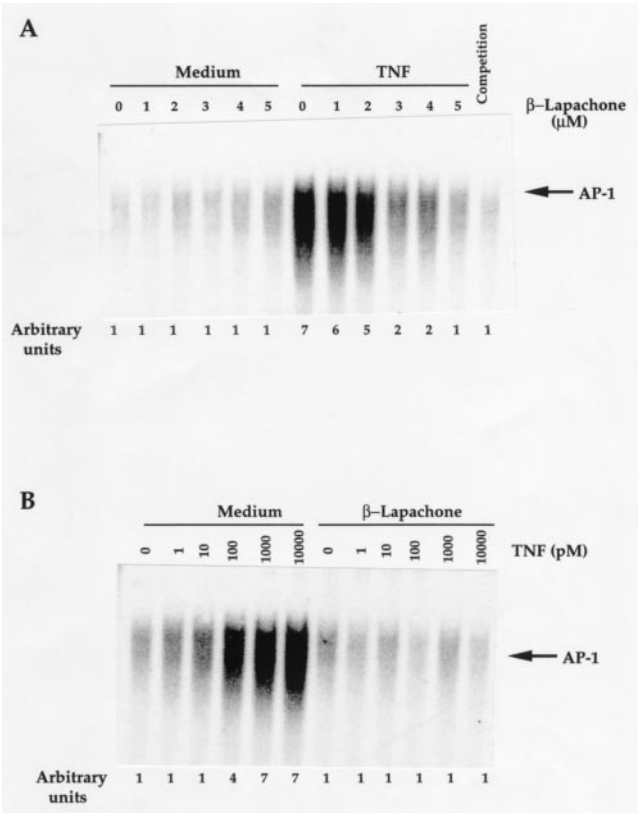


FIG. 8. Effect of β-lapachone on TNF-dependent AP-1 activation. (A) U937 cells (2×10^6) were treated with different concentrations of β-lapachone for 2 hr at 37°. After this, cells were stimulated with TNF (1 nM) for 1 hr and then assayed for AP-1 by EMSA. (B) Cells were treated with β-lapachone (5 μM) for 2 hr and then stimulated with different concentrations of TNF for 2 hr. AP-1 activity was determined by EMSA.

fect seen in cells treated with 5 μM β-lapachone. Thus, the inhibitory effect of β-lapachone on NF-κB activation may result from its effect on MEK.

Effect of β-Lapachone on TNF-Induced Cytotoxicity and Caspase-3 Activation

The activation of NF-κB, AP-1, and JNK represents an early cellular response to TNF. Induction of cytotoxicity to tumor cells represents a late response. Whether β-lapachone modulates TNF-mediated cytotoxicity was, therefore, also investigated. In this study, U937 cells were treated with variable concentrations of β-lapachone for 2 hr and then treated with 1 nM TNF. After 24 hr, cell viability was determined by the MTT method. Under these conditions, almost 80% of the cells were killed by TNF within 24 hr (Fig. 10A). When pretreated with β-lapachone, however, cells were protected from TNF-induced cytotoxicity by increasing concentrations of β-lapachone (Fig. 10A). These results suggest that the cytotoxic effects of TNF are also abolished by β-lapachone.

How TNF induces cytotoxicity is not fully understood, but activation of a protease, caspase-3, has been shown to be involved, and it precedes cytotoxicity. We examined the effect of β-lapachone on TNF-mediated activation of caspase-3. U937 cells were treated with variable concentrations of β-lapachone for 2 hr and then stimulated with TNF (1 nM) for 2 hr in the presence of cycloheximide (10 μg/mL) and assayed for caspase-3 by its ability to cleave PARP protein. A 2-hr treatment with TNF induced almost 80% cleavage of PARP (Fig. 10B). However, when cells were pretreated with β-lapachone, TNF-mediated PARP cleavage was inhibited by increasing concentrations of β-lapachone. These results suggest that β-lapachone also inhibits TNF-induced caspase-3 activation, and this may lead to inhibition of TNF-induced cytotoxicity.

Effect of DTT on the Inhibitory Effect of β-Lapachone

It has been shown that although agents such as TPCK, which modify the sulfhydryl group in NF-κB, inhibit NF-κB activation, this inhibition can be reversed by DTT [25]. To determine whether the inhibitory effect of β-lapachone on NF-κB could be reversed by this reducing agent, U937 cells were treated with β-lapachone in the presence or absence of DTT and then examined to determine whether TNF activated the NF-κB. As shown in the Fig. 11A, DTT reversed the inhibition produced by β-lapa-

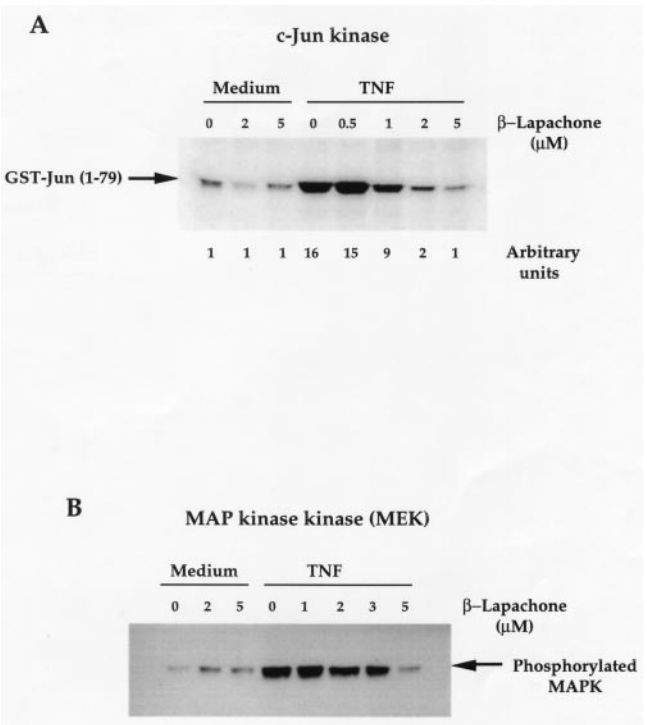


FIG. 9. Effect of β-lapachone on TNF-induced JNK and MEK activation. (A) U937 cells were treated with different concentrations of β-lapachone as shown in Fig. 2A and then stimulated with 1 nM TNF at 37° for 10 min. After this, the cells were washed, and pellets were extracted and assayed for JNK activation. (B) Cells were treated with different concentrations of β-lapachone for 2 hr, stimulated with 100 pM TNF for 30 min, and then assayed for MEK by western blot using the phospho-specific MAP kinase antibody.

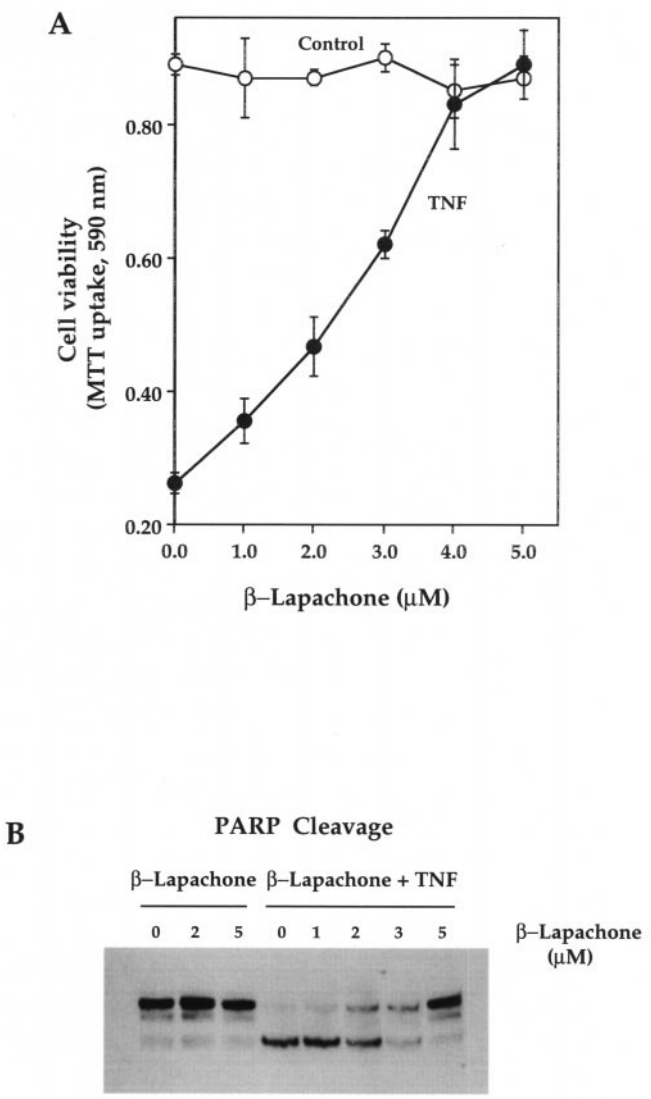


FIG. 10. Effect of β-lapachone on TNF-induced cytotoxicity and on caspase-3 activation. (A) U937 cells (5×10^3/0.1 mL) were treated with variable concentrations of β-lapachone for 2 hr and then exposed to 1 nM TNF for 24 hr at 37° in a CO_2 incubator. Cell viability was then determined by the MTT method. The results shown are the mean (± SEM) optical density of triplicate assays. (B) Cells were incubated with different concentrations of β-lapachone for 2 hr and then treated with 10 μg/mL of cycloheximide and TNF (1/mM) for 2 hr at 37° in a CO_2 incubator. Then the cells were washed, the pellet was extracted, and western blotting was performed to detect PARP cleavage. The uncleaved band appears at 116 kDa, which is degraded into 85 kDa.

chone, suggesting that β-lapachone suppresses NF-κB activation by blocking some critical sulfhydryl groups.

Effect of NAC on the Inhibitory Effects of β-Lapachone

NAC, PDTC, EDTA, and mannitol are known to quench superoxide anion, metal, Ca^{2+}, and hydroxyl radicals, respectively. Whether these intermediates play any role in the inhibitory effects of β-lapachone was also investigated.

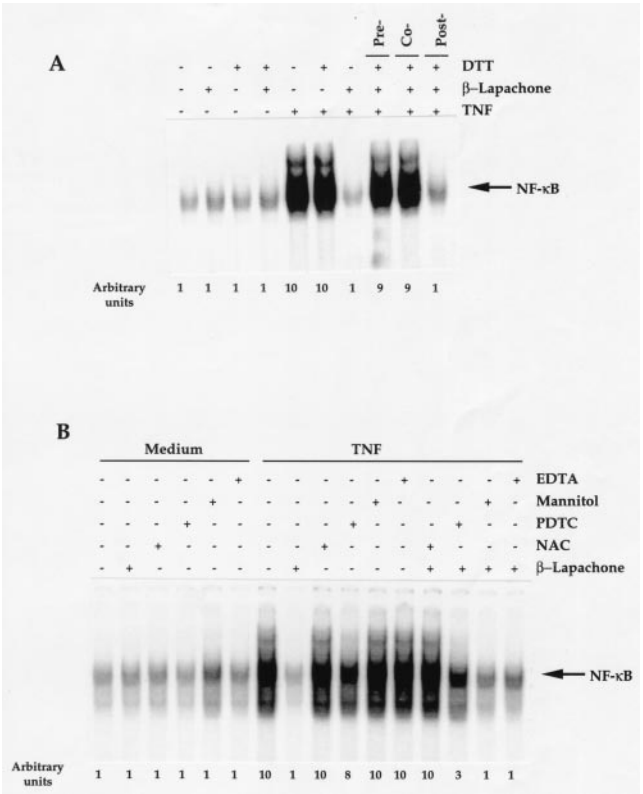


FIG. 11. Effect of DTT, NAC, EDTA, mannitol, and PDTC on β-lapachone-induced inhibition of NF-κB activation by TNF. (A) U937 cells (2×10^6/mL) were incubated at 37° for 60 min with DTT (100 μM) in the presence of β-lapachone (5 μM) for 2 hr or in the presence of other indicated combinations followed by incubation with 0.1 nM TNF for 30 min and then assayed for NF-κB activation. (B) Cells were pretreated with NAC (5 mM), EDTA (100 μM), mannitol (10 mM), or PDTC (100 μM) for 1 hr, next treated with 5 μM β-lapachone for 2 hr, and then exposured to 100 pM TNF for 30 min to activate NF-κB. After these treatments, nuclear extracts were prepared and assayed for NF-κB.

To determine this, cells were treated with one of the four quenchers for 1 hr and then treated with β-lapachone (5 μM) for 2 hr, after which the cells were stimulated with TNF (100 pM) for 30 min and NF-κB activity was examined. Figure 11B shows that only NAC had no effect on TNF-induced NF-κB activation but it completely abolished the inhibitory effects of β-lapachone. In contrast, PDTC reduced the TNF-induced NF-κB activation by 20% and reversed the inhibitory effects of β-lapachone by only 30%. Mannitol by itself slightly stimulated NF-κB. Both EDTA and mannitol had no effect either on TNF-induced stimulation or on β-lapachone-induced inhibition of NF-κB activation. It is therefore possible that NAC blocks the inhibitory effects of β-lapachone in the same way as DTT does.

DISCUSSION

β-Lapachone is a lipophilic *o*-naphthoquinone that exhibits antiviral, antitrypanosomal, and antitumor activities *in*

vivo [3, 7, 30]. β-Lapachone is also known to inhibit topoisomerase I and induce apoptosis in certain tumor cells [6, 8, 9]. In addition, this drug blocks gene expression directed by the LTR of HIV type I and blocks viral replication [7]. Although it is not known how β-lapachone inhibits these various activities, it is known that several of these activities also are suppressed by a structurally unrelated plant-derived compound, curcumin (diferuloylmethane). Specifically, curcumin has been shown to block the activation of NF-κB and AP-1. We therefore investigated whether β-lapachone also inhibits these transcription factors. Our results clearly showed that β-lapachone completely abolished the TNF-mediated activation of NF-κB and of AP-1 and that this correlated with inhibition of JNK and MEK, the kinases involved in activation of these transcription factors. β-Lapachone also abolished TNF-mediated cytotoxicity.

How β-lapachone abrogates TNF-mediated NF-κB activation is not clear, but our results point toward several possible mechanisms. For example, because TNF-induced IκBα degradation was also abolished by β-lapachone, this suggests that the site where β-lapachone acts is upstream in the NF-κB activation pathway. Because NF-κB activation by LPS, okadaic acid, and ceramide was also blocked by β-lapachone, this indicates a common signaling pathway. In contrast, NF-κB activation by PMA and H_2O_2 was affected minimally by β-lapachone, suggesting a difference in the signaling pathway for these agents. How these two signaling pathways differ, however, is not clear. This is consistent with reports that lipid peroxidation is needed for TNF-induced NF-κB activation but is not needed for IL-1-induced activation [31].

Recently, several kinases have been implicated as participating in the pathway leading to NF-κB activation (for references, see [32]). For instance, TNF and various other NF-κB activators are known to activate MAP kinase kinase kinase (also called MEKK or MKK), which activates a dual-specificity kinase, MEK, that in turn activates MAP kinase. There are also reports that MEKK activation is essential for TNF-induced NF-κB activation, whereas others have found that it is not essential (for references, see [32]). We found that β-lapachone inhibited the TNF-induced activation of MEK. Therefore, β-lapachone may inhibit NF-κB by inhibiting MEK.

Our studies also showed that β-lapachone blocks the NF-κB-dependent reporter gene expression induced by TNF. NF-κB is known to regulate the expression of several genes including those that encode cytokines (e.g. IL-1, TNF, lymphotoxin, granulocyte-monocyte colony stimulating factor), cytokine receptors (e.g. IL-2 receptor and TNF receptors), MHC-I, MHC-II, and endothelial cell adhesion proteins, cyclooxygenases, metalloproteases, and various viruses (e.g. HIV-1) (for references, see [13]). It is possible, therefore, that the anti-HIV-1 effects of β-lapachone that have been reported are, in part, due to the inhibition of NF-κB as reported here. Inhibition of NF-κB may also contribute to the antitumor and antiparasitic effects of β-lapachone. In addition, NF-κB activation is also required for expression of several gene products that induce apoptosis, including p53, c-myc, and caspase-1 [13]. Therefore, it is possible that inhibition of expression of these genes by β-lapachone blocks apoptosis. This is consistent with our results indicating that β-lapachone blocks TNF-induced apoptosis. There are, however, other NF-κB-regulated genes that block apoptosis, and this group includes genes that encode cellular inhibitors of apoptosis (cIAP), zinc finger protein A20, and Mn superoxide dismutase [33–35].

Like β-lapachone, curcumin also is known to block TNF-induced NF-κB activation [11]. Although curcumin is not structurally related to β-lapachone, like curcumin, β-lapachone also blocks TNF-induced AP-1 activation [12]. How curcumin blocks AP-1 activation is not clear, but our results indicate that JNK, one of the kinases that regulate AP-1, is suppressed by β-lapachone. Suppression of AP-1 activation, which is an anti-mitogenic signal, also may account for the apoptotic effects of β-lapachone. We found that β-lapachone also blocked TNF-mediated cytotoxicity and caspase-3 activation, suggesting that this drug interrupts a step that must be upstream of most of the TNF signaling processes. Because reactive oxygen intermediates have been implicated in TNF-mediated cytotoxicity and in NF-κB and AP-1 activation [36, 37], it is possible that β-lapachone is acting as an antioxidant. In this regard, the redox cycling of β-lapachone has been described [38], which may account for some of its effects.

We found that the inhibitory effects of β-lapachone could be abolished by DTT or NAC, results consistent with the observation that β-lapachone interferes with the cellular redox status. These findings also indicate the critical role played by a sulfhydryl group. Further, a cellular reducing catalyst, thioredoxin, is known to activate NF-κB by altering the redox state of a highly reactive cysteine at position 62 in the p50 subunit of NF-κB [39]. Thioredoxin does not interfere with IκBα degradation. Because in our studies β-lapachone blocked TNF-induced IκBα degradation and direct treatment of p50-p65 subunits with β-lapachone had no effect on DNA binding, it is unlikely that β-lapachone directly modifies the NF-κB proteins. In addition, because most protein tyrosine phosphatases (PTPases) contain a cysteine at their active site [40] and also because inhibition of PTPase has been shown to block NF-κB activation by TNF [41, 42], it is possible that β-lapachone acts by inhibiting the PTPase.

Overall, our results show that NF-κB, AP-1, MEK, and JNK are other novel targets for β-lapachone, and this may explain some of the previously reported pharmacologic effects of this drug.

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