BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **226,** 214–221 (1996)
ARTICLE NO. 1335

Inhibition of NF-κB Activation by Panepoxydone

Gerhard Erkel,*[,1] Timm Anke,* and Olov Sterner†

**Lehrbereich Biotechnologie der Universität, Paul-Ehrlich-Strasse 23, D-67663 Kaiserslautern, Germany; and †Division of Organic Chemistry 2, Chemical Center, University of Lund, S-221 00 Lund, Sweden*

Received July 15, 1996

In a search for new inhibitors of NF-κB mediated signal transduction in COS-7 cells using the Secreted Alkaline Phosphatase as reporter gene, panepoxydone has been isolated from fermentations of the basidiomycete *Lentinus crinitus.* Panepoxydone inhibits the NF-κB activated expression of the SEAP with an IC_{50} of 1.5 - 2 μg/ml (7.15 - 9.52 μM). No inhibition of AP-1 mediated expression of the reporter gene could be observed at a concentration up to 5 μg/ml panepoxydone. Panepoxydone strongly reduced the TPA, TNF-α and ocadaic acid mediated binding of NF-κB to the high affinity consensus sequence in COS-7 and HeLa S3 cells as confirmed by EMSA's. Panepoxydone inhibits the IκB phosphorylation and therfore sequesteres the NF-κB complex in an inactive form.

NF-κB is an inducible, ubiquitous transcription factor which regulates the expression of various cellular genes involved in immune response, inflammation, acute phase response and several viral genes (1). The active DNA-binding form of NF-κB are dimeric complexes, composed of various combinations of the Rel/NF-κB family of polypeptides (2). In non-stimulated cells NF-κB is located in the cytoplasm complexed with an inhibitory protein IκB-α. Upon stimulation of the cells with TNF-α, TPA, Ocadaic acid (OA) or cytokines, IκB-α is phosphorylated and becomes susceptible to proteolysis which leads to the dissociation from the NF-κB dimer and allows the translocation of the factor to the nucleus, DNA-binding of the active heterodimers composed of p50 and p65 subunits and the activation of transcription. Because NF-κB is involved as an immediate-early transcriptional activator of genes encoding inflammatory cytokines and cell adhesion molecules, inhibitors of NF-κB activation may therefore have broad application as novel therapeutics (3).

Basidiomycetes provide an interesting source of novel secondary metabolites with a variety of biological activities (4). In order to search for new inhibitors of the NF-κB mediated gene expression, COS-7 cells have been transiently transfected with a reporter gene construct, which contained the reporter gene secreted alkaline phosphatase (SEAP) under the control of five copies of the NF-κB binding consensus sequence. A screening of some 500 strains of basidiomycetes, ascomycetes and fungi imperfecti resulted in the isolation of panepoxydone from cultures of the basidiomycete *Lentinus crinitus.* The identification was done by spectroscopic methods. Panepoxydone (see Fig. 1) and several related derivatives have been previously reported as secondary metabolites from *Panus rudis, Panus conchatus* (5) and *Penicillium urticae* (6). Beside cytotoxic effects on mouse P815 mastocytoma cells, no biological activities of panepoxydone have been reported (5). In the present study we investigated the effect of Panepoxydone on NF-κB activation in mammalian cells.

[1] To whom correspondence should be addressed. Fax: (49)-631-205-2999. E-mail: erkel@rhrk.uni-kl.de.

Abbreviations: SEAP, secreted alkaline phosphatase; TNF-α, tumor necrosis factor-α; TPA, 12-o-tetradecanoylphorbol-13-acetate; OA, ocadaic acid; PDTC, pyrrolidine dithiocarbamate.

0006-291X/96 $18.00

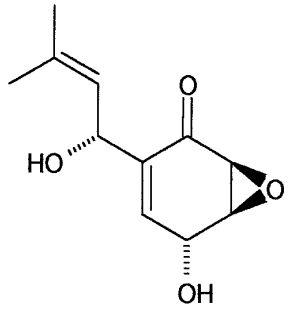

FIG. 1. Structure of panepoxydone.

MATERIALS AND METHODS

Lentinus crinitus strain 9325. Mycelial cultures were obtained from tissue plugs of fruiting bodies collected on wood. The strain is deposited in the culture collection of the LB Biotechnologie, Universität Kaiserslautern. For maintenace and production of panepoxydone, the fungus was cultivated in YMG medium composed of : glucose 0.4 %, yeast extract 0.4 %, malt extract 1 % and agar 1.5 % for solid media. Fermentations were carried out in a Biolafitte C-6 fermenter containing 20 l of YMG medium with aeration (3 l air/min) and agitation (120 rpm) at 22 °C. After four days the culture fluid was separated by filtration and applied onto Mitsubishi DIAION HP 21 resin. The resin was washed with water and the active compound was eluted with acetone. The eluate was concentrated and the remaining aqueous residue extracted with EtOAc. The solvent was evaporated and the crude product was separated by chromatography on silica gel (Merck 60) with cyclohexane:EtOAc (20:80) as eluent. Preparative HPLC (Merck LiChrosorb CN, 7 μm) with cyclohexane:*t*-buthylmethylether (40:60) as eluent yielded 30 mg of panepoxydone.

Plasmid construction. The reporter plasmids pGE2-NF1 and pGE2-AP1 have been constructed by replacing the CMV-promoter of the plasmid pCMV/SEAP (Tropix) with a Hind III/Mlu I fragment containing the enhancerless SV 40 promoter of the pGL2-promoter vector (Promega). Double stranded synthetic oligonucleotides (Promega) containing either the high affinity consensus binding site for the NF-κB complex or the TPA response element (TRE) were multimerized by phosphorylation and selfligation and cloned immediately upstream of the SV 40 promoter after digestion of the vectors with Nhe I and filled in with Klenow polymerase. After transformation of *E. coli* JM109 (7) clones were selected that contained 5× NF-κB or 3× AP-1 binding sites. The plasmid pGE3-NF1 has been constructed by replacing the luciferase gene of the promoterless pGL3-basic vector (Promega) with a Hind III/Xba I fragment containing the SEAP gene (8). A Hind III/BamH I fragment containing the thymidine kinase promoter subcloned in the pTZ18R (Pharmacia) vector was then transferred to the pGL3 derived vector containing the SEAP gene. 5× binding site for the NF-κB complex has been cloned in the Nhe I site of the resulting vector as described above to form pGE3-NF1.

The sequences of the oligonucleotides were: NF-κB binding site: 5′-AGTTGAGGGGACTTTCCCAGGC-3′, AP-1 binding site: 5′-CGCTTGATGAGTCAGCCGGAA-3′.

Cell Culture, transfection, and SEAP-Assay. Cos-7 cells (ATCC CRL 1651) and HeLa S3 cells (ATCC CCL 2.2) were maintained in DMEM-Medium supplemented with 10 % fetal calf serum (FCS) and 65 μg/ml penicillin G and 100 μg/ml streptomycin sulfate in a humidified atmosphere containing 5 % CO_2 at 37 °C. Transfections of COS-7 or HeLa S3 cells were performed by electroporating 3×10^6 cells suspended in 1 ml phosphate buffered saline (PBS) containing 30 μg of the reporter constructs at 500 V/cm and τ = 20 - 23 ms using a gene pulser apparatus (BioRad). After electroporation the cells were seeded at 1×10^5 cells/ml Opti-MEM (GIBCO, BRL) containing 10 % FCS in a 24 well tissue culture plate and allowed to recover for 16 hrs. For induction of SEAP expression, cells were treated with 50 ng/ml TPA or TNF-α (2 ng/ml) with or without test compounds in Opti-MEM containing 0.5 % FCS. The activity of the SEAP in the culture medium was determined 60 hrs after transfection using the Phospha-Light chemiluminiscent reporter gene assay (TROPIX, MA) according to the manufacturer's instructions with a liquid scintillation counter.

Electrophoretic mobility shift assays (EMSAs) and western blots. COS-7 and HeLa S3 cells were starved for 16 hrs in DMEM-medium with 0.5 % FCS, treated for the indicated times with test compounds and induced with 2 ng/ml TNF-α, 50 ng/ml TPA or 500 nM OA. Total cell extracts were prepared using a high-salt detergent buffer and tested for binding to the NF-κB and AP-1 binding sequence as recently described (9). For western-blotting, cell extracts (10 μg protein) were subjected to 10 % SDS-PAGE, transferred onto a nitrocellulose membrane, probed with a anti-IκB-antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and then with a goat anti-rabbit antibody conjugated

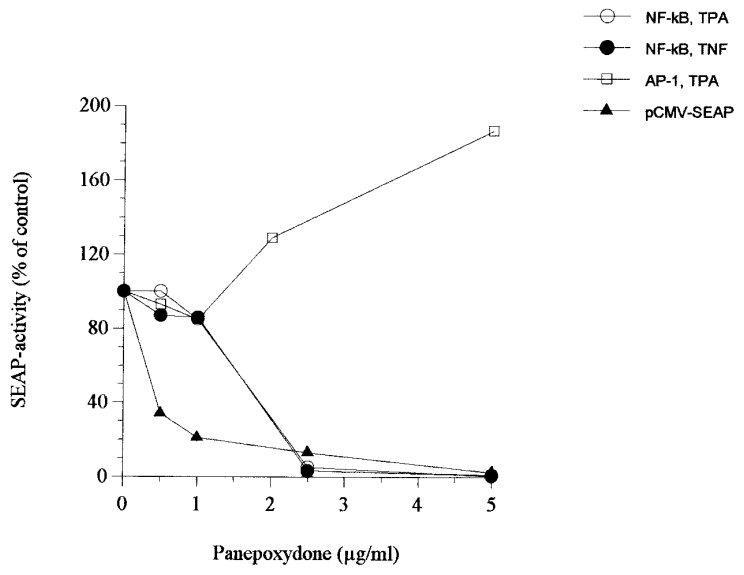

FIG. 2. Inhibition of NF-κB and AP-1 mediated SEAP-expression in COS-7 cells. Cos-7 cells were transfected with pCMV-SEAP or a reporter gene construct containing the SEAP gene under the control of the SV40 minimal-promotor and 5 $\times$ NF-κB or 3 $\times$ AP1 consensus sequences and stimulated with TPA (50 ng/ml) or TNF-α (2 ng/ml) for 48 hrs with or without panepoxydone. Control (100%): stimulation only.

to alkaline phosphatase (TROPIX, MA). Immunoreactive proteins were visualized by the Western-Light chemoluminiscent detection system (TROPIX, MA).

Cytotoxicity and macromolecular syntheses. Cytotoxicity and macromolecular syntheses in whole COS-7 cells were measured as described previously (10).

RESULTS AND DISCUSSION

Transfection of COS-7 cells with an SEAP reporter plasmid containing a SV-40 minimal promoter and 5 $\times$ NF-κB (pGE2-NF1) and induction with TPA (50 ng/ml) or TNF-α (2 ng/ml) resulted in a 20 fold activation over the basal level of SEAP expression (data not shown). In order to examine the effect of panepoxydone on AP-1 mediated SEAP expression, the reporter plasmid pGE2-AP1 had been constructed which increased the SEAP activity eight fold after stimulation with 50 ng/ml TPA. Panepoxydone inhibited the TPA and TNF induced NF-κB mediated SEAP-expression with an IC_{50} of 1.5 - 2 μg/ml (7.15 - 9.52 μM). At the same concentration a slight stimulation of AP-1 mediated SEAP expression after induction with TPA could be observed which might be due to the complete shutdown of the NF-κB induced pathway (Fig.2). Stimulation of the cells with 2 μg/ml panepoxydone without addition of TPA showed no increase in the AP-1 mediated SEAP expression. In addition, the SEAP expression in COS-7 cells, transfected with the pCMV-SEAP vector (Promega) which is regulated by the constitutive CMV-promoter was also inhibited to 50 % by 0.5 μg/ml panepoxydone. This might be due to the occurrence of multiple enhancer sequences in the CMV-promoter (11).

The effect of panepoxydone on NF-κB mediated SEAP expression in HeLa S3 cells has been investigated using a reporter plasmid which expresses the reporter gene under the control of five copies of the NF-κB binding site immediatly cloned upstream of the thymidine kinase promoter (pGE3-NF1). Induction of SEAP expression in HeLa S3 cells with 50 ng/ml TPA or 2 ng/ml TNF-α resulted in an increase of the SEAP activity between 15 and 20 fold

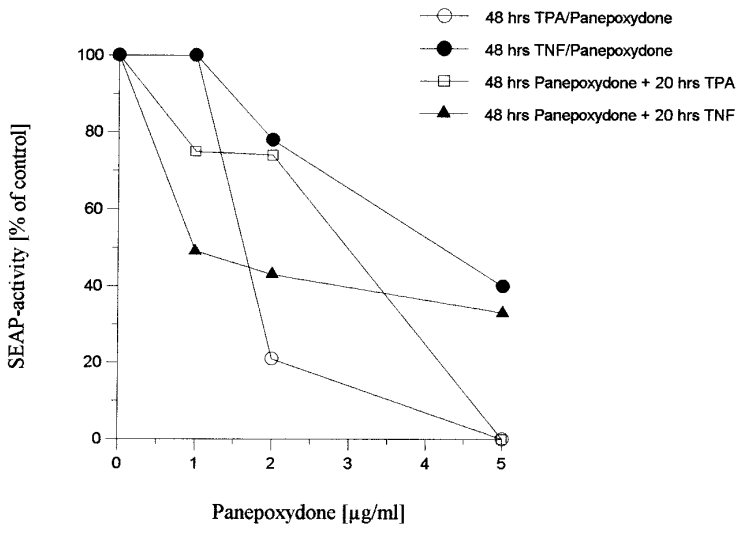

FIG. 3. Inhibition of TPA and TNF induced SEAP-expression in HeLa S3 cells by panepoxydone. HeLa S3 cells transfected with a reporter gene construct containing the SEAP gene under the control of the TK-promotor and 5 × NF-κB consensus sequence were stimulated with TPA (50 ng/ml) or TNF-α (2 ng/ml) in the presence of panepoxydone for 48 hrs or treated with panepoxydone for 24 hrs and stimulated for additional 24 hrs with TPA or TNF-α. Control (100%): stimulation by TPA or TNF-α without additions.

compared to the uninduced control. Treatment of HeLa S3 cells for 48 hrs with TPA and panepoxydone resulted in a similar inhibition of the NF-κB mediated SEAP-expression as observed in COS-7 cells (IC_{50}: 1.5 - 2 μg/ml). Preincubation with panepoxydone for 24 hrs and stimulation with TPA for additional 20 hrs resulted in a increase of the IC_{50} to 3 μg/ml (Fig. 3). The inhibitory effect of panepoxydone on the TNF activated SEAP expression was much more pronounced after preincubation with panepoxydone and stimulation for 20 hrs with TNF-α (IC_{50}: 1.5 μg/ml).

Cellular DNA-, RNA-, and protein syntheses were examined in COS-7 cells by determing the incorporation of (^{14}C)-thymidine, (^{14}C)-uridine and (^{14}C)-leucine into acid-insoluble fractions. Up to a concentration of 20 μg/ml of panepoxydone no effect on macromolecular syntheses could be observed (data not shown). These results indicate that panepoxydone does not interfere with the replication, transcription or translation in a general manner.

In order to determine the effect of panepoxydone on activation of NF-κB, EMSA's with whole cell extracts were performed with a high affinity binding site. Stimulation of COS-7 and HeLa S3 cells with TPA, an activator of protein kinase C, for 48 hrs resulted in the appearance of a band which could be eliminated by a 10 fold excess of unlabelled NF-κB probe (Fig. 4). Incubation of the cells with TPA (50 ng/ml) and panepoxydone lead to a dose dependent reduction of NF-κB binding. Quantification by densiometry scanning revealed that the inhibition of NF-κB activation corresponded well to the results from the reporter gene assays. The IC_{50} for the inhibition of NF-κB binding to the consensus sequence was determined to 2 μg/ml for both cell lines (Fig.4). EMSA's using the same cell extract with a high affinity binding site for the transcription factor AP-1 (data not shown) revealed no inhibition of AP-1 DNA binding by panepoxydone indicating a preferential target within the NF-κB activating pathway.

Stimulation of Hela S3 cells with 2 ng/ml TNF-α for 20 min resulted in a strong increase in NF-κB activation (Fig. 5). Pretreatment of HeLa S3 cells with 2 μg/ml (9.52 μM) of

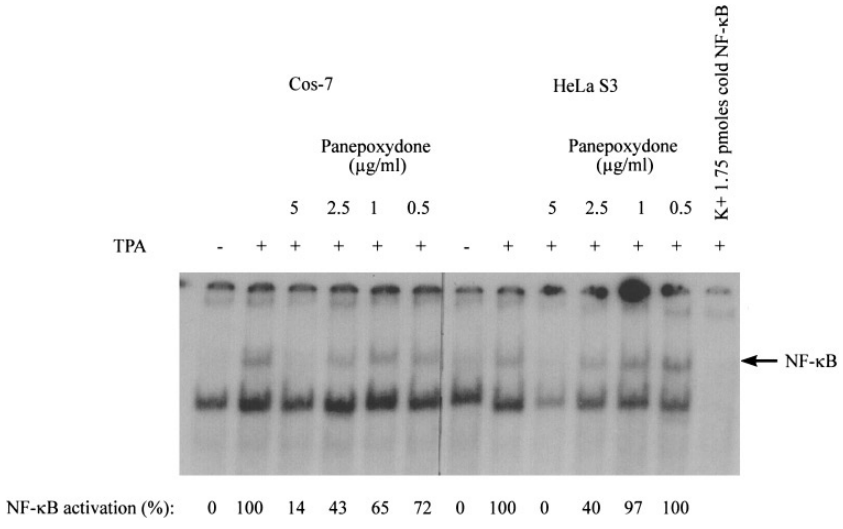

FIG. 4. Effect of panepoxydone on NF-κB activation in COS-7 and HeLa S3 cells. COS-7 and HeLa S3 cells were treated for 48 hrs with TPA (50 ng/ml), without or with various concentrations of panepoxydone. Total cell extracts containing equal amounts of protein (10 μg) were then analyzed by EMSA for DNA binding activity of NF-κB using a ^{32}P-labelled oligonucleotide with the high affinity site 5′-AGTTGAGGGGACTTTCCCAGGC-3′. The inducible NF-κB complex is indicated. A section of the fluorogram from a native gel is shown.

panepoxydone for 2 hrs before stimulation showed a strong reduction in NF-κB binding activity (Fig.5) which was comparable to 100 μM of the antioxidant PDTC, a known inhibitor of NF-κB activation (12,13). In addition, NF-κB activation mediated by the phosphatases 1, and 2A inhibitor OA (14) was also markley inhibited by pretreatment of the cells with 2μg/ml panepoxydone for 2 hrs. Similar results have been obtained with

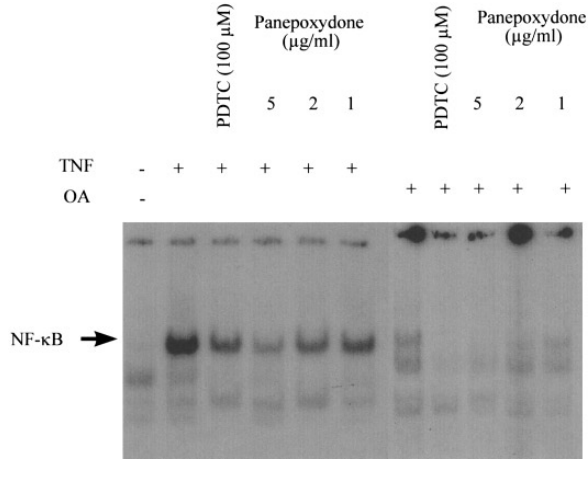

FIG. 5. Effect of panepoxydone on NF-κB activation in HeLa S3 cells. Hela S3 cells have been pretreated for two hrs with various concentrations of panepoxydone and stimulated for 20 min with TNF-α (2 ng/ml) or 30 min with OA (500 nM). Whole cell extracts (10 μg of protein) were analyzed by EMSA using a ^{32}P-labelled NF-κB consensus motif. The inducible NF-κB complex is indicated. A section of the fluorogram from a native gel is shown.

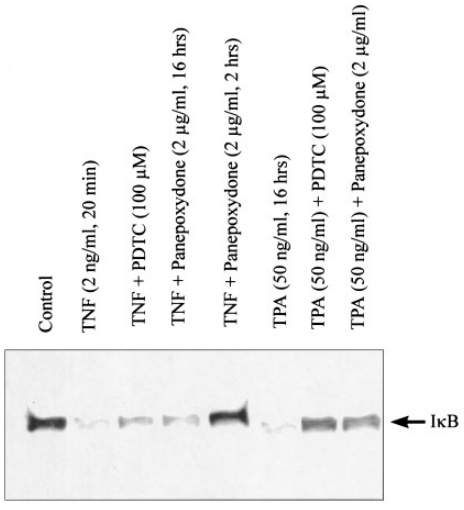

FIG. 6. Effect of panepoxydone on degradation of IκB in Hela S3 cells. Hela S3 cells were treated with paneopxydone for the indicated times and stimulated for 20 min with TNF-α or 16 hrs with TPA. Total cell extracts were prepared and western blotting was performed as described under Material and Methods using an IκB antibody.

preincubating the cells for 16 hrs and stimulation with TNF-α and OA for 20 min and 30 min respectively (data not shown).

HeLa cells were pretreated with panepoxydone for the indicated times and stimulated with TPA and TNF. Total cell extracts were analyzed by western blotting using a polyclonal IκB antibody. The immunoblotting demonstrated that activation of Hela S3 cells with TPA or TNF resulted in a loss of IκB (Fig. 6). The degradation of IκB was deminished in the presence of either panepoxydone (2 μg/ml) or the antioxidant PDTC (100 μM). These results suggest that panepoxydone interrupts the signalling pathway by inhibiting the phosphorylation or degradation of IκB.

To characterize whether panepoxydone prevents NF-κB activation by inhibiting the phosphorylation and proteolytic degradation of IκB, HeLa cells were preincubated with 2 μg/ml panepoxydone for 2 hrs and stimulated with OA (500 nM). At the indicated times total cell extracts were prepared and immunoblotted with an IκB antibody. After 30 min of OA treatment almost all IκB disappeared. The loss of IκB after 30 and 60 min of OA treatment was efficiently prevented by panepoxydone (Fig. 7A). It has been shown that proteasome inhibitors like PSI (Cbz-Ile-Glu(O-t-Bu)-Ala-leucinal) or N-Acetyl-Leu-Leu-norleucinal (Ac-LLnL) led to the accumulation of the phorphorylated form of IκB-α, which could be detected by its reduced mobility in SDS gels (15, 16). Pretreatment of HeLa cells with 10 or 100 μM of the peptide inhibitor Ac-LLnL for two hours and stimulation with 10 ng/ml TNF-α for 20 min resulted in the stabilization of the hyperphosphorylated form of IκB (Fig. 7B). After treatment of HeLa S3 cells with Ac-LLnL in the presence of 2 μg/ml panepoxydone, no stabilization of phosphorylated IκB (Fig. 7B) could be observed (17). This results indicate that panepoxydone inhibits NF-κB activation by preventing the phosphorylation of IκB and therefore sequestering the complex in an inactive form.

Reactive oxygen intermediates have been directly implicated as second messengers in the activation of NF-κB by various stimuli based upon the ability of different antioxidants to inhibit NF-κB activation in intact cells (1-3, 18, 19). These include N-acetyl-L-cysteine (NAC), vitamine E derivatives, 2-mecaptoethanol, dithiocarbamates, butylated hydroxyanisol, α-lipoic

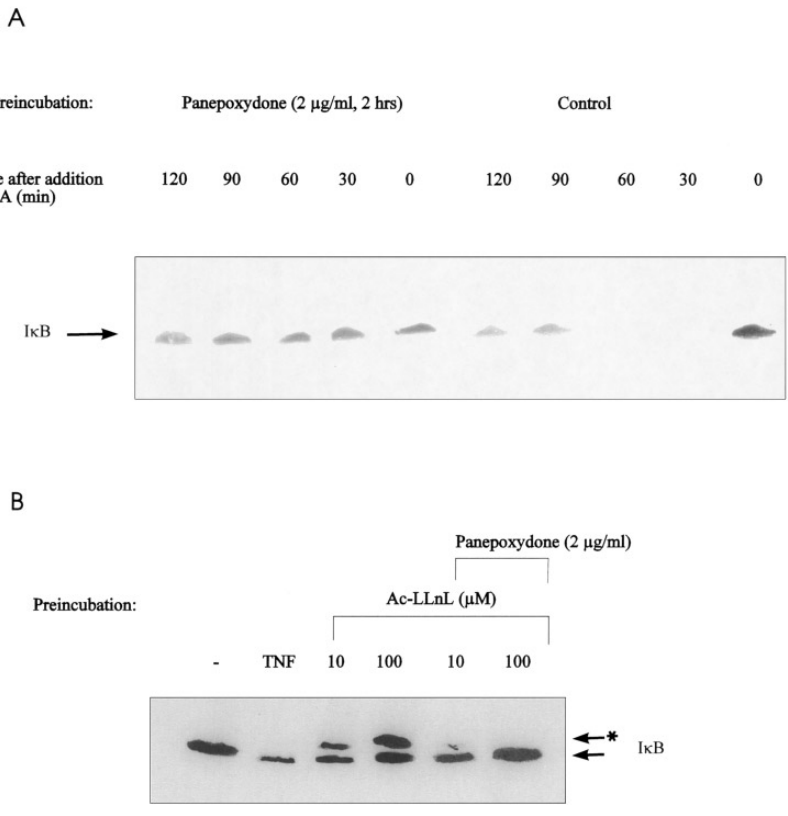

FIG. 7. Effect of panepoxydone on the stability of IκB in HeLa S3 cells. (A) Hela S3 cells were pretreated for 2 hrs with 2 μg/ml panepoxydone followed by stimulation with 500 nM OA for the indicated periods of time. Total cell extracts were prepared and western blotting was performed as described in material and methods using an IκB antibody. (B) Hela S3 cells were left untreated or pretreated for 2 hrs with 10 μM or 100 μM Ac-LLnL with or without 2 μg/ml panepoxydone and stimulated with 10 ng/ml TNF-α for 20 min. Total cell extracts were prepared and western blotting was performed as described under Material and Methods using an IκB antibody. The asterik marks the more slowly migrating phosphorylated IκB variant.

acid and chelators of iron and copper ions (3, 20). The mode of action of these inhibitors seems to be the result of their ability to scavange free radicals and not of a special structure-related acitivity. Recently it has been shown that antioxidants like PDTC and NAC strongly activate the TRE-dependent transactivation of an AP-1 tkCAT reporter gene construct as well as the DNA-binding activity of AP-1 in HeLa S3 cells while the activation of the transcription factor NF-κB by antioxidants alone were ineffective (21). In contrast, our results indicate that panepoxydone does not inhibit NF-κB activation via an antioxidant mode of action since panepoxydone alone failed to stimulate the AP-1 mediated SEAP expression and the activation of DNA binding of the AP-1 transcription factor complex in COS-7 and HeLa S3 cells (data not shown). Our data suggest that panepoxydone inhibits NF-κB activiation by preventing the phosphorylation of the IκB protein and thereby interrupting the signalling pathway.

ACKNOWLEDGMENT

We thank M. Saul for skillful technical assistance.

REFERENCES

1. Bäuerle, P. A., and Henkel, T. (1994) *Annu. Rev. Immunol.* **12,** 141–179.
2. Siebenlist, U., Franzoso, G., and Brown, K. (1994) *Annu. Rev. Cell Biol.* **10,** 405–455.
3. Manning, A. M., and Anderson, D. C. (1994) *Annu. Rep. Med. Chem.* **29,** 235–244.
4. Erkel, G., and Anke, T. (1996) *in* Biotechnology (Rehm, H. J., Reed, G., Pühler, A., and Stadler, P., Eds.), Vol. 10.
5. Kis, Z., Closse, A., Sigg, H. P., Hruban, L., and Snatzke, G. (1970) *Helv. Chem. Acta* **53,** 1577–1597.
6. Sekiguchi, J., and Gaucher, G. M. (1979) *Biochem. J.* **182,** 445–453.
7. Yanisch-Perron, C., Viera, J., and Messing, J. (1985) *Gene* **33,** 103–119.
8. Cullen, B. R., and Malim, M. H. (1992) *in* Methods Enzymol. **216,** 362–368.
9. Pahl, H. L., and Bäuerle, P. A. (1995) *EMBO J.* **14,** 2580–2588.
10. Weber, W., Anke, T., Stefan, B., and Steglich, W. (1990) *J. Antibiot.* **47,** 1017–1024.
11. Boshart, M., Weber, F., Jahn, G., Dorsch-Häsler, K., Fleckenstein, B., and Schaffner, W. (1985) *Cell* **41,** 521–530.
12. Henkel. T., Machleidt, T., Alkalay, I., Krönke, M., Ben-Neriah, Y., and Baeuerle, P. A. (1993) *Nature* **365,** 182–185.
13. Schreck, R., Meier, B., Männel, D. N., Dröge, W., and Baeuerle, P. A. (1992) *J. Exp. Med.* **175,** 1181–1194.
14. Thévenin, C., Jim, S. C., Rieckmann, P., Fujiki, H., Norcross, M. A., Sporn, M. B., and Kehrl (1991) *New Biol.* **2,** 793–800.
15. Traenckner, E. B-M., Wilk, S., and Baeuerle, P. A. (1994) *EMBO J.* **13,** 5433–5441.
16. DiDonato, J. A., Mercurio, F., and Karin, M. (1995) *Mol. Cell. Biol.* **15,** 1302–1311.
17. Traenckner, E. B-M., Pahl, H. L., Henkel, T., Schmidt, K. N., Wilk, S., and Baeuerle, P. A. (1995) *EMBO J.* **14,** 2876–2883.
18. Schreck, R., Rieber, P., and Baeuerle, P. A. (1991) *EMBO J.* **10,** 2247–2258.
19. Schulze-Osthoff, K., Bakker, A. C., Vanhaesebroeck, B., Beyaert, R., Jacob, W. A., and Fiers, W. (1992) *J. Biol. Chem.* **267,** 5317–5322.
20. Suzuki, Y. J., Aggarwal, B. B., and Packer, L. (1992) *Biochem. Biophys. Res. Commun.* **189,** 1709–1715.
21. Meyer, M., Schreck, R., and Baeuerle, P. A. (1993) *EMBO J.* **12,** 2005–2015.