

Dithiocarbamates as Potent Inhibitors of Nuclear Factor κ B Activation in Intact Cells

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Summary

Dithiocarbamates and iron chelators were recently considered for the treatment of AIDS and neurodegenerative diseases. In this study, we show that dithiocarbamates and metal chelators can potentially block the activation of nuclear factor κ B (NF- κ B), a transcription factor involved in human immunodeficiency virus type 1 (HIV-1) expression, signaling, and immediate early gene activation during inflammatory processes. Using cell cultures, the pyrrolidine derivative of dithiocarbamate (PDTC) was investigated in detail. Micromolar amounts of PDTC reversibly suppressed the release of the inhibitory subunit I κ B from the latent cytoplasmic form of NF- κ B in cells treated with phorbol ester, interleukin 1, and tumor necrosis factor α . Other DNA binding activities and the induction of AP-1 by phorbol ester were not affected. The antioxidant PDTC also blocked the activation of NF- κ B by bacterial lipopolysaccharide (LPS), suggesting a role of oxygen radicals in the intracellular signaling of LPS. This idea was supported by demonstrating that treatment of pre-B and B cells with LPS induced the production of O₂⁻ and H₂O₂. PDTC prevented specifically the κ B-dependent transactivation of reporter genes under the control of the HIV-1 long terminal repeat and simian virus 40 enhancer. The results from this study lend further support to the idea that oxygen radicals play an important role in the activation of NF- κ B and HIV-1.

When cells are exposed to life-threatening conditions, many genes are newly activated with products that serve to warn other cells and defend the organism. For instance, UV light, γ rays, elevated temperature, oxygen radicals, pro- and eukaryotic parasites, viruses, and cytokines (which are released by pathogen-stimulated cells) can elicit such an emergency program of gene activation. Among the newly synthesized proteins are cytokines, such as IFN- β and TNF, cell surface receptors with immunological functions, acute phase proteins, and growth factors. This kind of response is not restricted to cells of the immune system but can probably occur in most cell types of the organism. One would expect that there are multiple receptors for pathogens that elicit various intracellular signal transducing systems. These finally reach many different transcription factors in the nucleus which then activate the genes encoding signaling and defense proteins.

It was thus surprising to find that one multiprotein complex, the nuclear factor κ B (NF- κ B),¹ has apparently specialized

during evolution in the reception of many diverse pathogenic signals and, also, in cytoplasmic/nuclear signal transduction and immediate early activation of a multitude of genes encoding signaling and defense proteins (for review, see references 1 and 2). Pathogenic signals lead to the activation of a preexisting, cytoplasmic form of NF- κ B that, after translocation into the nucleus, initiates transcription of genes in synergy with other factors. The activation of NF- κ B has its molecular basis in the release of an inhibitory subunit, called I κ B, from a heterodimer of two DNA binding subunits, p50 and p65 (3, 4). It is a puzzling observation that this reaction can be triggered by very diverse agents. Some of them, such as phorbol esters, induce protein kinase C (PKC). But this kinase seems not to be a universal activator because other NF- κ B-activating agents, such as TNF and IL-1, do apparently not require PKC (5, 6).

We and others have recently observed that the activation of NF- κ B can be blocked by thiol compounds (7–9). 20–30 mM *N*-acetyl-L-cysteine (NAC) (8, 7) or 0.5 mM L-cysteine (9) could block NF- κ B induction upon PMA or TNF treatment of cells. The chemical basis for the inhibiting effect of the compounds seemed to lie in an oxygen radical-scavenging effect of the thiol groups. An involvement of reactive oxygen intermediates (ROI) in the effects of PMA and TNF was

¹ *Abbreviations used in this paper:* DDTC, diethyl derivative of dithiocarbamate; DOC, sodium deoxycholate; EMSA, electrophoretic mobility shift assays; NAC, *N*-acetyl-L-cysteine; PDTC, pyrrolidine dithiocarbamate; PGA, pyroglutamic acid; ROI, reactive oxygen intermediates; SOD, superoxide dismutases.

further evident from depletion of glutathione levels (8, 10) and release of H_2O_2 and O_2^- when cells are stimulated with PMA or TNF (11–15). More direct evidence that ROI play a role in the activation of NF- κ B came from the demonstration that exposure of T cells to micromolar amounts of H_2O_2 can activate the transcription factor and the κ B-dependent induction of a reporter gene under control of the HIV-1 LTR (7). Subsequently, it was shown that induction of NF- κ B by many other inducers, such as cycloheximide, double-stranded RNA, bacterial LPS, IL-1, lectin, calcium ionophore (7), and the viral transactivator tax from HTLV-1 (R. Schreck, R. Grassmann, B. Fleckenstein, and P. Baeuerle, manuscript in preparation), was also blocked by thiol reagents, suggesting that ROI serve as an important and widely used messenger in the activation of NF- κ B (16).

The metal-chelating properties of the diethyl derivative of dithiocarbamate (DDTC) were exploited for decades for the treatment of metal poisonings (17 and references therein). In 1983, DDTC was administered for the first time to AIDS patients (18). In the meantime, many clinical trials showed that the drug can retard the onset of AIDS symptoms in some but not all infected individuals (19–21 and references therein). The molecular basis for this activity of DDTC is unknown, but thought to be related to the previously described radical-scavenging, antioxidative properties of dithiocarbamates (22–24). The recent observation that exposure to oxidative stress of cell cultures latently infected with HIV-1 induces activation of the provirus and viral replication (7, 25, 26) would explain the antiviral effect of antioxidants. Also consistent with an important role of oxidative stress in the progression of AIDS are the observations that HIV-infected patients have decreased levels of plasma cysteine (27, and reviewed in reference 28), and that antioxidants, such as vitamin C (29), NAC (30), and glutathione (and esters) (31), suppress HIV-1 replication. Because NF- κ B is an important activator of the HIV-1 provirus (32, 33) and is specifically activated upon oxidant stress (7), we have tested here whether the transcription factor is a target for dithiocarbamates.

Other agents that can interfere with production of oxygen radicals in intact cells are desferrioxamine (34–37) and *o*-phenanthroline (38). The chelators can complex Fe^{3+} and other metal ions that, by the Fenton reaction, participate in the production of hydroxyl radicals from H_2O_2 . A recent study suggests that desferrioxamine may slow down clinical progression of the dementia associated with Alzheimer's disease (39). For these reasons, we also tested nonsulfur metal chelators for inhibition of NF- κ B induction.

Using cell culture experiments, dithiocarbamates and metal chelators are shown here to be potent inhibitors of NF- κ B activation. Three dithiocarbamate derivatives and two metal chelators could inhibit NF- κ B induction at an up to two orders of magnitude lower concentration than the sulhydryl compound NAC. Pyrrolidine dithiocarbamate (PDTC) seemed to be suited best for cell culture experiments. The effect of PDTC seemed to be specific for NF- κ B, and the drug was active with all NF- κ B inducers and cell lines tested. Early withdrawal of PDTC did not affect the responsiveness of cells,

but overdosage or chronic treatment desensitized the cells for the inhibiting effect of PDTC. The specific and dose-dependent inhibitory effect of PDTC on the inducibility of NF- κ B DNA binding was reflected in the drug's effect on the transactivation of various reporter genes. While κ B-dependent gene induction was inhibited by micromolar concentrations of PDTC, the basal activities of reporter constructs were virtually unaffected by the drug. Because PDTC did not affect the level of the cytoplasmic NF- κ B-I κ B complex and could not interfere with DNA binding or nuclear uptake of NF- κ B, it most likely blocked a reaction required for release of I κ B from NF- κ B in intact cells. This reaction seemed to require metal ions and hydroxyl radicals. Superoxide radicals, which are rapidly eliminated in cell lines overexpressing Mn-dependent superoxide dismutase (MnSOD), seem not to be directly involved in NF- κ B activation. PDTC also blocked the activation of NF- κ B by LPS, suggesting that the endotoxin induced oxidative stress in cells. This was directly demonstrated by measuring the production of H_2O_2 and O_2^- in pre-B and B cells stimulated with LPS.

Materials and Methods

Cell Culture and Treatments. Jurkat T (subclone JR, kindly provided by Dr. Th. Hünig, Würzburg, Germany), BJA-B, and MCF-7 cell lines, and an EBV-transformed human B cell line, were grown in RPMI 1640 supplemented with 10% FCS and 1% (wt/vol) penicillin/streptomycin (all purchased from Gibco Laboratories, Grand Island, NY) 70Z/3.12 cells (TIB 158; American Type Culture Collection, Rockville, MD) were cultured in RPMI 1640 containing 50 μM 2-ME. Ltk^- cells were kept in DME containing 10% FCS and 1% antibiotics. TNF- α and IL-1 β were purchased from Genzyme (Boston, MA), and LPS, PHA, NAC, pyrrolidine, glutathione, PDTC, DDTC, disulfiram, desferrioxamine, FeCl_3 , *o*-phenanthroline, pyroglutamic acid (PGA), cycloheximide, and PMA were from Sigma Chemical Co. (St. Louis, MO). H_2O_2 was purchased from Merck and 2-ME from Roth. PGA and NAC were adjusted to pH 7.4 by addition of 1 N NaOH. LPS, pyrrolidine, glutathione, PDTC, and DDTC were dissolved in PBS, and *o*-phenanthroline in 20% EtOH. Cycloheximide and desferrioxamine were dissolved in H_2O , disulfiram in 100% EtOH, and PMA in DMSO. The respective solvents were added to control cultures. The use of metal spatulas was avoided. Preincubation periods and concentrations of compounds are indicated in the figure legends.

Oligonucleotides and Plasmid Constructs. Oligonucleotides were synthesized on a cell synthesizer (A380; Applied Biosystems, Inc., Foster City, CA) by the phosphoroamidate method and purified on OPC cartridges (Applied Biosystems, Inc.) according to the instructions provided by the manufacturer. The sequences of the double-stranded oligonucleotides used to detect the DNA binding activities of NF- κ B, octamer binding proteins (oct), AP-1/c-Fos (TRE), CRE binding proteins, and Sp1 were the following (the binding site is underlined). NF- κ B: 5'-AGCTTGGGCC-GGGGAATCCCGCTAAG-3' (this motif is from the promoter of the IRF-1 transcription factor [40] containing a high affinity binding site for NF- κ B [R. Schreck and P. Baeuerle, manuscript in preparation]); Oct: 5'-AGCTTTGGGTAATTTGCATTTCTAAG-3'; AP-1: 5'-AGCTTAAAAAAGCATGAGTCAGACACCTG-3'; CREB: 5'-TCGACTGCTGACGTCAGAGGGAGGAGTCAGTCTCCA-GCT-3'; Sp1: 5'-TCGACCTGGGGCGGGGCAGGAGCT-3'. The

complementary strands created 5'-overhanging ends (SalI or HindIII), which allowed labeling by the Klenow polymerase (Boehringer Mannheim Biochemicals, Indianapolis, IN) using two α -[^{32}P]-dNTPs (3,000 Ci/mmol) (Amersham Corp., Arlington Heights, IL) and the respective other two dNTPs in unlabeled form. The labeled DNA probe was purified on push columns (Stratagene Corp.). The wild-type and κB mutant forms of the plasmids, called here HIV-1-LTR-CAT, contain HIV-1 sequences from -453 to +80 from the transcription start site of the viral genome upstream of a CAT reporter gene (32, 41). The plasmid pSVtkCAT contains two 72-bp repeats from the SV40 enhancer in front of the tk promoter (42).

Electrophoretic Mobility Shift Assays. Cytoplasm and nuclear extracts were prepared from cultured cells as described (3). Equal proportions of cell fractions with 2–8 μg of protein were used in the assays. DNA binding conditions for NF- κB and electrophoretic mobility shift assays (EMSAs) were described in detail elsewhere (43). Briefly, binding reactions (20 μl) contained 2 μg poly(dI-dC) (Pharmacia Fine Chemicals, Piscataway, NJ), 5–10,000 cpm (Cerenkov) for ^{32}P -labeled DNA probe (>3,000 Ci/mmol), 1 μl buffer D containing 1% (vol/vol) NP-40, 20 μg BSA, and binding buffer. Reactions were started by the addition of cell extracts and allowed for 30 min before electrophoresis. Samples were analyzed on native 4% polyacrylamide gels. Dried gels were exposed to Kodak XR5 film on intensifying screens (DuPont Co., Wilmington, DE) for 5–15 h at -70°C .

Transfections and CAT Assays. Transfections were performed by the DEAE-dextran method according to Pomerantz et al. (44). 10^7 cells were washed with PBS and resuspended in Tris-buffered saline containing 200 $\mu\text{g}/\text{ml}$ DEAE-dextran (Pharmacia Fine Chemicals) and 15 $\mu\text{g}/\text{ml}$ of plasmid DNA in a total volume of 1 ml. 20 h after transfection, aliquots from batchwise-transfected cells were incubated with the indicated amounts of PDTC or PGA. 1 h later, cells were stimulated for 9 h with a combination of PMA (50 ng/ml) and PHA (5 $\mu\text{g}/\text{ml}$; both from Sigma Chemical Co.). Cell extracts were prepared by three cycles of freeze-thawing and equal amounts of protein determined by the method of Bradford (Bio-Rad Laboratories, Richmond, CA) used for CAT assays (45). A reaction mix of 150 μl contained 20 mM acetyl CoA (Sigma Chemical Co.) and 0.3 μCi ^{14}C -chloramphenicol (Amersham Corp.). Chloramphenicol acetylation was allowed with 60 μg of protein for 3.5 h (HIV-1-LTR-CAT) and with 40 μg for 1 h (pSVtkCAT) at 37°C , respectively. Transfections were performed in duplicate. Values deviated <10% from the mean. Mock transfections showed a chloramphenicol conversion of <0.3%. In unstimulated cells, the chloramphenicol conversion with HIV-1-LTR-CAT was 4.2% and the basal conversion of pSVtkCAT was 4.6%.

SOD-overexpressing Cell Line. The stable transfection of the human breast carcinoma cell line MCF-7 with the expression plasmid pExneo-SOD was described in detail by Kraft (46). Northern analysis was performed as described (47), and MnSOD activity in crude cell homogenates was determined in native 10% polyacrylamide gels by the staining method of Beauchamp and Fridovich (48).

Determination of H_2O_2 and O_2^- . H_2O_2 released from 2×10^5 70Z/3 and pre-B EBV-transformed B cells was determined fluorometrically by the peroxidase-catalyzed oxidation of scopoletin (49). Cell cultures contained 40 nM scopoletin and 1 μM peroxidase. A SFM-23 fluorimeter (Kontron) was used at an excitation wave length of 381 nm. Emission was measured at 436 nm. Plots were standardized with H_2O_2 . O_2^- released by the cell cultures was determined by the reduction of cytochrome *c* (50 μM) (50). A double-beam photometer (Uvikon 820; Kontron) with automated cuvette exchanger was used. Basal production was measured for 1 h before

stimulation. The specificity of cytochrome reduction was tested by the addition of SOD (1 μM).

Results

Diverse Thiol Compounds and Metal Chelators Interfere with Activation of NF- κB . We have recently observed that a Jurkat T cell subclone activates NF- κB when exposed to oxidative stress by treatment with H_2O_2 (7). H_2O_2 can passively cross the plasma membrane and, within the cell, is either converted by catalases into H_2O and O_2 , or, by the Fenton reaction, into hydroxyl radicals ($\text{OH}\cdot$). If the effect of H_2O_2 relies on the production of $\text{OH}\cdot$ radicals, two types of agents should be able to prevent the activation of NF- κB : (a) chelators absorbing metal ions required for the Fenton reaction, and (b) thiol compounds that can efficiently react with radicals. Here, metal chelators and noncysteine sulfur reagents were tested for inhibition of NF- κB activation.

NF- κB in a strongly H_2O_2 -responsive subclone of Jurkat T cells (called JR) was induced by treatment of cells with 100 μM H_2O_2 . This is shown by the appearance in nuclear extracts of the DNA binding activity of NF- κB using electrophoretic mobility shift assays (EMSAs) with a ^{32}P -labeled κB DNA probe (Fig. 1 A, lane 4). The induction was prevented when 500 U/ml of catalase were present in the cell culture medium (data not shown). As shown in Fig. 1 A (compare lanes 4 and 5), cells pretreated with 100 μM of the iron chelator desferal showed a 70% reduction in their capability to activate NF- κB . Other unrelated binding activities (characterized in reference 7) were unaffected. Desferal was ineffective if preloaded with an equimolar amount of Fe^{3+} (Fig. 1, lane 6), suggesting that the inhibiting effect relied on the iron chelating property of the agent. Also, 100 μM *o*-phenanthroline, a chelator with high affinity for copper ions, gave a strong inhibition of the activation of NF- κB by H_2O_2 (Fig. 1 A, compare lanes 9 and 10).

Under the same conditions, 30 mM NAC gave only a partial inhibition of NF- κB activation (Fig. 1 B, compare lanes 1 and 5). 14 mM 2-ME had a much stronger effect (Fig. 1 B, lane 2), but was also ineffective at concentrations <1 mM (data not shown). Glutathione (10 mM) barely suppressed NF- κB activation, most likely, because it is not taken up very well by cells (lane 3). DDTC gave a very strong inhibition of NF- κB activation (lane 4). DDTC, disulfiram (a disulfide-linked form of DDTC), and PDTC were tested at lower concentrations. At 100 μM , all three dithiocarbamates potently blocked the induction of NF- κB , while nonspecific DNA binding activities were barely influenced by the agents (Fig. 1 B, lanes 7–9). Because DDTC and disulfiram caused an increase in the pH of the culture medium, long-term treatments of cell cultures could not be performed with these two agents (for toxicity of the two agents in cell culture, see reference 51). We therefore concentrated on characterizing the pyrrolidine derivative PDTC, which did not detectably change the pH of the culture medium.

Pyrrolidine alone and PGA, a cyclic molecule structurally related to PDTC, had no inhibitory effect on the activation of NF- κB in cells, even at a concentration of 10 mM (Fig.

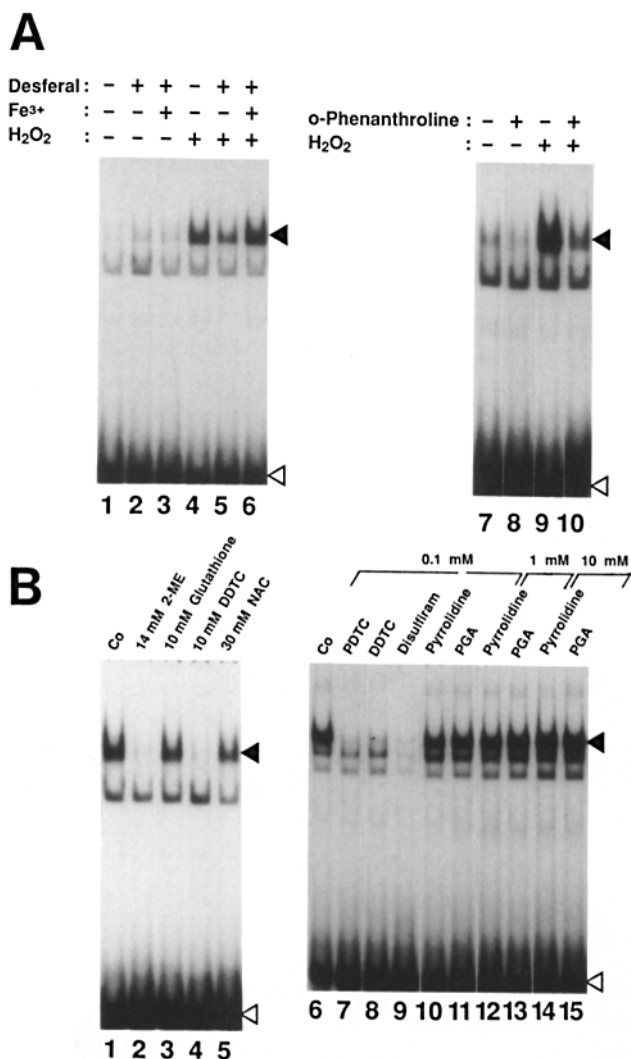


Figure 1. The effect of metal chelators and thiol compounds on the activation of NF- κ B by H₂O₂. Cells of the Jurkat T cell subclone JR were stimulated with 100–150 μ M H₂O₂ for 1 h in the absence and presence of the indicated concentrations of compounds. Nuclear extracts were prepared and equal amounts of protein analyzed for κ B-specific DNA binding in EMSAs using a ³²P-labeled DNA probe (see Materials and Methods). Fluorograms of native gels are shown. The H₂O₂-inducible complex (filled arrowhead) was characterized in detail earlier (7). The unbound DNA probe is indicated by an open arrowhead. Faster migrating complexes were not specific. (A) The effect of metal chelators. As indicated on top of the lanes, cells were preincubated with 100 μ M desferrioxamine for 17 h (desferal; left) or with 100 μ M o-phenanthroline for 2 h (right). (B) The effect of thiol compounds. Cells were preincubated for 2.5 h with compounds having a single SH group, such as 2-ME, glutathione and NAC, and DDTC at the indicated concentrations (left). PDTC, DDTC, and disulfiram were tested at a concentration of 100 μ M during a 1.5 h-preincubation period (right). The effects of pyrrolidine and PGA on activation of NF- κ B were tested between 0.1 and 10 mM, as indicated.

1 B, lanes 10–15), suggesting that the dithiocarboxy group was responsible for the activity of PDTC. The observation that metal chelators and chemically diverse sulfur compounds inhibit the activation of NF- κ B indicates that the production of hydroxyl radicals from H₂O₂ via the Fenton reaction was required for activation of NF- κ B in intact cells.

PDTC Shows a Biphasic Dose-Response Curve. Stimulation of Jurkat T cells with human TNF- α strongly induced the DNA binding and nuclear appearance of NF- κ B (Fig. 2 A, compare lanes 2 and 4; see reference 52). Cells treated for 1 h with PDTC before stimulation with TNF showed a dose-dependent suppression of the appearance of NF- κ B activity in nuclear extracts. 10 μ M PDTC reduced the κ B-specific signal by 40% (lane 5) and treatment with 100 μ M PDTC resulted in a complete inhibition; the signal was reduced to the background level observed in uninduced cells (Fig. 2 A, compare lanes 2 and 6). At concentrations of PDTC >500 μ M, the inhibitory effect was partially relieved (lanes 7–9). 5 mM PDTC was only as efficient as 10 μ M PDTC in preventing the activation of NF- κ B. Cells treated with 5 mM PDTC showed a pattern of DNA binding activities that was not distinct from that of control cells stimulated with TNF (Fig. 2 A, compare lanes 4 and 9). This suggests that PDTC is specific and did not simply inhibit the DNA binding activity of NF- κ B, induced proteolysis, or led to a desintegration of cells. It is also unlikely that PDTC blocked TNF binding to its receptor. 1 mM PDTC alone could not induce NF- κ B binding activity (Fig. 2 A, lane 3).

We tested the toxicity of PDTC in cell cultures. Jurkat cells were incubated for up to 24 h with various concentrations of PDTC, and the number of trypan blue-excluding cells was determined. At concentrations between 10 and 250 μ M of PDTC, the number of living cells stayed constant for at least 24 h (Fig. 2 B). There was, however, no increase, as observed with control cells. The stagnation in cell number in the presence of PDTC was most likely due to an arrest in cell cycle rather than cell death because there was no increase in the number of trypan blue-stained cells. Only a 24-h incubation in the presence of 0.5 mM PDTC resulted in a reduction of cell number. These results show that cells can be kept alive for at least 24 h within a closed cell culture system at concentrations of PDTC that are optimal for inhibition of NF- κ B.

PDTC Is a Specific Inhibitor. The specificity of PDTC was tested by assaying nuclear extracts from PDTC-treated Jurkat cells for the DNA binding activity of other transcription factors. Treatment of Jurkat cells with PMA and the lectin PHA induced the binding of NF- κ B (Fig. 3, compare lanes 1 and 2), which was potently blocked by 100 μ M PDTC (lane 3). In the same extracts, the induction of AP-1 by PMA was detected by assaying with a TRE probe (Fig. 3, compare lanes 4 and 5). The newly induced protein-DNA complex was specific, as it was the only complex competed for by an excess of unlabeled TRE probe (data not shown). PDTC could not prevent the induction of the AP-1 binding activity (Fig. 3, lane 6), indicating that PDTC did not interfere with other PKC-regulated events in intact cells. PDTC had also no significant influence on the binding activity of oct-1 (lane 9) and proteins binding to a cAMP-response element (CRE; lane 12) and the GC-rich binding motif of Sp1 (lane 15). Also, various nonspecific DNA binding activities detected by the various DNA probes were not affected by a treatment of cells with 100 μ M PDTC. A single nonspecific activity binding to the TRE probe seemed to be impaired upon stimulation

A

TNF : - - + + + + + +
 PDTC : - 1 - 0.01 0.1 0.5 1 5 [mM]

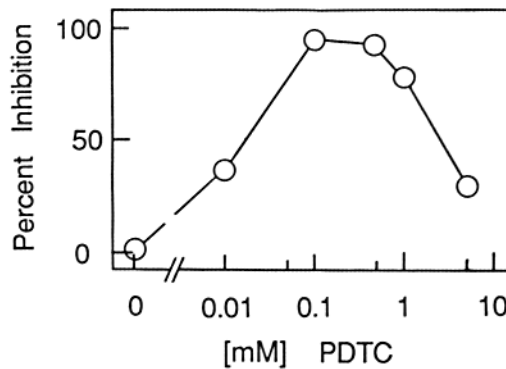
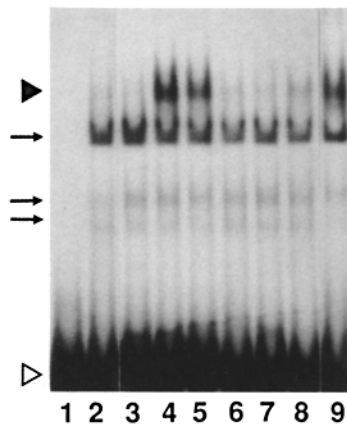
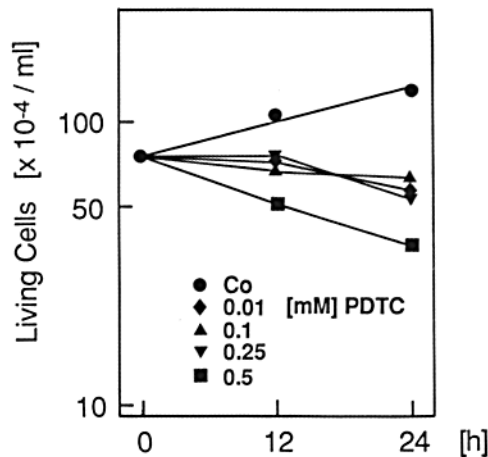


Figure 2. Dose dependence of PDTC effects. (A) The inhibiting effect of PDTC on the TNF-induced NF- κ B activation. Jurkat T cells were preincubated for 1.5 h with the indicated concentrations of PDTC followed by stimulation with recombinant human TNF- α (270 U/ml). For details, see legend to Fig. 1. The filled arrowhead (left) indicates the position of TNF-induced NF- κ B, the small arrows indicate the positions of nonspecific binding activities, and the open arrowhead indicates the position of unbound DNA probe. A quantitation of the inhibiting effect of PDTC on the TNF-induced κ B-binding activity is shown on the right. The band corresponding to the NF- κ B-DNA complex was excised from the dried gel and the 32 P radioactivity determined by Cerenkov counting. The value obtained in lane 4 was set to 0% inhibition. (B) The effect of PDTC on cell viability. Jurkat cell cultures were treated for the indicated periods of time with the indicated concentrations of PDTC. Total cell numbers were counted microscopically by phase contrast and the number of dead cells determined by trypan blue uptake.

B

and PDTC treatment (lane 6). In conclusion, PDTC seems to be a fairly specific inhibitor of the induction of NF- κ B.

PDTC Blocks NF- κ B Activation Independently of the Inducing Agent and Cell Line. The activity of PDTC was tested in other cell types and with other agents known to induce NF- κ B. PDTC suppressed the NF- κ B induction in Jurkat T cells after TNF- α or PMA/PHA treatments (Fig. 4, lanes 5 and 6). In the mouse pre-B cell line 70Z/3, NF- κ B was induced by treatments of cells with bacterial LPS, PMA, or IL-1 (Fig. 4, lanes 8-10). The strong induction of NF- κ B by all three agents was suppressed by PDTC to a residual binding activity of 10-20% (lanes 11-13), which was slightly above the activity of unstimulated cells (lane 7). Unstimulated mouse fibroblasts (Ltk⁻) showed an elevated basal activity of NF- κ B (lane 14), which was augmented three- to fourfold after

treatment of cells with PMA or TNF- α (lanes 15 and 16). Treatment of cells with PDTC reduced the basal as well as the induced NF- κ B activity to a very low level (lanes 17-19). PDTC was also active with the human monocytic cell line Mono-mac-6 stimulated with derivatives of bacterial lipopolysaccharide (R. Schreck, D. Bevec, P. Baueerle, and G. Bahr, manuscript in preparation).

In cell lines derived from mature B lymphocytes, NF- κ B can be present in a constitutive form (53). Unlike in fibroblasts, the nuclear presence of NF- κ B in B cells could not be completely suppressed by PDTC (Fig. 4, lane 21). The NF- κ B complex was reduced by ~50%, as determined by scintillation counting of protein-DNA complexes. This indicates that the constitutive nuclear NF- κ B in B cells is, at least in part, kept active by a mechanism distinct from that

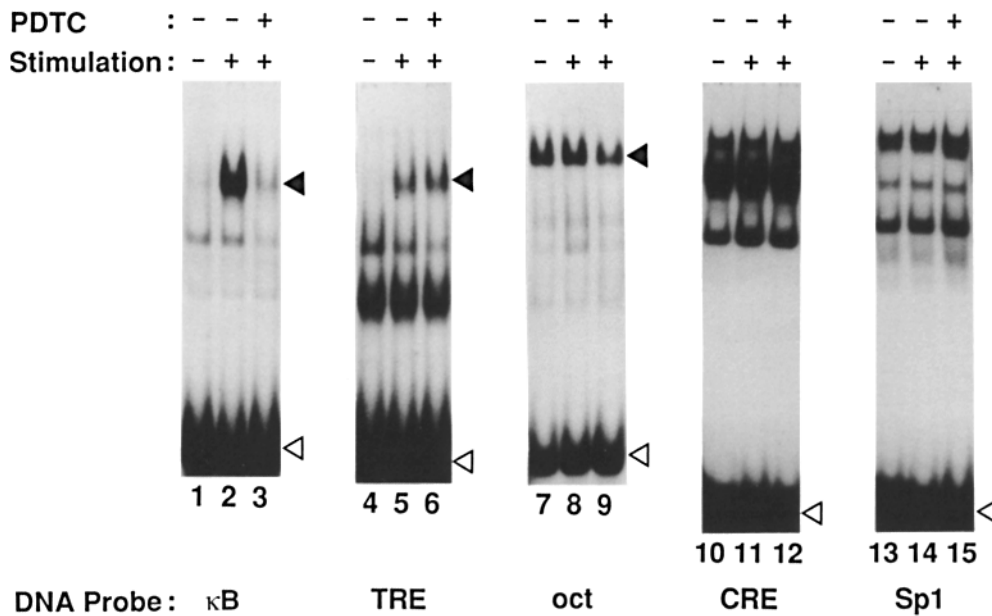


Figure 3. The effect of PDTC on other DNA-binding activities. Jurkat T cells were pretreated for 1 h with 100 μ M PDTC, as indicated, followed by a 3-h stimulation with either PMA (50 ng/ml)/PHA (2.5 μ g/ml) (lanes 2, 3, 5, 6, 8, and 9), or 250 U/ml human TNF- α (lanes 11, 12, 14, and 15). Nuclear extracts were analyzed by EMSA with 32 P-labeled DNA probes detecting κ B-binding activities (κ B), AP-1/jun/fos-like activities (TRE), octamer binding proteins (oct), cAMP-response element binding activities (CRE), and Sp1 (Sp1). Fluorograms of native gels are shown. Complexes tested for specific binding are indicated by filled arrowheads. The open arrowhead indicates the positions of unlabeled DNA probes.

inducing NF- κ B after extracellular treatments. In conclusion, PDTC seems to be a versatile agent allowing interference with the mobilization of NF- κ B in various cell types.

The Effect of PDTC Is Reversible. PDTC was added to cultures of Jurkat T cells 20 h before stimulation with PMA/PHA. If the PDTC was washed out after 1.5 h, a PMA/PHA treatment could strongly induce the nuclear appearance of NF- κ B 18.5 h later (Fig. 5, lane 1). This shows that the PDTC treatment did not irreversibly inhibit the activation of NF- κ B. Cells pretreated with PDTC could still respond to PDTC when the same amount of the drug was

added shortly before the PMA/PHA stimulus, as shown by a strong inhibition of NF- κ B activation (lane 2). However, in cells where PDTC was not washed out, the activation of NF- κ B by PMA/PHA after 18.5 h was barely suppressed (lane 3). Moreover, chronically PDTC-treated cells became completely insensitive if additional drug was added shortly before PMA/PHA stimulation (lane 4). Very similar results were obtained when cells were stimulated with TNF- α . None of the various PDTC treatments could induce NF- κ B when no PMA/PHA or TNF was added (data not shown). A possible explanation for the observed desensitization will be discussed.

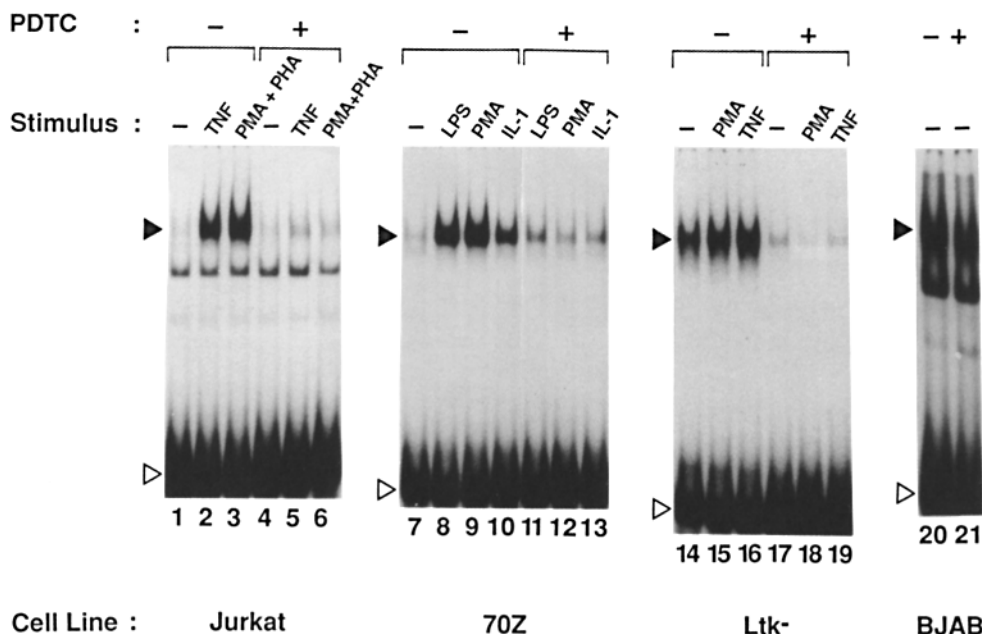


Figure 4. The effect of PDTC on cell lines treated with various inducers of NF- κ B. Jurkat T cells (lanes 1-6), 70Z/3 pre-B cells (lanes 7-13), and mouse fibroblasts (Ltk⁻; lanes 14-19) were pretreated for 1 h with 100 μ M PDTC followed by a 3-h treatment with PMA (50 ng/ml) plus PHA (2.5 μ g/ml), 10 μ g/ml LPS, 50 ng/ml PMA alone, 50 U/ml IL-1, or 300 U/ml human or mouse TNF- α , as indicated on top of the lanes. Human B cells (BJAB; lanes 20 and 21) were treated for 2 h with 100 μ M PDTC without subsequent stimulation. Nuclear extracts were analyzed on EMSA. Fluorograms of native gels are shown. The filled arrowheads indicate the positions of NF- κ B-DNA complexes. The open arrowheads indicate the position of unbound DNA probe.

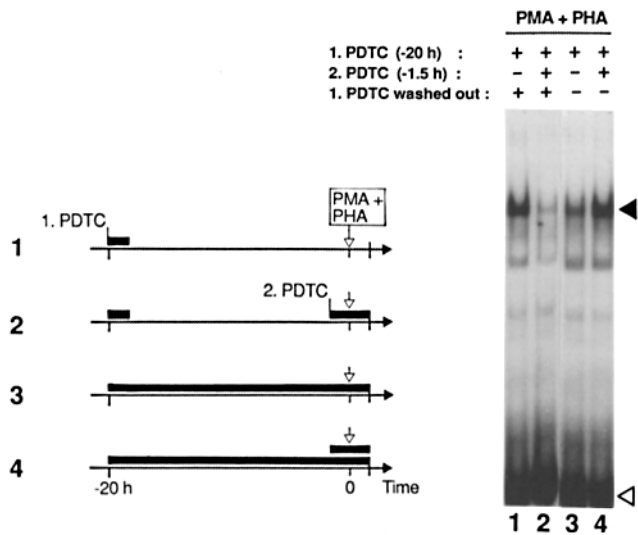


Figure 5. Reversibility of PDTC treatments. The scheme on the left illustrates the time course of the four experiments shown on the right. Jurkat T cells were treated with 150 μ M PDTC for 1.5 h. After that period, the culture medium was either exchanged (wash out; lanes 1 and 2) or not (lanes 3 and 4). 20 h after the first addition (1.) of PDTC, cells were stimulated with PMA (50 ng/ml) plus PHA (2.5 μ g/ml) for 1 h. 1.5 h before stimulation, one dish of pretreated (lane 2) and chronically treated cultures (lane 4) received a second time 150 μ M PDTC (2.). No activation of NF- κ B was observed in the various control cultures if stimulation with PMA/PHA was omitted (data not shown). Nuclear extracts were analyzed by EMSA. A fluorogram of a native gel is shown. The filled arrowhead indicates the position of the NF- κ B-DNA complex, the open arrowhead the position of unbound DNA probe.

PDTC Suppresses κ B-dependent Transactivation. We transfected Jurkat T cells with κ B-dependent CAT reporter constructs and tested whether PDTC can suppress the induction of CAT activity after a PMA/PHA treatment of cells. Both

reporter constructs are under inducible control of NF- κ B binding sites (32, 54–56). Cells transfected with HIV-1-LTR-CAT showed a strong increase in CAT activity after a treatment with PMA/PHA (Fig. 6, compare lanes 1 and 2). Treatment of cells with 30 and 90 μ M PDTC resulted in a dose-dependent suppression of the induction of CAT activity (lanes 3 and 4). This effect was in the same concentration range as the inhibiting effect of PDTC on NF- κ B activation seen by EMSA. 30 and 90 μ M PGA had no effect (lanes 5 and 6). Also, with the construct pSVtkCAT, a strong CAT enzyme induction was observed after PMA/PHA stimulation of Jurkat cells (Fig. 6, lanes 7 and 8) for which, most likely, NF- κ B is responsible (56). PDTC had an even stronger inhibitory effect on the induction of CAT activity with this construct (lanes 9 and 10).

In the presence of 30 and 90 μ M PDTC, the basal activity of HIV-1-LTR-CAT was slightly increased (Fig. 6, lanes 11–13). In a related reporter construct with two mutated κ B sites (32, 41), which is mainly dependent on three Sp1 sites for basal activity, the levels of CAT activity were virtually unchanged if cells were treated with 30 or 90 μ M PDTC (lanes 14 to 16). Also, the basal activity of the pSVtkCAT reporter constructs was unaffected by the PDTC treatment of cells (lanes 17–19). These data suggest that PDTC is an inhibitor of NF- κ B-dependent transactivation and does not influence gene regulation by various other factors.

PDTC Appears to Prevent the Release of I κ B from NF- κ B. The inactive cytoplasmic complex of NF- κ B and I κ B can be dissociated in vitro by treatment of cytoplasmic fractions with sodium deoxycholate (DOC) (57). This allows detection and quantitation of the NF- κ B-I κ B complex by EMSA. In cytoplasm from unstimulated cells only very low amounts of NF- κ B activity were detected before a DOC treatment (Fig. 7 A, lane 1). The cytoplasmic NF- κ B activity was slightly

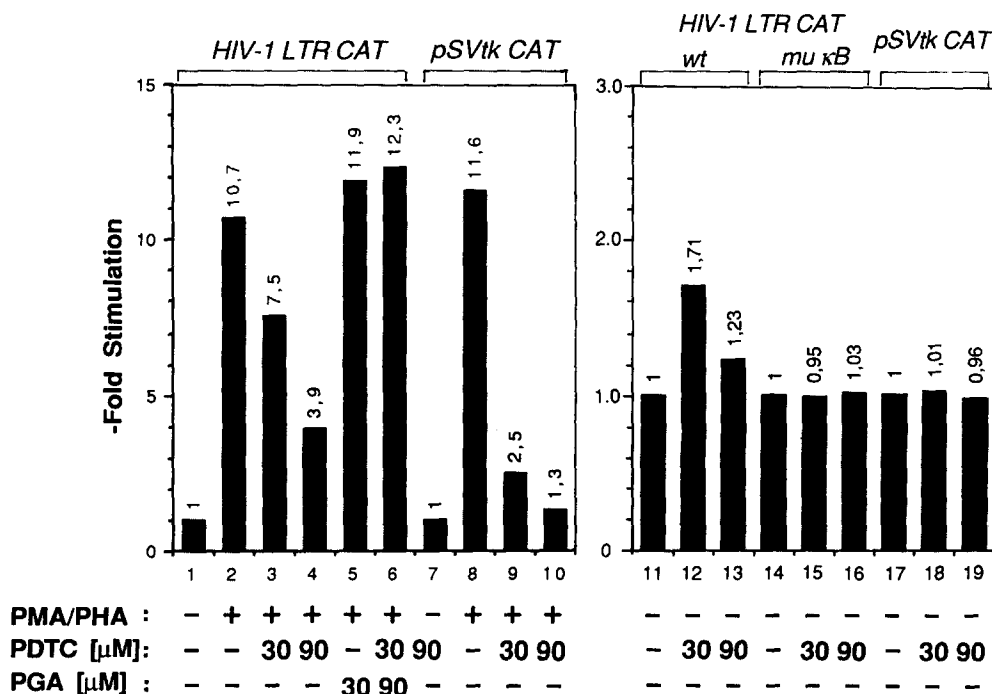


Figure 6. The effect of PDTC on the transcriptional activity of various reporter genes. Jurkat T cells were transfected with chloramphenicol acetyltransferase (CAT) reporter plasmids controlled by a wild-type HIV-1 LTR, a mutant HIV-1 LTR in which both NF- κ B binding sites are altered (32, 41), and by two copies of the 72-bp repeat of the SV40 enhancer (42). 24 h after transfection, cultures were treated for 10 h with the indicated amounts of PDTC or PGA. 1 h after addition of PDTC or PGA, cells were treated for the remaining 9 h with PMA (50 ng/ml) plus PHA (2.5 ng/ml). Cell extracts were analyzed for CAT activity as described in Materials and Methods. Mean values from two independent transfection experiments are shown. Deviations were <10%.

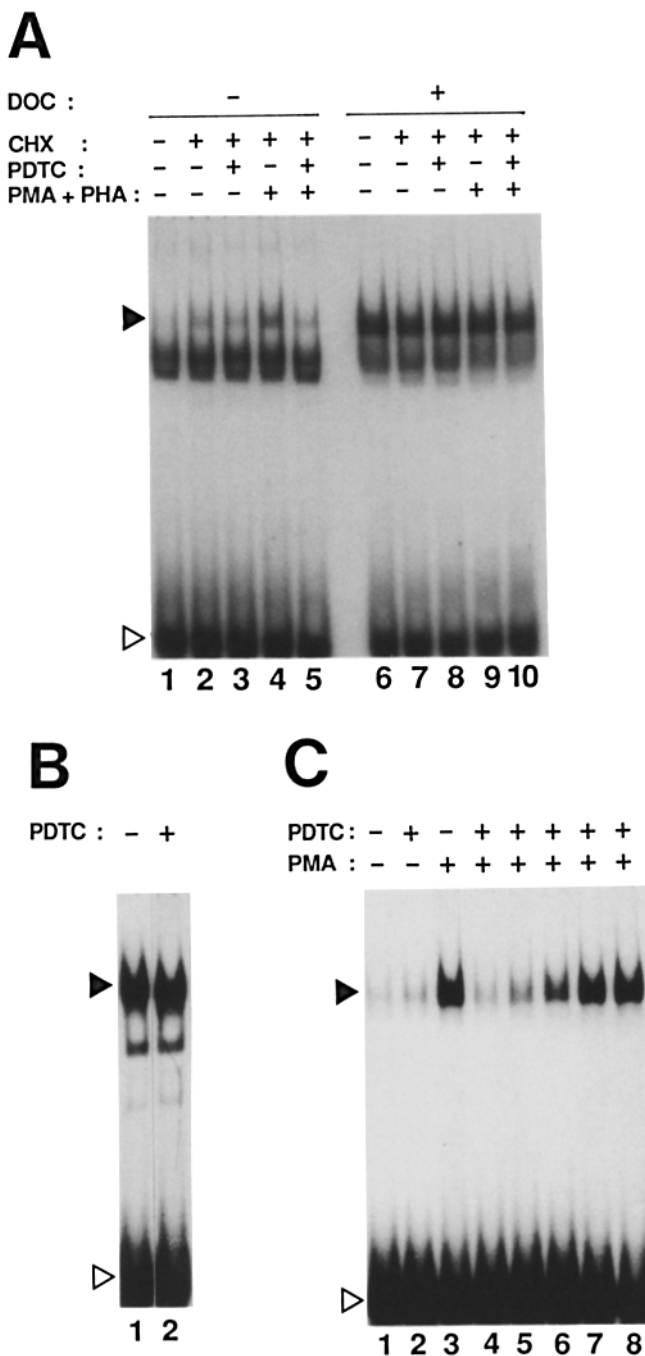


Figure 7. The effect of PDTC on the mobilization of cytoplasmic NF- κ B. (A) The effect of PDTC on the in vitro activation of cytoplasmic NF- κ B-I κ B complex. As indicated on top, cultures of Jurkat T cells were left untreated (lanes 1 and 6) or preincubated with 20 μ g/ml cycloheximide and PDTC (150 μ M). Where indicated, cells were stimulated with 50 ng/ml PMA plus 2.5 μ g/ml PHA. Cytoplasmic fractions from Jurkat T cells were analyzed by EMSA without (lanes 1–5) or after treatment with DOC (lanes 6–10), as described earlier (57). The filled arrowheads indicate the positions of NF- κ B binding activities, the open arrowheads the position of the κ B DNA probe. Fluorograms of native gels are shown. (B) The effect of PDTC on the DNA binding activity of NF- κ B in vitro. Nuclear extracts from PMA/PHA-stimulated Jurkat cells were incubated in the absence (lane 1) and presence (lane 2) of 1 mM PDTC for 30 min and analyzed by EMSA. (C) Kinetics of the PDTC effect. 70Z/3 pre-B cells were stimulated with 50 ng/ml PMA (lane 3). 250 μ M PDTC was added

enhanced if cells were treated with cycloheximide or PMA/PHA. Cycloheximide was used to avoid effects coming from transcriptional upregulation of NF- κ B-I κ B in stimulated T cells (R. Schreck and P. Baeuerle, unpublished observation). NF- κ B-I κ B complex could still be detected in cytoplasm from PDTC-treated cells, as seen by a strong increase in NF- κ B binding activity after DOC treatment (Fig. 7 A, lanes 8 and 10). There was no accumulation of active NF- κ B in the cytoplasm of stimulated cells pretreated in the presence of PDTC (Fig. 7 A, lane 5), indicating that PDTC did not interfere with nuclear uptake of NF- κ B. Only minor differences were noted in the amounts of DOC-activated NF- κ B between control and stimulated cells, showing that, upon stimulation, only small portions of the cytoplasmic NF- κ B were activated and translocated to the nucleus. The data are consistent with the idea that PDTC prevents a reaction required to release I κ B from NF- κ B in intact cells.

From the experiment shown in Fig. 2 A, it is already obvious that PDTC does not interfere with the DNA binding activity of NF- κ B: even at a concentration of 5 mM PDTC in the medium, a TNF treatment could induce NF- κ B activity. Also, incubation of a nuclear extract from stimulated Jurkat cells with 1 mM PDTC could not significantly inhibit the DNA binding of NF- κ B (Fig. 7 B, lane 2).

We investigated the kinetic of the PDTC effect. Only if pre-B cells were incubated for a minimum of 30 min with the agent before stimulation with PMA, a strong inhibitory effect was seen (Fig. 7 C, lanes 4 and 5). 50% inhibition was observed if PMA and PDTC were given at the same time to the cell culture (Fig. 7 A, lane 6), and none if PDTC was added 30 or 60 min after the PMA stimulus (lanes 7 and 8). PDTC seemed to be only active if taken up by the cells before stimulation.

Superoxide Radicals Are Not Directly Involved in NF- κ B Activation. A frequent species of oxygen radicals in cells is the superoxide anion, O $_2^-$. The radical can be eliminated by superoxide dismutases (SODs). A previous study indicated that the cytotoxic effects of TNF rely on the production of O $_2^-$ because cell lines overexpressing MnSOD were more resistant to the cytotoxic effects of TNF (58). We have used such an MnSOD-overexpressing cell line, MCF-7/SOD, and the respective parental line, MCF-7, to investigate whether overexpression of the same type of superoxide dismutase influences the activation of NF- κ B by TNF, IL-1, and PMA. As shown in Fig. 8 A, MCF-7/SOD had high constitutive levels of MnSOD mRNA, whereas the mRNA was barely detectable in the parental line MCF-7, in which the MnSOD gene is under inducible control (59). The high MnSOD mRNA level in MCF-7/SOD cells correlated with an increased level of the enzymatic activity of MnSOD, as determined by the method of Beauchamp and Fridovich (48) (data not shown). Additional evidence that MCF-7/SOD cells overexpressed the

1.5 h (lane 4) or 0.5 h (lane 5) before stimulation, at the same time (lane 6), or 0.5 h (lane 7) and 1 h after stimulation (lane 8) to cells. Lane 1, unstimulated cells; lane 2, cells treated with PDTC without subsequent stimulation. Nuclear extracts were prepared and analyzed by EMSA for NF- κ B binding activity.

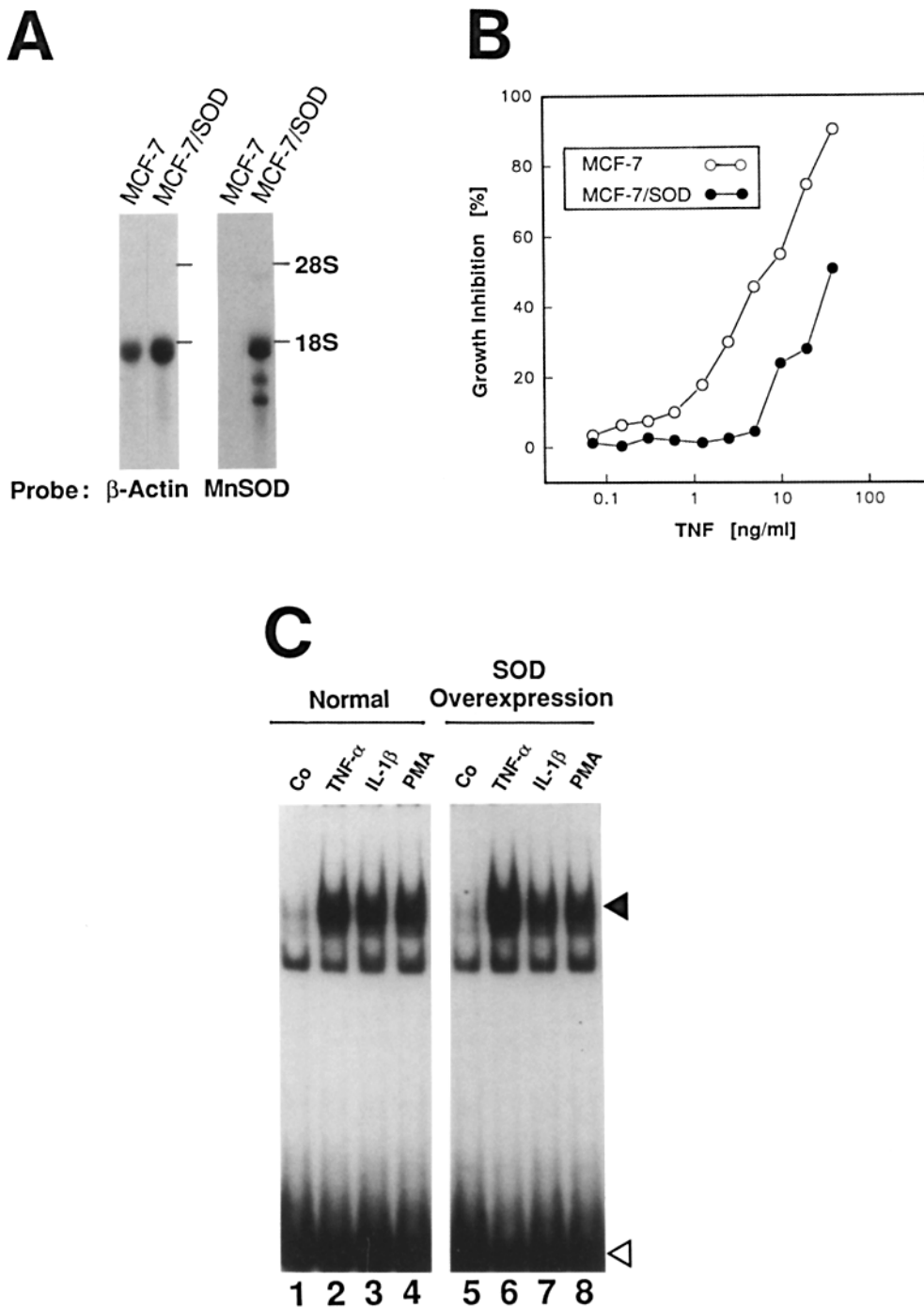


Figure 8. The effect of MnSOD overexpression on the activation of NF- κ B by various inducers. (A) A Northern blot analysis comparing the MnSOD mRNA levels in the human mammary carcinoma cell line MCF-7 and a MnSOD-overexpressing subclone, called MCF-7/SOD, derived from stable transfection of an MnSOD expression vector (46). Total RNA from 3×10^5 cells was used for electrophoresis. As indicated, filters were probed with cDNA fragments detecting β -actin or human MnSOD. The positions of 18S and 28S rRNAs were detected by methylene blue staining and are indicated at the right. (B) The effect of MnSOD overexpression on the growth of MCF-7 cells in the presence of TNF. Parental and MnSOD-overexpressing cells (10^4 cells in 0.1 ml) were cultured for 24 h in the presence of the indicated amounts of recombinant human TNF- α . [3 H]Thymidine incorporated into DNA within a 6-h pulse was determined by liquid scintillation. (C) The effect of MnSOD overexpression on mobilization of NF- κ B. Cultures of MCF-7 (lanes 1-4) and MCF-7/SOD were treated for 3 h with 250 U/ml human TNF- α , 50 U/ml IL-1, and 66 ng/ml PMA, and nuclear extracts were analyzed by EMSA. A fluorogram of a native gel is shown. The filled arrowhead indicates the position of the inducible NF- κ B-DNA complex and the open arrowhead indicates the position of unbound DNA probe.

MnSOD protein came from the observation that the cells were more resistant towards the cytotoxic effect of TNF than parental cells (Fig. 8 B).

The NF- κ B in the MCF-7/SOD line was induced upon treatments with TNF- α , IL-1 β , and PMA as efficiently as that in the control cell line MCF-7 (Fig. 8 C). We consistently observed that TNF could more strongly induce NF- κ B in the MnSOD overexpressor. This might be due to an increased production of H₂O₂ (and subsequently OH \cdot) from O₂⁻ by the activity of the constitutive MnSOD. The data

indicate that O₂⁻ radicals, which are more rapidly eliminated in MnSOD-overexpressing cells, are not directly involved in the activation of NF- κ B by various inducing agents, although they seem to be required for cytotoxic effects of TNF. The inhibiting effect of metal chelators (see Fig. 1 A) suggests rather that hydroxyl radicals originating from H₂O₂ are required.

LPS Induces the Production of Reactive Oxygen Intermediates. Various cell types can respond to a treatment with TNF, IL-1, or PMA with the production of reactive oxygen intermediates

(for references, see Introduction). We therefore tested whether also LPS can induce the production of ROI, which would provide an explanation for the inhibitory effect of PDTC on the NF- κ B activation by LPS. The pre-B cell line 70Z/3 is strongly responsive to LPS (60) (see Fig. 4). Before stimulation, cells released only very low amounts of O_2^- and H_2O_2 (Fig. 9, A and B). After addition of 100 ng/ml LPS to cell cultures, 70Z/3 pre-B and an EBV-transformed human B cell line immediately started to produce O_2^- (Fig. 9 A) and H_2O_2 (Fig. 9 B). The induced signals were specific because they were abolished if SOD and catalase, respectively, were added to the culture medium (data not shown). LPS caused a between 5- and 10-fold induction in the rate of ROI release from these cells. Because H_2O_2 is fully and O_2^- is partially membrane permeable, the reactive oxygen species released into the medium upon LPS stimulation are likely to reflect an intracellular production of the compounds. ROI may thus play an important role in the signal transduction by LPS.

Discussion

In this study, we tested whether the pyrrolidine derivative of dithiocarbamate is useful as inhibitor of NF- κ B in cell cultures. By all criteria tested, PDTC proved to be a potent agent allowing interference with the mobilization of NF- κ B in intact cells. (a) PDTC could be used at micromolar concentra-

tions. In this respect, it is approximately 200-fold more potent than NAC. (b) PDTC was of low toxicity for at least 24 h when used in cell cultures. (c) PDTC did not inhibit the DNA binding activity of all other factors tested, nor the inducibility of AP-1 by PMA. (d) PDTC interfered in a specific manner with the κ B-dependent transactivation of genes. (e) PDTC could prevent activation of NF- κ B by all inducers and in all cell lines tested. The agent might thus find application as an experimental tool in cell cultures to study, for instance, the role of NF- κ B in the induction of genes containing potential NF- κ B binding sites.

Because dithiocarbamates are established pharmaceuticals, in vivo studies also seem possible. The suppressing effect of PDTC on the activation of the HIV-1 LTR might stimulate further studies exploring effects of DDTC and other derivatives on the progression of AIDS. Because NF- κ B is involved in T cell and macrophage activation and, presumably, in many other regulatory processes involving cytokine production, it seems also worth testing whether the drugs can suppress the induction of NF- κ B-controlled cytokine genes in vivo, for instance, those encoding the GM-CSF (61) and TNF- α (62, 63). Both GM-CSF and TNF- α are potent inducers of HIV-1 (for review, see reference 64).

The Mode of PDTC Action. PDTC has at least two chemical properties. One is a chelating activity for heavy metals, which is why the diethyl derivative is used for the treatment of heavy metal poisoning. The second is an antioxidative activity of its dithiocarboxy group. Because nonthiol metal chelators, such as *o*-phenanthroline and desferrioxamine, also can inhibit NF- κ B activation, it is possible that the inhibitory effect of dithiocarbamates relies on both of its properties. This could provide an explanation why dithiocarbamates are much more potent than sulfhydryl compounds, which are not as strong binding certain metal ions. Alternatively, or in addition, PDTC, DDTC, and disulfiram might reach higher or longer-lasting intracellular concentrations than the sulfhydryl compounds tested so far. This could come from a longer half-life within the cell or a more efficient uptake.

We could not observe that PDTC: (a) Interfered with the DNA-binding properties of NF- κ B in vitro, (b) blocked the nuclear uptake of NF- κ B, (c) diminished the amount of NF- κ B-I κ B complex in cycloheximide-treated cells, or (d) interfered with the release of I κ B in the presence of deoxycholate. Thus, the most likely explanation for the effect of PDTC is suppression of a reaction required for release of I κ B from NF- κ B in the cytoplasm. Because PDTC could block this reaction in response to every NF- κ B inducer tested so far (see also reference 7), the antioxidant and metal chelator seems to interfere with the activity of a common messenger produced by apparently different signaling pathways. As proposed earlier, this messenger might be a ROI (7, 16).

In this study, we have analyzed in more detail the nature of the ROI involved in NF- κ B activation. Major species of ROI in cells are H_2O_2 , $OH\cdot$, and O_2^- (reviewed in reference 65). The abundance of the various ROI species in cells can be manipulated, which allows testing to determine which one is required for nuclear appearance of activated NF- κ B in intact cells. H_2O_2 can be taken up by cells from the medium;

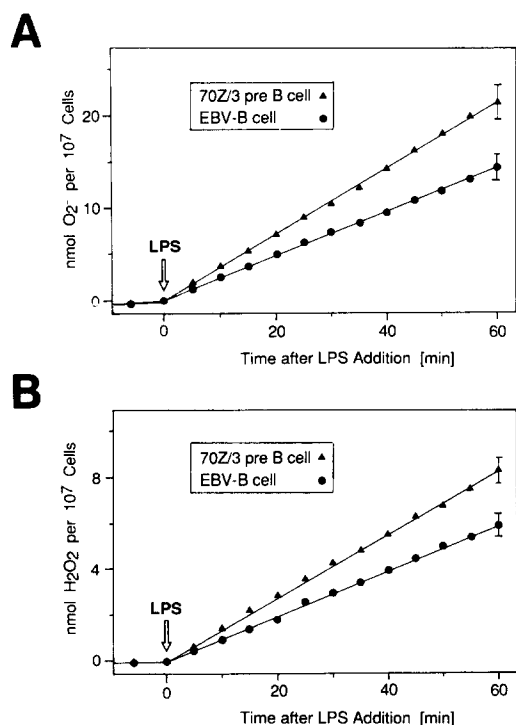


Figure 9. The effect of LPS on the production of reactive oxygen intermediates in pre-B and B cells. The release of superoxide anions (A) and H_2O_2 (B) from 70Z/3 pre-B cells and an EBV-transformed human B cell line was determined before and after treatment of cultures with 100 ng/ml bacterial LPS (see Materials and Methods). Measurements were performed for 1 h before LPS addition without observing a significant production of ROI. The 60-min value represents the mean of three independent measurements. Bars indicate the SD.

O_2^- can be eliminated by overexpression of SOD; the production of $OH\cdot$ from H_2O_2 can be specifically blocked with low concentrations of metal chelators; and both $OH\cdot$ and O_2^- radicals can be scavenged by thiol reagents, which react poorly with H_2O_2 . Data obtained from these kinds of experiments suggest that production of $OH\cdot$ is required for activation of NF- κ B. The level of O_2^- , which seems to increase in response to TNF stimulation of cells (58), was not required for NF- κ B activation by TNF, as shown with a MnSOD-overexpressing cell line. Overexpression of MnSOD rather stimulated NF- κ B induction by TNF, presumably, by increasing the level of intracellular H_2O_2 .

The molecular mechanism by which radicals release I κ B from NF- κ B is not yet understood. One can envision a direct damage of I κ B by radicals. Because $OH\cdot$ is highly reactive and therefore restricted in its diffusion within the cell, an in situ production of the radical from H_2O_2 seems to be required. Alternatively, oxygen radical-sensitive messenger proteins could be involved, for instance a redox-regulated tyrosine kinase such as Ltk (66), or a specific protease. In that context, the analysis of a consensus tyrosine phosphorylation site conserved in the NH_2 terminus of recently cloned I κ B proteins is of great interest (67, 68).

The induction of AP-1 by PMA in the presence of PDTC suggests that the activity of PKC is not impaired in PDTC-treated cells. This idea is strongly supported by our finding that, in intact cells, the activity and subcellular redistribution of PKC after PMA treatment is virtually unaffected by PDTC (R. Schreck and P. Baeuerle, manuscript in preparation). Therefore, PKC is most likely not directly involved in NF- κ B-I κ B phosphorylation, which is in contrast to the in vitro situation (69). A messenger function of ROI produced in response to PMA would provide a plausible explanation for the inhibiting effect of dithiocarbamates and other chemically distinct thiol compounds on the activation of NF- κ B by PMA.

An important observation might be that overdosage or chronic treatment with PDTC could desensitize cells for the drug's inhibiting effect. It is conceivable that chronic treatment with the antioxidant creates a hypoxic status in the cell (as during ischemia) under which radical-eliminating enzymes are downregulated. This could lead to a more permissive effect of the radicals produced in response to strong inducers, despite the presence of the scavenger. Alternatively, high amounts of radical scavengers might also lead to stabilization of radicals within the cell. Biphasic dose-responses are not unique to PDTC. Also, the antioxidants α -tocopherol, ascorbic acid, and rutin show biphasic dose-response curves when tested for protection from lipid peroxidation in lymphoid cells (70). A consequence of these findings is that long-term incubations with PDTC (and perhaps other antioxidants) or overdosage of the agent should be avoided in cell culture experiments. These effects might deserve particular attention in clinical trials with antioxidants.

How Specific Is PDTC? We cannot exclude the possibility that the antioxidant PDTC also affects other transcription factors and signal transducing systems. In bacteria, a transcriptional activator protein of oxidative stress-inducible genes,

called oxyR, is activated upon direct oxidation (71). Also in eukaryotic cells, oxidative stress-responsive transcription factors and messenger proteins unrelated to NF- κ B are therefore likely to exist. Their activation should also be suppressed by PDTC and other antioxidants. An important function of such regulatory proteins might be to allow a cellular response to changes in the oxidant state, for instance, by controlling the level of radical-eliminating enzymes. An important enzyme involved in oxygen radical metabolism is the Mn-dependent mitochondrial SOD. Its mRNA is transcriptionally induced when cells are stimulated with TNF and LPS (58). Because both agents activate NF- κ B and induce oxidant stress, it is possible that NF- κ B is involved in the oxidative stress-responsive induction of the MnSOD gene. Future studies have to explore a role of NF- κ B as regulator of ROI homeostasis.

Recent reports showed that c-Jun mRNA is induced by low H_2O_2 concentrations in HeLa cells (72) and in an osteoblastic cell line (73). In HeLa cells, treatment with 250 μ M H_2O_2 for several hours could also elicit AP-1 binding activity. Since it is not known that κ B-like proteins control c-Jun expression and because the H_2O_2 effect apparently required an intact TRE element in the c-Jun promoter (72), the effect was likely to be mediated by c-Jun itself. However, in the Jurkat T cell line used in our study, the induction of AP-1 by PMA was not influenced by PDTC and we could not observe induction of AP-1 by H_2O_2 (7). Apart from cell type-specific differences, this could indicate that PMA and H_2O_2 induce AP-1 by distinct intracellular pathways. PMA and PKC are thought to activate c-Jun by triggering a dephosphorylation reaction in its DNA binding domain (74). The involvement of redox reactions in c-Jun activation are currently a matter of debate because it has also been proposed that the DNA binding activities of Jun and Fos are downregulated by oxidation of conserved cysteine residues (75).

ROI and the Intracellular Signaling of LPS. There is experimental evidence that a variety of NF- κ B inducers elicit oxidant stress in cells. These include PMA, IL-1, TNF, hydrogen peroxide (reviewed in reference 16), γ rays (76), and UV light (77). Here we tested LPS, another strong inducer of NF- κ B, for that property. Indicative for the induction of oxidant stress, two cell lines showed an increased release of O_2^- and H_2O_2 into the medium when triggered with low amounts of LPS. LPS is thought to signal intracellularly by G proteins (78). As indicated by the inhibiting effect of pertussis toxin and a decrease in cAMP levels, a G_i -like protein is thought to be involved in LPS-induced IL-1 production. Because there are no effects of intracellular cAMP levels on the activation of NF- κ B (6, 79; P. Baeuerle, unpublished observation), we consider it unlikely that G_i proteins and cAMP levels are involved in NF- κ B activation or induction of oxidative stress. Rather, our present data suggest that LPS activates a variety of messenger systems. The induction of oxidative stress by LPS could come from activation of PKC (80). Because no phosphatidyl inositol breakdown was observed in response to LPS, a breakdown of phosphorylcholine could provide the diacylglycerol necessary for activation of PKC. This situation was observed in cells treated with the NF- κ B inducer IL-1 (81).

We are grateful to Dr. Georg Arnold for oligonucleotide synthesis, Dr. Marc Muller for providing the pSVtkCAT construct, Markus Meyer for the CRE and Sp1 oligonucleotides, Dr. Loms Ziegler-Heitbrock for stimulating discussions, and Prof. E.-L. Winnacker for his continuing support.

This work was supported by grants from the Deutsche Forschungsgemeinschaft awarded to P. Baeuerle (Ba 957/1-2 and SFB 217) and Bundesministerium für Forschung Technologie awarded to P. Baeuerle and W. Dröge (FG 5-1075 KS/DA), and partially fulfils the requirements for the doctoral thesis of R. Schreck.

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Received for publication 5 December 1991 and in revised form 22 January 1992.

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