

2-Mercaptoethanol restores the ability of nuclear factor κ B (NF κ B) to bind DNA in nuclear extracts from interleukin 1-treated cells incubated with pyrrolidine dithiocarbamate (PDTC)

Evidence for oxidation of glutathione in the mechanism of inhibition of NF κ B by PDTC

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The metal chelator and anti-oxidant pyrrolidine dithiocarbamate (PDTC) has been used extensively in studies implicating reactive oxygen intermediates in the activation of nuclear factor κ B (NF κ B). In agreement with other studies, we have shown that PDTC inhibits NF κ B activation in response to the pro-inflammatory cytokines interleukin 1 (IL1) and tumour necrosis factor (TNF). However, we have found that the inhibition was reversed by treatment of inhibited nuclear extracts with the reducing agent 2-mercaptoethanol. This was observed in extracts prepared from IL1-treated EL4.NOB-1 thymoma cells and TNF-treated Jurkat E6.1 lymphoma cells. These results suggested that the inhibition was caused by oxidation of NF κ B on a sensitive thiol, possibly on the p50 subunit (which was detected in NF κ B

complexes in both cell types), and not by inhibition of the activation pathway. The possibility that PDTC was acting as a pro-oxidant was therefore investigated. PDTC caused an increase in oxidized glutathione, suggesting that it acts as an oxidizing agent in the cells tested rather than as an anti-oxidant. Similar results were obtained with diamide, a compound designed to oxidize glutathione. Finally, an increase in the ratio of oxidized to reduced glutathione was shown to inhibit NF κ B–DNA binding *in vitro*. On the basis of these results we suggest that, while NF κ B activation is unaffected by PDTC, DNA binding is inhibited through a mechanism involving a shift towards oxidizing conditions, and that this is the mechanism of action of both PDTC and diamide in the cells tested here.

INTRODUCTION

Nuclear factor κ B (NF κ B) is a transcription factor involved in the expression of a wide range of genes, most of which code for proteins which play a role in immunity and inflammation (see [1] for a review). It is activated by several factors, including the pro-inflammatory cytokines interleukin 1 (IL1) and tumour necrosis factor (TNF) [2]. The regulatory regions of many of the genes induced by these agents contain NF κ B-binding motifs, and NF κ B activation is considered a key event in the signalling pathways for both IL1 and TNF [2]. The family of DNA-binding subunits of NF κ B is currently known to comprise five members in mammals: p50, p65 (RelA), c-Rel, p52 and RelB. The commonest form of NF κ B, however, comprises a heterodimer of p50 and RelA [1]. In resting cells, NF κ B occurs in a latent form, complexed to an inhibitory subunit I κ B. Upon activation with such agents as IL1, I κ B becomes phosphorylated by an as yet unidentified kinase, and is subsequently degraded by the proteasome [3–5]. This releases NF κ B, allowing it to translocate to the nucleus. The signalling events that occur prior to this are still unclear. A model has been proposed suggesting that reactive oxygen intermediates may play a ubiquitous signalling role in the process [6]. Three separate sets of observations have been made which support this model. Firstly, hydrogen peroxide has been shown to activate NF κ B in cells [7]. Secondly, compounds with anti-oxidant properties such as *N*-acetylcysteine and vitamin E have been shown to inhibit NF κ B activation [7,8]. Finally, in cells which overexpress catalase (which metabolizes hydrogen

peroxide), NF κ B activation was suppressed, whereas it was superinduced in cells overexpressing Cu/Zn superoxide dismutase [6].

These reports have led to the widespread use of one particular compound, pyrrolidine dithiocarbamate (PDTC), as an anti-oxidant inhibitor of NF κ B. Little work has been done, however, on the precise mechanism of action of PDTC, and none of the studies have attempted to demonstrate that it inhibits NF κ B by decreasing oxygen radicals in cells. We have previously reported that PDTC is an effective inhibitor of NF κ B in IL1-stimulated EL4.NOB-1 thymoma and TNF-stimulated Jurkat E6.1 cells [9]. However, another anti-oxidant, *N*-acetylcysteine, had no effect, which suggested that in these cells the inhibitory effect of PDTC may not have involved its anti-oxidant properties. In the present study we have explored the basis for the effect of PDTC further. The results suggest that, rather than acting as an anti-oxidant, PDTC has a pro-oxidant effect in the cells which leads to the oxidation of NF κ B and thereby inhibits DNA binding directly.

EXPERIMENTAL

Materials

The murine thymoma cell line EL4.NOB-1 and the human lymphoblast line Jurkat E6.1 were obtained from the European Collection of Animal Cell Cultures (Salisbury, U.K.). RPMI 1640 medium, fetal calf serum and cell culture consumables were from Greiner (Frickenhausen, Germany). Recombinant IL1 α

Abbreviations used: IL1, interleukin 1; NF κ B, nuclear factor κ B; PDTC, pyrrolidine dithiocarbamate; TNF, tumour necrosis factor.

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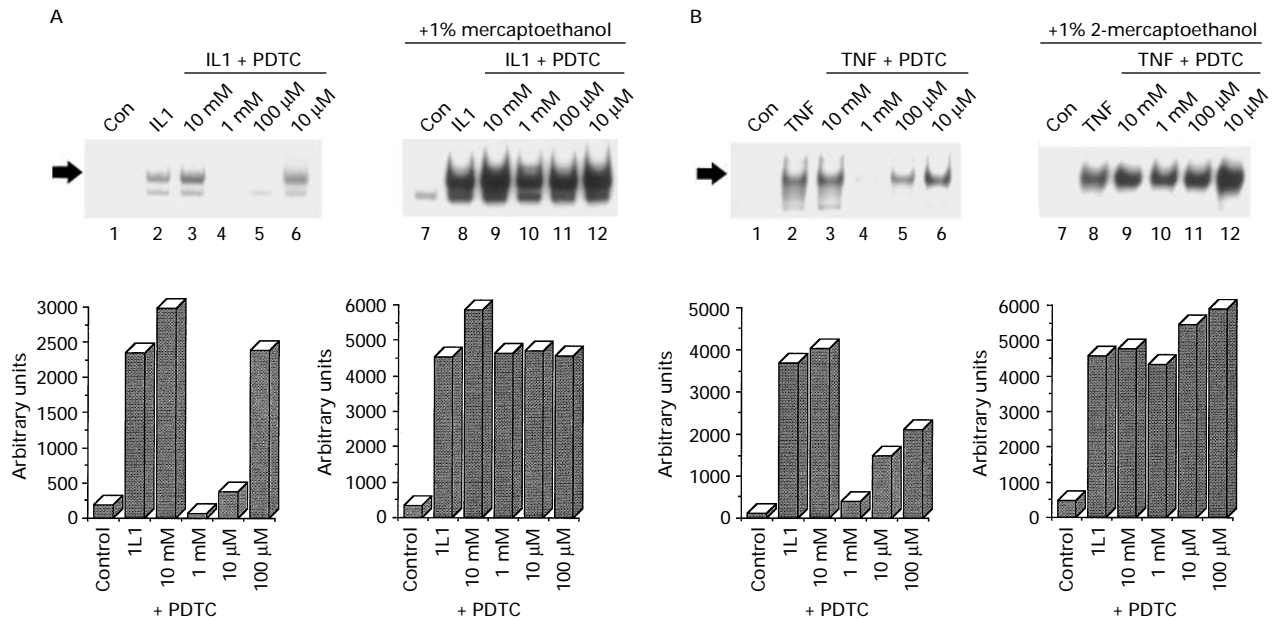


Figure 1 Effect of the reducing agent 2-mercaptoethanol on PDTC inhibition of NF κ B in EL4.NOB-1 and Jurkat cells

EL4.NOB-1 (A) and Jurkat (B) cells were pretreated for 1 h with the indicated concentrations of PDTC. Cells were then treated with IL1 or TNF (each at 10 ng/ml) for 1 h. Nuclear extracts were prepared as detailed in the Experimental section. A 4 μ g sample of each nuclear extract was treated with 1% 2-mercaptoethanol for 15 min on ice as indicated. The samples were then assayed for NF κ B binding. DNA-protein complexes are indicated (arrows). Con, control. Densitometric analysis was also performed, the results of which are shown beneath the corresponding autoradiographs. All results shown are representative of three experiments.

was a gift from Dr. Jeremy Saklatvala (Babraham Institute, Babraham, U.K.). Recombinant TNF α was a gift from Dr. Steve Foster (Zeneca Pharmaceuticals, Macclesfield, Cheshire, U.K.). T4 polynucleotide kinase, the 22 bp oligonucleotide containing the NF κ B consensus sequence (underlined) (5'-AGTTGAG GGGACTTCCCCAGGC-3'), the 22 bp oligonucleotide containing the consensus sequence (underlined) for the immunoglobulin enhancer oligonucleotide factor 1 (Oct1) (5'-TGTCGAA TGCAAATCACTAGAA-3') and poly(dI-dC) were from Promega (Madison, WI, U.S.A.). Specific antisera to p50, p65/RelA and c-Rel were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). [γ - 32 P]ATP was from Amersham International (Amersham, Bucks., U.K.). All other reagents, including nuclease-free BSA and Nonidet P40, were supplied by Sigma (Poole, Dorset, U.K.).

Cell culture

EL4.NOB-1 and Jurkat T-cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin and 100 μ g/ml streptomycin. Cells were maintained at 37 $^{\circ}$ C in a humidified atmosphere of 5% CO $_2$.

Preparation of subcellular fractions

Crude subcellular fractionation was performed as described previously [9]. Cells were treated with the indicated stimuli and inhibitors, as indicated, at 5×10^6 cells/ml (1 ml per sample) in RPMI 1640 medium with 10% fetal calf serum. Stimulation was terminated by adding 5 ml of ice-cold PBS. Each sample was centrifuged at 163 g in a swing-out rotor and then resuspended in 1 ml of buffer A (10 mM Hepes, pH 7.9, 1.5 mM MgCl $_2$, 10 mM KCl, 10 mM PMSF) and centrifuged at 12000 g for 10 min in a

benchtop Microfuge at 4 $^{\circ}$ C. Cells were then lysed by addition to 20 μ l of buffer A containing 0.1% Nonidet P40 and left on ice for 10 min, after which time they were centrifuged at 12000 g for 10 min. The nuclear extract was prepared by adding 15 μ l of buffer C (20 mM Hepes, pH 7.9, 420 mM NaCl, 1.5 mM MgCl $_2$, 25% glycerol, 0.5 mM PMSF) to the supernatant. The mixture was left on ice for 15 min. It was then centrifuged at 12000 g for 10 min and the supernatant was added to 75 μ l of buffer D (10 mM Hepes, pH 7.9, 50 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM PMSF). The protein in these crude extracts was determined using the method of Bradford [10]. The extracts were assayed immediately for NF κ B activity or stored at -20 $^{\circ}$ C until further use.

Electrophoretic mobility shift assay

Samples of 4 μ g of protein were incubated with 10000 c.p.m. of a 32 P-labelled oligonucleotide containing the consensus sequence for NF κ B in binding buffer (40% glycerol, 1 mM EDTA, 10 mM Tris, pH 7.5, 100 mM NaCl, 0.1 mg/ml nuclease-free BSA, 50 mM dithiothreitol) and 2 μ g of poly(dI-dC) at room temperature for 30 min, as described previously [9]. In some experiments, unlabelled oligonucleotides containing either the NF κ B or Oct1 consensus sequences were added to the extracts before incubation with the labelled oligonucleotide. In experiments involving antisera to NF κ B subunits, 0.5 μ l of a specific antiserum to p50, p65/RelA or c-Rel was incubated with 4 μ g of nuclear extract for 30 min prior to the binding reaction. When samples were treated with 2-mercaptoethanol, nuclear extracts were incubated with 1% 2-mercaptoethanol for 15 min on ice prior to the binding reaction. All incubations were subjected to electro-

phoresis on native 5% polyacrylamide gels that were subsequently dried and autoradiographed at -86°C overnight. Densitometric analysis was carried out on the autoradiographs using 'NIH Image'.

Assay of total and oxidized glutathione

Total glutathione was determined using a modification of the method of Tietze [11] for use with a Dynatech MR5000 plate reader. Cells were suspended at 5×10^6 cells/ml (1 ml of cell suspension per sample). Cells were incubated for 15 min at 37°C and then treated with the indicated concentrations of PDTC for 1 h. Incubations were terminated by addition of 5 vol. of ice-cold PBS per sample, and were then centrifuged for 2 min at 364 *g*. The pellet was resuspended in 200 μl of 2.5% sulphosalicylic acid and centrifuged at 12000 *g* for 2 min. The supernatant was removed to a clean Microfuge tube and assayed for total and oxidized glutathione.

Total glutathione was assayed by the addition of 190 μl of freshly prepared assay buffer [100 μM NADPH, 5 mM 5,5'-dithiobis-(2-nitrobenzoic acid), 1 unit/ml glutathione reductase, 1 mM EDTA and 50 mM phosphate buffer, pH 7] to 10 μl of sample. The change in absorbance was measured after 5 min using an MR5000 Dynatech plate reader at 405 nm and compared with a standard curve, from 0 to 80 μM , determined at the same time. To measure oxidized glutathione (GSSG), 70 μl of sample was derivatized with 2-vinylpyridine to remove reduced glutathione (GSH) [12]. This involved adding 4 μl of 2-vinylpyridine and 3.2 μl of triethanolamine per sample. Samples were left at room temperature for 1 h to allow the reaction to occur. The GSSG that remained was then assayed in the same manner as for total glutathione. Standards were prepared and treated in the same fashion.

In vitro treatment of nuclear extracts with glutathione

In vitro experiments with glutathione involved incubating 4 μg of nuclear extract from IL1-treated EL4.NOB-1 cells with various ratios of total to oxidized glutathione for 15 min on ice. The samples were then assayed for NF κ B as described above. The total glutathione (i.e. GSH + GSSG) concentration in each sample was 100 μM , with the concentration of GSSG increasing at the expense of that of GSH to obtain each ratio. GSH and GSSG stock solutions were prepared in 2.5% sulphosalicylic acid. The nuclear extracts which were used had undetectable ($< 0.1 \mu\text{M}$) levels of glutathione.

RESULTS

Reversal of the inhibitory effect of PDTC by 2-mercaptoethanol

Figure 1 demonstrates that pretreatment of either EL4.NOB-1 (Figure 1A) or Jurkat (Figure 1B) cells with PDTC inhibited NF κ B in cells treated with IL1 or TNF respectively. As previously demonstrated, PDTC concentrations of 100 μM and 1 mM were inhibitory (Figures 1A and 1B, lanes 4 and 5), while 10 mM had no effect (lane 6). When the nuclear extracts from stimulated cells were treated with 1% 2-mercaptoethanol for 15 min, DNA-binding activity was restored (lanes 10 and 11). 2-Mercaptoethanol also caused a slight increase in DNA binding in each sample.

As shown below the results from each electrophoretic mobility shift assay in Figure 1, densitometric analysis was performed in order to quantify the observed effects and ensure that the reversal by 2-mercaptoethanol was not simply an overall increase in DNA binding for each sample. 'NIH Image' was used to analyse

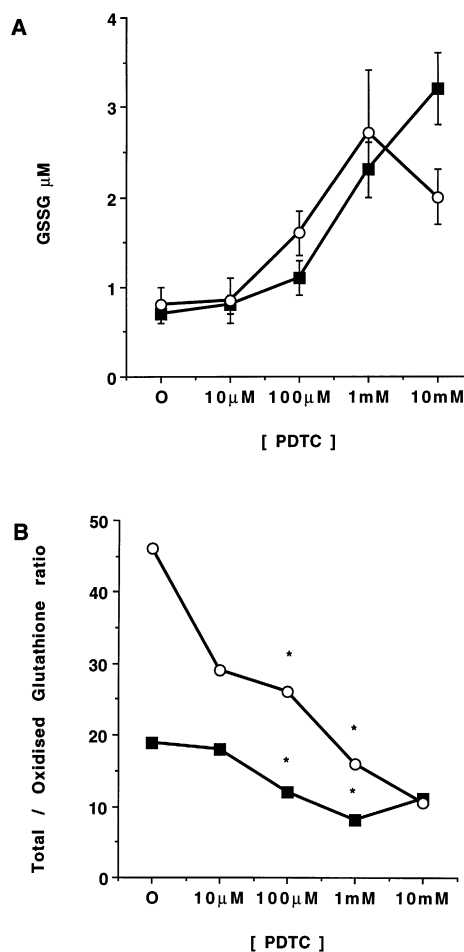


Figure 2 Effect of PDTC on glutathione in EL4.NOB-1 and Jurkat cells

Jurkat (○) and EL4.NOB-1 (■) cells were treated for 1 h with PDTC as indicated. (A) Cells were assayed for GSSG as detailed in the Experimental section. Results represent means \pm S.E.M. from four experiments. (B) Cells were assayed for total glutathione and GSSG as detailed in the Experimental section. Results show the ratio obtained by dividing the total glutathione for each sample by the GSSG for each sample. Asterisks indicate concentrations of PDTC that inhibited NF κ B binding.

each scan, and in the present study densities of up to 10000 arbitrary units were detected. For the 2-mercaptoethanol-treated samples in Figure 1(A), there was no decrease in the signal in samples from cells treated with 1 mM or 100 μM PDTC (right-hand graph), but a decrease was clearly evident in samples that had not been treated with 2-mercaptoethanol (left-hand graph). Similarly, in Figure 1(B), a clear decrease in signal was seen in samples from cells treated with 1 mM PDTC (left-hand graph) which was not evident when the samples had been treated with 2-mercaptoethanol (right-hand graph). It can therefore be concluded that 2-mercaptoethanol restores DNA binding in samples from PDTC-treated cells.

PDTC increases the level of GSSG in cells by oxidizing GSH

The reversal of inhibition of NF κ B by 2-mercaptoethanol suggested that PDTC was causing an oxidative shift in the cells which inhibited NF κ B. We next tested the effect of PDTC on the redox balance in cells by measuring the ratio of total to oxidized glutathione. PDTC was found to cause an increase in GSSG (Figure 2A) in both EL4.NOB-1 and Jurkat cells. A concentration

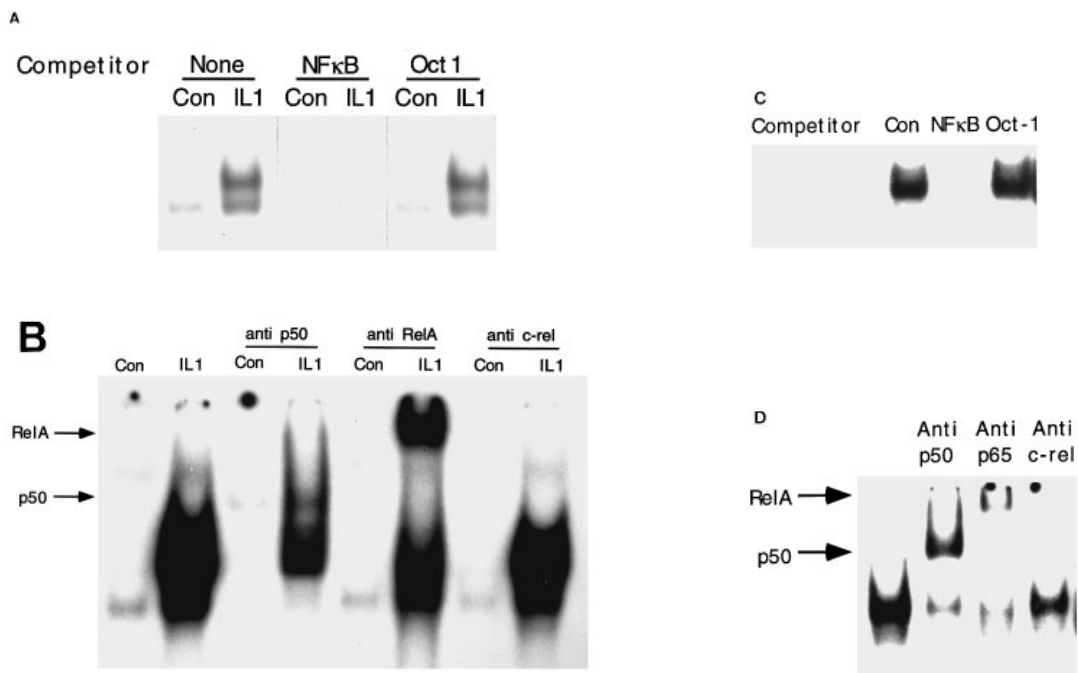


Figure 3 Competition and supershift analysis of NF κ B in EL4.NOB-1 and Jurkat cells

(A) Samples of 4 μ g of nuclear extract prepared from control (Con) or IL1-treated (10 ng/ml; 1 h) EL4.NOB-1 cells were incubated in the absence (none) or presence of unlabelled oligonucleotide competitor (1.75 pmol) containing the NF κ B or Oct1 consensus sequences prior to assaying for NF κ B binding activity as described in the Experimental section. DNA–protein complexes are shown. (B) Samples of 4 μ g of nuclear extract protein from control (Con) or IL1-treated (10 ng/ml; 1 h) EL4 cells were incubated with the indicated antisera prior to assaying for NF κ B binding activity as described in the Experimental section. DNA–protein complexes are shown. Supershifted complexes corresponding to p50 and p56/RelA are indicated. (C) Samples of 4 μ g of nuclear extract prepared from Jurkat cells stimulated with TNF (10 ng/ml) for 1 h were incubated in the absence (Con) or presence of unlabelled oligonucleotide competitor (1.75 pmol) containing the NF κ B or Oct1 consensus sequences prior to assaying for NF κ B binding activity as described in the Experimental section. DNA–protein complexes are shown. (D) Samples of 4 μ g of nuclear extract protein from Jurkat cells stimulated with TNF (10 ng/ml) for 1 h were incubated with the indicated antisera prior to assaying for NF κ B binding activity as described in the Experimental section. DNA–protein complexes are shown. Supershifted complexes corresponding to p50 and p56/RelA are indicated.

of 1 mM PDTC led to a 2–3-fold increase in GSSG in both cell types. To ensure that the effect was due to the oxidation of GSH, the ratio of total to oxidized glutathione was also measured. A progressive increase in GSSG relative to total glutathione occurred in both Jurkat and EL4.NOB-1 cells (Figure 2B). It therefore appeared that, instead of acting as an anti-oxidant in Jurkat and EL4.NOB-1 cells, PDTC was having a pro-oxidant effect.

p50 and p65/RelA are the major NF κ B subunits in IL1-activated EL4.NOB-1 and TNF-activated Jurkat cells

Because PDTC appeared to be having a pro-oxidant effect, we analysed the NF κ B complexes in more detail, as the p50 subunit is known to be particularly susceptible to oxidation on cysteine-62 [13]. Competition studies with unlabelled oligonucleotides demonstrated that the complexes detected in EL4.NOB-1 and Jurkat cells were specific for NF κ B (Figures 3A and 3C respectively), as the unlabelled NF κ B oligonucleotide competed for binding to the probe whereas the unlabelled Oct1 sequence did not. Generally, two major complexes were detected in extracts from IL1- and TNF-treated cells. Untreated EL4.NOB-1 cells also contained low levels of the faster migrating complex (Figure 3A and 3B). This complex contained predominantly p50, as an antibody to p50 further retarded the complex in the gel (Figure 3B). It was therefore most probably composed of p50p50 homodimers. p50 was also present in the IL1-activated sample, which was also shown to contain p65/RelA, as an antibody to this subunit caused a major supershift in the complex (Figure 3B,

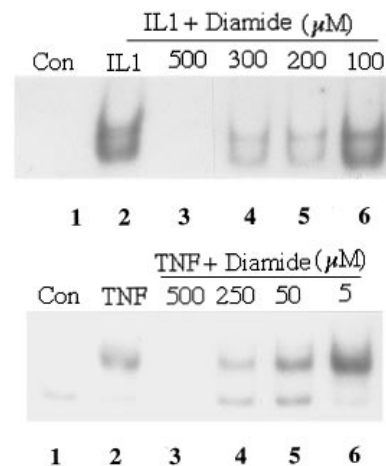


Figure 4 Effect of diamide on NF κ B binding in EL4.NOB-1 and Jurkat cells

EL4.NOB-1 (upper panel) and Jurkat (lower panel) cells were pretreated for 1 h with a range of concentrations of diamide, as indicated. Cells were then treated with IL1 or TNF (10 ng/ml) for 1 h (Con, control). Nuclear extracts were prepared and the samples were assayed for NF κ B binding as detailed in the Experimental section. DNA–protein complexes are shown. The results are representative of three experiments.

lane 6). This autoradiograph has been overexposed in order to clearly demonstrate the presence of p65/RelA. No c-Rel was detected in the cells.

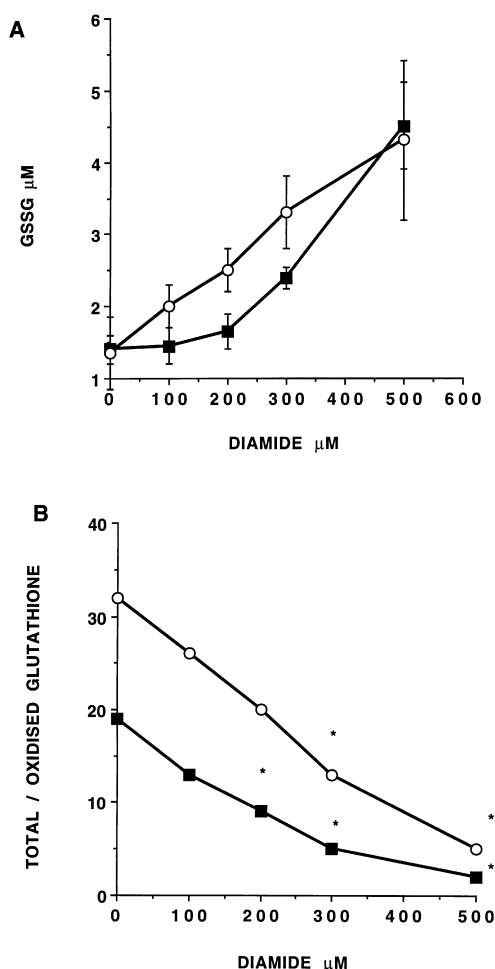


Figure 5 Effect of diamide on glutathione in EL4.NOB-1 and Jurkat cells

Jurkat (○) and EL4.NOB-1 (■) cells were treated for 1 h with diamide as indicated. (A) Cells were assayed for GSSG as detailed in the Experimental section. Results are means \pm S.E.M. from four experiments. (B) Cells were assayed for total glutathione and GSSG as detailed in the Experimental section. Results show the ratios obtained by dividing the total glutathione for each sample by the GSSG for each sample. Asterisks indicate the concentrations of diamide that inhibited NF κ B binding.

With Jurkat cells, control samples displayed negligible levels of NF κ B. Supershift analysis of extracts from TNF-treated cells demonstrated the presence of both p50 (Figure 3D, lane 2) and p65/RelA (lane 3). A slight decrease in binding in anti-c-Rel-treated samples suggested that at least part of the complex contained the c-Rel subunit (lane 4).

These results therefore indicated that the NF κ B complexes detected predominantly contained p50 and p65/RelA, the fastest migrating complexes comprising p50p50 homodimers and the slower complexes comprising p50p65 heterodimers.

Diamide inhibits NF κ B and increases the level of GSSG in EL4.NOB-1 and Jurkat cells

In order to explore the link between NF κ B and glutathione further, we carried out similar studies with diamide, which was originally designed as a glutathione-oxidizing agent. Similar to PDTDC, diamide was found to inhibit NF κ B in IL1-treated

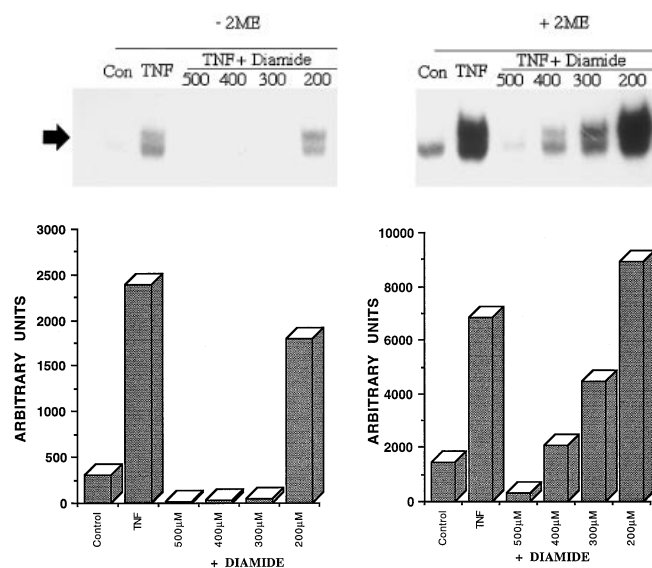


Figure 6 Effect of the reducing agent 2-mercaptoethanol on diamide inhibition of NF κ B in Jurkat cells

Jurkat cells were pretreated for 1 h with the indicated range of diamide concentrations. Cells were then treated with TNF (10 ng/ml) for 1 h (Con, control). Nuclear extracts were prepared as described in the Experimental section. Samples of 4 μ g of each nuclear extract were treated (+2ME) or not (-2ME) with 1% 2-mercaptoethanol for 15 min on ice. The samples were then assayed for NF κ B binding as described in the Experimental section. DNA-protein complexes are shown. Densitometric analysis was also performed, the results of which are shown beneath the corresponding autoradiograph. All results are representative of three separate experiments.

EL4.NOB-1 and TNF-treated Jurkat cells, at concentrations of 200–500 μ M (Figure 4, lanes 3–5). These concentrations were also found to increase the level of GSSG in the cells, with 500 μ M diamide increasing GSSG 3-fold in both EL4.NOB-1 and Jurkat cells (Figure 5A). The increase in GSSG was due to the oxidation of GSH, as we also observed a progressive increase in GSSG relative to GSH in both cell types (Figure 5B), similar to that observed with PDTDC.

We also attempted to restore DNA binding in samples from TNF-treated Jurkat cells inhibited by diamide. Recovery of binding was achieved at lower concentrations, particularly 200 μ M and 300 μ M diamide, and to a lesser extent at 400 μ M (Figure 6, compare the left-hand and right-hand panels). The effect of diamide may have been more difficult to reverse because of its stronger pro-oxidant properties compared with PDTDC, which could result in other protein targets being oxidized leading to an impairment in the NF κ B activation process.

Increasing the ratio of GSSG to total glutathione inhibits NF κ B DNA binding *in vitro*

Finally, we tested the effects of various ratios of total to oxidized glutathione on NF κ B DNA binding *in vitro*. The total glutathione (i.e. GSH + GSSG) concentration in each sample was 100 μ M, with the concentration of GSSG increasing at the expense of that of GSH to give each ratio. Decreasing ratios were found to inhibit NF κ B binding activity in nuclear extracts (Figure 7). The extracts themselves had undetectable levels of glutathione (< 0.1 μ M). A ratio of total to oxidized glutathione of 100:1 proved inhibitory, with increasing inhibition occurring at 11:1 and 2:1. This suggests that the ratios that were induced in intact cells by PDTDC and diamide would be sufficient to inhibit NF κ B.

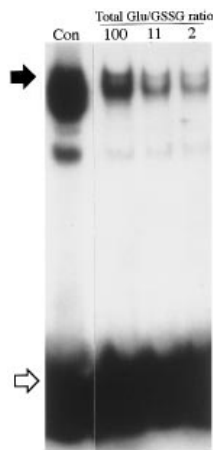


Figure 7 Effect of the ratio of total to oxidized glutathione on NF κ B binding *in vitro*

Samples of 4 μ g of nuclear extract from IL1-treated EL4.NOB-1 cells were incubated with the indicated ratios of total to oxidized glutathione for 15 min on ice. The total glutathione ('Glu', i.e. GSH + GSSG) concentration in each sample was 100 μ M, with the concentration of GSSG being increased at the expense of that of GSH to give each ratio. The samples were then assayed for NF κ B binding as described in the Experimental section. 'Con' indicates control extracts from IL1-treated cells not incubated with glutathione. The filled arrow indicates DNA-protein complexes, and the open arrow indicates unbound DNA. The results are representative of three experiments.

DISCUSSION

Our results demonstrate that the mechanism of inhibition of NF κ B by PDTC in IL1- or TNF-stimulated lymphocytes involves a pro-oxidant effect on NF κ B rather than an inhibition of NF κ B activation. Several studies into the mechanism of activation of NF κ B have utilized PDTC because of its anti-oxidant properties [14–16]. It has been shown to block NF κ B in a range of cell types and in response to diverse stimuli, and this has been presented as evidence for oxygen radicals being the key second messengers in NF κ B activation [9,14,15,17]. However, no studies to our knowledge have demonstrated that PDTC decreases the level of oxygen radicals in cells. Anti-oxidant effects have been reported in chemical systems [18], and the assumption that similar effects are occurring in cells has been widely made. Any anti-oxidant effect of PDTC may be due to its metal-chelating properties [19]. Apart from metal chelation, dithiocarbamate compounds have been shown to have a range of other effects [20]. The free thiol group can react with other thiol groups and interact covalently with protein thiols [20]. More importantly, there have been reports describing a pro-oxidant effect, thereby generating intracellular oxidative stress [21]. Our data suggest that it is the pro-oxidant properties of PDTC, rather than any anti-oxidant effect, that are responsible for the inhibition of NF κ B.

This conclusion is based on the observation that DNA binding can be restored in inhibited nuclear extracts by 2-mercaptoethanol. This was also observed using diamide (a compound designed to oxidize glutathione) instead of PDTC. Furthermore, both PDTC and diamide increased glutathione oxidation and thereby increased the ratio of oxidized to total glutathione. In addition, we demonstrated that altering this ratio *in vitro* inhibited NF κ B DNA binding. It is possible that the increased oxidation of GSH to GSSG leads to the formation of a mixed disulphide with NF κ B, resulting in inhibition of DNA

binding. The disulphide formed would be reduced by 2-mercaptoethanol and DNA binding restored. DNA binding was more difficult to restore in cells treated with higher concentrations of diamide (400–500 μ M). At these concentrations, diamide would be expected to have a strong pro-oxidant effect, which in the present study was evident in its marked effect on GSSG and on the ratio of total to oxidized glutathione. Indeed, we found higher concentrations of diamide to be more powerful at oxidizing glutathione than was PDTC. The stronger pro-oxidant effect may have resulted in additional targets being oxidized, which possibly impeded the NF κ B activation process.

A thiol likely to be targeted by PDTC is cysteine-62 on the p50 subunit of NF κ B. We demonstrated here that the NF κ B complex in IL1-treated EL4.NOB-1 and TNF-treated Jurkat cells contains p50. Cysteine-62 on p50 has been shown to be critical for DNA binding since, if it is mutated to a serine, the affinity of p50 for DNA decreases 10-fold [13]. Furthermore, other thiol-reactive compounds such as iodoacetate have been shown to block DNA binding by reacting with cysteine-62 [13]. Most recently, we have shown that the thiol-reactive tyrosine kinase inhibitor herbimycin A blocks NF κ B action in IL1-treated EL4 cells by reacting with cysteine-62 [22]. It is also possible, however, that p65/RelA could be a target, as it has a cysteine in an analogous position to that in p50 [23] and is the other major NF κ B family member present in these extracts. Similar mutational analysis has not been carried out on p65/RelA. The X-ray crystal structure of p65/RelA is not known, unlike p50 where it has been demonstrated that cysteine-62 is close to the DNA interaction domain and, if modified, would be very likely to interfere with binding. However, given that the cysteine is conserved in p65/RelA, it is possible that it is also a target. The effect of PDTC on the NF κ B system may be somewhat specific. Schreck et al. [19] have shown that, under conditions where PDTC inhibits NF κ B, other transcription factors such as Oct1 or Sp1 are unaffected, indicating that the compound does not have a generalized effect on all transcription factors.

PDTC itself is unlikely to react directly with NF κ B, since adding PDTC to nuclear extracts directly did not inhibit DNA binding (results not shown). This suggests either that a metabolite of PDTC generated in cells is responsible for the effect or, alternatively, that the pro-oxidant effect of PDTC drives the formation of a mixed disulphide between NF κ B and a cellular thiol group. Since GSH is the most abundant thiol in cells, and since PDTC increases oxidation of GSH, it is possible that a glutathione-NF κ B adduct is formed. Increasing the ratio of oxidized to reduced glutathione *in vitro* inhibited DNA binding by NF κ B. This would be consistent with a model involving a direct effect of glutathione on NF κ B DNA binding in intact cells. The ability of PDTC to increase GSSG in cells could be due to a general pro-oxidant effect, which may involve copper transport into cells. Recently, Nobel et al. [21] have shown that PDTC induces apoptosis in cells through a pro-oxidant effect involving copper transport. Alternatively, dithiocarbamates have been shown to have glutathione peroxidase-like activity, and it has been demonstrated that xenobiotic thiols such as PDTC can be converted enzymically into sulphenic acid, which would oxidize GSH to GSSG. In agreement with our work, others have demonstrated that PDTC increases the level of GSSG in intact cells [24]. A range of studies therefore indicate that, instead of acting as an anti-oxidant, PDTC has an overall pro-oxidant effect. It is tempting to speculate that an increase in GSSG mediates many of the inhibitory effects of PDTC, including inhibition of apoptosis and NF κ B.

An unusual feature of the inhibitory effect of PDTC was the lack of an effect at concentrations of > 1 mM. This has been

observed by others [19]. At these higher concentrations, however, GSSG levels were still elevated. One explanation for this could be that, at high concentrations, PDTC has saturated the mono-oxygenase which converts it into sulphenic acid, and becomes an important thiol in the cell. Under these conditions, PDTC could act as a reducing agent, counteracting the effect of GSSG and thereby protecting the sensitive thiol on p50. This would explain the 'bell-shaped' dose-response curve for the effect of PDTC on NF κ B in cells.

As DNA binding could be restored in nuclear extracts, PDTC did not appear to interfere with the nuclear translocation of NF κ B stimulated in response to IL1 or TNF. A similar conclusion was reached by Galter et al. [24], who proposed that NF κ B is under dual regulation. In the cytosol, a pro-oxidant signal is required to activate NF κ B. In the nucleus, however, where conditions are much more reducing, NF κ B must be maintained in a reduced state for DNA binding to occur. GSSG would therefore have a positive effect on NF κ B activation and translocation to the nucleus, whereas it would inhibit DNA binding. We did not observe any activation of NF κ B by PDTC on its own, which would argue against oxidative activation by GSSG. It is possible, however, that other signals are needed for optimal activation, which could be induced by IL1 and TNF. In spite of this, the inhibitory effect of PDTC on NF κ B supports the view that nuclear NF κ B must be kept in a reduced state in order for DNA binding to occur, with a specific requirement for maintaining cysteine-62 of p50 in a reduced state.

In conclusion, the present results indicate that modulation of NF κ B by the widely used agent PDTC is unlikely to involve an anti-oxidant effect in the cells tested here. We would therefore advise caution in the use of this compound to implicate reactive oxygen species in NF κ B activation and in the induction of NF κ B-regulated genes. The work further argues that a pro-oxidant effect which increases GSSG relative to GSH will not interfere with the activation and nuclear translocation of NF κ B, but rather will oxidize NF κ B and prevent DNA binding.

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