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Effects of Antioxidant Enzyme Modulations on Interleukin-1-Induced Nuclear Factor Kappa B Activation

Patricia Renard,† Marie-Denise Zachary,* Catherine Bougelet,* Marc-Edouard Mirault,‡ Guy Haegeman,§ José Remacle* and Martine Raes**

*Laboratoire de Biochimie Cellulaire, 61, rue de Bruxelles, Facultés Universitaires, B-5000 Namur, Belgium; ‡Centre Hospitalier de l'Université de Laval, 2705 Boul Laurier, Quebec G1V4G2, Canada; §Laboratorium voor Molekulaire Biologie, 35, Ledeganckstraat, Rijksuniversiteit Gent, B-9000 Ghent, Belgium

ABSTRACT. Nuclear factor kappa B (NF-κB) is a potent and pleiotropic transcription factor that can be activated by a wide variety of inducers, including interleukin-1 (IL-1). Although the detailed activation mechanism of NF-κB is still under investigation, it requires both phosphorylation and degradation of its inhibitory subunit IκB and the presence of an oxidative environment. In this study, we systematically evaluated the influence of glutathione peroxidase, glutathione reductase and catalase on IL-1-induced NF-κB activation by analysing the effect of specific inhibitors of these enzymes. For the three antioxidant enzymes mentioned, their inhibition correlated with an overactivation of NF-κB, particularly for glutathione peroxidase. Inversely, we tested the response of glutathione peroxidase-transfected cells on NF-κB activation, which was lower as compared with the parental cells. Furthermore, interleukin-6 production also correlated perfectly with the reduced level of NF-κB activation in these experiments. The results clearly show that NF-κB activation is, strongly dependent on the antioxidant potential of the cells, especially on the activity of reduced glutathione-dependent enzymes such as glutathione peroxidase. The results support the hypothesis that the level of the oxidised glutathione:reduced glutathione ratio and the activity of intracellular antioxidant enzymes play a major role in NF-κB fine tuning. *Copyright © 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 53;2:149–160, 1997.

KEY WORDS. nuclear factor kappa B; interleukin-1; glutathione peroxidase; catalase; glutathione reductase

In 1986, Sen and Baltimore discovered several nuclear factors that interact with the immunoglobulin-enhancer sequences. One factor, initially detected in B cells, was associated with the kappa light chain enhancer [1] and was therefore called NF-κB.[‖] NF-κB was subsequently though an ubiquitous transcription factor present in an inactive form in many resting cells [2] and in a constitutively active form in differentiated B cells [3], neurons [4] and vascular smooth muscle cells [5].

The most frequent form of NF-κB is a dimer composed of two DNA-binding proteins: p50 and RelA (formerly called p65). In its cytoplasmic inactive form, NF-κB is bound to one inhibitory subunit of the IκB family. These inhibitory subunits can be considered cytoplasmic anchors for the transcription factor because they prevent nuclear uptake of NF-κB, probably through the masking of the nuclear localisation sequence located on both p50 and RelA [2].

NF-κB can be activated by a wide variety of agents that either signal or are themselves a threat to the organism: e.g., TNFα, IL-1, lipopolysaccharides, several viruses and double-stranded RNA (for a review, see [6]). These agents induce a cascade of events leading to the phosphorylation of IκB and its further degradation [7, 8]. As a consequence, the p50–RelA heterodimer is released from its cytoplasmic anchor and translocates into the nucleus, where it binds to different gene promoters, thus inducing the transcription of a large number of genes such as those encoding IL-6 and several adhesion molecules (for a review, see [9]). NF-κB is a privileged mediator of the immune and inflammatory reactions and of the acute phase response.

Polypeptides p50 and RelA belong to the Rel/dorsal family [10], which presently includes RelA, RelB (or I-Rel), the oncogene product of the avian virus REV-T (v-Rel), its cellular counterpart c-Rel, the *Drosophila* factors dorsal and dif, p50, and p52 [11]. The different subunits of the family can form homo- or heterodimers and have different DNA-binding affinities and different transactivating properties.

† Corresponding author: Patricia Renard, Laboratoire de Biochimie Cellulaire, Facultés Universitaires, 61, rue de Bruxelles, B-5000 Namur, Belgium. TEL: 32-81-724321; FAX: 32-81-724135.

‖ *Abbreviations:* BCNU, bischloroethylnitrosourea; GSH, reduced glutathione; GSSG, oxidised glutathione; IL-1, interleukin-1; IL-6, interleukin-6; IOD, integrated optical density; Mn-SOD, manganese superoxide dismutase; NF-κB, nuclear factor kappa B; PDTC, pyrrolidine dithiocarbamate; PMA, phorbol myristate acetate; ROI, reactive oxygen intermediates; TNFα, tumour necrosis factor α.

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Moreover, the inactivation of NF-κB is performed by different IκB proteins such as IκB-α, -β, -γ, cactus, p100 and p105 (for reviews, see [12, 13]). These subunits also bind to the Rel/dorsal proteins with different affinities. The presence of and possible interactions among all these proteins make the system much more versatile than initially expected and account for, at partly, the variation in the cellular responses observed with different stimuli and within different tissues.

The activation mechanism of NF-κB is under intensive investigation and probably requires both phosphorylation and the presence of oxygen-derived species [14, 15]. NF-κB has been reported to be activated *in vitro* through IκB phosphorylation by protein kinase C, protein kinase A and heme-regulated kinase eIF-2 [16], by Raf-1 kinase [17] and by a double-stranded RNA-dependent protein kinase [18]. *In vivo*, kinases involved in NF-κB activation apparently depend on both the inducer used and the cell type considered. Kinases are currently the object of intensive research. Phosphorylation of IκB is followed by its polyubiquitination, which tags the inhibitory subunit to the proteasome 26S degradative pathway [8, 19].

Besides the search for the IκB kinase(s), reactive oxygen intermediates have been proposed as second messengers that could switch on a particular kinase cascade leading to the activation of NF-κB [20]. This hypothesis is mainly based on three observations. Firstly, oxidising conditions such as incubations in the presence of low doses of peroxides (H_2O_2 and tert-butyl-hydroperoxide for example), ultraviolet light, ionising radiations or hyperoxia induce NF-κB activation in several cell types [21]. Secondly, NF-κB activation can be inhibited by many antioxidants, metal chelators or oxygen scavengers (for a review, see [21]). Thirdly, the presence of oxygen-derived species following cell stimulation has been reported, even in noninflammatory cells, for different NF-κB inducers, such as TNFα or IL-1 [22–24].

The activity of NF-κB is clearly under the control of an oxidant/antioxidant balance, even if the identity of the reactive oxygen species involved and their mechanism of action remain to be defined. Schutze-Osthoff *et al.* [25] showed that, in the case of L929 fibrosarcoma cells stimulated by TNFα, the activation of NF-κB was clearly dependent on the mitochondrial oxidative metabolism, although this is not necessarily true for inducers other than TNFα. There are many experimental data supporting the notion that reactive oxygen species serve as second messengers at low doses, even though at high or prolonged concentrations they are undoubtedly toxic for mammalian cells [26]. The level of free radicals withstood by a cell directly depends on the level of the antioxidant defences and include nonenzymatic defenses (glutathione, vitamins E and C, etc.) and three main antioxidant enzymes: superoxide dismutase (dismutating two superoxide ions into H_2O_2), catalase-destroying H_2O_2 and glutathione peroxidase, which reduces both hydrogen and organic peroxides. Glutathione reductase is also involved because it contributes to the recycling of glutathione that will again be used as a substrate by glutathione peroxidase.

The involvement of reactive oxygen species in NF-κB activation has been investigated mostly in human cell lines such as Jurkat and HeLa cells [20] by incubating the cells with antioxidising molecules such as metal chelators or –SH-protecting molecules [21]. In contrast, there have been fewer attempts to modulate the intracellular levels of antioxidant enzymes. Firstly, the effect of Mn-SOD overexpression on NF-κB DNA-binding activity was investigated in human epithelial breast carcinoma cells MCF-7 [20]. Activation of NF-κB by PMA or IL-1 was not inhibited in the Mn-SOD overexpressing cell line as compared with the parental cell line, supporting the idea that superoxide anions were not directly involved in the activation. TNFα-induced NF-κB activation was more important in the cells transfected with Mn-SOD than in the nontransfected cells. Secondly, TNFα-induced NF-κB activation has been investigated in mouse epidermal JB6 cells overexpressing either the human Cu/Zn-SOD or the human catalase gene. NF-κB activation was lower in catalase-overexpressing cells and increased in Cu/Zn-SOD-transfected cells, again indicating that superoxide anions are not directly involved in NF-κB activation, although they may be a source for H_2O_2, which could then act as a messenger molecule activating NF-κB [27]. Thirdly, the construction of a chloramphenicol acetyltransferase reporter gene under the control of four cis-acting κB elements showed a higher NF-κB transactivating activity in the presence of BCNU, a glutathione reductase inhibitor [28].

In the present study, using a model of SV40-transformed human WI-38 fibroblasts, we compared the NF-κB activation in cells treated with inhibitors that affect three major antioxidant enzymes, glutathione peroxidase, glutathione reductase and catalase. We then compared the IL-1β-induced NF-κB activation in cells transfected with the cDNA of glutathione peroxidase and in their untransfected counterpart. Our data suggest that the modulation of GSH-associated antioxidant enzymes strongly affects NF-κB activation.

MATERIALS AND METHODS

Cell Culture

The cell line WI-38 VA13, a SV40 virus-transformed human fibroblastic cell line, was purchased from the American Type Culture Collection and plated in 25 or 75 cm^2 flasks at 60,000 cells/cm^2. Cells were serially cultivated in minimum essential medium (Gibco, UK) supplemented with 10^{-7} M Na_2SeO_3 in the presence of 10% fetal bovine serum. For each experiment, cells were rinsed for at least 30 min and further cultured in the same medium without serum but in the presence of 0.2% lactalbumin hydrolysate as a serum substitute during stimulation in the presence (or absence for the controls) of IL-1β.

Vector Construction and Transfection

The WI-38 VA13 cell line was transfected using the calcium phosphate precipitation method as modified by Chen and Okoyama [29], with the plasmid pML-MT-GPx-Hyg+. This expression vector derives from the pML hygro plasmid [30], a modified pBR322 enriched with the multiple cloning sequence of pSPT19 and the gene coding for hygromycin B phosphotransferase under the control of the SV40 early promoter. The hygromycin B phosphotransferase gene is a positive marker for the selection of cell transfectants [31]. In this plasmid, the cDNA-encoding human glutathione peroxidase, flanked by 41 nucleotides of the 5′-noncoding region and 126 nucleotides of the 3′-noncoding region, was inserted under control of the human metallothionein promoter. Among the 8 viable clones obtained after transfection and selection, the clone expressing the highest level of glutathione peroxidase, i.e. WI-38 MT08, was selected and used for all experiments.

Enzymatic Assays

Aminotriazol was purchased from Janssen Chimica (Geel, Belgium); and mercaptosuccinate and PDTC were purchased from Sigma (St. Louis, MO, USA). Before use, these inhibitors were dissolved in culture medium. BCNU, obtained from Bristol-Myers (Paris, France) [32], was first dissolved in ethanol and then diluted at least 1000 times in culture medium. Cells, grown to confluence, were incubated for 30 min in these solutions at 37°C. Cells were then rinsed twice with cold phosphate buffered saline, harvested with a rubber policeman and homogenised with a Dounce in cold phosphate buffered saline.

The enzymatic assay for glutathione reductase activity was performed according to the method of Mbemba *et al.* [33]. Briefly, cell homogenates in the presence of Triton X-100 0.1% (Merck, Darmstad, Germany) were incubated in the presence of 3 mM GSSG and 1.1 mM NADPH. The disappearance of NADPH was measured at 340 nm.

Similarly, glutathione peroxidase was assayed by measuring the disappearance of NADPH at 366 nm when tert-butylhydroperoxide was added to a mixture containing cell homogenates, 1.1 mM NADPH, 1.3 mM GSH and glutathione reductase [33].

Catalase activity was determined by incubating the cell homogenates with H_2O_2 for 45 min at room temperature before adding $TiSO_4$, an acid that reacts with H_2O_2 to generate a coloured product measured at 420 nm [34].

The total protein content present in the homogenates was determined according to the method of Bradford [35] to express the results as specific activities, i.e. in units of enzymatic activity/mg total protein.

Electrophoretic Mobility Shift Assay

Confluent cells were stimulated for 30 min with 5 ng/mL IL-1β (Bachem, Bubendorf, Switzerland) in the presence or absence of different inhibitors. In the case of aminotriazole, mercaptosuccinate or BCNU, cells were preincubated for 30 min in the presence of the inhibitor alone, and PDTC was given at the same time as IL-1β. After stimulation, cells were rinsed twice with cold phosphate buffered saline before being scraped and centrifuged for 10 min at 1000 rpm. The pellet was then resuspended in a 100 μL lysis buffer (Hepes 20 mM, NaCl 0.35 M, glycerol 20%, NP-40 1%, $MgCl_2.6H_2O$ 1 mM, EDTA 0.5 mM, EGTA 0.1 mM). After 10 min on ice, the lysate was centrifuged for 20 min at 14,000 rpm. The supernatant constitutes the total protein extract and can be kept frozen at −70°C. The binding reaction occurs in a binding mixture of 20 μL containing 2 mM Hepes (pH 7.5), 5% glycerol, 75 mM KCl, 2.5 mM dithiotreitol, 2 μg poly-dIC, 20 μg bovine serum albumin, cell extract (25 μg protein determined according to the Bradford assay) and a ^{32}P-labelled oligonucleotide (±1 ng or 20,000 cpm). The cold double-stranded probe (5′-AGTTGAGGGGACTTTCCCAGGC) was purchased from Promega (Madison, WI, USA), labelled with γ-^{32}P using the T4 polynucleotide kinase and purified on a Sephacryl S-200 column. After 30 min of incubation, the binding mixture was analysed on a native 4% acrylamide gel in 0.5 × TBE (Tris 0.9 M, boric acid 0.9 M, EDTA 0.02 M). After autoradiography, the film was scanned by an image analysis system (Visage 101, Millipore, USA) that allows quantification of the integrated optical density (IOD) contained in the shifted bands.

A similar procedure was followed for the supershift experiments, except that 1 μL antisera raised against p50 or p65 was added to the protein extracts on ice 30 min before addition of the ^{32}P-labelled probe. The antisera were generously provided by Prof. Piette, Université de Liège, Belgium.

IL-6 Assay

Cells were cultivated in 96-well plates at a density of 4000 cells/well in 200 μL Minimum essential medium + 0.2% lactalbumin hydrolysate. Cells were stimulated with 0.25 ng IL-1β/mL in the presence or absence of PDTC. IL-6 was assayed by ELISA (Eurogenetics, Tessenderlo, Belgium) in the culture medium collected after a 6-hr stimulation.

RESULTS

Activation of NF-κB by IL-1β in SV-40-transformed WI-38 Human Fibroblasts

First, we tested the effect of IL-1β on the activation of the transcription factor NF-κB in the WI-38 VA13 cell line and characterised the activated forms of NF-κB, because all the data on human fibroblasts obtained so far had been limited to non-virus-transformed cells [36]. NF-κB activation was followed with the electrophoretic mobility shift assay. Different incubation times and IL-1β concentrations were tested to optimise the working conditions for the virus-transformed WI-38 VA13 cells. IL-1β clearly induced

the retardation of the ^{32}P-labelled double-stranded DNA probe containing the NF-κB binding site in a dose- and time-dependent manner. The shifted band only appeared in lanes corresponding to protein extracts of cells stimulated with IL-1β, indicating that unstimulated fibroblasts had no constitutive NF-κB DNA-binding activity, unlike other cell types [3, 4]. NF-κB DNA-binding activity slightly increased with incubation time, reaching its maximal activity after 60 min of incubation in the presence of 5 ng/mL of IL-1. The intensity of the shifted bands was quantified by an image analysis system and expressed as IOD. This quantitative analysis (Fig. 1B) confirmed that NF-κB activation was induced by IL-1β in a time- and dose-dependent manner.

To characterise the subunits present in the NF-κB complex activated by IL-1β in WI-38 VA13 cells, supershift experiments were conducted. Figure 2 shows that the addition of anti-p50 (lanes 5–6) or anti-p65 (lanes 7–8) antibodies induced a further shift of the specific complex, indicating that the NF-κB factor activated in this cellular model was composed of the p50–p65 heterodimer. Moreover, addition of 50 μg/mL cycloheximide during incubation with IL-1β did not affect the intensity of the specific complex (Fig. 2, lanes 9–10), indicating that the IL-1β-induced NF-κB activation was a posttranslational event. Cycloheximide alone did not effect NF-κB activation in this cell line (data not shown).

IL-1-induced NF-κB activation was a reactive oxygen intermediates (ROI)-dependent mechanism in WI-38 VA13 fibroblasts, because the antioxidant PDTC clearly inhibited NF-κB activation in a dose-dependent manner (Fig. 3A). Quantitative analysis (Fig. 3B) of the autoradiography indicated that 10^{-4} M PDTC inhibited up to 66% of the NF-κB activation as compared with cells stimulated with IL-1 alone.

In the subsequent experiments, the antioxidant potential of the cells was modulated either by reducing the activity of three different antioxidant enzymes using specific inhibitors or by using cells overexpressing glutathione peroxidase. To follow the variations in the level of NF-κB activation under these conditions, suboptimal stimulating conditions were chosen, with stimulation for 30 min with 5 ng/mL IL-1β (see Fig. 1).

Decrease in the Cellular Antioxidant Potential

A systematic study of different antioxidant enzyme inhibitors on human WI-38 fibroblasts has already been performed to evaluate the relative protection of the corresponding enzymes in oxidative stress [37]. It turned out that mercaptosuccinate, BCNU and aminotriazole inhibited glutathione peroxidase and glutathione reductase in a rather specific way. In contast, diethyldithiocarbamate, considered a potent reversible inhibitor of superoxide dismutase, also inhibited glutathione peroxidase and was thus regarded as a more general and nonspecific inhibitor and therefore not used in this section of our study.

The effect of three specific antioxidant enzyme inhibitors on their enzymatic activity and, correspondingly, on IL-1β-induced NF-κB activation as measured by electrophoretic mobility shift assays was tested directly. The inhibitory effects obtained with mercaptosuccinate (Fig. 4), BCNU (Fig. 5) and aminotriazole (Fig. 6) on the enzymatic activity are shown in panel A of Figs. 4–6 and on NF-κB DNA-binding activity in panel B of Figs. 4–6. The residual specific activity of the different enzymes is expressed in percentages vs. the enzymatic activity measured in cells incubated without inhibitors arbitrarily set to 100%. NF-κB activity was tested by gel shift analysis and the autoradiography quantitatively analysed, as described in Fig. 1. Results are expressed as the percentage of the IOD compared with the IOD of cells stimulated with IL-1β without inhibitors, arbitrarily set to 100%. The inhibitors had no effect on cell viability or on basal NF-κB DNA-binding activity (data not shown).

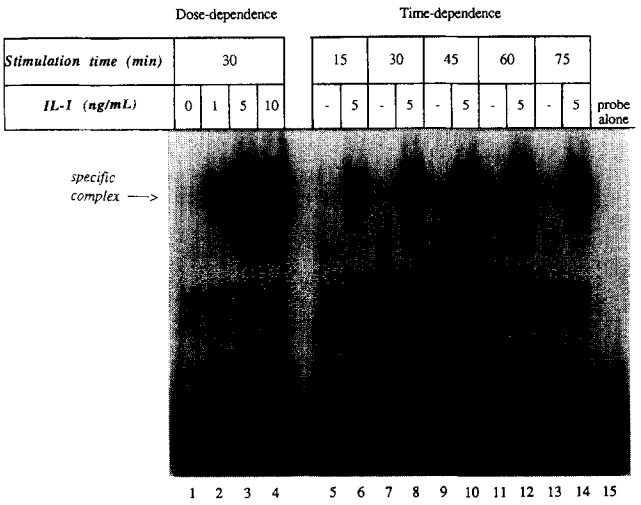
The three inhibitors tested reduced the catalytic activity of the corresponding antioxidant enzymes in the WI-38 VA13 cell line, although the efficiency of inhibition was lower than that obtained in the nontransformed cell line [37], e.g. 10^{-5} M mercaptosuccinate inhibited 30% of the glutathione peroxidase activity in WI-38 fibroblasts but only 5% in the WI-38 VA13 cell line.

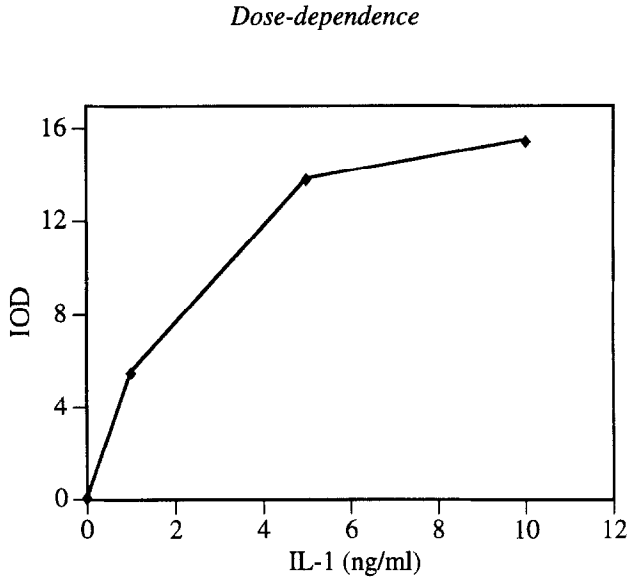
The results shown in Fig. 4 suggest that even a limited inhibition of glutathione peroxidase by mercaptosuccinate correlates with a significant increase in the activation of NF-κB induced by IL-1β: a 6% inhibition of glutathione peroxidase activity after a 30-min incubation in the presence of 10^{-5} M mercaptosucciante corresponded to a 45% overactivation of NF-κB in IL-1β-stimulated cells.

The importance of glutathione metabolism for NF-κB activation was further confirmed by the effect of the glutathione reductase inhibitor, BCNU, on NF-κB activation (Fig. 5). BCNU is degraded into two metabolites, one of which is a carbamylating agent that reacts with and inhibits the reduced form of glutathione reductase [32]. At 10^{-4} M, BCNU inhibits approximately 52% of glutathione reductase, with a corresponding overactivation of NF-κB (28%).

The first experiments suggesting a role for reactive oxygen intermediates in NF-κB activation, published by Baeuerle and Baltimore [2], showed that low concentrations of H_2O_2 could activate the transcription factor NF-κB in Jurkat cells, and this observation was subsequently confirmed [21, 38]. Therefore, an inhibition of catalase, the enzyme responsible for H_2O_2 degradation, was also expected to affect NF-κB activation. Figure 6 shows that IL-1β-induced NF-κB activation increased concomitantly with the inhibition of catalase. However, this effect was obtained in the range of 10^{-2} to 5×10^{-2} M of aminotriazole, which is a rather high concentration. We verified that aminotriazole, glutathione peroxidase and reductase were not affected at these concentrations (data not shown) but cannot exclude some other side effects.

As highlighted by these results, the activity of different antioxidant enzymes seems to influence IL-1β-mediated





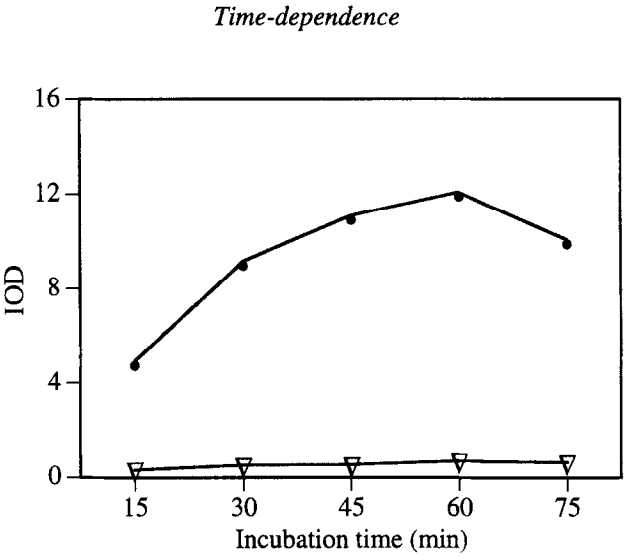


FIG. 1. Effect of IL-1 on NF-κB activation. (A) Gel shift mobility assay of NF-κB extracted from WI-38 VA13 cells stimulated or not stimulated with IL-1: cells were stimulated for 30 min with 0, 1, 5 or 10 ng/mL IL-1 (dose dependance, lanes 1–4) and for 15, 30, 45, 60 or 75 min in the presence or absence of 5 ng/mL IL-1 (time dependence, lanes 5–14). (B) Quantitative analysis of the gel shown in A. IOD represents the integrated optical density in arbitrary units of the band shifted by the binding of NF-κB to the probe, as analysed by the Visage 101 system. The upper part of the figure represents the dose-dependent activation of NF-κB by IL-1. The bottom part shows the time-dependent activation of NF-κB. Black circles and white triangles represent stimulations in the presence or absence of IL-1, respectively.

NF-κB activation. The use of chemicals, in this case enzyme inhibitors, always has to be interpreted with caution, because they may also react directly with other cell components essential for NF-κB activation, which may be true for mercaptosuccinate, even if it apparently does not affect the activity of other antioxidant enzymes. To confirm further the role of glutathione peroxidase in NF-κB activation, we designed a direct experiment by transfecting the cell line with a glutathione peroxidase gene expression vector and analysed the effect of glutathione peroxidase overexpression on NF-κB activation under the same experimental conditions.

Increase in the Intracellular Antioxidant Potential

WI-38 VA13 cells were transfected as described in Materials and Methods with a plasmid containing the human glutathione peroxidase cDNA under the control of the human metallothionein promoter [30]. Among the different transfectants isolated, the best expressing clone, WI-38 MT08, was selected and characterised for its antioxidant enzyme activity in comparison with parental cells. No major difference was observed between the two cell lines when glutathione reductase and catalase specific activities were considered; however, the transfected cells showed a higher level of glutathione peroxidase specific activity, i.e. 0.097 units/mg protein compared with 0.058 for the parental WI-38 VA13 cells, an increase of approximately 70% (Fig. 7).

We first compared the NF-κB DNA-binding activity in the two cell lines stimulated with IL-1β (Fig. 8). Cells overexpressing glutathione peroxidase (columns in black) showed lower levels of NF-κB activation (70%) then did the parental nontransfected cells (arbitrarily fixed to 100%) (columns in white).

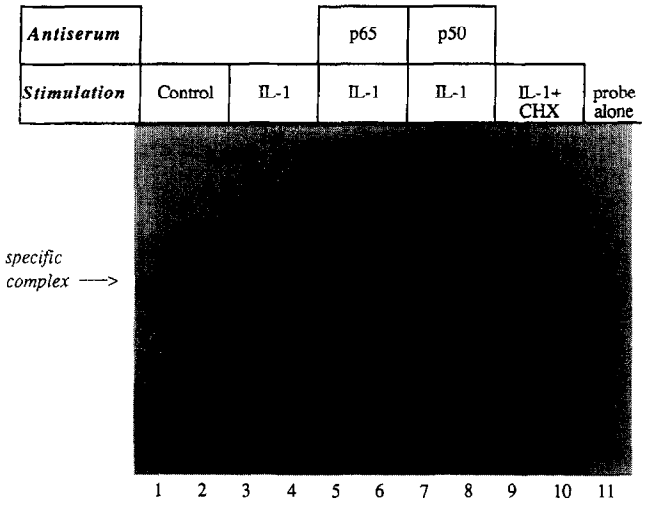


FIG. 2. Identification of the NF-κB subunits bound to NF-κB-binding site. Cells were stimulated for 30 min in the absence (lanes 1–2) or presence (lanes 3–10) of 5 ng/mL IL-1. Protein extracts were incubated either directly with the ^{32}P-labelled probe (lanes 1–4, 9–10) or preincubated with antisera against p65 (lanes 5–6) or p50 (lanes 7–8). In lanes 9–10, cycloheximide was added at 50 μg/mL to the solution containing IL-1. Lanes 1–2, 3–4, 5–6, 7–8, 9–10 are duplicates.

The effect of PDTC on NF-κB activation was also tested. PDTC is an antioxidant well known for inhibiting NF-κB activation under different stimulatory conditions [12, 39, 40] and is probably one of the most potent inhibitors of NF-κB activation [20]. This molecule did inhibit IL-1β-induced NF-κB activation in the two cell variants WI-38 VA13 and WI-38 MT08 in a dose-dependent manner (Fig. 8). However, there was a clear shift in the dose–response curve, NF-κB activation being systematically lower in transfected cells.

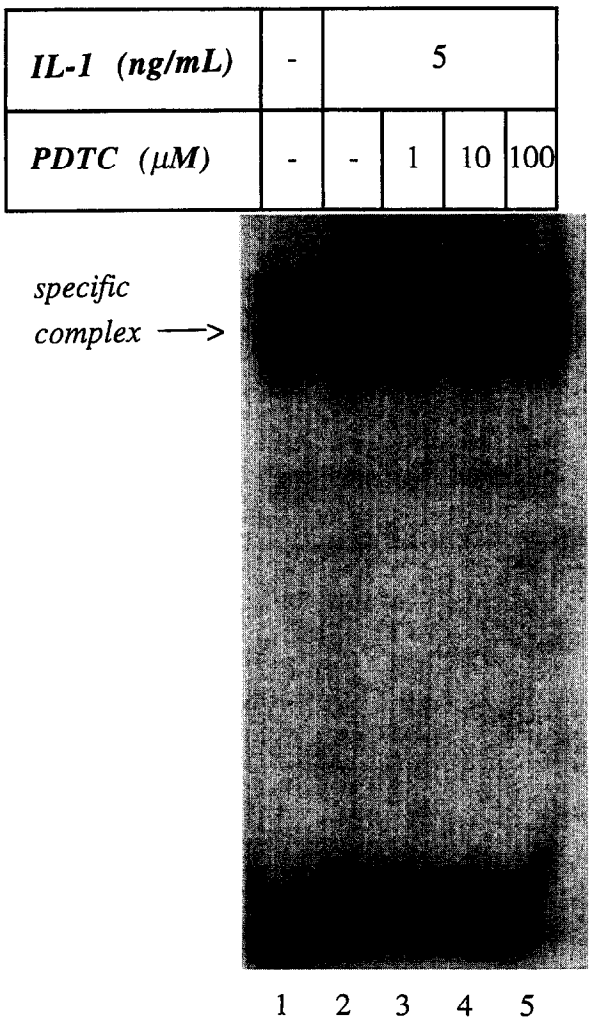
We also wondered whether this modulating effect of glutathione peroxidase could be observed on the expression of a gene under the control of NF-κB. NF-κB is a potent and pleiotropic transcriptional activator that controls a large number of genes, including the IL-6 gene. IL-6, a major activator of the acute phase response, is synthesised by fibroblasts in response to IL-1β [36]. Therefore, IL-6 production was assayed in supernatants of WI-38 VA13 and WI-38 MT08 cells stimulated with IL-1β in the absence or the presence of PDTC (Fig. 9). The influence of PDTC on IL-6 synthesis followed the pattern of NF-κB activation presented in Fig. 8, although the inhibitory effect of PDTC appeared to be stronger on IL-6 release than on NF-κB activation. The effect exerted by PDTC on the transcription factor was amplified by the protein synthesis machinery when considering the release of IL-6. IL-6 synthesis was also lower in glutathione peroxidase overexpressing cells than in nontransfected cells: a 6-hr stimulation with 5 ng/mL IL-1β induced a release of 2.1 ng/mL of IL-6 in transfected cells compared with 3.8 ng/mL in nontransfected cells. In summary, PDTC inhibited not only NF-κB activation but also IL-1β-induced IL-6 production in a dose-dependent manner.

DISCUSSION

The implication of different oxygen-derived species in the activation pathway of NF-κB has been studied by many investigators and is now largely accepted [6, 20, 21, 41]. Experimental data supporting this hypothesis are, however, essentially based on the addition into the culture medium of different molecules that have either prooxidant effects, such as H_2O_2, or antioxidant effects, such as PDTC. In this paper, we have investigated the influence of the endogenous cell antioxidant defences by modulating antioxidant enzyme activity. Cell antioxidant potential was first decreased by using specific enzyme inhibitors. A clear relationship was seen between the inhibition of different antioxidant enzymes and the increased activation of NF-κB. The overstimulation of NF-κB was especially pronounced when glutathione peroxidase was inhibited with mercaptosuccinate, although direct effects of mercaptosuccinate on the NF-κB activation pathway cannot be ruled out. There was, however, no effect of mercaptosuccinate on the basal level of NF-κB activation. We also increased the antioxidant potential by expressing the glutathione peroxidase cDNA. Correspondingly, NF-κB displayed lower activation levels in cells overexpressing glutathione peroxidase.

H_2O_2 is a potent NF-κB activator in Jurkat cells [42], HeLa cells [38] and in the lymphocytic ACH-2 cell line [43]. The fact that we observed an enhancement of IL-1β-induced NF-κB activation in the presence of aminotriazole and mercaptosuccinate also argues in favour of the involvement of H_2O_2 in the WI-38 VA13 cells. Moreover, cells that overexpress catalase are deficient in TNFα-induced NF-κB activation [27]. Nevertheless, several investigators have also shown that H_2O_2 by itself fails to activate NF-κB in many cell types, e.g. MRC-5 fibroblasts [15] or the lymphocytic cell line Molt-4 [28]. NF-κB responsiveness to H_2O_2 treatments obviously depends on the cell type studied; moreover, the composition of the culture medium is also important because in the Molt-4 cell line NF-κB is not generally activated in response to H_2O_2 unless cells are cultured in a modified medium containing more physiological amino acid concentrations, including cystine but no cysteine [44]. In the standard medium, the cell content in GSSG was much higher than in the modified medium, and consequently GSSG levels were barely increased in the presence of H_2O_2, which suggests that variations in responses to H_2O_2 in regard to NF-κB activation may simply reflect differences in the endogenous redox status of the cells investigated [15].

The results obtained with glutathione peroxidase and reductase inhibitors (Figs. 4 and 5) are consistent with the idea that glutathione metabolism plays a role in NF-κB activation. Although mercaptosuccinate exerts only a lim-



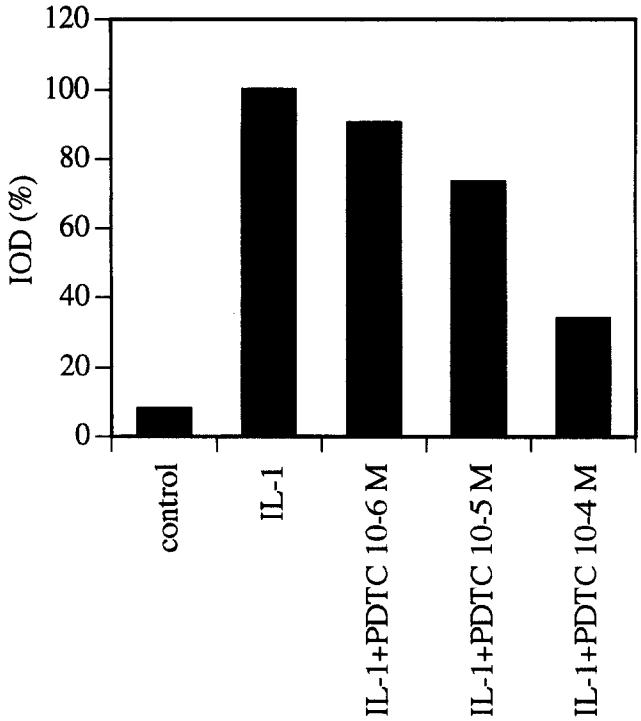


FIG. 3. Effects of PDTC on NF-κB-binding activity. (A) Gel shift mobility assay of NF-κB extracted from WI-38 VA13 cells, stimulated for 30 min without (lane 1) or with 5 ng/mL IL-1 in the absence (lane 2) or presence of different concentrations of PDTC (lanes 3–5). (B) Quantitative analysis of the gel shown in A. As analysed by the Visage 101 system, IOD represents the integrated optical density of the band shifted by binding of NF-κB to the probe expressed as the percentage of the IOD of cells stimulated with IL-1 alone.

ited inhibition on glutathione peroxidase, its effect on NF-κB activation is impressive (50%). We cannot rule out a glutathione peroxidase-independent effect of mercaptosuccinate, but the overall results presented in Fig. 8 and 9 confirm the importance of glutathione peroxidase in regulating levels of NF-κB activation. Glutathione peroxidase-overexpressing cells showed a lower level of NF-κB activation and of IL-6 release (Fig. 9) than did nontransfected cells (Fig. 8). Our results on the whole are in agreement with the data available in the literature, confirming that glutathione peroxidase is a key enzyme in regulating the cell redox status, hence influencing NF-κB activation. Firstly, selenium supplementation of T-lymphocytic ACH-2 cells can increase glutathione peroxidase activity and decrease NF-κB activation induced by H_2O_2, TNFα or PMA [45]. Secondly, ebselen, a peptide with glutathione peroxidase activity, inhibits NF-κB activation [21]. In addition, small changes in glutathione peroxidase activity can drastically affect cellular physiology. Raes and co-workers compared the protective role of glutathione peroxidase, superoxide dismutase and catalase in fibroblasts exposed to oxidative stress by microinjecting the cells with these enzymes. Glutathione peroxidase was 7 times more efficient than catalase and 2000 times more efficient than superoxide dismutase in protecting cells against hyperoxia-induced cell mortality [46, 47]. Moreover, glutathione peroxidase overexpression confers remarkable oxidoresistance to human T47D cells exposed to exogenous and intracellular sources of H_2O_2, with lower levels of cytotoxicity and DNA damage and enhancement of heat-shock gene induction [30].

The fact that glutathione metabolism influences NF-κB activation is also shown by its overactivation when glutathione reductase is inhibited by BCNU (Fig. 5). The involvement of glutathione metabolism in NF-κB activation has been suggested by several experimental results, such as those obtained with N-acetyl-cystein, a precursor of glutathione and an effective inhibitor of NF-κB activation [42, 48]. In contrast, buthionine sulfoximine, an inhibitor of glutathione synthesis, leads to enhancement of NF-κB activation [44]. In addition, TPA (12-O-tetradecanoylphorbol 13 acetate) not only activates NF-κB but also induces an increase in the GSSG level [49]. Taken together, these observations suggest that NF-κB activation is favoured by a high level of GSSG or rather by an increase in the GSSG:GSH ratio [28].

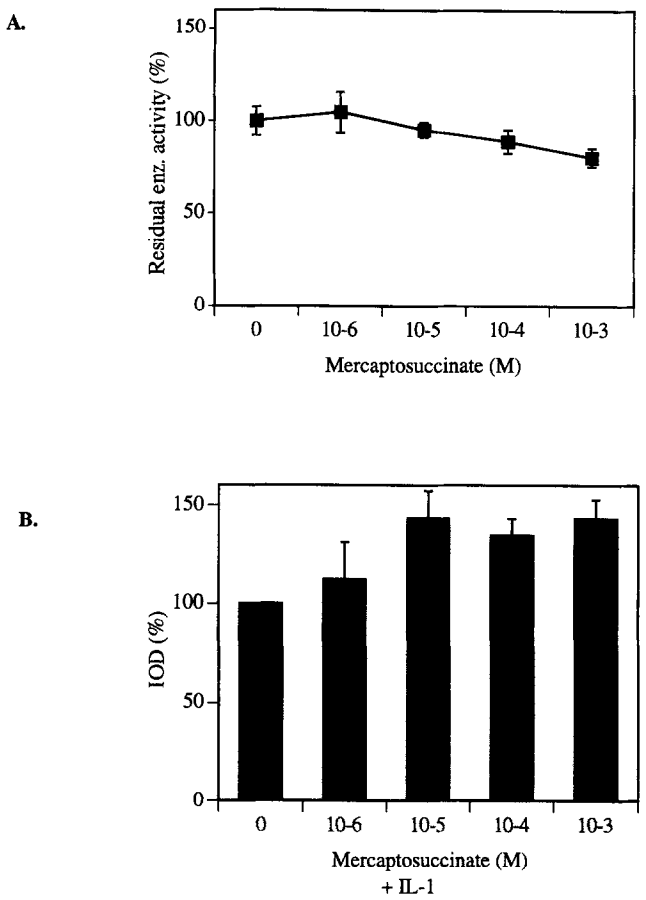


FIG. 4. Effect of mercaptosuccinate on glutathione peroxidase activity (A) and on NF-κB binding activity (B) in the WI-38 VA13 cell line. (A) Enzymatic activity was assayed after 30 min of incubation in the presence or absence of different concentrations of mercaptosuccinate. Residual enzymatic activity is expressed as a percentage of the total activity measured in cells incubated without inhibitor. Each point represents the mean ± standard deviation of experiments performed in triplicate. (B) For the electrophoretic mobility shift assay, cells were preincubated for 30 min in the presence of mercaptosuccinate and then stimulated for 30 min with 5 ng/mL IL-1 in the continuous presence of the inhibitor. The autoradiographs were quantified, and the IOD data from the cells incubated in the presence of the inhibitor are expressed as the percentage of the IOD of cells stimulated with IL-1 alone. The bars represent the mean values ± standard deviation of experiments performed in triplicate.

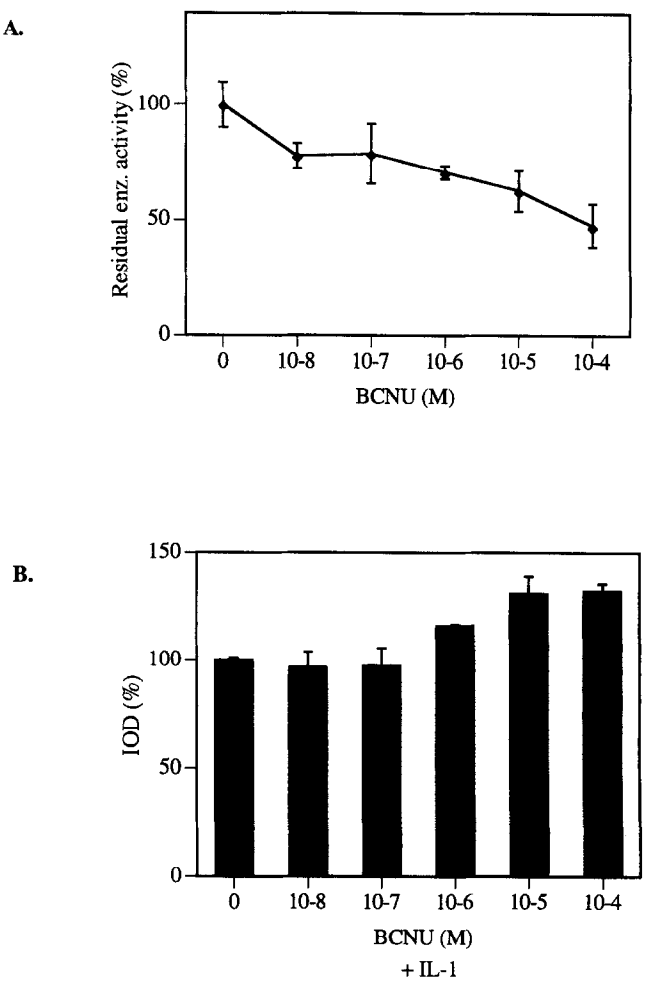


FIG. 5. Effect of BCNU on glutathione reductase activity (A) and on NF-κB activation (B) in the WI-38 VA13 cell line. (A) Enzymatic activity was assayed after 30 min of incubation in the presence of different concentrations of BCNU. Residual enzymatic activity is expressed as a percentage of the corresponding activity measured in cells incubated without inhibitor and represents the mean value ± standard deviation of 3 experiments. (B) For the electrophoretic mobility shift assay, cells were preincubated for 30 min in the presence of BCNU and then stimulated for 30 min with 5 ng/mL IL-1 in the continuous presence of the inhibitor. The resulting autoradiographs were quantified, and the IOD data from the cells incubated in the presence of BCNU are expressed as the percentage of the IOD value from cells stimulated with IL-1 alone.

However, GSSG can have two antagonist effects towards NF-κB activation: very low levels of GSSG prevent cytoplasmic activation of NF-κB, whereas very high doses of GSSG prevent the DNA-binding capacity of NF-κB in the nucleus [44]. At the nuclear level, the transcription factor NF-κB apparently has to be in a reduced form to present full DNA-binding activity. For instance, diamide (a sulfhydryl oxidizing agent) inhibits its DNA-binding activity, a phenomenon that can be reversed by the addition of mercaptoethanol [48, 50, 51]. The free -SH group of the conserved cystein 62 in the DNA-binding domain turned out to be essential for DNA-binding activity of all the nuclear Rel/Dorsal family members [18, 28, 52], as shown by mutational analysis [51, 53]. Under normal conditions, the oxidation of this conserved cystein would be prevented by a nuclear enzyme called Ref-1 [52], which was confirmed by Galter *et al.* [28] who found that BCNU could have two different effects, depending on subcellular localisation. They showed that cytoplasmic NF-κB activation was facilitated in the presence of H_2O_2 or BCNU but that the same molecules could inhibit the DNA-binding activity of the transcription factor. This antagonist effect is not reflected in our results, probably because we routinely used dithiotreitol, a powerful reducing agent, in the gel shift

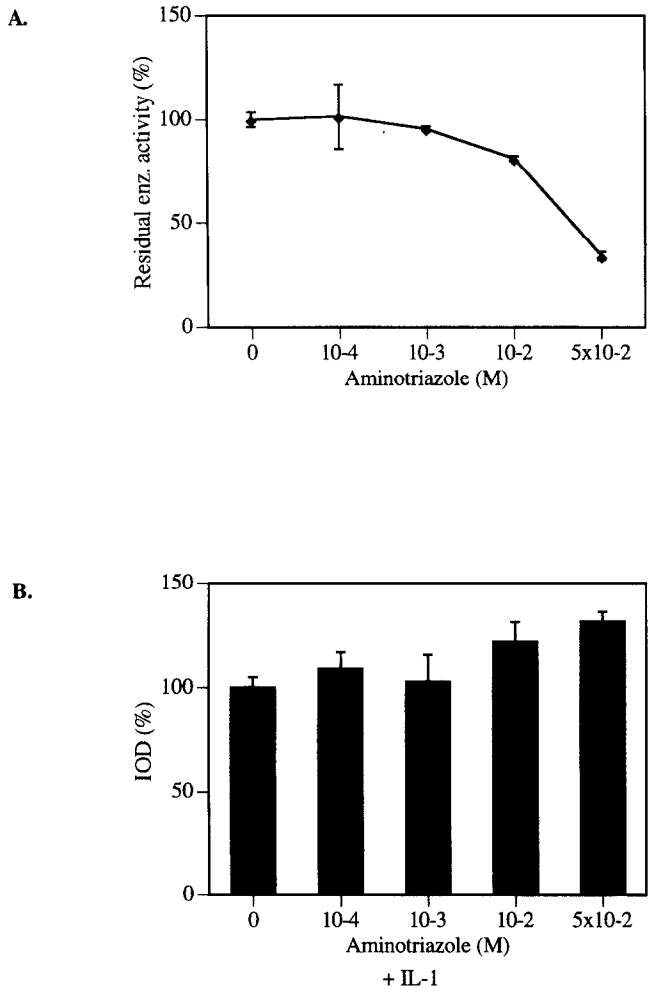


FIG. 6. Effect of aminotriazole on catalase activity (A) and on NF-κB binding activity (B) in the WI-38 VA13 cell line. (A) Enzymatic activity was assayed after 30 min of incubation in the presence of different concentrations of aminotriazole. The residual enzymatic activity is expressed as the percentage of the corresponding activity measured in cells incubated without inhibitor and represents the mean value ± standard deviation of 3 experiments. (B) For the electrophoretic mobility shift assay, cells were preincubated for 30 min in the presence of aminotriazole and then stimulated for 30 min with 5 ng/mL IL-1 in the presence of the inhibitor. The resulting autoradiographs were quantified and the IOD data from the cells incubated in the presence of aminotriazole are expressed as the percentage of the IOD value from the cells stimulated with IL-1 alone.

assay, whereas Galter *et al.* used a dithiotreitol-free binding assay to show the inhibitory effect of GSSG on DNA-binding; this oxidative inhibition of DNA-binding activity was reversed by dithiotreitol in our study.

At the cytoplasmic level, data have accumulated to provide an understanding of the importance of the GSH: GSSG ratio on NF-κB activation and to lead to a hypothetic mechanism of NF-κB activation. Firstly, H_2O_2-treated cells have increased levels of GSSG [54], which could be the oxidant mediating the activation. Secondly, the activity of several enzymes is sensitive to variations in GSSG:GSH levels, as is the case for some phospholipase C

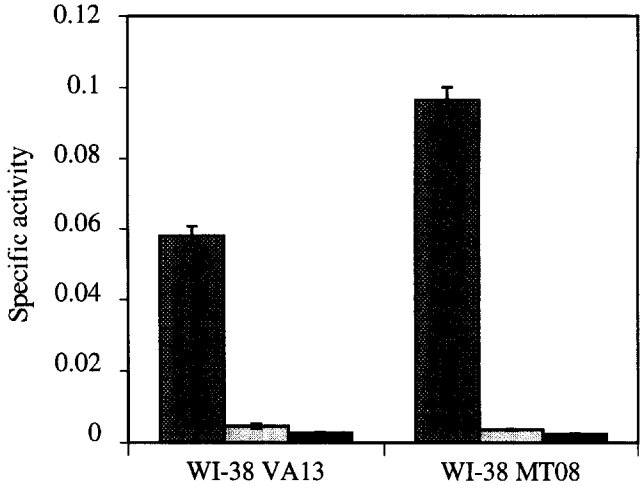


FIG. 7. Comparison of the specific activities of antioxidant enzymes in the stably transfected cell line WI-38 MT08 and in the parental cells WI-38 VA13. The enzymatic activity of glutathione peroxidase (shaded columns), catalase (grey columns) and glutathione reductase (black columns) was assayed as described in Materials and Methods in both the WI-38 VA13 and WI-38 MT08 cell lines (WI-38 VA13 cells were transfected with the glutathione peroxidase cDNA). The values presented are the mean of experiments performed in triplicate.

isozymes, thioredoxin or for a putative tyrosine kinase [44, 55]. Thirdly, the pattern of protein phosphorylation increases after H_2O_2 treatment, because H_2O_2 can induce protein tyrosine kinase activity and inhibit protein tyrosine

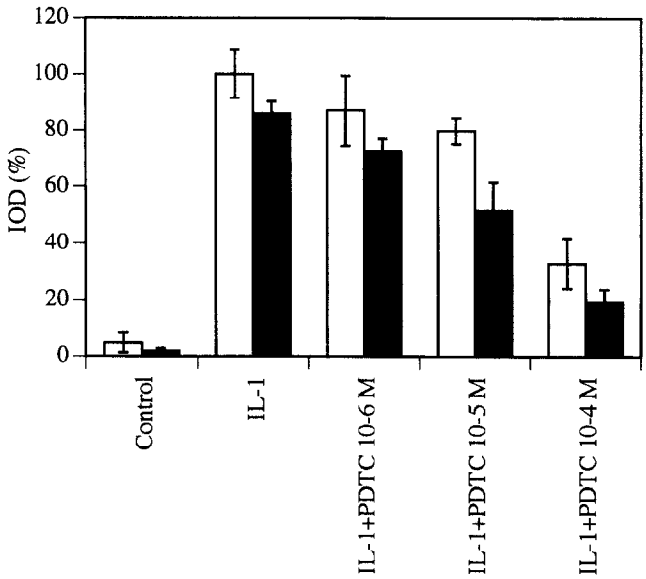


FIG. 8. Effects of PDTC on IL-1-induced NF-κB binding activity, in WI-38 VA13 cells (white columns) and in glutathione peroxidase overexpressing WI-38 MT08 cells (black columns). Cells were stimulated for 30 min with IL-1 (5 ng/mL) in the presence or absence of PDTC and lysed for the electrophoretic mobility shift assay. The gels (not shown) were analyzed quantitatively, and the IOD values of the shifted bands are expressed as the percentage of the IOD value obtained for the active NF-κB complex induced by IL-1 alone in WI-38 VA13 cells, arbitrarily set to 100%.

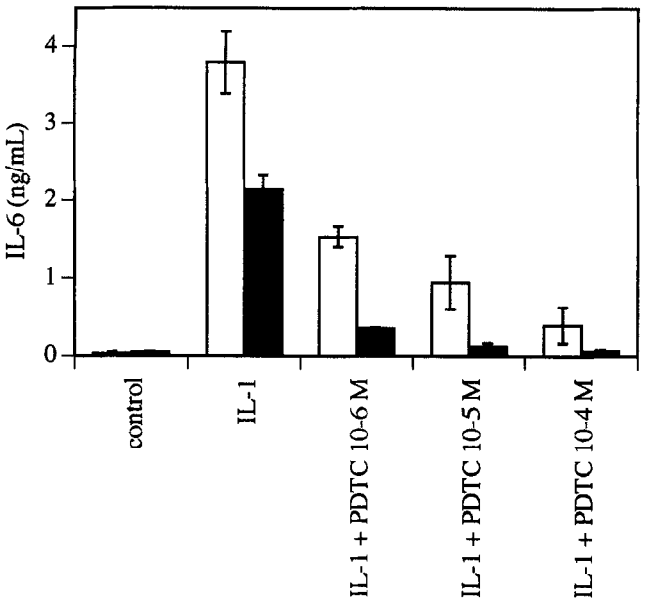


FIG. 9. Effect of PDTC on IL-1-induced IL-6 release in the nontransfected WI-38 VA13 cells (white columns) and in the overexpressing WI-38 MT08 cells (black columns). Cells were stimulated for 6 hr with 5 ng/mL IL-1 in the presence or absence of PDTC, and the secreted IL-6 was quantified in the supernatant by ELISA.

phosphatase activity [14, 54, 56, 57]. Because many transduction pathways contain tyrosine kinases located upstream in the activation cascade, one of these kinase could be redox sensitive, i.e. sensitive to H_2O_2 or to the GSSG:GSH level and could then trigger the NF-κB activation cascade. Eventually, the GSSG level could induce disulfide bridge formation, leading to protein dimerisation necessary for enzymatic activity [58]. High GSSG levels could also affect proteases involved in IκB degradation. Chen *et al.* did not rule out the possibility that oxidative conditions could make proteins, e.g. IκB, more susceptible to the ubiquitine degradative pathway [59]. Clearly, the expression of NF-κB-dependent genes needs a well-balanced level of GSSG.

The link between reactive oxygen species and the phosphorylation of IκB has not yet been found. The kinases involved in NF-κB activation can be different according to the stimulus and the cell type studied and have not yet been clearly identified. One should not forget that the global protein phosphorylation pattern is the result of both protein kinases and protein phosphatases [56, 60]. In this context, Anderson *et al.* proposed an activation mechanism composed of stimuli-specific signals followed by a common pathway leading to IκB phosphorylation [41]. The different pathways switched on by different inducers would converge towards a common redox-sensitive pathway, triggering the activation of kinase cascade(s) leading to the phosphorylation of IκB.

Other transcription factors have been reported to be sensitive to redox changes *in vitro* and *in vivo*, which is the case for AP-1 [38, 61], generally considered an antioxidant-responsive transcription factor, and Sp1 [62].

In conclusion, modulation of antioxidant cell defences affects NF-κB activation. The more protected the cells are against oxygen species by their level of antioxidant enzyme activity, the lower the NF-κB activation under an identical IL-1β stimulating challenge. NF-κB activation is thus directly related to the intracellular and molecular mechanisms that control in particular peroxide levels and the GSSG:GSH ratio. Thus, the level of antioxidant enzyme activity has to be considered to interpret accurately the different (and sometimes contraditory) observations obtained on the effect of oxidants on NF-κB activation in different cell types.

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