

Inhibition of I κ B- α Phosphorylation and Degradation and Subsequent NF- κ B Activation by Glutathione Peroxidase Overexpression

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Abstract. We report here that both κ B-dependent transactivation of a reporter gene and NF- κ B activation in response to tumor necrosis factor (TNF α) or H₂O₂ treatments are deficient in human T47D cell transfectants that overexpress seleno-glutathione peroxidase (GSHPx). These cells feature low reactive oxygen species (ROS) levels and decreased intracellular ROS burst in response to TNF α treatment. Decreased ROS levels and NF- κ B activation were likely to result from GSHPx increment since these phenomena were no longer observed when GSHPx activity was reduced by selenium depletion. The cellular contents of the two NF- κ B subunits (p65 and p50) and of the inhibitory subunit I κ B- α were unaffected by GSHPx overexpression, suggesting that increased GSHPx activity interfered with the activation, but not the synthesis or stabil-

ity, of NF- κ B. Nuclear translocation of NF- κ B as well as I κ B- α degradation were inhibited in GSHPx-overexpressing cells exposed to oxidative stress. Moreover, in control T47D cells exposed to TNF α , a time correlation was observed between elevated ROS levels and I κ B- α degradation. We also show that, in growing T47D cells, GSHPx overexpression altered the isoform composition of I κ B- α , leading to the accumulation of the more basic isoform of this protein. GSHPx overexpression also abolished the TNF α -mediated transient accumulation of the acidic and highly phosphorylated I κ B- α isoform. These results suggest that intracellular ROS are key elements that regulate the phosphorylation of I κ B- α , a phenomenon that precedes and controls the degradation of this protein, and then NF- κ B activation.

THE transcription factor NF- κ B plays a pivotal role in the regulation of a wide variety of cellular genes, particularly those involved in immune and inflammatory responses, and also participates in the regulation of viral promoters, including the human immunodeficiency virus long terminal repeat (HIV-1 LTR) (3, 7, 29, 51, 66). Five different subunits of NF- κ B have been described that can homo- and heterodimerize (21, 53). These polypeptides belong to the rel family of transcription factors, and the more frequent and therefore prototypical form of NF- κ B is a heterodimer complex containing the p50 and p65/RelA subunits (6, 11, 28, 59, 67). Unlike most transcription factors, these proteins reside in the cytoplasm in a latent form and must therefore translocate into the nucleus to function (5). In unstimulated cells, the nuclear import of the NF- κ B DNA-binding dimer p65/RelA-p50 is prevented by high-affinity association of the p65/RelA subunit with a cytoplasmic inhibitor called I κ B (4, 13, 57, 71). The inhibitory subunit I κ B belongs also to a

family of distinct proteins (62) and interacts, through its ankyrin-like repeats, with the nuclear localization signals of p50 and p65/RelA (9, 34). The prototypical I κ B protein involved in cytoplasmic retention of NF- κ B dimers is I κ B- α , encoded by the *MAD-3* gene (32). The inactive NF- κ B-I κ B- α complexes are dissociated in response to a variety of extracellular stimuli, thereby allowing free NF- κ B dimers to translocate to the nucleus and activate transcription of genes containing κ B regulatory elements. Phosphorylation of I κ B- α is required for NF- κ B activation (10, 27, 35) but does not induce I κ B dissociation from the inactive NF- κ B-I κ B- α complex (20, 25, 39). This complex is more probably disrupted because of the selective degradation of phosphorylated I κ B- α in response to extracellular signals (14, 33, 44). Recently, it has been shown that I κ B- α turnover was regulated by phosphorylation at serine residues 32 and 36 (12, 14, 74). A multiprotease complex, the proteasome (2, 22), as well as I κ B- α ubiquitination, is involved in this process (16, 37, 56, 74, 75). Stimulation results in a rapid loss of I κ B- α and the rapid nuclear translocation of NF- κ B. Transactivation by this factor, in turn, induces high levels of I κ B- α synthesis that probably restore the unstimulated inhibited state (1, 13, 17, 71). The proteasome also appears involved in the proteolytic processing of p50 from a 105-kD precursor protein (p105) (24, 44, 45).

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Transcription factors are usually activated by a restricted number of specific extracellular stimuli. In contrast, NF- κ B is activated by an extraordinarily large number of conditions and agents (3, 7, 29, 73). Of great interest was the discovery that most inducers of NF- κ B seem to rely on the production of intracellular reactive oxygen species (ROS)¹ as evidenced by the inhibitory effect of several antioxidants, including *N*-acetylcysteine (63, 70) and the activation induced by hydrogen peroxide (63). ROS include superoxide radicals ($O_2^{\cdot-}/O_2H$), hydrogen peroxide (H_2O_2), organic hydroperoxides, and hydroxyl radical ($OH\cdot$). Eukaryotic cells produce ROS continuously as side products of the mitochondrial electron transfer chain reactions (31), but also upon exposure to different stimuli that can activate NF- κ B, including UV light, hydrogen peroxide, and inflammatory cytokines, such as tumor necrosis factor α (TNF α) and interleukin 1 (18, 19, 43, 64).

The intracellular balance between ROS formation and detoxification is regulated by nonenzymatic as well as enzymatic defenses. In mammalian cells, major antioxidant enzymes include superoxide dismutases (SOD), catalase, and a family of selenium-dependent glutathione peroxidases (68). In addition to the classical seleno-glutathione peroxidase (GSHPx) (26), which can reduce H_2O_2 and a variety of organic hydroperoxides in the presence of glutathione, this family of enzymes includes a plasmatic, a gastrointestinal, and a phospholipid hydroperoxide glutathione peroxidase. In the cytoplasm and in the mitochondria, H_2O_2 is mainly detoxified by GSHPx; catalase, which has a much higher Michaelis-Menten constant (K_m) for H_2O_2 than GSHPx, is found predominantly in peroxisomes. GSHPx is a homotetramer, and each subunit contains one selenocysteine residue in its active site (40). In higher eukaryotes, selenium (Se) is a trace element that is essential for the activity of these glutathione peroxidases. Lowering Se cellular contents, either *in vivo* by dietary manipulations or *in vitro* by using selenium-depleted growth media, decreased the selenoperoxidase-mediated cytoprotection against oxidative stress (26, 69, 72). On the other hand, cells grown in selenium-supplemented growth media showed increased GSHPx activity that resulted in a decreased NF- κ B activation by oxidative stress and reduced HIV-1 reactivation in HIV-1 latently infected T lymphocytes exposed to oxidative stress (60). Moreover, Se deficiency that results in reduced glutathione peroxidase activities has been detected in HIV-infected patients (23, 54), suggesting that, *in vivo*, Se levels may be an important determinant of the progression and pathology of AIDS.

Until recently, most studies aimed to demonstrate a link between NF- κ B activation and ROS were performed with chemicals and antioxidants that were often used at very high inhibitory doses and not devoid of possible side effects. To overcome this problem, Schmidt et al. (61) used cell lines that overexpress SOD or catalase to modulate intracellular ROS levels. These authors pointed out the essential role of ROS, probably H_2O_2 , in NF- κ B activation.

1. *Abbreviations used in this paper:* DOC, sodium desoxycholate; EB, ethidium bromide; ECL, enhanced chemiluminescence; GSHPx, seleno-glutathione peroxidase; HE, hydroethidine; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF α , tumor necrosis factor α .

In the present study, the overexpression of glutathione peroxidase was used to further demonstrate the implication of ROS in NF- κ B activation by hydrogen peroxide and TNF α . To this end, human breast carcinoma T47D cells, which are characterized by low endogenous GSHPx levels, were stably transfected with a cDNA gene encoding human GSHPx (49, 50). GSHPx overexpression induced oxidoresistance status and decreased intracellular ROS levels. Here, we show that the overexpression of GSHPx abolished NF- κ B-I κ B- α activation by TNF α or hydrogen peroxide. This phenomenon was characterized, *in vitro*, by an inhibition of NF- κ B DNA-binding activity and, *in vivo*, by a strong decrease in NF- κ B nuclear translocation, I κ B- α phosphorylation and subsequent degradation, and κ B-dependent transcription. Moreover, a time correlation was observed between the TNF α -mediated intracellular burst of ROS and I κ B- α degradation. The data presented suggest that I κ B- α phosphorylation and subsequent degradation are controlled by intracellular ROS levels.

Materials and Methods

Cell Cultures

The transfectant derivatives HCMV-GSHPx-2 and T47D-Hygro-3 of human breast T47D cell line have been described elsewhere (49, 50). The names of these cells were abbreviated in T47D-GPx and T47D-Hygro cells. Cells were grown at 37°C in the presence of 5% CO₂ in Hepes-buffered RPMI medium (Sigma Chimie, St. Quentin Fallavier, France) supplemented with 10% FCS (GIBCO BRL, Cergy Pontoise, France), 0.1 μ M fresh sodium selenite, 2 mM L-glutamine, 0.5 μ g/ml insulin, 100 U/ml penicillin, and 0.1 mg/ml streptomycin.

Reagents and Plasmids

Murine recombinant TNF α (10⁷ U/mg) was from Boehringer Mannheim (Meylan, France). Hydrogen peroxide, t-butylhydroperoxide, and sodium desoxycholate were from Sigma Chimie. Anti-hsp70 serum was from Amersham Corp. (Little Chalfont, UK). Glutathione peroxidase activity was tested in cell extracts in the presence of t-butylhydroperoxide, as previously described (49, 50). The specificity of anti-glutathione peroxidase antibody was previously described (49). Anti-p65/RelA, anti-p50, and anti-I κ B- α /MAD-3 were from Santa Cruz Biotechnology (Santa Cruz, CA). pTKluc and κ B6-TKluc vectors (a kind gift from P.A. Baeuerle [Freiburg University, Germany]) were described by Pahl and Baeuerle (1995) (55). Briefly, pTKluc contains the luciferase gene under the control of the herpes simplex thymidine kinase promoter. The construct κ B6-TKluc was obtained by insertion of a double-stranded oligonucleotide representing six NF- κ B binding sites into the pTKluc plasmid. pCMV- β plasmid (Clontech, Palo Alto, CA) contains the gene encoding β -galactosidase under the control of the cytomegalovirus promoter.

Transfection, Luciferase, and β -Galactosidase Assays

Hygro and GPx cells were seeded out the day before transfection at a density of 2.5×10^6 cells per 100-mm dishes. 8 μ g of pTKluc or κ B6-TKluc and pCMV- β were cotransfected using Gibco's Lipofectamine reagent (GIBCO BRL) according to the manufacturer's instructions. 8 h after transfection, cells were trypsinized and replated into four 60-mm dishes. 12 h later, cells were treated for 2 h with hydrogen peroxide or TNF α , and then the medium was changed and the cells were allowed to recover for 14 h before harvesting; i.e., 36 h after transfection. Cells were lysed in 400 μ l of BLUC lysis buffer (25 mM Tris/H₃PO₄, pH 7.8, 10 mM MgCl₂, 1% Triton X-100, 15% glycerol, and 1 mM EDTA). 150 μ l of the lysates were then added to 100 μ l of BR LUC reacting buffer (1.2 mM ATP and 0.33 mM luciferin in BLUC lysis buffer), and after mixing the reagents, the emission of light was measured during 10 s using a luminometer (LUMAT LB 9501; Becton Dickinson, Le Pont de Claix, France) (52). The percentage of cells expressing β -galactosidase was monitored by 5-bromo-chloro-3-indolyl β -D-galactosidase staining (38).

Estimation of Intracellular Reactive Oxygen Species

Estimation of intracellular ROS in living cells was performed by using the sodium borohydride-reduced form of ethidium bromide (EB), hydroethidine (HE) fluorescent probe (Molecular Probe-Interchim, Montluçon, France) (15, 58). This probe freely penetrates inside cells and is specifically oxidized by ROS. 2.5×10^5 cells (T47D-GPx-2 or T47D-Hygro-3) were washed twice with PBS and incubated for 10 min with 40 $\mu\text{g}/\text{ml}$ hydroethidine before being analyzed by flow cytometry using a FACS[®] can flow cytometer (Becton Dickinson). Excitation wavelength was 488 nm, and emission filter specific for oxidized hydroethidine (EB) fluorescence was 610 nm bandpass.

Preparation of Whole Cell Extracts and Cell Fractionation

T47D cells growing on 60-mm dishes (Falcon Labware, Oxnard, CA) were washed with cold PBS, scraped from the dishes, and pelleted for 5 min at 1,000 g. The cellular pellet was then either lysed and boiled in Laemmli sample buffer (whole cell extract) or lysed at 4°C in a buffer containing 10 mM Tris, pH 7.4, 10 mM NaCl, 1.5 mM MgCl₂, and 0.5% Triton X-100. The lysates were then clarified for 10 min at 12,000 g. The procedure was repeated until the nuclei present in the pellet fraction were free of cytoplasmic contaminations, as judged by microscopical analysis with a TMS inverted photomicroscope (Nikon Inc., Garden City, NY) equipped with phase-contrast. The crude nuclear pellets and the resulting cytoplasmic supernatants were then resuspended or diluted in similar amounts in Laemmli SDS buffer before being boiled and analyzed by SDS-PAGE.

Gel Electrophoresis and Immunoblotting

One and two-dimensional gel electrophoresis and immunoblots were performed as already described (41, 42), except that the isoelectrofocusing gels were made up with 60% of pH 4–6 and 40% of pH 3–10 ampholines (Sigma Chimie). Isoelectrofocusing sample buffer contained pH 6–8 ampholine. p65/RelA, p50, I κ B- α , and GSHPx antisera were used as primary antibodies, and the revelation of immunoblots was performed with the ECL kit from Amersham Corp. The duration of the exposure was calculated as to be in the linear response of the film. The bands on the films, representing the levels of the different proteins, were scanned with the Bioprofil system (Vilber Lourmat, France).

Electrophoretic Mobility Shift Assays

Extraction of DNA-binding proteins and binding conditions have been previously described (36). In brief, 10 μg of protein from nuclear extracts was incubated with a 20,000 cpm (Cerenkov) ³²P-labeled κB DNA probe in the presence of 4 μg poly (dI-dC) (Pharmacia Biotech, Orsay, France) and 1 μl $10\times$ BB buffer (50 mM Tris, pH 7.5, 5 mM DTT, 5 mM EDTA, 250 mM NaCl, and 10% Ficoll 400). Reaction was for 15 min at room temperature

after the addition of the ³²P-labeled κB probe. The double-stranded oligonucleotide used to detect the NF- κB DNA binding activity was as previously described (36, 77). Native 4% polyacrylamide gels were used to analyze the samples. Autoradiographs of the gels were recorded onto BioMax MR films (Eastman-Kodak Co., Rochester, NY). For the competition experiments, 10 or 40 ng of unlabeled competitive κB probe were added to the binding mix including proteins 5 min before the incubation with 0.1 ng of the ³²P-labeled κB probe. Supershift experiments were performed by adding 2 μg of an antiserum directed against the p65/RelA subunit of NF- κB to the binding mix including proteins, 30 min before the incubation with the ³²P-labeled κB probe. Sodium desoxycholate (DOC) treatment (5) was performed by incubating the cytosolic fractions from unstimulated Hygro and GPx cells for 15 min with 0.8% DOC and 1% NP-40 (final concentrations) before electrophoretic separation of protein-DNA complexes.

Results

κB -dependent Transactivation of a Reporter Gene by TNF α and Hydrogen Peroxide Is Inhibited in T47D Cells that Overexpress GSHPx

The role of ROS formation and intracellular GSHPx activity in κB -dependent gene transactivation was investigated in T47D transfectant cell lines that overexpress exogenous GSHPx (T47D-GPx-2, -16, and -10) as well as in control cell lines (e.g., T47D-Hygro-3) that express low endogenous GSHPx level and activity (4 mU/mg of cellular proteins) (49, 50). In contrast, the T47D-GPx-2 cells most often used in this study have a 70-fold higher GSHPx activity (270 mU/mg) than T47D or T47D-Hygro-3 cells but similar levels of Cu/Zn-SOD and catalase (49). On the other hand, a sevenfold-decreased GSHPx activity was observed in GSHPx-expressing T47D cells grown 6 d in selenium-depleted medium, a trace element that is essential for GSHPx activity.

The efficiency of κB -dependent gene transactivation, as induced by TNF α and hydrogen peroxide, was therefore compared in control Hygro-3- and GPx-2-T47D cells that were transiently transfected with a plasmid vector p $\kappa\text{B6-TKluc}$ containing a luciferase gene reporter placed under control of a thymidine kinase promoter coupled to six κB elements (55). Luciferase expression from this construct was compared to that from a similar plasmid but without κB elements (pTKluc). This was assessed by measuring lu-

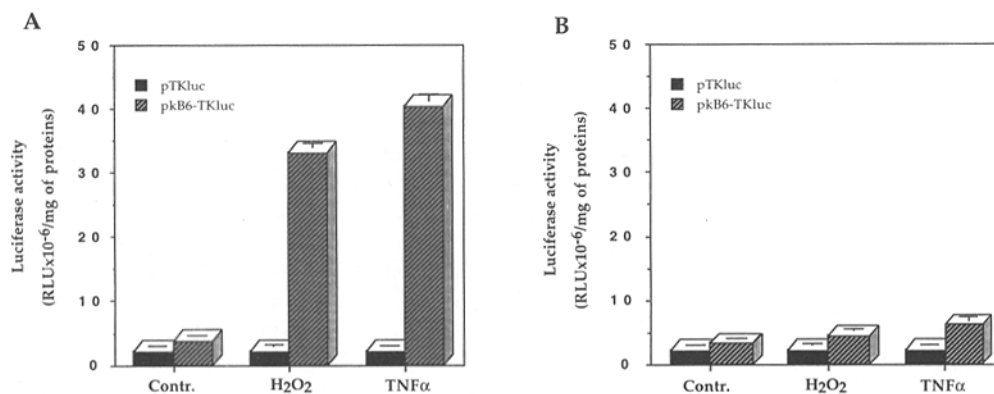


Figure 1. Overexpression of glutathione peroxidase decreases NF- κB -mediated transcriptional activation induced by TNF α or hydrogen peroxide. Control T47D-Hygro-3 (A) and GSHPx-expressing T47D-GPx-2 (B) cells were cotransfected with either pTKluc (dark plots) and pCMV β or with p $\kappa\text{B6-TKluc}$ (hatched plots) and pCMV β as described in Materials and Methods. 20 h after transfection and replating, cells were

either kept untreated (Contr.) or treated for 2 h with 250 μM H₂O₂ or 2,000 U/ml TNF α . The medium was changed, and the cells were allowed to recover for 14 h before harvesting. The determination of luciferase and β -galactosidase activities was performed in parallel cultures (see Materials and Methods). Transfections presenting identical efficiency, estimated as the number of cells expressing β -galactosidase, were further analyzed. Luciferase transcriptional activation is represented by arbitrary relative light unit (RLU) per mg of proteins. The histograms shown are representative of three identical experiments; SD are presented ($n = 3$). Note the strong stimulation of luciferase activity in Hygro-3 cells treated with hydrogen peroxide or TNF α , which was only faintly detectable in GPx-2 cells.

ciferase activity in cell extracts (see Materials and Methods). Fig. 1 *A* shows that H₂O₂ and TNF α strongly increased luciferase activity (up to 10-fold) in T47D-Hygro-3 cells transfected with κ B6-TKluc vector but not in cells transfected with pTKluc. This result clearly indicates that in T47D cells the transcription of genes controlled by κ B elements is inducible by TNF α and bona fide oxidants. In contrast, the induction of luciferase by these agents was remarkably lower in T47D-GPx-2 cells transfected with κ B6-TKluc plasmid (Fig. 1 *B*). This inhibitory effect was strongly attenuated when the same experiment was performed with cells grown for 6 d in selenium-depleted medium before being transfected (not shown). Note that luciferase activity after transfection with the noninducible pTKluc vector was similar in T47D-Hygro-3 and GPx-2 cells, and that the transfection efficiency was similar in both cell lines (about 7%), as determined by the fraction of cells expressing β -galactosidase in transfection experiments performed with a plasmid containing the β -galactosidase gene controlled by the cytomegalovirus promoter (pCMV- β). Hence, these results suggest that high levels of GSHPx activity can interfere negatively on NF- κ B activation, synthesis, or stability.

The trivial possibility that T47D-GPx-2 cells were deficient in NF- κ B synthesis, or that this factor was abnormally unstable in these cells after exposure to TNF α or hydrogen peroxide, was thus investigated. The immunoblots presented in Fig. 2 show that the cellular contents of both p65/RelA and p50 subunits of NF- κ B were very similar in T47D-Hygro-3 and -GPx-2 cells. This figure also shows that the level of neither NF- κ B subunits nor GSHPx was affected by a 2-h treatment with 2,000 U/ml of TNF α . Similar observations were made when cells were treated for 2 h

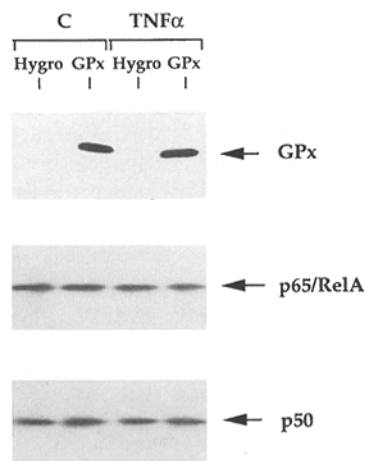


Figure 2. Intracellular levels of glutathione peroxidase and NF- κ B subunits p65 and p50 in T47D-Hygro-3 and T47D-GPx-2 cells treated or not with TNF α . Control T47D-Hygro-3 (*Hygro*) and GSHPx-expressing T47D-GPx-2 (*GPx*) cells were treated (TNF α) or not (C) for 2 h with 2,000 U/ml of TNF α . The cellular contents of glutathione peroxidase and NF- κ B subunits p65/RelA and p50 were analyzed in immunoblots probed with antibodies that recognize specifically these proteins, as revealed by enhanced chemiluminescence (ECL). Note that the overexpression of glutathione peroxidase or the treatment with TNF α did not induce significant differences in the cellular concentrations of the NF- κ B subunits p50 and p65.

with 250 μ M H₂O₂ (not shown). Therefore, it is unlikely that deficient synthesis or increased instability of NF- κ B was responsible for the inhibition of κ B-dependent gene transactivation observed in T47D-GPx-2 cells (Fig. 1). Also note that endogenous GSHPx in control T47D-Hygro-3 cells was not detected, confirming that these cells contain very low level of this enzyme. Moreover, neither GSHPx overexpression nor the treatment with TNF α did induce a stress response in T47D cells (41). Similar results were obtained with the other GSHPx-expressing cell lines (not shown).

The Endogenous Levels of ROS As Well As the Burst of ROS Induced by TNF α Are Decreased in GSHPx-overexpressing T47D Cells

Antioxidants such as *N*-acetylcysteine, a precursor of glutathione, are able to prevent NF- κ B-mediated gene transactivation (63, 70). We have thus investigated whether the inhibition of κ B-dependent gene transactivation observed in T47D-GPx-2 cells (Fig. 1 *B*) was due to an altered cellular redox state linked to GSHPx overexpression, which could confer higher reducing capacity than in T47D-Hygro-3 cells. This hypothesis was verified by comparing ROS levels in both cell lines, exposed or not to TNF α or H₂O₂. Intracellular pools of ROS were assessed by FACS[®] analysis of EB fluorescence resulting from ROS-mediated oxidation of HE (15, 58). Fig. 3 shows that, in T47D-Hygro-3 cells, a 10-min treatment with 2,000 U/ml of TNF α increased the mean EB fluorescence index by \sim 30%, indicating that this cytokine induced a rapid burst of HE-oxidizing ROS in these cells. In contrast, in T47D-GPx-2 cells, the basal EB fluorescence index was below that observed in control T47D-Hygro-3 cells, and only a slight increase was observed after TNF α treatment. Similar results were obtained with the other T47D cell lines that express similar levels of GSHPx. A decreased burst of ROS was also observed in T47D-GPx-2 cells in response to H₂O₂ treatment (not shown). To confirm the link between GSHPx activity and intracellular ROS levels detected with the HE probe, control experiments were performed to depress GSHPx levels in T47D-GPx-2 cells by selenium depletion. Culturing these cells for 6 d in selenium-depleted medium depressed their GSHPx activity to \sim 15% of that normally found in GPx-2 cells. In such cells, EB fluorescence was reversed to the level observed in T47D-Hygro-3 cells (Fig. 3). Selenium depletion had no detectable effect on HE oxidation in T47D-Hygro-3 cells (containing barely detectable GSHPx, not shown). These results therefore indicate that the difference in ROS levels observed in control and GSHPx-expressing T47D cells was likely to result from GSHPx overexpression. They also suggest that the decreased accumulation of ROS in T47D-GPx-2 cells exposed to TNF α or H₂O₂ was a likely cause of κ B dependent gene transactivation inhibition.

GSHPx Overexpression Suppresses the TNF α - and Hydrogen Peroxide-mediated Binding of NF- κ B to DNA

The effect of GSHPx overexpression on the activation of NF- κ B by TNF α or H₂O₂ was analyzed by DNA binding and electrophoretic shift assays. Nuclear extracts were prepared from control T47D-Hygro-3 and GSHPx-expressing

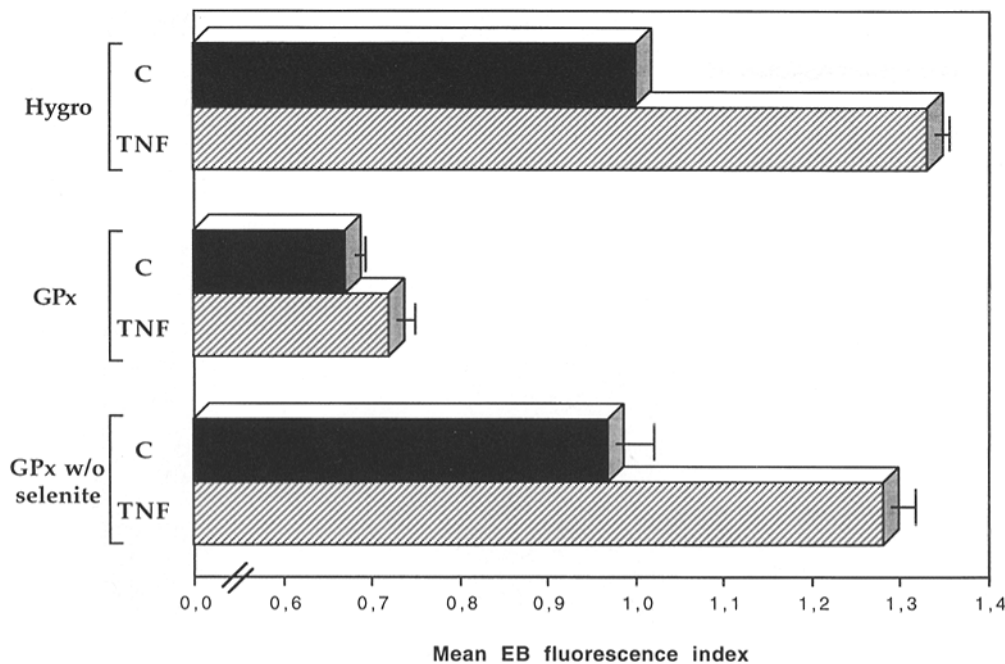


Figure 3. Reactive oxygen species levels in T47D-Hygro-3 and T47D-GPx-2 cells exposed or not to TNF α . ROS levels were determined by FACS[®] analysis of the oxidation of HE. T47D-Hygro-3 (Hygro) or T47D-GPx-2 (GPx) cells were incubated at 37°C for 10 min with HE in the presence (TNF) or absence (C) of 2,000 U/ml of TNF α added at the same time than HE. EB fluorescence was measured as described in Materials and Methods. Results are presented as mean EB fluorescence indexes that were expressed as mean EB fluorescence of each sample divided by that measured in control untreated T47D-Hygro-3 cells. (GPx w/o selenite) The mean EB fluorescence

index of T47D-GPx-2 cells grown for 6 d in selenium-depleted medium before being treated (TNF) or not (C) with TNF α . The histograms shown are representative of three identical experiments; SD are presented ($n = 3$).

T47D cells that were either left untreated or exposed to hydrogen peroxide or TNF α , and electrophoretic mobility shift assays were performed using a DNA probe encompassing the κ B motif (see Materials and Methods). As seen in Fig. 4 A, in T47D-Hygro-3 cells, a 2-h treatment with 250 μ M hydrogen peroxide induced the binding of a protein factor to κ B DNA. A similar result was observed when cells were treated for only 1 h with H₂O₂ (not shown). Competition experiments show that the binding to the radioactive κ B DNA was no longer detectable when increasing concentrations of nonradioactive κ B DNA were added to the binding mixture. A supershifted band was also observed when the reaction mixture was incubated with an antibody that recognizes the p65/RelA subunit of NF- κ B. Hence, these observations indicate that in T47D-Hygro-3 cells, H₂O₂ induces the binding of NF- κ B to the “ κ B” oligonucleotide. In contrast, in similarly treated T47D-GPx-2 cells, no protein factor was found to interact specifically with this oligonucleotide, indicating that, in these cells, NF- κ B activation or NF- κ B binding to DNA was abolished. A similar analysis was performed with cells treated for 2 h with 2,000 U/ml TNF α . The results, shown in Fig. 4B, indicate that GSHPx overexpression also blocked the TNF α -mediated binding of NF- κ B to DNA. Similar results were observed with the other GSHPx-overexpressing cell lines and when the TNF α treatment was for 1 h. Control experiments were therefore performed to determine whether the observed inhibition of NF- κ B binding to DNA was a direct consequence of GSHPx activity increment in T47D-GPx-2 cells. This was assessed by analyzing NF- κ B activation in T47D-GPx-2 cells grown for 6 d in a medium devoid of selenium to strongly decrease their GSHPx activity and reestablish a “normal” level of intracellular ROS (see above). As seen in Fig. 4 C, normal induction of NF- κ B DNA binding activity by TNF α was restored in selenium-deprived T47D-

GPx-2 cells. A similar result was observed when such cells were exposed to H₂O₂ (not shown). Hence, the observed inhibition of NF- κ B activation in T47D-GPx-2 cells grown in complete (selenium-supplemented) medium appears to be a direct consequence of the high levels of GSHPx activity and low levels of ROS in these cells.

The DNA Binding Ability of NF- κ B Is Not Affected by Elevated Levels of Intracellular GSHPx Activity

We then investigated the mechanism by which glutathione peroxidase overexpression and concomitant decreased levels of ROS inhibited NF- κ B activation in T47D-GPx-2 cells. The observation that the level of the two NF- κ B subunits (p65-p50) was not affected by GSHPx overexpression (Fig. 2) suggests that the inhibition of NF- κ B activation was posttranslational. In nonactivated cells, NF- κ B is cytoplasmic, in the form of the NF- κ B-I κ B- α complex that is unable to bind DNA. However, the DNA binding property of this factor can be restored by treating the cytosolic fraction with DOC, which, in the presence of a nonionic detergent, NP-40, dissociates I κ B- α from NF- κ B (5). This DOC treatment therefore allows us to determine the amount of inducible NF- κ B present in the cytoplasm by electrophoretic mobility shift assay. It is seen in Fig. 5 that DOC treatments promoted similar levels of NF- κ B binding to κ B DNA in T47D-Hygro-3 and -GPx-2 cytoplasm. This indicates that GSHPx activity increment did not alter the intrinsic ability of NF- κ B to bind DNA, but rather did inhibit the process that leads to the activation of this factor by oxidative stress.

NF- κ B Is No Longer Recovered in the Nucleus of GSHPx-Overexpressing Cells Exposed to TNF α or Hydrogen Peroxide

NF- κ B activation by oxidative stress is a multistep process that results in the translocation of a fraction of the p65-p50

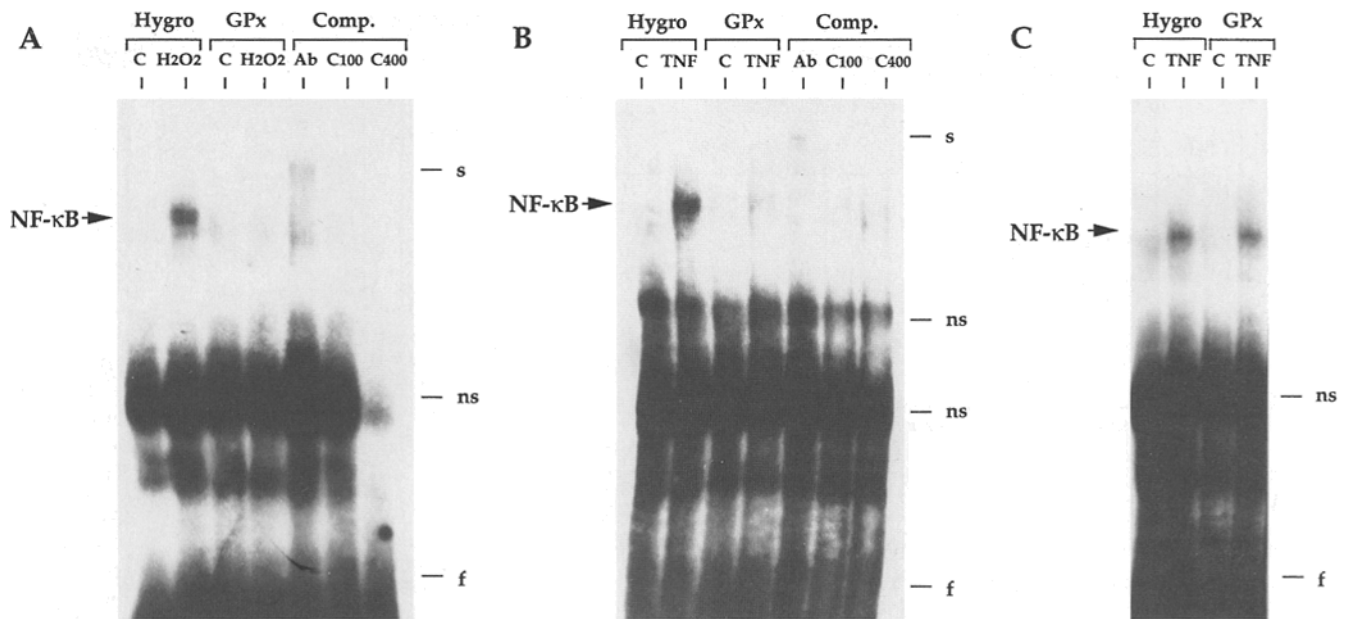
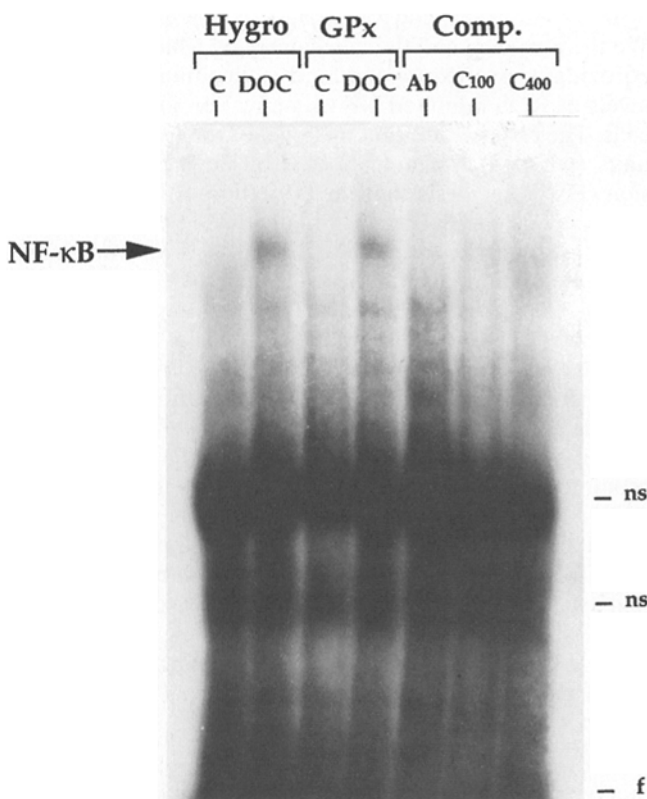


Figure 4. Effect of GSHPx overexpression on the TNF α - or hydrogen peroxide-mediated activation of the DNA-binding activity of the transcription factor NF- κ B. (A) The activation of NF- κ B by intracellular ROS was measured in T47D-Hygro-3 (*Hygro*) and T47D-GPx-2 (*GPx*) cells that were either left untreated (C) or incubated for 2 h at 37°C with 250 μ M of hydrogen peroxide (H_2O_2). Nuclear extracts were prepared and equal amounts (10 μ g) of nuclear proteins were incubated with a ^{32}P -labeled DNA probe encompassing the κ B motif as described in Materials and Methods. Samples were analyzed on native 4% polyacrylamide gels. An autoradiograph of the gel is presented. (Comp.) Competition experiments: (Ab) supershift performed by adding an antiserum recognizing the p65 subunit of NF- κ B to the binding mixture of H_2O_2 -treated T47D-Hygro-3 cell extracts; (C100 and C400) competition performed with either 10 or 40 ng of unlabeled κ B probe added to the binding mixture of H_2O_2 -treated T47D-Hygro-3 cell extracts. (B) Same experiment as A but in this case, the treatment was for 2 h with 2,000 U/ml of TNF α (*TNF*). (C) Same experiment as B but in this case, cells were grown for 6 d in selenium-depleted medium before being analyzed. In this case, the competition reactions are not shown. The position of the supershifted (s) and nonspecific (ns) complexes as well as the free probe (f) are indicated. Note the sodium selenite-dependent decreased activation of NF- κ B binding to κ B DNA in glutathione peroxidase-overexpressing T47D cells.



heterodimer in the nucleus where it binds to DNA (5). We therefore investigated whether the GSHPx-mediated inhibition of p65-p50 binding to κ B oligonucleotide was due to a deficient translocation of this transcription factor in the nucleus. To this end, Hygro-3 and GPx-2-T47D cells were incubated or not for 2 h with 250 μ M hydrogen peroxide or 2,000 U/ml TNF α , and then lysed and fractionated as described in Materials and Methods. The distribution of the p65 subunit in the resulting nuclear and soluble fractions was analyzed in immunoblots probed with a specific antiserum. It is seen in Fig. 6 that \sim 30% of the cellular

Figure 5. Glutathione peroxidase increment in T47D cells does not alter the intrinsic ability of NF- κ B to bind DNA. Equal amounts (10 μ g) of cytoplasmic extracts of either Hygro-3 (*Hygro*) and GPx-2 T47D (*GPx*) cells were incubated with a ^{32}P -labeled DNA probe encompassing the κ B motif. The mixtures were either left untreated (C) or treated for 15 min with 0.8% sodium desoxycholate (DOC) in the presence of 1% NP-40 before electrophoretic separation of the protein-DNA complexes on native 4% polyacrylamide gels. An autoradiograph of a typical experiment is presented. Competition experiments (Comp.), performed as described in Fig. 4, show the specificity of the NF- κ B/DNA complex. In this figure the supershifted complex is not visible. Nonspecific complexes (ns) and free probe (f) are indicated. Note that DOC similarly activates the DNA-binding property of NF- κ B present in the cytoplasm of both control and GSHPx-expressing T47D cells.

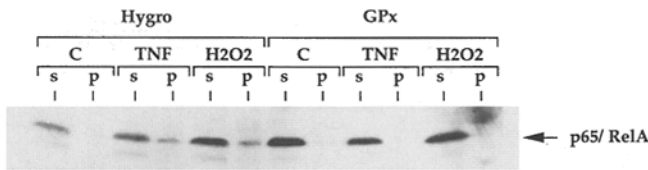


Figure 6. The presence of p65 in the nucleus of T47D cells treated with TNF α or hydrogen peroxide is abolished by glutathione peroxidase overexpression. Hygro-3 (*Hygro*) and GPx-2 T47D (*GPx*) cells were either left untreated (C) or incubated for 2 h at 37°C with 250 μ M hydrogen peroxide (H_2O_2) or 2,000 U/ml of TNF α (TNF). Cells were harvested, lysed, and fractionated as described in Materials and Methods, and the p65 subunit of NF- κ B content present in the cytoplasmic (s) and nuclear (p) fractions was analyzed by immunoblots probed with a specific antiserum. Immunoblots were revealed with ECL. Note that NF- κ B is no longer recovered in the nucleus of GSHPx-overexpressing cells exposed to TNF α or H_2O_2 .

content of p65 was present in the nuclear fraction of control Hygro-3 cells in response to TNF α or H_2O_2 treatments. In contrast, this phenomenon was not observed in the GSHPx-overexpressing cells. Similar results were observed when the immunoblots were probed with anti-p50 antiserum (not shown). Hence, the inhibition of κ B-dependent gene transactivation (Fig. 1) and the absence of NF- κ B binding to DNA observed in T47D-GPx-2 cells (Fig. 4) probably resulted from an interference at or upstream of NF- κ B translocation into the nucleus.

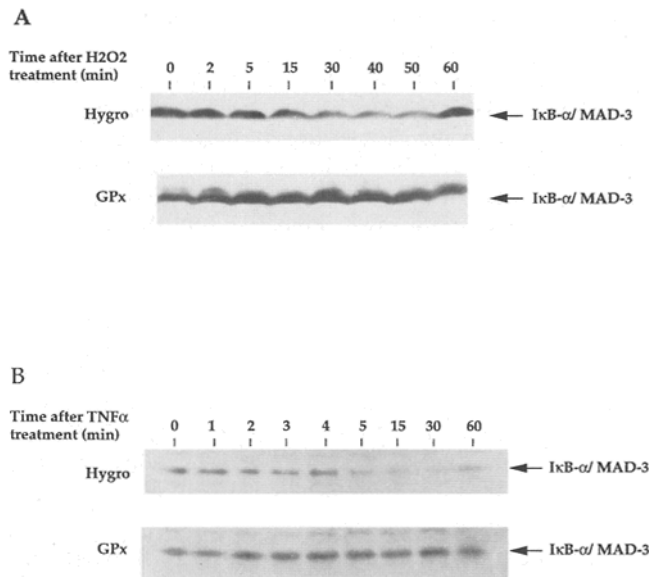


Figure 7. I κ B- α degradation is abolished in glutathione peroxidase-overexpressing T47D cells exposed to TNF α or hydrogen peroxide. (A) Hygro-3 (*Hygro*) and GPx-2 T47D (*GPx*) cells were either left untreated (0) or treated with 250 μ M of hydrogen peroxide during various times ranging from 2–60 minutes. (B) Same experiment as in A but in this case, cells were treated for various times with 2,000 U/ml of TNF α . Whole cellular extracts were prepared, and the cellular contents of I κ B- α /MAD-3 inhibitory subunit of NF- κ B were analyzed by immunoblots probed with an antibody specific for I κ B- α and revealed by ECL. Note the inhibition of I κ B- α transient degradation during TNF α and hydrogen peroxide treatments in glutathione peroxidase-overexpressing T47D cells.

GSHPx Overexpression Abolishes the TNF α - and Hydrogen Peroxide-mediated Degradation of the Inhibitory Subunit I κ B- α

The dissociation of the p65-p50 NF- κ B heterodimer from phosphorylated I κ B is accompanied by proteolytic degradation of I κ B (4, 13, 20, 33, 44). We therefore analyzed the kinetics of degradation of the major I κ B protein, I κ B- α /MAD-3, in Hygro-3 and GPx-2 cells exposed to oxidative stress. Cells were incubated with 250 μ M of H_2O_2 or 2,000 U/ml of TNF α , harvested at different times, and whole cell extracts were analyzed in immunoblots probed with an antibody that recognizes specifically I κ B- α /MAD-3. Fig. 7 shows that these treatments induced a transient degradation of I κ B- α in Hygro-3 cells. This phenomenon was detectable already after 5 min of treatment with TNF α , while 30 min of incubation were necessary in the case of H_2O_2 . After 60 min of treatment with TNF α , a resurgence of I κ B- α was observed while in H_2O_2 -treated cells, the level of this protein was almost back to normal. In contrast, in GPx-2 cells, no degradation of I κ B- α was observed in response to either TNF α or H_2O_2 . A quantitative analysis of

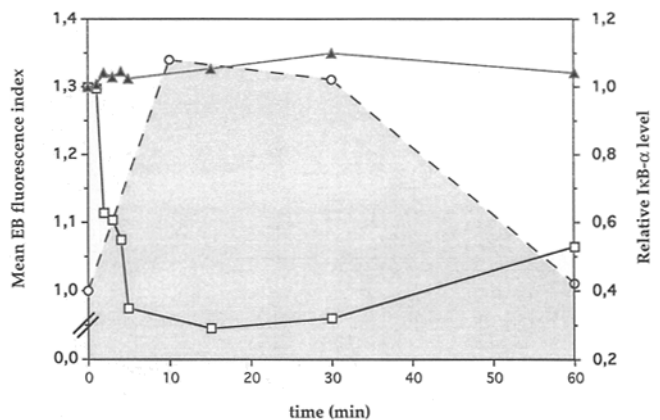


Figure 8. Kinetics of I κ B- α degradation and ROS accumulation after TNF α treatment. Hygro-3 and GPx-2 T47D cells were treated with 2,000 U/ml of TNF α during various times ranging from 1–60 min. The cellular content of I κ B- α was analyzed as described above in Fig. 7, and the immunoblots were quantified as a graph representing the relative I κ B- α levels as a function of the duration of TNF α treatment: (\blacktriangle), I κ B- α from GPx-2 cells; (\square), I κ B- α from Hygro-3 cells. The relative levels of I κ B- α were expressed as the levels of I κ B- α measured after treatment divided by that of untreated Hygro-3 or GPx-2 cells. (Shaded area) Kinetics of ROS accumulation after TNF α treatment of T47D-Hygro-3 cells. Before and at different times (10, 30, and 60 min) after the addition of 2,000 U/ml of TNF α , ROS levels were estimated by the conversion of HE into EB fluorescence (see Materials and Methods and Fig. 3). Since an incubation of 10 min with HE was necessary to observe a detectable conversion of HE in EB, the first measurement (after 10 min of incubation with TNF α) was performed by adding simultaneously HE and TNF α to the culture medium. For the other time points, HE was added during the last 10 min of the TNF α treatment. SD between experiments ($n = 3$) were $<5\%$. Note the rapid and transient increase of EB fluorescence in T47D-Hygro-3 cells treated with TNF α that correlates with the rapid and transient degradation of I κ B- α . In GPx-2 cells EB fluorescence is only weakly increased (not shown, see also Fig. 3).

the transient degradation of I κ B- α in TNF α -treated Hygro-3 cells is presented in Fig. 8. This figure shows that, in Hygro-3 cells, up to 70% of the total content of I κ B- α was degraded after 10 min of TNF α treatment, while the amount of this protein remained almost the same in GPx-2 cells. Of great interest is that in TNF α -treated Hygro-3 cells, the rapid degradation of I κ B- α occurred concomitantly with the burst of intracellular ROS. The kinetics

presented in Fig. 8 also show that the I κ B- α content of Hygro-3 cells appears inversely related to that of ROS. This suggests that the TNF α -mediated burst of ROS triggers the degradation of I κ B- α .

GSHPx Overexpression Abolishes the TNF α -mediated Transient Accumulation of an Acidic and Slow Migrating Isoform of the Inhibitory Subunit I κ B- α

Several reports have described the rapid appearance, in SDS polyacrylamide gel, of a slow migrating phosphorylated form of I κ B- α that precedes the degradation of this protein in response to TNF α (10, 14, 20, 25, 74, 75). This transient phosphorylation of I κ B- α appears to control the degradation of this protein (75). We therefore investigated, by two-dimensional immunoblot analysis, the kinetics of appearance of a more acidic, with apparent higher molecular weight, isoform of I κ B in response to TNF α treatment. Fig. 9 shows that in untreated control Hygro-3 cells, I κ B- α is resolved in two major isoforms; the *b* isoform has a slightly higher apparent molecular weight and is more acidic than the major *a* isoform. After 1 min of treatment with TNF α , the level of the *b* isoform drastically increased while that of the *a* isoform concomitantly decreased. Hence, during the first minutes of TNF α treatment, the *b* isoform becomes the major isoform of I κ B- α , suggesting that a large fraction of this protein is rapidly phosphorylated. After 3 min of treatment, the *b* isoform disappeared and the total level of I κ B- α decreased. Until 5 min of treatment, the level of I κ B- α , mainly in the form of the *a* isoform, continued to decrease. Thereafter, a gradual and slow increase of the *a* and then *b* isoforms was observed. After 60 min, the level, as well as the distribution, of I κ B- α isoforms was almost back to normal and resembled that observed in untreated cells. This confirms that a drastic change in the distribution of I κ B- α isoforms, presumably due to phosphorylation, precedes the degradation of this protein. In sharp contrast, in GSHPx-expressing cells only the *a* isoform was detected, and this particular distribution, as well as the level of this isoform, was unaffected in response to TNF α . These results suggest that, in growing and TNF α -treated T47D cells, GSHPx overexpression induced drastic inhibition of I κ B- α phosphorylation. This phenomenon correlated and was probably responsible for the lack of transient degradation of this protein in response to TNF α .

Discussion

We have observed that the overexpression of exogenous glutathione peroxidase in T47D cells inhibited the oxidative stress-mediated transcriptional activation of a luciferase gene placed under the control of six κ B regulatory elements. This observation demonstrates that the transcriptional activity of genes controlled by κ B elements can be modulated by the activity of intracellular GSHPx and suggests that reactive oxygen species play an important role in this phenomenon. Our findings also confirm earlier observations that were performed with anti-oxidant drugs (63, 70) or by using catalase-overexpressing mouse cells (61).

Our results show that elevated levels of GSHPx significantly decreased the basal level of intracellular ROS as

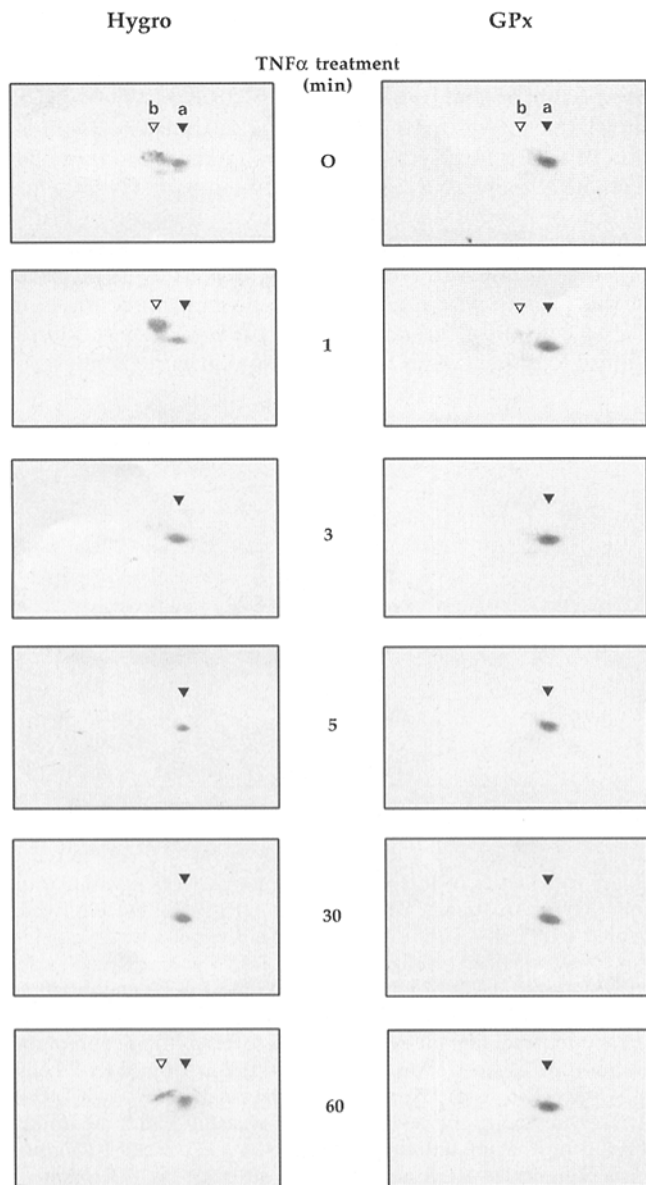


Figure 9. Kinetics analysis of I κ B- α isoforms in control and glutathione peroxidase-overexpressing T47D cells exposed to TNF α . Total proteins of Hygro-3 (*Hygro*) and GPx-2 T47D (*GPx*) cells, treated or not with 2,000 U/ml of TNF α during various time periods, were analyzed in two-dimensional immunoblots probed with anti-I κ B- α antibody and revealed by ECL as described in Materials and Methods. As indicated in the figure, analyses were performed before and after 1, 3, 5, 30, and 60 min of treatment with TNF α . The more acidic, with apparent higher molecular weight, I κ B- α phospho-isoform is indicated as the *b* isoform. The less acidic and faster migrating I κ B- α isoform is indicated as the *a* isoform. Note that GSHPx expression inhibited the TNF α -mediated redistribution and degradation of I κ B- α isoforms.

measured by FACS[®] analysis of HE fluorescence. The rapid and transient burst of intracellular ROS induced by TNF α was also abolished by GSHPx overexpression. These effects of GSHPx were abolished when cells were grown in the absence of selenium, suggesting that small variations in the level of ROS drastically modulate NF- κ B activity. TNF α is known to stimulate O₂⁻ production in mitochondria, and NF- κ B activation is reduced in cells depleted of the mitochondrial respiratory chain (65). Therefore, the burst of ROS detected with HE could have been triggered by superoxide production. It is not known, however, whether HE was oxidized directly by superoxide (O₂⁻) and/or by more downstream ROS metabolites such as H₂O₂, fatty acid (hydro)peroxides, hydroxyl radicals, or singlet oxygen. The latter possibility is supported by the fact that a strong burst of ROS was detected by HE in T47D-Hygro-3 cells treated with H₂O₂. It is not excluded that, although both GPx-2 and Hygro-3 cell lines contain similar levels of Cu/Zn-SOD (49), GSHPx overexpression could also stimulate the conversion of superoxide into peroxide since a higher capacity to eliminate H₂O₂ appears to protect Cu/Zn-SOD from inactivation by H₂O₂ (8, 76).

Several reports have shown that anti-oxidant drugs inhibit the activation of NF- κ B by oxidative stress (47, 48, 63, 64) and that H₂O₂ can induce this factor in some cell lines (47, 48, 63). These observations led to the conclusion that ROS act as second messengers in the activation of NF- κ B. Recently, Schmidt et al., (61) showed that, in mouse JB6 cells, the overexpression of Cu/Zn-SOD, which dismutates superoxide into H₂O₂ and O₂ in the cytosol, increases NF- κ B activation by TNF α . In contrast, overexpression of catalase, which detoxifies H₂O₂, decreased NF- κ B activation (61). These results demonstrate a causal link between H₂O₂ production and NF- κ B activation. Our results showing that the overexpression of another major H₂O₂-detoxifying enzyme, glutathione peroxidase, inhibits NF- κ B activation in response to TNF α or H₂O₂ support the model that intracellular H₂O₂ is the likely precursor of the putative free radicals that activate NF- κ B. Hence, the primary cause leading to NF- κ B inhibition in GSHPx-overexpressing T47D cells appears to be an increased depletion of H₂O₂.

Several reports have pointed out the importance of GSHPx *in vivo*. For example, in peripheral blood mononuclear cells from elderly subjects, the redox equilibrium is shifted toward a prooxidant state as a consequence of a 30 to 40% decreased GSHPx activity (46). Our results also clearly demonstrate that in cells that overexpress GSHPx, NF- κ B activation may be controlled by the concentration of selenium, a trace element required for GSHPx activity. In T lymphocytes, selenium has also been reported to act as a key regulator of NF- κ B activation (60). Hence, *in vivo*, the level of selenium as well as that of GSHPx directly participate in the fine tuning of intracellular ROS, and any change in their cellular contents may have dramatic consequences for the organism. The observation that NF- κ B activation by TNF α can also be suppressed by transition metal chelating agents (e.g., desferoxamine) or free radical trapping agents (64) suggests that activation of NF- κ B is likely mediated by free radicals and not by H₂O₂ directly. It is of interest to note that oxidative DNA damage induced in T47D cells by menadione (an intracellular

source of O₂⁻/H₂O₂) could also be suppressed by GSHPx overexpression or preincubation with desferoxamine, which chelates catalytic Fe³⁺ ions (49). In this case, DNA damage was strongly suspected to be caused by hydroxyl radicals formed by iron-dependent Fenton-type reactions. Whether NF- κ B activation by menadione, H₂O₂, or TNF α will also be suppressed in T47D Hygro-3 cells preincubated with desferoxamine remains to be determined.

The question remains as to how ROS activate NF- κ B. We have shown that GSHPx overexpression leading to decreased levels of ROS inhibited the oxidative stress-mediated p65-p50 nuclear translocation. This suggests that low ROS levels inhibited the activation of the cytoplasmic NF- κ B-I κ B- α complex. Several reports have described that I κ B- α degradation precedes NF- κ B activation and that transactivation by this factor, in turn, promotes I κ B- α synthesis, restoring the unstimulated inhibited state (13, 17, 71). Kinetics experiments revealed that, in parental and control transfectant T47D cells, I κ B- α was already degraded after 5 min of treatment with TNF α , while, in the presence of H₂O₂, 30 min appeared necessary. This is consistent with the fact that the kinetics of NF- κ B induction by H₂O₂ are rather slow as compared with those mediated by TNF α (61, 63), probably because oxidative damage induced by H₂O₂ slows down the mechanism of NF- κ B activation. A kinetic analysis of the burst of ROS generated by TNF α in T47D Hygro-3 cells showed that this phenomenon occurred concomitantly with the transient degradation of I κ B- α . This suggests that I κ B- α degradation is the target that is controlled by ROS. Decreased ROS levels would therefore inhibit I κ B- α degradation. Recently, I κ B- α phosphorylation has been reported to precede (10, 20, 25) and to be necessary for the rapid degradation of this protein by the proteasome (16, 37, 56, 74, 75). Analysis of this phenomenon in T47D Hygro-3 cells revealed that TNF α induced the rapid accumulation of an acidic phospho-isoform of I κ B- α that preceded the degradation of this protein. This transient accumulation of this isoform was not observed in TNF α -treated cells that overexpress GSHPx, confirming that the phosphorylation of I κ B- α precedes its degradation. Moreover, we show that, while untreated T47D-Hygro-3 cells already contain a small amount of the *b* phospho-isoform, this is not the case in untreated T47D cells that overexpress GSHPx. This favors the hypothesis that the intracellular levels of ROS control the level of the *b* phospho-isoform of I κ B- α by activating a kinase or inactivating a phosphatase that is specific to this protein. Hence, the GSHPx-mediated low levels of ROS, by inhibiting I κ B- α phosphorylation, probably abolish the specific proteolysis of phosphorylated I κ B- α that results in NF- κ B activation.

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