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GPx-1 Gene Delivery Modulates NFκB Activation Following Diverse Environmental Injuries Through a Specific Subunit of the IKK Complex

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ABSTRACT

Numerous environmental stimuli alter cell functions by the induction of intracellular reactive oxygen species, such as superoxide and hydrogen peroxide (H_2O_2). These redox alterations can change the activity of kinases and phosphatases responsible for controlling intracellular signal transduction cascades important in determining how cells react to their environment. One such well known pathway includes nuclear factor-κB (NFκB); however, the exact redox-sensitive factors important in controlling H_2O_2-mediated activation of NFκB remain unclear. In the present study, we have investigated how intracellular clearance of H_2O_2, using a recombinant adenovirus expressing glutathione peroxidase-1 (GPx-1), modulates NFκB activation following UV irradiation, tumor necrosis factor-α, or H_2O_2 treatment of MCF-7 cells. Findings from these studies demonstrate that GPx-1 overexpression can down-regulate NFκB DNA binding, and transcriptional activation of an NFκB-dependent luciferase reporter, to varying extents following these environmental stimuli. Studies using dominant negative adenoviral vectors expressing IKKα(KM) and IKKβ(KA) suggest that GPx-1-mediated H_2O_2 clearance appears to preferentially inhibit the activity of IKKα, but not IKKβ. These studies demonstrate for the first time that redox regulation of NFκB activation by intracellular H_2O_2 may be specific for a unique subunit in the IKK complex. Such findings suggest that IKK kinases or IKK phosphatases may have unique redox-regulated components. These studies have shed mechanistic insight into the potential application of redox-modulating gene therapies aimed at altering NFκB activation following environmental injury. Antioxid. Redox Signal. 3, 415–432.

INTRODUCTION

ALL CELLULAR ORGANISMS have evolved complex redox (reduction–oxidation) regulating systems to afford protection from injury and disease (16). Changes in the intracellular redox state following environmental stresses are mediated by the production of reactive oxygen species (ROS). ROS are implicated in disease processes such as inflammation (27), neurodegeneration (51), aging (43), and many stress responses, including exposure to UV or ionizing radiation (50, 68). ROS are also considered to be important modulators of cellular responses to tumor necrosis factor-α (TNF-α), a multifunctional proinflammatory cytokine involved in numerous environmental injuries (26, 34, 38, 59). There is ample evidence showing that ROS are signaling intermediates in a variety of signal transduction cascades, which in turn alter gene transcription patterns in response to environmental stresses.

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ROS are oxygen-containing molecules that have a higher chemical reactivity than the ground-state molecular oxygen. ROS include superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$), and singlet oxygen ($^1O_2$). The main ROS that determine the intracellular redox environment are $O_2^{\cdot -}$ and H_2O_2, in cooperation with the ratios of GSH/GSSG and NADPH/$NADP^+$ (49). ROS are constantly produced during normal metabolic processes in the human body. In living cells, H_2O_2 can be generated by the dismutation of $O_2^{\cdot -}$ by the protein superoxide dismutase (SOD). H_2O_2 is small and can easily diffuse into surrounding cytosolic compartments (13). Moreover, it is considered a key effector in ROS-mediated cell injuries (16).

The redox state in living cells is controlled by various antioxidants that remove ROS as well as their metabolic products. Such antioxidants include glutathione (GSH), vitamins C and E, and enzymes like SOD, catalase, glutathione peroxidase (GPx), and thioredoxin-dependent peroxidase. Among them, GPx is recognized as playing a main role in removing intracellular H_2O_2. Overexpression of GPx-1 protects against oxidative damage in some cell types (40, 53) and suppresses apoptosis induced by H_2O_2 (24). At least four GPx isoforms have been described: GPx-1 (cytoplasmic GPx) (39), GPx-2 (GPx-GI) (11), GPx-3 (plasma GPx) (58), and GPx-4 (PhGPx) (61). Cytoplasmic GPx (GPx-1, EC 1.11.1.9) plays a critical role in protecting cells from free radical damage. Although localized mainly in the cytoplasm, GPx-1 has also been suggested to reside in the nucleus (41), and is the only member of this family that is expressed in all tissues. The gene encoding GPx-1 (also called *cGPx*) has been localized in human chromosome 3 (10). As a substrate, GPx-1 strongly prefers H_2O_2, but a wide variety of hydroperoxides, such as lipid hydroperoxides, can also serve as substrates (2). In contrast, GPx-4 preferentially degrades organic hydroperoxides over H_2O_2. The main function of GPx-1 is to catalyze the reduction of H_2O_2 to water by using GSH as a source of reducing equivalents. GPx-1 is a tetramer composed of four identical subunits of 23 kDa. Its function requires GSH, NADPH, and glucose-6-phosphate as cofactors, and glutathione reductase and glucose-6-phosphate dehydrogenase as secondary enzymes. Glucose-6-phosphate dehydrogenase generates NADPH to recycle GSH. GPx-1 is also a selenoprotein (52). Each subunit has a selenocysteine, which is localized within the enzyme's active site. Selenocysteine functions as an electron donor and is oxidized by the enzyme's peroxide substrates during the reaction. GSH serves as the electron donor to regenerate the reduced form of selenocysteine (62).

ROS not only determine the cellular redox environment, they also act as second messengers in numerous signal transduction pathways that determine cellular responses to a number of environmental stresses (55, 57). These pathways include nuclear factor-κB (NFκB) (18, 50) and activator protein-1 (37). NFκB is a ubiquitous transcription factor that regulates an array of host genes responsive to environmental stresses. Activation of NFκB is known for its ability to induce a proinflammatory state by the induction of cytokines, and its ability to inhibit apoptotic pathways (4, 19, 44, 65). NFκB is composed of homo- and heterodimers of the Rel proteins. In unstimulated cells, NFκB is sequestered in an inactive form in the cytoplasm by association with members of the IκB family of inhibitory proteins, such as IκBα (6, 46). Stimuli that activate NFκB lead to the phosphorylation of IκBα at two N-terminal serines (Ser^{32} and Ser^{36}). This results in the ubiquitination and degradation of IκBα, releasing NFκB to the nucleus as an active transcription factor (7, 9). Two IκB kinases (IKK), IKKα and IKKβ, that specifically phosphorylate IκBα have been characterized (45, 66, 67). IKKα is an 85-kDa serine/threonine kinase composed of 745 amino acids. It has a serine/threonine kinase activity domain, a leucine zipper helix, and a helix–loop–helix domain. IKKβ is also a serine/threonine kinase and has the same topology as IKKα but is a slightly larger protein with an 11-amino acid extension at the C-terminus. The two kinases share 52% identity (35, 67). In the cell, IKKs interact to form a large 900-kDa multicomponent signaling protein complex termed the IKK signalsome (15, 45). This molecular complex contains two additional factors, including IKKγ (also termed NEMO or IKKAP1), which is a regula-

tory adaptor protein (36), and IKAP, which functions as a scaffolding protein (12). The kinase activities of IKKα and IKKβ are themselves up-regulated by phosphorylation. The target of phosphorylation is a MAPKK activation loop motif within the leucine zipper domain near the N-terminus of both IKKs. Kinases known to phosphorylate IKKα and IKKβ include the NFκB-inducing kinase (NIK), which has been proposed as a downstream component of the TNF-α signal pathway (21), MEKK1 (MAPK/ERK kinase kinase1), which is a key player in the activation of both c-Jun and NFκB (28), NFκB-activating kinase (NAK) (60), and Cot/Tpl-2, which is involved in CD3-CD28 activation of NFκB (31).

In the present study, we have investigated the role of H_2O_2 in the activation of NFκB using recombinant adenoviral vectors to modulate GPx-1 and IKK activity. Environmental stimuli, including UV irradiation, H_2O_2, and TNF-α treatment, were used to induce NFκB in MCF-7 cells. Our results demonstrated that intracellular clearance of H_2O_2 by GPx-1 reduced NFκB activation by these environmental insults to variable extents. Further analysis of the H_2O_2-sensitive components of the NFκB pathway revealed that overexpression of GPx-1 preferentially inhibited IKKα and had little or no effect on IKKβ activity.

MATERIALS AND METHODS

Construction of recombinant GPx-1 adenovirus

The *GPx-1* cDNA was generated from pcDNA3.1/Zeo(+)GPx-1 (5) by PCR with two specific primers. The forward primer harbored a c-Myc epitope (N-terminal in frame fusion with the GPx-1 coding sequence) and both primer encoded restriction sites for further cloning into the adenoviral proviral plasmid pAd.CMVlink. Primer sequences were as follows: EL522 forward primer: 5′-CGCGAGATCTACC*ATG*GCCGAACAAAAACTCATCTCAGAAGAGGATCTGTGTGCTGCTCGGCTAGCGG-3′, with the order of sequence sites from 5′ → 3′ as follows, BglII (single underline), Kozak/ATG (no underline, start methionine codon italic), c-Myc epitope (double underline), and GPx-1 homology (dotted underline). EL523 reverse primer: 5′-CGCGAAGCTTGCTGACACCCGGCACTTTATTA-3′, with the order of sequences from 5′ → 3′ as follows, *Hin*dIII (single underline) and *GPx-1* homology (dotted underline). PCR-generated *GPx-1* clones included the entire coding region and 208 nucleotides downstream of the stop codon, which includes the entire 3′-untranslated sequence (23). The 3′-untranslated region is necessary for selenocysteine incorporation during translation, which is a requirement for *GPx-1* enzyme activity (52). A recombinant adenoviral proviral plasmid was generated by inserting the PCR clone cDNA between *Bgl*II and *Hin*dIII sites in pAd.CMVLink, which contains the cytomegalovirus (CMV) enhancer/promoter and a simian virus 40 polyadenylation site for efficient expression of the transgene (17). Recombinant *GPx-1* adenovirus (Ad.GPx-1) was generated by cotransfection of 293 cells with the recombinant pAd proviral plasmid linearized with *Eco*R1 together with *Cla*1-cut Ad5.sub360 (E3-deleted) viral DNA (32). Following transfection, plates were overlaid with agar, and two rounds of plaque purification were performed by screening for c-Myc expression. Ad.GPx-1 was determined to be free of wild-type adenovirus contamination.

Cell culture and viral infection

MCF-7 cells (a human breast cancer cell line obtained from ATCC) was chosen for the majority of studies due to their low level of endogenous GPx-1. MCF-7 cells were grown in minimum essential medium with Eagle's salts and L-glutamine, 1% minimum essential medium nonessential amino acids, 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 30 n*M* sodium selenite. RAW 264.7 cells, 293 cells, HeLa cells, and primary human fibroblasts (also obtained from ATCC) were grown in Dulbecco's modified Eagle's medium with 10% FBS and 1% penicillin and streptomycin. Adenoviral infections were performed in serum-free medium for 2 h at a multiplicity of infection (MOI) of 500 particles/cell, followed by the addition of an equal volume of fresh medium containing 20% FBS. Incubation was continued for a total of 24 h. The medium was replaced 24 h after infection, and cells were

analyzed at 48 h after infection. These conditions produced >90% transduction with recombinant adenovirus, as assessed with Ad.CMV-GFP reporter gene expression. Five different types of recombinant adenoviruses were used, including: Ad.BglII (empty control vector that does not express a transgene), Ad.NFκBLuc (an NFκB-driven luciferase reporter vector) (47), Ad.GPx-1 (construction described above), Ad.IKKα(KM) (a dominant negative mutant form of IKKα) (47), and Ad.IKKβ(KA) (a dominant negative mutant form of IKKβ) (47).

Cell treatments and modulation of NFκB activation

MCF-7 cells were infected with Ad.GPx-1, Ad.IKKα(KM), and/or Ad.IKKβ(KA) each at an MOI of 500 particles/cell 48 h prior to treatment with each of the environmental stimuli. For luciferase assays, MCF-7 cells were further infected with Ad.NFκBLuc at an MOI of 500 particles/cell 24 h prior to treatment with each of the environmental stimuli. For UV irradiation, most of the spent medium was removed from the plates, leaving enough to just cover the cells. The cells were then irradiated with UV-C light at 50 J/m^2 for 3 s, and then fresh medium was quickly applied to the plates. For H_2O_2 treatments, concentrated H_2O_2 (30%) was diluted to 1 *M* with deionized H_2O and added to fresh medium at a final concentration of 1 m*M*. The spent medium was removed from MCF-7 cells and quickly replaced with medium containing 1 m*M* H_2O_2. After 1 h of incubation at 37°C, the medium was changed to fresh medium without H_2O_2 and the incubation was continued. For TNF-α induction experiments, human TNF-α was diluted in fresh medium to 0.5 ng/ml and used to replace the spent medium on MCF-7 cells. The cells were incubated in TNF-α-containing medium until harvest. Control MCF-7 cells were also fed with fresh medium, but did not receive any treatment. Cells were harvested for luciferase assays 6 h after each treatment. MCF-7 cells were washed twice with ice-cold phosphate-buffered saline and prepared for each assay accordingly.

Analysis of GPx-1, IKKα(KM), and IKKβ(KA) protein expression

Western blotting. MCF-7 cells were infected with recombinant adenoviruses at an MOI of 500 particles/cells as described above. Cell lysates were prepared at 48 h after infection and protein concentrations determined using a Bio-Rad kit (Bio-Rad, Philadelphia, PA, U.S.A.). Western blotting was performed using standard protocols. In brief, 50 μg of proteins for each condition was separated on a denaturing 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose (Hybond C; Amersham, Piscataway, NJ, U.S.A.). The transfer efficiency was monitored by staining with 0.5% Ponceau S. For GPx-1, membranes were blocked and probed with a 1:2,000 dilution of anti-myc-horseradish peroxidase (HRP) antibody (Invitrogen, Carlsbad, CA, U.S.A.) for 1 h at room temperature. The immunoreactive myc-tagged proteins were then detected using enhanced chemiluminescence (ECL; Amersham) and exposure to x-ray film. To detect IKKα (KM) and IKKβ (KA) expressions, blots were incubated for 1 h in rabbit anti-IKKα antibody or anti-IKKβ antibody (Santa Cruz Biotech, Santa Cruz, CA, U.S.A.), followed by peroxidase (HRP)-labeled anti-rabbit IgG (Santa Cruz Biotech).

GPx-1 enzyme assays. A GPX-340 kit from R&D Systems (Minneapolis, MN, U.S.A.) was used to test GPx-1 enzyme activity. In brief, cells infected with Ad.BglII or Ad.GPx-1 were sonicated and diluted with assay buffer (5 m*M* EDTA, 50 m*M* Tris-HCl, pH 7.6) to 1 μg/μl. Seventy microliters of each sample was added to 350 μl of assay buffer, and then 350 μl of NADPH reagent (3 m*M* GSH, 0.5 U of glutathione reductase, and 0.5 m*M* reduced β-nicotinamide adenine dinucleotide phosphate) and 350 μl of 0.007% *tert*-butyl hydroperoxide (substrate) were added to the mixture. The absorbance of each sample at 340 nm was recorded for a total of 3 min at 10-s intervals at room temperature. The GPx-1 activity in the sample is directly proportional to the rate of decrease in the absorbance at A_{340}, which re-

flects the oxidation of NADPH. One GPx-1 enzyme activity unit is defined as oxidation of 1 μM NADPH/min.

GPx-1 enzyme activity gels. Two hundred micrograms of protein from each sample was separated in an 8% native polyacrylamide Tris-glycine gel (gel buffer: 1 m*M* EDTA, 25 m*M* Tris-base, 190 m*M* glycine, pH 8.5) with a 4% stacking gel. After electrophoresis, the gel was rinsed with 1 m*M* GSH two times for 10 min (56). The gel was then incubated with 0.008% cumene hydroperoxide, 1 m*M* GSH for another 10 min. The gel was briefly washed with water two times and stained with 1% ferric chloride, 1% potassium ferricyanide solution. Achromatic bands correspond to proteins demonstrating GPx-1 activity. A duplicate native gel was blotted to nitrocellulose, and GPx-1 was detected with anti-bovine GPx-1 antibody using western blotting methods described above (30).

Electrophoretic mobility shift assays (EMSA)

Nuclear extracts were prepared with the following modifications to a previously published protocol (1). After washing with ice-cold phosphate-buffered saline twice, 60-mm dishes of cells were scraped into buffer A [10 m*M* HEPES, pH 7.9, 1.5 m*M* $MgCl_2$, 10 m*M* KCl, 0.5 m*M* dithiothreitol (DTT), and 0.2 m*M* phenylmethylsulfonyl fluoride (PMSF)] and incubated at 4°C for 10 min. Cell suspensions were then centrifuged at 14,000 *g* for 1 min and the supernatants discarded. The pellets were washed with buffer A once again to remove contamination with cytoplasmic organelles. Washed nuclear pellets were resuspended in 20 μl of buffer C (20 m*M* HEPES, pH 7.9, 25% glycerol, 420 m*M* NaCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* EDTA, 0.5 m*M* DTT, and 0.5 m*M* PMSF) and incubated at 4°C for 20 min to extract nuclear proteins. After centrifugation at 14,000 *g* for 2 min, the supernatant containing the nuclear extract was collected and the protein concentration determined using the Bio-Rad assay. All samples were normalized to a final concentration of 1 $\mu g/\mu l$ with buffer C.

EMSA was performed using a NFκB oligonuleotide (catalog no. E3292, Promega, Madison, U.S.A.) end-labeled with [γ-^{32}P]ATP (Amersham) and T4-polynucleotide kinase (New England Biolabs, Beverly, MA, U.S.A.), according to the manufacturer's instructions. Four micrograms of nuclear extract and 1 μl of ^{32}P-labeled probe (10^5 cpm) were added to 15 μl of reaction buffer (1 $\mu g/\mu l$ bovine serum albumin, 1 $\mu g/\mu l$ poly(dI/dC), 50 m*M* KCl, 20 m*M* HEPES, pH 7.9, 25% glycerol, 1 m*M* EDTA, and 1 m*M* DTT) and incubated for 25 min at room temperature. DNA binding was assessed by running samples on a 4% polyacrylamide native Tris-glycine gel containing 2.5% glycerol (gel buffer: 1 m*M* EDTA, 25 m*M* Tris-base, 190 m*M* glycine, pH 8.5) for ~3 h at 180 V. Gels were dried under vacuum and exposed to x-ray film.

Luciferase assays

Luciferase activity was measured using a kit (catalog no. E4030, Promega) according to the manufacturer's instructions. MCF-7 cells were infected with Ad.NFκBLuc 24 h prior to treatments by UV, TNF-α, or H_2O_2. Ad.NFκBLuc is an NFκB-responsive luciferase reporter vector. This construct contains the luciferase gene driven by four tandem copies of the NFκB consensus sequence fused to a TATA-like promoter from the Herpes simplex virus thymidine kinase gene (47). Five micrograms of total protein from each sample was used to perform the luciferase assays.

RESULTS

Adenoviral mediated GPx-1 transgene expression and functional characterization

With the goal of generating a recombinant adenoviral vector capable of expressing the human GPx-1, a 30-bp myc-tag coding sequence was fused in frame to the human *GPx-1* cDNA using a PCR strategy. This modification allowed for the differentiation of exogenous and endogenous GPx-1 protein expression in cells by immunologic methods. Four clones were sequenced in their entirety,and one clone with no Taq errors was used for construction of recombinant adenovirus. A recombinant adeno-

virus encoding the c-myc-*GPx-1* cDNA (Ad.GPx-1) was tested for its ability to express functional GPx-1 protein using western blots and enzyme activity assays. MCF-7 and 293 cells were infected with recombinant Ad.GPx-1 or Ad.CMVLacZ as a negative control. Adenoviral mediated GPx-1 protein expression was detected in western blots of whole cell lysates with anti-c-Myc antibody (Fig. 1). In MCF-7 cells, there was a clear 24-kDa band only in the Ad.GPx-1-infected, but not in the Ad.CMVLacZ-infected sample. This band represented the c-Myc-GPx-1 fusion protein (the GPx-1 monomer is 23 kDa, and the c-Myc epitope 1 kDa). A 43-kDa nonspecific band appeared in all sample lanes at equal density. The influence of selenium on the expression of GPx-1 was also evaluated in 293 cells. Ad.GPx-1-infected 293 cells grown in medium supplemented with sodium selenite (30 m*M*) demonstrated approximately twofold higher levels of GPx-1 protein than cells grown in normal medium (Fig. 1). Similar

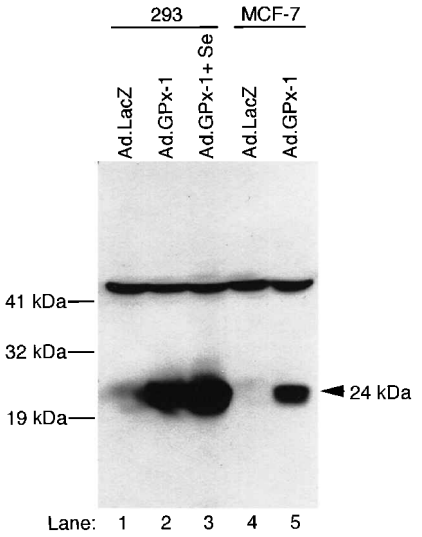

FIG. 1. Ad.GPx-1 mediates recombinant GPx-1 protein expression in 293 and MCF-7 cells. 293 cells (lanes 1–3) were infected with purified Ad.GPx-1 or Ad.LacZ (MOI of 25 particles/cell) and were grown in the presence or absence of added sodium selenite (30 n*M*). MCF-7 cells (lanes 4 and 5) were infected at an MOI of 500 particles/cell and were always grown in the presence of 30 n*M* sodium selenite. Twenty-one hours (for 293 cells) or 48 h (for MCF-7 cells) after infection, total cell lysates were prepared and electrophoresed on a 12% SDS-PAGE. Western blotting was performed using an anti-cMyc-HRP antibody and ECL detection. Molecular mass standards are indicated to the left of the gel, and the arrow on the right indicates the position of the 24-kDa cMyc-tagged GPx-1 monomer.

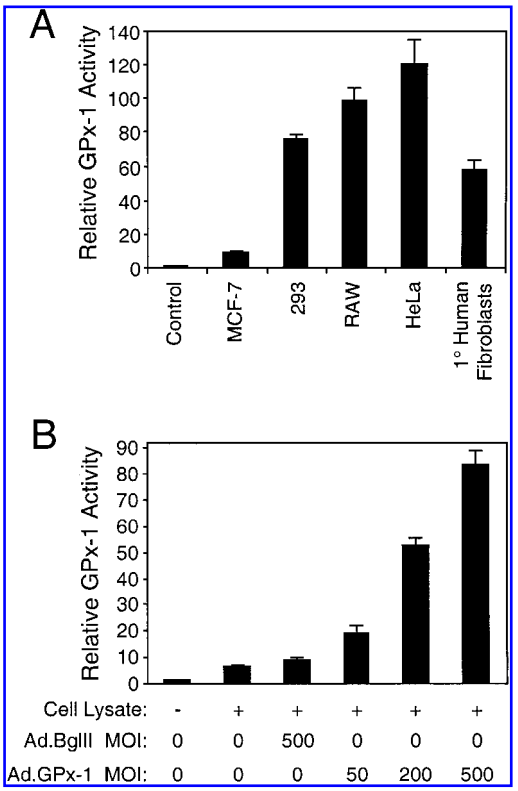

FIG. 2. Different cell types demonstrate variable expression of GPx-1 enzyme activity. (**A**) Endogenous GPx-1 enzyme activity was evaluated in primary human fibroblasts, HeLa, RAW 264.7, 293, and MCF-7 cells with an assay kit from R&D Systems (see Materials and Methods). The control sample indicates baseline values in the absence of cellular lysate. (**B**) The same method was also used to assess GPx-1 enzyme activity in MCF-7 cells following infection with Ad.GPx-1 at MOIs of 0, 50, 200, or 500 particles/cell or with the empty control virus Ad.BglII (500 particles/cell) 48 h prior to harvest and enzyme analysis. All values represent the mean relative GPx-1 activity for each cell type (± SEM, $n = 3$).

results have been previously reported for some T-cell lines (33, 48). These results demonstrate that recombinant Ad.GPx-1 expresses the predicted sized myc-GPx-1 fusion protein in both 293 and MCF-7 cells, and that selenium supplementation enhances recombinant GPx-1 expression.

As a major goal of this research was to use recombinant expression of GPx-1 to understand how intracellular H_2O_2 levels act to induce NFκB activity, choosing a cell line with low endogenous GPx-1 activity was preferable to allow for maximal modulation of GPx-1 levels. To this end, the endogenous GPx-1 enzyme activity in primary human fibroblasts, MCF-7, 293, RAW 264.7, and HeLa cells was evaluated

(Fig. 2A). Among these cell types, the human breast cancer cell line MCF-7 demonstrated the lowest level of endogenous GPx-1 activity. All other cell types tested had endogenous GPx-1 levels ranging from sixfold (fibroblasts) to 12-fold (HeLa cells) higher than seen in MCF-7 cells.

We next sought to evaluate whether infection with Ad.GPx-1 could increase the level of GPx-1 enzyme activity in MCF-7 cells. MCF-7 cells were infected with Ad.GPx-1 or the empty control virus, Ad.BglII, at various MOIs. The level of GPx-1 enzyme activity was dose-dependent on the MOI of the Ad.GPx-1 vector and increased 10-fold over the baseline at an MOI of 500 particles/cell (Fig. 2B). Control infection of MCF-7 cells with Ad.BglII at 500 particles/cell produced no significant increase in GPx-1 activity.

The final method used to confirm functionality of the recombinant Ad.GPx-1 vector was a native activity gel. Two identical native gels were used for activity assays and western blotting (Fig. 3). As an antibody against human GPx-1 was not available, a bovine GPx-1 antibody with cross-reactivity for both bovine and human GPx-1 proteins was used for detecting GPx-1. Bovine GPx-1 protein was used as a positive control for western blotting. Both GPx-1 protein expression and enzyme activity were increased in MFC-7 cells following Ad.GPx-1 infection, and the increases were dose-dependent with MOI (Fig. 3, lanes 5–8). Uninfected and Ad.CMVLacZ-infected MCF-7 cells exhibited undetectable endogenous GPx-1 protein expression and enzyme activity (Fig. 3, lanes 3 and 4, respectively). The positive control, bovine GPx-1, showed strong GPx-1 activity, but a slightly retarded migration in comparison with human GPx-1 in the native gel due to its larger size.

In summary, analysis of the Ad.GPx-1 vector using three criteria has confirmed that the vector produces functional GPx-1 protein. Furthermore, MCF-7 cells have a low level of endogenous GPx-1 and can be readily infected with Ad.GPx-1. Thus, MFC-7 cells appear to possess the appropriate characteristics for mechanistic studies of NFκB activation using this GPx-1 vector and were used in all the experiments described below.

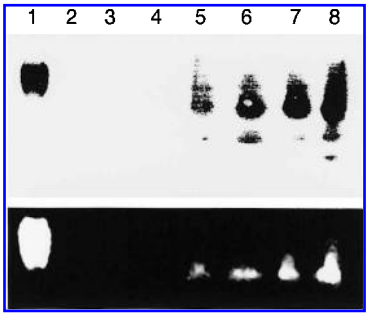

FIG. 3. Analysis of adenovirally produced GPx-1 protein activity. MCF-7 cells were infected with Ad.GPx-1 at MOIs of 20, 50, 100, or 500 particles/cell, or Ad.CMVLacZ at an MOI of 500 particles/cell as a control. At 48 h after infection, cells were harvested and cell lysates prepared. Two hundred micrograms of each cell lysate was run on an 8% native gel and either blotted against anti-bovine GPx-1 antibody (**upper panel**) or processed for in-gel GPx-1 enzyme activity (**lower panel**) as described in Materials and Methods. Anti-bovine IgG-HRP antibody and ECL detection were used to visualize immunoreactive GPx-1 in the upper panel. Zones of clearing in the lower panel indicate GPx-1 enzyme activity. Lane 1: bovine GPx-1 protein control; lane 2: no cell lysate; lane 3: MCF-7 cells without infection; lane 4: MCF-7 cells infected with Ad.CMVLacZ (MOI 500 particles/cell); lanes 5–8: samples from MCF-7 cells infected with Ad.GPx-1 at MOIs of 20, 50, 100, and 500 particles/cell, respectively.

NFκB activation by UV, H_2O_2, and TNF-α treatments in MCF-7 cells

With the goal of understanding how H_2O_2 regulates NFκB activation following environmental stress, we chose to study UV irradiation and TNF-α treatment, which are known to induce NFκB DNA binding and transcriptional activity in the nucleus. Exogenous addition of H_2O_2 has often been used to mimic the effect of H_2O_2 production during signal transduction processes (3, 25, 64). Thus, in our studies, H_2O_2 treatment was used to mimic the intracellular production of H_2O_2, which mediates, at least in part, the NFκB activation induced by UV, TNF-α, or other stresses. As a prelude to mechanistic studies with GPx-1, we first examined the time course of NFκB activation using two functional assays: (1) DNA binding using EMSA, and (2) transcriptional activation using an NFκB-responsive luciferase reporter assay.

The time courses of changes in NFκB DNA binding activity as a result of UV, H_2O_2, and TNF-α treatments in MCF-7 cells were first examined by EMSA (Fig. 4A). As previously reported, each of these three treatments induced

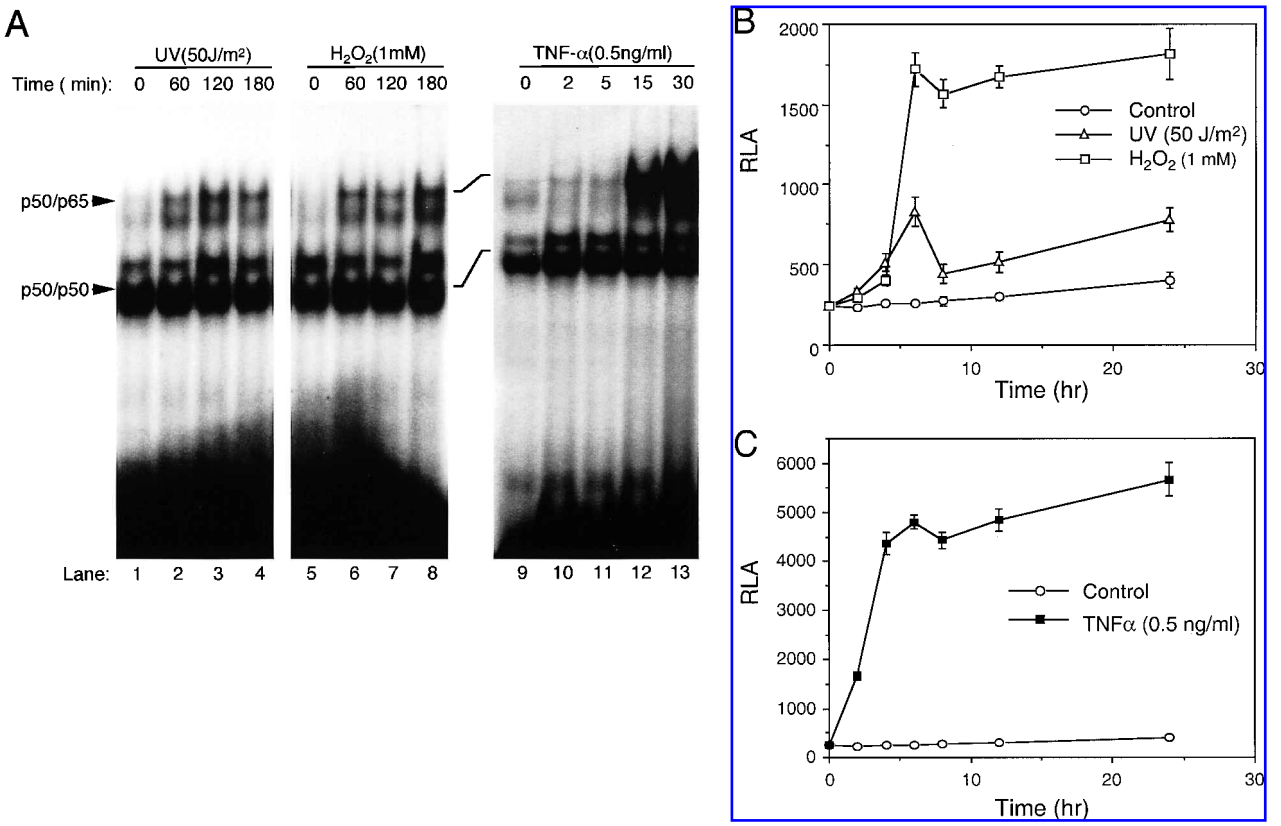

FIG. 4. Exposure to UV, H_2O_2, and TNF-α induces NFκB activity in MCF-7 cells. MCF-7 cells were treated with UV (50 J/m^2), H_2O_2 (1 m*M*), or TNF-α (0.5 ng/ml) and were harvested at different time points after treatment for analysis of NFκB DNA binding using EMSA (**A**) or NFκB transcriptional activity using a luciferase reporter (**B** and **C**). EMSA analyses of NFκB DNA binding activity in nuclear extracts (4 μg) were performed at selected time points (min) after treatment (A). Treatments and time points are indicated above the lanes. p50/p65 and p50/p50 NFκB dimers indicated to the left of the gel were determined by supershifts with anti-p50 and anti-p65 antibodies (data not shown). MCF-7 cells were also infected with a recombinant adenovirus (Ad.NFκB.Luc), encoding the luciferase reporter gene driven by an NFκB-specific binding sequence, 24 h prior to exposure to UV, H_2O_2, or TNF-α at time zero (B and C). Luciferase assays were performed on cellular lysates harvested from 0 to 24 h following exposure to the various environmental injuries. Results depict the mean relative luciferase activity (RLA) (± SEM, $n = 3$) and are normalized for protein concentration.

NFκB DNA binding activity, which appeared as a shifted doublet representing the NFκB p50/p65 heterodimer. No change in the level of p50/p50 homodimers was seen for any of the stimuli. Supershift assays with antibodies to p65 and p50 confirmed the identity of shifted bands (data not shown). The data suggest that although each of these three stimuli could induce NFκB DNA binding activity, the time course of activation varied. Maximal NFκB DNA binding activity was evident at 2 h after UV treatment, 3 h after H_2O_2 treatment, and only 30 min after TNF-α treatment. Another difference was the magnitude of the induced DNA binding activity. TNF-α treatment produced a much higher level of NFκB activation than either UV or H_2O_2, with UV treatment exhibiting the lowest level.

To compare changes in NFκB DNA binding directly with transcriptional activation, a luciferase reporter assay was also used. In these studies, an adenoviral vector was used to deliver an NFκB-responsive luciferase transgene to MCF-7 cells 24 h prior to treatments with the stimuli. A luciferase assay was then used to quantify NFκB transcription activation (Fig. 4B and C). As was seen with EMSA, the increase in NFκB transcriptional activity resulting from TNF-α treatment was two- to threefold higher than with H_2O_2, and was close to 10-fold higher than that following UV irradiation. NFκB activation first peaked at 6 h for all treatments.

These data are consistent with the findings evaluating NFκB DNA binding activity by EMSA, and indicate a delay of 3–5 h between peak DNA binding and peak protein expression. Hence, 6-h time points, representing the peak response for all stimuli, were chosen for all studies evaluating effects of GPx-1 expression on NFκB transcriptional activation.

Overexpression of GPx-1 in MCF-7 cells reduces NFκB activity

Having determined the parameters for NFκB induction in MCF-7 cells following UV, H_2O_2, and TNF-α treatments, the next focus of our studies was to evaluate effects of GPx-1-mediated reduction of intracellular H_2O_2 on the ability of various stimuli to induce NFκB. In these studies, MCF-7 cells were infected with Ad.GPx-1 2 days prior to exposure of the cells to UV, H_2O_2, or TNF-α. Both uninfected and Ad.BglII-infected samples were used as controls. EMSA analyses demonstrated that overexpression of GPx-1 attenuated the induction of NFκB DNA binding by each of these stimuli (Fig. 5). However, the extent of this reduction was specific for each individual stimulus. Overexpression of GPx-1 in the H_2O_2 treated sample demonstrated the most extensive reduction in NFκB DNA binding activity, and this effect was primarily evident in the p50/p65 NFκB heterodimer (upper shifted band). In contrast, for both UV and TNF-α, the reduction in NFκB DNA binding was mainly associated with decreased density in the lower shifted band corresponding to the p50/p50 homodimer.

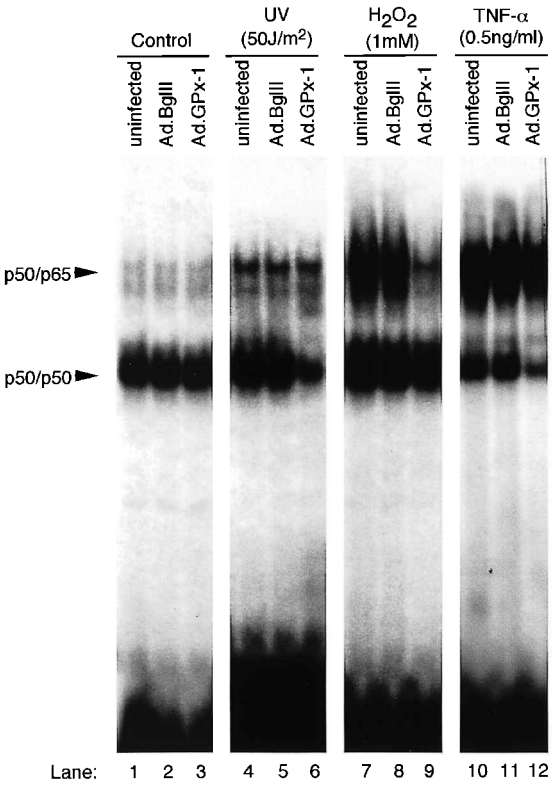

FIG. 5. Overexpression of GPx-1 protein reduces NFκB DNA binding activity induced by UV, H_2O_2 or TNF-α in MCF-7 cells. MCF-7 cells were infected with Ad.GPx-1 (lanes 3, 6, 9, and 12) or the control virus Ad.BglII (lanes 2, 5, 8, and 11) at 500 particles/cell. Uninfected controls were also analyzed for baseline activity (lanes 1, 4, 7, and 10). At 48 h after infection, cells were either untreated or treated with UV (50 J/m^2), H_2O_2 (1 m*M*), or TNF-α (0.5 ng/ml). Cell samples exposed to environmental stimuli were harvested at the peak time point of NFκB induction (UV, 120 min; H_2O_2, 180 min; TNF-α, 30 min), and 4 μg of nuclear protein from each sample was analyzed by EMSA for NFκB binding activity. p50/p65 and p50/p50 NFκB dimers are indicated to the left of the gel, and treatment conditions are described above each lane.

Luciferase reporter assays also demonstrated GPx-1-mediated reductions in UV, H_2O_2, and TNF-α stimulated NFκB transcription activity (Fig. 6). Following H_2O_2 treatment, NFκB transcriptional activation was reduced by more than half by overexpression of GPx-1. GPx-1 expression inhibited NFκB transcriptional activation by TNF-α or UV treatments to a lesser extent (25–30%). These results substantiate GPx-1-mediated reductions seen in DNA binding following H_2O_2, TNF-α, or UV treatments.

In summary, these experiments have demonstrated that overexpression of GPx-1 can reduce NFκB activation stimulated by UV, H_2O_2, and TNF-α treatments. GPx-1-mediated reductions in NFκB activation were greatest with H_2O_2 treatment, suggesting that GPx-1 overexpressed by Ad.GPx-1 effectively degrades the intracellular H_2O_2 required for NFκB activation. Taken together, these results suggest that UV irradiation and TNF-α treatment may induce changes in the intracellular level of H_2O_2, which is, in part, required for activation of NFκB. We hypothesized that the observed differences in the ability of GPx-1 to attenuate NFκB activation following these three stimuli might reflect mechanistic differences in the contribution of H_2O_2 to activation of NFκB by

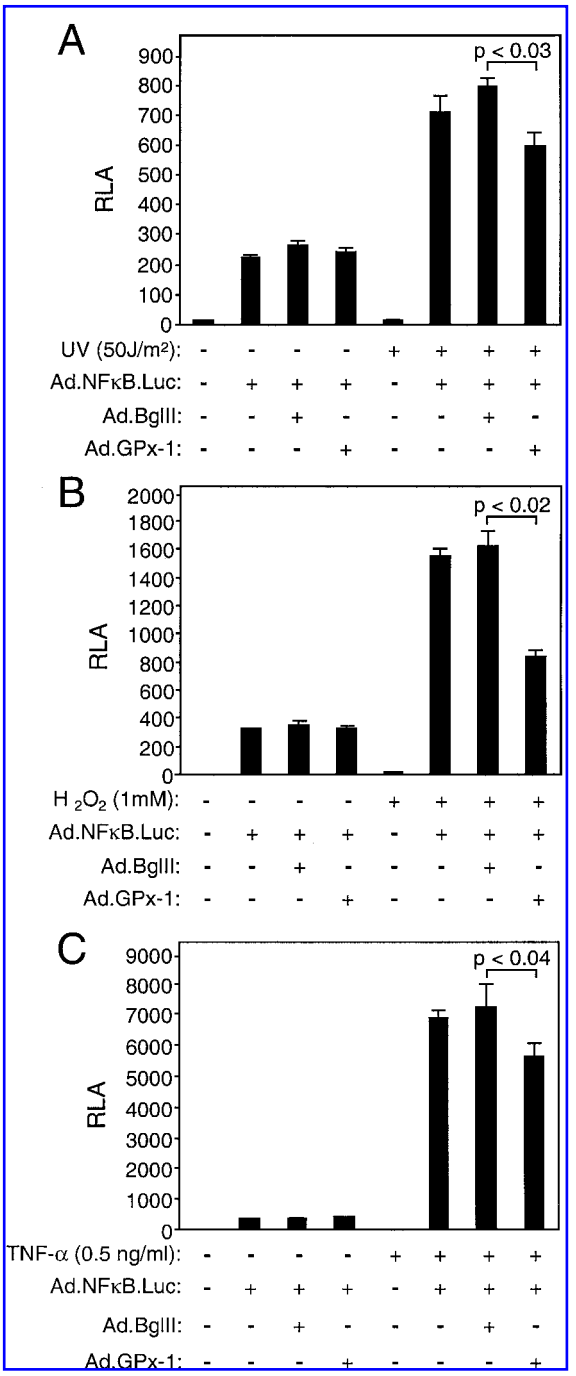

FIG. 6. GPx-1 overexpression reduces NFκB transcriptional activity following UV, H_2O_2, or TNF-α treatment of MCF-7 cells. MCF-7 cells were infected with Ad.GPx-1 or the control virus Ad.BglII at 500 particles/cell. At 24 h after infection, cells were then infected with Ad.NFκBLuc (500 particles/cell). Twenty-four hours after the second infection, cells were treated with UV (50 J/m^2), H_2O_2 (1 m*M*), or TNF-α (0.5 ng/ml). Cells were harvested for luciferase activity assays 6 h after UV (**A**), H_2O_2 (**B**), or TNF-α (**C**) treatments to quantify NFκB transcription activity. Conditions for infection and treatment are indicated below each graph. Results depict the mean relative luciferase activity (RLA) (± SEM, $n = 3$) and are normalized for protein concentration. Statistical comparisons using the Student's *t* test are marked as *p* values above the control vector and Ad.GPx-1-treated groups.

these distinct pathways. Of interest to our present study was understanding what factors in the NFκB activation cascade might be regulated by H_2O_2 and are common to a number of environmental stimuli. Several potential redox-regulated factors might control NFκB activation. One obvious candidate was the IKK complex, which is responsible for phosphorylation of IκB (inhibitor of NFκB).

Overexpression of GPx-1 specifically inhibits the activity of IKKα

IKKα and IKKβ are two members of the IKK complex that are required for the phosphorylation of IκBα and β. Following phosphorylation on Ser^{32} and Ser^{36}, IκB is ubiquitinated and degraded by the proteasome pathway, thereby releasing NFκB to the nucleus to activate responsive genes. We hypothesized that H_2O_2 production following UV and TNF-α exposure might modulate NFκB activation by influencing IKKα/IKKβ kinase activities. To evaluate this hypothesis, two adenoviral constructs encoding dominant negative mutants of IKKα (Ad.IKKαKM) and IKKβ (Ad.IKKβKA) were used (47). Each of the recombinant vectors encodes a hemagglutinin (HA)-tagged IKK subunit.

The expression of HA-IKKα(KM) and HA-IKKβ(KA) proteins by these recombinant vectors was confirmed in MCF-7 cells by western blot analysis (Fig. 7). Western blotting with an anti-HA antibody demonstrated significant immunoreactivity of an 86–88-kDa recombinant protein in Ad.IKKα(KM)- and Ad.IKKβ(KA)-infected MCF-7 cells, but not in uninfected control cells. Western blotting with anti-IKKα and anti-IKKβ antibodies demonstrated that the recombinant IKK subunits were expressed at significantly higher levels than their endogenous counterparts (Fig. 7).

Having demonstrated our ability to express high levels of recombinant IKK mutant subunits in MCF-7 cells, we next sought to determine how IKKα and IKKβ might be regulated by intracellular H_2O_2 levels. Our approach was to evaluate the influence of GPx-1 overexpression under conditions where one or both of the IKK subunits were functionally blocked by expression of a dominant negative mutant subunit (Fig. 8). Infection of MCF-7 cells with either Ad.IKKα(KM) or Ad.IKKβ(KA) alone

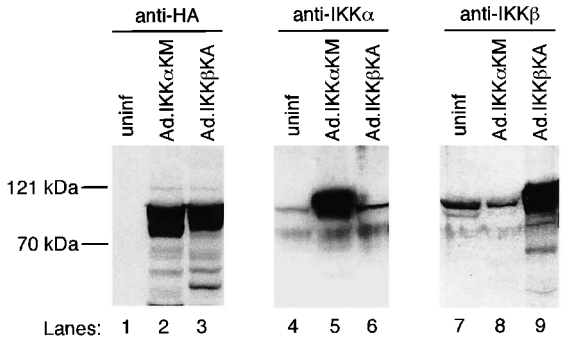

FIG. 7. IKKα(KM) and IKKβ(KA) expression mediated by adenoviral infection in MCF-7 cells. MCF-7 cells were infected with Ad.IKKα(KM) or Ad.IKKβ(KA) at an MOI of 500 particles/cell. Cells were harvested 48 h after infections, and 20 μg of total protein from each sample was separated by 8% SDS-PAGE and blotted to nitrocellulose. Immunoreactive IKK was detected using anti-HA-HRP antibody (Boehringer Mannheim), anti-IKKα (Santa Cruz), or anti-IKKβ (Santa Cruz) antibodies. Anti-HA-HRP detects only the recombinant forms of IKKα(KM) and IKKβ(KA) (**left panel**), whereas anti-IKKα (**middle panel**) and anti-IKKβ (**right panel**) detect both the recombinant and endogenous forms of IKKα and IKKβ, respectively. For the middle and right panels, immunoreactivity was detected using HRP-secondary antibodies. All blots were developed using an ECL detection system.

produced major reductions (40–60%) in NFκB transcriptional activation by all three forms of environmental stimuli. Blocking both IKKs by coinfection with both Ad.IKKα(KM) and Ad.IKKβ(KA) produced a larger decrease than seen with either alone. This maximal inhibition of NFκB by combined Ad.IKKα(KM) and Ad.IKKβ(KA) infection was an indication of the maximum influence of the IKK complex on NFκB activation (assuming the dominant inhibitory vectors are 100% effective in blocking activity of each of the IKK subunits). For H_2O_2 treatment, combined expression of both the mutant IKK subunits inhibited NFκB induction >95%. In contrast, for UV and TNF-α treatments, combined inhibition of both IKKα and IKKβ did not completely abolish NFκB induction, but rather led to 87% and 77% reductions, respectively. These findings suggest that alternative, non–IKK-regulated pathways may also contribute to the overall level of NFκB induction resulting from UV- and TNF-α-mediated injury.

To examine whether IKKα and/or IKKβ activities are modulated by the level of intracellular H_2O_2, we asked whether coinfection of MCF-7 cells with Ad.GPx-1 and Ad.IKKβ(KA) or Ad.IKKα(KM) had an additive effect on NFκB induction. Under these conditions, it was possible to dissect selectively the influences of H_2O_2 on IKKα or IKKβ activities. Interestingly, Ad.GPx-1 coinfection with Ad.IKKβ(KA), but not Ad.IKKα(KM), resulted in a level of NFκB inhibition nearly identical to that seen with combined Ad.IKKβ(KA) and Ad.IKKα(KM) coinfection (Fig. 8). No such influence of GPx-1 on IKKβ activity was observed, as indicated by the fact that Ad.GPx-1 and Ad.IKKα(KM) coinfection resulted in identical levels of NFκB inhibition as seen with Ad.IKKα(KM) infection alone (Fig. 8). In summary, GPx-1 overexpression had an additive inhibitory effect with Ad.IKKβ(KA), but no additive effect with Ad.IKKα(KM). These results suggest that GPx-1 expression, and presumably the intracellular H_2O_2 level, selectively influence IKKα activity. Furthermore, the selective influence of GPx-1 expression on IKKα activity was consistent across all environmental injuries tested.

GPx-1-mediated reduction in NFκB activity is limited to pathways controlling serine phosphorylation of IκBα

Data thus far have suggested that GPx-1 expression, and hence the level of H_2O_2, selectively influence the activity of IKKα, but not IKKβ. Important control data required for the interpretation of these results was the finding that combined infection with Ad.IKKβ(KA), Ad.IKKα(KM), and Ad.GPx-1 did not provide a greater level of inhibition in NFκB induction over coinfection with Ad.IKKβ(KA) and Ad.IKKα(KM) without Ad.GPx-1 (Fig. 8). These data begin to address whether other non–IKK-mediated, H_2O_2-responsive, pathways influencing NFκB induction might also be active following UV, H_2O_2, or TNF-α treatment. It is clear that other GPx-1 nonresponsive pathways capable of inducing NFκB are present to some extent following UV and TNF-α treatment. However, to provide further evidence that the IKK complex is the predominant H_2O_2-sensitive NFκB-inducing pathway, we sought to evaluate whether Ad.GPx-1 infection could modulate the inhibitory properties of the dominant negative IκB serine mutant (IκBαS32A/S36A) (22). The IKK complex is

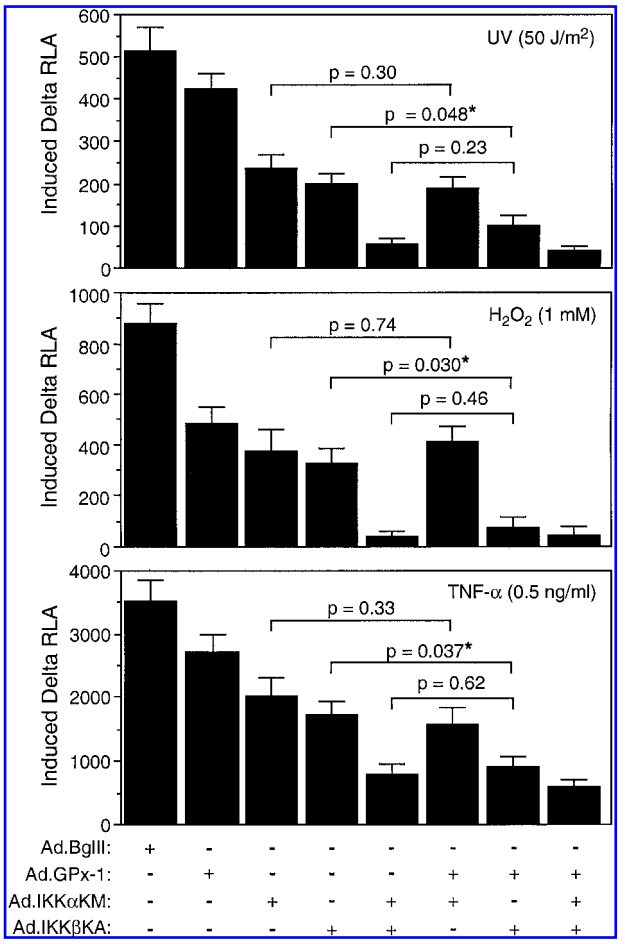

FIG. 8. NFκB activity in MCF-7 cells coexpressing GPx-1 and IKKα(KM) or IKKβ(KA) mutants. MCF-7 cells were coinfected with Ad.GPx-1 and Ad.IKKα(KM) or Ad.IKKβ(KA) at an MOI of 500 particles/cell of each virus. Ad.BglII infection was used as a negative control. At 24 h after infection, each cell sample was then infected with Ad.NFκBLuc at an MOI of 500 particles/cell. Twenty-four hours following the second infection, cell were treated with UV (50 J/m^2), H_2O_2 (1 m*M*), or TNF-α (0.5 ng/ml). Cells were harvested 6 h after treatment with UV (**top panel**), H_2O_2 (**middle panel**), or TNF-α (**bottom panel**), and luciferase activity was determined to quantify NFκB transcription activity. Conditions for infection are indicated below the bottom graph and are the same for all environmental stimuli. Results depict the mean change in relative luciferase activity (Delta RLA) (± SEM, $n \geq 3$) following stimulation with the various agents and are normalized for protein concentration. To calculate the Delta RLA following treatment with UV, H_2O_2, or TNF-α, the background level of luciferase activity (as determined from untreated Ad.BlgII infected cells) was subtracted from all experimentally induced values.

known for its ability to phosphorylate IκBα on Ser^{32} and Ser^{36}. In fact, it is the only pathway responsible for these phosphorylation events that target IκB to the proteasome and promote NFκB activation by facilitating its transport to the nucleus. Hence, if GPx-1 expression did not increase the inhibitory properties of IκBαS32A/S36A, one could safely conclude that effects of GPx-1 expression were likely limited to modulation of the IKK complex. In contrast, if H_2O_2 influences pathways other than IKK, we would expect to observe a synergistic reduction of NFκB activation following combined Ad.IκBαS32A/S36A (22) and Ad.GPx-1 expression in MCF-7 cells. As seen in Fig. 9, combined expression of IκBαS32A/S36A and GPx-1 did not further reduce NFκB activation following H_2O_2, UV, or TNF-α treatments, as compared with expression of IκBαS32A/S36A alone. These data suggest that the predominant influence of H_2O_2 on NFκB activation likely occurs as a result of changes in the activity of the IKK complex. Based on subunit inhibition studies, this interaction more specifically appears to lie at the level of IKKα subunit activation.

DISCUSSION

Intracellular redox environment is increasingly being recognized as an important regulator of cell responses to environmental stimuli. These redox changes can occur directly by modulation of intracellular ROS, such as $O_2^{\cdot -}$, H_2O_2, and ·OH radicals, or indirectly through the modification of redox-sensitive molecules, such as GSH, NADPH, and thioredoxin (16, 49). The induction of intracellular ROS can occur through receptor-mediated mechanisms that induce NADPH oxidases at the membrane, with subsequent production of $O_2^{\cdot -}$, and enzymatic conversion to H_2O_2 by SOD. The biological effects of UV irradiation are, in part, also mediated by ROS. The major ROS formed following UV irradiation is H_2O_2, which is mainly synthesized in the mitochondria (20). In the present study, we have attempted to evaluate the contribution of H_2O_2 in NFκB regulation following exposure of cells to TNF-α and UV irradiation, which likely induce ROS formation by several independent mechanisms. As each of these stimuli likely alters other ROS in addition to H_2O_2, direct comparison with cells treated with exogenous H_2O_2 was used as a more selective strategy for altering intracellular ROS.

Using recombinant adenoviral vectors to

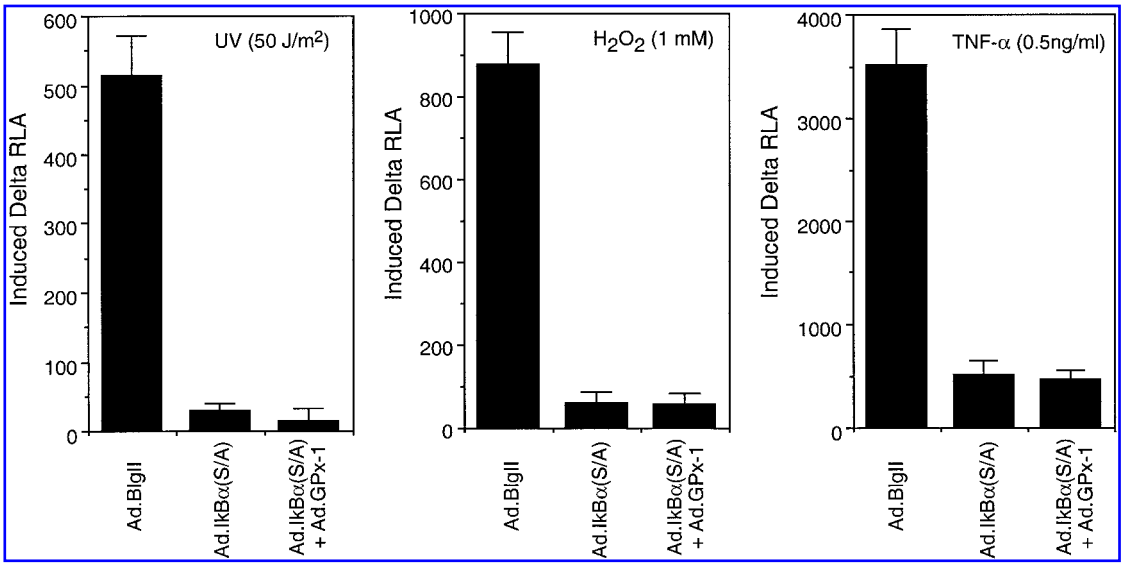

FIG. 9. GPx-1 expression does not enhance the inhibitory properties of the IκBαS32A/S36A serine mutant. MCF-7 cells were infected with Ad.BglII, Ad.GPx-1, and/or Ad.IκBαS32A/S36A expressing a dominant serine mutant of IκBα termed Ad.IκBα(S/A) in the graph. Infections were performed at an MOI of 500 particles/cell of each virus. After 24 h, each cell sample was reinfected with Ad.NFκBLuc at 500 particles/cell. Following another 24 h of incubation, cell samples were treated with UV (50 J/m^2), H_2O_2 (1 m*M*), or TNF-α (0.5 ng/ml). Cells were harvested 6 h after treatments, and luciferase activity was determined. Results depict the mean change in relative luciferase activity (Delta RLA) (± SEM, $n \geq 3$) following stimulation with the various agents and are normalized for protein concentration.

overexpress GPx-1 and thereby modulate intracellular H_2O_2 levels, we have explored the role of H_2O_2 in the activation of NFκB. MCF-7 cells were used as the model system for these experiments, because their low endogenous GPx-1 level allowed for a greater range of manipulation. The results demonstrate that GPx-1 overexpression can attenuate NFκB DNA binding and transcriptional activation following TNF-α, UV, and H_2O_2 treatments. As anticipated, GPx-1 expression exhibited the greatest effect on NFκB activity induced by direct H_2O_2 treatment. These results were expected because both TNF-α and UV irradiation likely induce NFκB through more than one pathway. In support of this notion, GPx-1-mediated alterations in NFκB DNA binding were quite diverse following these three stimuli. For example, following H_2O_2 treatment, p50/p65 heterodimers were most significantly reduced with little change in the level of p50/p50 homodimer. In contrast, following UV and TNF-α treatments, GPx-1-mediated reductions in the level of p50/p50 homodimers were significantly greater than those seen in p50/p65 heterodimers. These findings suggest that the mechanisms for NFκB induction by these three stimuli all involve H_2O_2; however, distinct characteristics in these pathways also exist.

In an effort to investigate further the induction of NFκB by TNF-α, UV, and H_2O_2 treatments, we focused on how alterations in the intracellular H_2O_2 levels might alter the activity of the IKK complex. Through its function in phosphorylating IκB, the IKK complex is recognized as a key mediator of NFκB activation. Using recombinant adenoviruses to overexpress dominant negative mutants of IKKα and IKKβ and selectively block each of the IKK subunits individually, we were able to explore the function of H_2O_2 in the activation of IKK. Hence, coinfection of MCF-7 cells with Ad.GPx-1 and Ad.IKKα(KM) or Ad.IKKβ(KA) allowed for the differential determination of how intracellular H_2O_2 might alter the activity of each of these IKK subunits. Interestingly, this analysis provided a clear delineation of mechanistic similarities in the regulation of NFκB by the three independent environmental stimuli tested. In all cases, GPx-1 overexpression significantly reduced ($p < 0.05$) activation of NFκB only in the presence of IKKβ(KA) expression, but not in the presence of IKKα(KM) expression ($p > 0.3$). These findings suggest

several possibilities for the selective action of H_2O_2 on the IKK complex. First, GPx-1-mediated reductions in intracellular H_2O_2 might enhance the inhibitory properties of IKKβ(KA). However, given the fact that IKKα(KM) and IKKβ(KA) coexpression gave levels of NFκB inhibition that were not significantly different ($p > 0.23$) from coexpression of GPx-1 and IKKβ(KA), our results seem to suggest the alternative hypothesis that GPx-1 expression selectively inhibits the IKKα subunit. If this second hypothesis were true, one would expect that the level of NFκB inhibition following coexpression of GPx-1, IKKα(KM), and IKKβ(KA) would not be significantly different from expression of IKKα(KM) and IKKβ(KA) in the absence of GPx-1. Results from these experiments supported this hypothesis and suggest that indeed GPx-1 expression is likely directly modulating IKKα but not IKKβ activity. Despite these similarities in the mechanism of IKKα regulation following UV, TNF-α, and H_2O_2 treatments, subtle differences in the pathways regulating NFκB likely also exist. For example, following H_2O_2 treatment, NFκB transcriptional activation was nearly completely blocked by inhibiting the IKK complex with either combined expression of both mutant IKK subunits or GPx-1/IKKβ(KA) coexpression. In contrast, residual NFκB activation existed following these same treatment conditions in cells stimulated with TNF-α. These findings suggest that other H_2O_2-insensitive pathways also play a role in TNF-α-stimulated NFκB activity. Furthermore, following UV irradiation, GPx-1/IKKβ(KA) coexpression did not provide maximal inhibition as seen in cells coexpressing IKKα(KM)/IKKβ(KA) or GPx-1/IKKα(KM)/IKKβ(KA). One interpretation is that the action of GPx-1 to inhibit IKKα was slightly less effective following this environmental stimulus. Despite these small differences, the overall similarities in the ability of GPx-1 expression to inhibit NFκB activation in the presence of IKKβ(KA) but not IKKα(KM), suggest that IKKα activity may be directly activated by increased intracellular H_2O_2 levels following UV, TNF-α, and H_2O_2 treatments (Fig. 10).

IKKα and IKKβ are serine/threonine kinases whose functions are to specifically phosphorylate IκBα on Ser^{32} and Ser^{36}, leading to IκBα ubiquitin-dependent degradation, NFκB translocation to the nucleus, and transcriptional activation of NFκB-dependent genes.

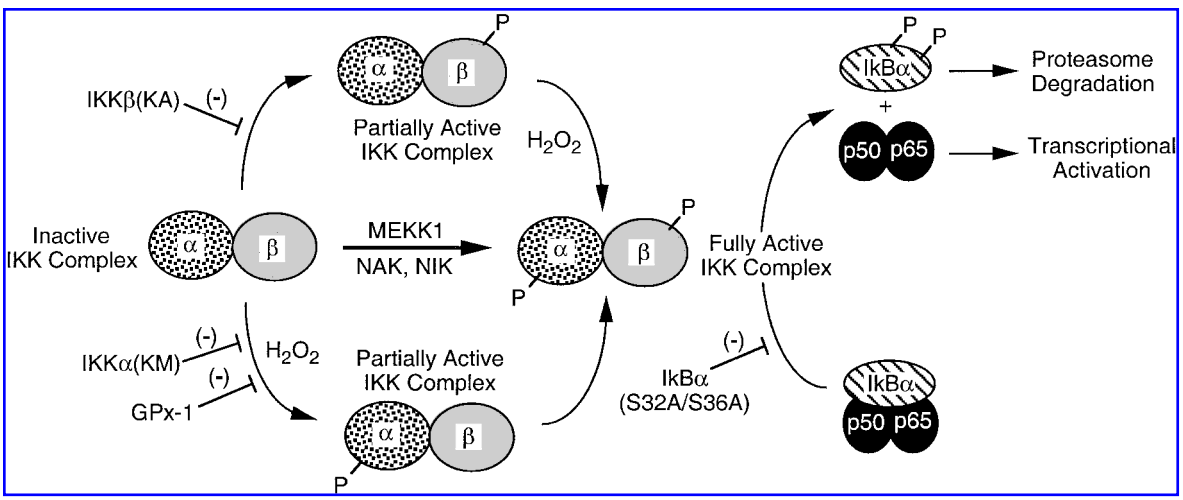

FIG. 10. Schematic summary of GPx-1 regulation of the IKK complex following environmental stimuli. Several phosphorylated forms of the IKK complex can be formed following treatment with environmental stimuli such as UV and TNF-α. These include dual phosphorylated, fully active IKK (both IKKα and IKKβ are phosphorylated), and hemiphosphorylated, partially active IKK complexes (either IKKα or IKKβ is phosphorylated). The enzymes NIK, NAK, and MEKK1 facilitate phosphorylation of the IKK complex by acting on individual IKK subunits. The type of environmental stimulus can influence the specificity of this interaction. Dominant mutants IKKβ(KA), IKKα(KM), and IκBα(S32A/S36A) inhibit induction of NFκB at different points in the activation cascade. Ectopic overexpression of GPx-1 appears to alter NFκB activity by inhibiting the activation of IKKα, but not IKKβ. These results suggest that H_2O_2 plays a selective role in the induction of the IKK complex by activating IKKα.

Our current hypotheses regarding how GPx-1 expression modulates the IKK complex would predict that GPx-1 does not alter the activity of other IKK-independent pathways that modify NFκB activity. Data from coexpression of IKK mutants and/or GPx-1 have suggested that H_2O_2 levels (inferred by responsiveness to GPx-1 expression) primarily influence IKKα activity in the IKK complex. To address conclusively whether GPx-1 acts at the level of IκBα serine phosphorylation by the IKK complex, a series of experiments were performed to evaluate whether GPx-1 expression resulted in increased inhibition of NFκB in the presence of an IκBαS32A/S36A dominant negative mutant. This IκBα serine mutant acts downstream from IKK to inhibit NFκB induction through this pathway. Under all stimuli tested, coexpression of GPx-1 and the IκBαS32A/S36A mutant did not increase the level of NFκB inhibition above that seen in the presence of the IκBαS32A/S36A mutant alone. Such findings conclusively demonstrate that GPx-1 expression does not influence NFκB induction through pathways other than those controlled by serine phosphorylation of IκBα. As seen in earlier studies with the IKK mutants, residual non–GPx-1-sensitive pathways of NFκB induction are primarily evident in TNF-α-treated cells. However, these pathways appear to be independent of IKK and serine phosphorylation of IκBα and represent a minor component of induction following all stimuli.

Although IKKα regulation by GPx-1 infers a selective influence of H_2O_2 on the activity of the IKK complex (Fig. 10), the exact mechanism responsible for this regulation remains unclear. In addition to being kinases themselves, IKKα and IKKβ are also regulated by phosphorylation. Upstream kinases such as NIK, NAK, and MEKK1 play unique roles in the phosphorylation of IKKα and/or IKKβ and can have distinct properties in this activation cascade, depending on the type of environmental stimulus. For example, NIK preferentially phosphorylates IKKα, whereas MEKK1 prefers to phosphorylate IKKβ (42). Although much less is known about potential phosphatases regulating the IKK complex, it is reasonable to hypothesize that specific or nonspecific phosphatases could participate in regulating this complex by dephosphorylating IKKα and/or IKKβ to an inactive state. Our data demonstrating that expression of GPx-1 inhibits activation of the IKKα subunit suggest that H_2O_2 may either activate an IKKα kinase or inactivate an IKKα phosphatase. Alternatively, the regulatory events controlling phosphorylation and the activity of upstream mediators required for activation of IKK kinases might also be influenced by H_2O_2, and hence GPx-1 expression. Previous studies have suggested that H_2O_2 may influence the activity of phosphatases through the oxidation of specific cysteine residues, which alter secondary structure and activity (14). In this regard, H_2O_2 has been shown to inhibit the activity of certain protein tyrosine and protein serine/threonine phosphatases (8, 29). H_2O_2 has also been shown to activate certain kinases (54, 63).

In summary, we have demonstrated for the first time that the action of intracellular H_2O_2 as a major mediator in NFκB activation occurs through the IKKα subunit. This ROS-sensitive pathway appears to play a dominant role in three independent environmental stimuli. The exact site of action of H_2O_2 remains to be determined, but could include either upstream kinases (IKK kinases) or phosphatases (IKK phosphatases) with a selective preference for regulating IKKα. These studies provide insight into the potential therapeutic application of redox-modulating GPx-1 gene therapy vectors aimed at altering NFκB activation.

ACKNOWLEDGMENTS

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ABBREVIATIONS

DTT, dithiothreitol; ECL, enhanced chemiluminescence; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; GPx1, glutathione peroxidase; GSH, glutathione; HA, hemagglutinin; H_2O_2, hydrogen peroxide; HRP, horseradish peroxidase; IKK, IκB kinase;

MEKK1, MAPK/ERK kinase kinase 1; MOI, multiplicity of infection; NAK, NFκB-activating kinase; NFκB, nuclear factor κB; NIK, NFκB-inducing kinase; $O_2^{\cdot -}$, superoxide anion radical; ·OH, hydroxyl radical; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α.

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