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Original Research Communication

Hydrogen Peroxide Activates NFκB and the Interleukin-6 Promoter Through NFκB-Inducing Kinase

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ABSTRACT

Aging is associated not only with oxidant stress, but also with increased interleukin-6 (IL-6) levels. To determine if oxidative stress could contribute to the age-associated increase IL-6 expression, we exposed LNCaP prostate carcinoma cells and HeLa cervical carcinoma cells to H_2O_2 as an oxidant challenge. We found that H_2O_2 induced IL-6 expression through activation of the IL-6 promoter. Furthermore, H_2O_2-induced activation of the promoter was mediated through nuclear factor-κB (NFκB) secondary to H_2O_2-induced phosphorylation and degradation of IκBα. NFκB-inducing kinase (NIK) is upstream of the IκB kinase complex that induces IκBα degradation. Accordingly, we explored if H_2O_2 induces IL-6 expression through NIK. In addition to H_2O_2 inducing NIK autophosphorylation, transfection of LNCaP cells with a dominant negative NIK diminished H_2O_2-mediated NFκB and IL-6 promoter activity. Taken together, these results demonstrate that H_2O_2 induces the IL-6 promoter by activating NFκB through NIK. These data provide a candidate mechanism through which oxidant challenge induces IL-6 gene expression with age. Antioxid. Redox Signal. 3, 493–504.

INTRODUCTION

THE CYTOKINE INTERLEUKIN-6 (IL-6) increases with age in a variety of species (reviewed in 23). IL-6 is associated with many age-related disorders, including Alzheimer's disease, prostate cancer, and osteoporosis. Thus, understanding how IL-6 is regulated with age may provide important therapeutic targets. However, the mechanism responsible for age-associated increase of IL-6 is currently unknown.

One potential mechanism through which IL-6 expression is increased with age is oxidant challenge (25). Reactive oxygen species (ROS) act as secondary messengers to induce signal transduction and activation of several transcription factors, including nuclear factor-κB (NFκB) (1, 2, 20, 49, 50, 55). This has direct relevance to IL-6 expression because NFκB is a potent activator of the IL-6 promoter (38, 48). However, it is unknown if and how oxidant challenge induces IL-6 expression.

NFκB is a homo- or heterodimer of the Rel family of proteins that include p65, RelB, p52, p50, and c-Rel subunits (reviewed in 58). NFκB is sequestered in the cytoplasm by binding to an inhibitory protein, IκBα. Upon cellular exposure to a variety of stimuli, including phorbol esters, cytokines, and viruses, IκBα is phosphorylated, targeting it for ubiquination followed by proteolysis. IκBα then releases NFκB, which translocates to the nucleus, binds its cognate response element and transactivates

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the target gene's promoter. A high-molecular-weight complex, IκB kinase (IKK), is the main mediator of IκBα phosphorylation (8). The IKK complex consists of several proteins, including IKKα (or IKK1), IKKβ (or IKK2), and IKKγ (or NEMO) (11, 12, 29, 39, 60–62). IKK is activated by NFκB-inducing kinase (NIK), a mitogen-activated protein kinase kinase kinase (28, 52).

The goal of the present study was to determine the extent and mechanism of ROS-mediated induction of IL-6 expression using H_2O_2. Our results demonstrate that ROS increase IL-6 expression through NIK-mediated activation of NFκB that induces the IL-6 promoter.

MATERIALS AND METHODS

Plasmids

Luciferase reporter vectors pGL2-basic, pGL2-control, and pGL2-IL-6p(-225) containing no promoter, simian virus 40 (SV40) promoter, or 225 bp of the IL-6 promoter, respectively, have been described (22). The NFκB site of pGL2-IL-6p(-225) was mutated using a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, U.S.A.) as directed by the manufacturer. Primers to create the mutation were IL-6NFKBM-Upper (5′-GATTTATCAAATGCGGAATCCTCCCATGA GTCTCAAT ATTAG-3′) and IL-6NFKBM-Lower (5′-CTAATATTGAGACTCATGGGAGGATTCCGCATTTGATAAATC-3′). Underlines indicate the mutation sites. The resulting plasmid was designated pGL2-IL-6p(-225)-NFκB-mut. The presence of the mutated sequence was confirmed by sequencing. Efficiency of the mutation was confirmed by observing the inability of pGL2-IL-6p(-1200)/(NFκB-M) to respond to cotransfected NFκB p65 and p50 expression vectors, which induce pGL2-IL6p(-1200) (data not shown). The reporter vector NFκB2x-lux, which contains two NFκB response elements in tandem with a thymidine kinase minimal promoter driving the luciferase cDNA, has been described (36) and was generously provided by Dr. S. Ghosh (National Institutes of Health, Bethesda, MD, U.S.A.). The NIK wild-type and dominant negative expression vectors have been described (28) and were generously provided by Dr. D. Wallach (Weizmann Institute of Science, Rehovot, Israel). The IκBαS32G and IκBαS36A expression vectors have been described (6) and were kindly provided by Dr. U. Siebenlist (National Institutes of Health). The FLAG epitope-tagged wild-type and kinase inactive mutant NIK plasmids, FLAG-NIK and FLAG-NIK(KA), respectively, have been described (26) and were kindly provided by Dr. David Goeddel (Tularik, Inc., San Francisco, CA, U.S.A.).

Cell culture and transfections

LNCaP cells, a human prostate cancer cell line, and HeLa cells, a human cervical carcinoma cell line, were maintained at 37°C, 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Gaithersburg, MD, U.S.A.) supplemented with 10% (vol/vol) fetal bovine serum (FBS; Hyclone, Logan, UT, U.S.A.), 100 IU/ml penicillin, 100 mg/ml streptomycin, and 2 m*M* glutamine (Life Technologies). Cells were transfected using calcium phosphate precipitation as described (22) using a total of 8 μg of indicated plasmids including 1 μg of β-gal expression vector as an internal control. Twenty-four hours after transfection, cells were treated as indicated and then cultured for another 24 h and harvested. Protein extracts were prepared and assessed for luciferase activity as described (22). Luciferase activities were normalized to the internal control β-gal activity and reported as the means ±SE of two separate experiments that each contained duplicates (*i.e.*, four determinations).

Measurement of IL-6 protein concentration

After treatment of cells with H_2O_2, cell supernatants were collected and frozen at −80°C until assayed. IL-6 concentration was measured in the supernatants by enzyme-linked immunosorbent assay (ELISA; Quantikine Immunoassay Kit, R&D Systems, Minneapolis, MN, U.S.A.) according to the manufacturer's directions. The ELISA demonstrated a <5% coefficent of variation for intraassay precision.

Quantification of IL-6 mRNA levels

After treatment with H_2O_2, total RNA was extracted using the guanidinium thiocyanate

method (10). The total RNA was reverse-transcribed and subjected to polymerase chain reaction using primers for IL-6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Nuclear protein extraction

Nuclear protein was obtained as described (22). In brief, HeLa cells were scraped with a rubber policeman into 5 ml of ice-cold phosphate-buffered saline (PBS) and centrifuged at 250 *g* for 10 min at 4°C twice. The resulting cell pellet was resuspended in 5 volumes of buffer A [10 m*M* HEPES (pH 7.9), 10 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.5 m*M* dithiothreitol (DTT), 0.5 m*M* phenylmethylsulfonyl fluoride], incubated on ice for 10 min, centrifuged at 250 *g* for 10 min, resuspended in 3 volumes of buffer A with Nonidet P-40 added to 0.05% (vol/vol). Nuclei were then released by homogenizing the cells with 30 strokes in a tight-fitting Dounce homogenizer. Successful lysis was confirmed by phase-contrast microscopy. Nuclei were pelleted by centrifugation at 250 *g* for 10 min, resuspended in 500 μl of buffer C [5 m*M* HEPES (pH 7.9), 26% glycerol (vol/vol), 1.5 m*M* $MgCl_2$, 300 m*M* NaCl, 0.2 m*M* EDTA, 0.5 m*M* DTT, 0.5 m*M* phenylmethylsulfonyl fluoride] and incubated on ice for 30 m. After incubation, the lysed nuclei were centrifuged at 24,000 *g* for 20 min at 4°C, and the supernatant was snap-frozen in dry ice and ethanol and frozen at −80°C until use. Protein concentration was determined with the bicinchoninic acid protein assay kit (Pierce Biochemicals, Rockford, IL, U.S.A.).

Electrophoretic mobility shift assay (EMSA)

Two oligomers consisting of the sequences 5′-TCGACATGTGGGATTTTCCCATGAC-3′ and 5′-TCGAGTCATGGGAAAATCCCACATG-3′ were annealed to form the NFκB response element present within the IL-6 promoter from −43 to −61 bp (based on numbering system in 37). The probe was ^{32}P-end-labeled with T4 kinase and purified on a Chromaspin 10 column (Clontech, Palo Alto, CA, U.S.A.). Probe (10,000 cpm) was incubated with 10 μg of LNCaP nuclear extract in EMSA binding buffer 1 [10 m*M* HEPES (pH 7.9), 50 m*M* KCl, 0.2 m*M* EDTA, 2.5 m*M* DTT, 10% glycerol (vol/vol), 0.05% NP40 (vol/vol)] for 30 min at room temperature. For competition studies, prior to addition of labeled probe, nuclear protein extracts were incubated for 10 min at room temperature with 100× unlabeled specific probe consisting of the consensus NFκB response element (Promega), or nonspecific probe consisting of consensus Oct 1 response element (Promega). Protein/DNA complexes, without the addition of loading dye, were resolved on a 7% nondenaturing polyacrylamide gel (37.5:1 acrylamide/bisacrylamide) run in TG buffer (5 m*M* Tris, 38 m*M* glycine). The gels were then subjected to autoradiography at −80°C for 1–4 h. The autoradiographs were then scanned into computer files and band densities were quantified using NIH Image software (version 3.2). Gel images adjacent to each band were used to quantify background, which was subtracted from the density of each individual band.

Immunoblot

Cells were plated at 2×10^6 cells/well in six-well plates and allowed to adhere over 24 h. Cells were treated with H_2O_2 as indicated, medium was aspirated, and cells were washed with PBS. Cells were then lysed with 100 μl of sodium dodecyl sulfate (SDS) sample buffer [62.5 m*M* Tris-HCl (pH 6.8), 2% (wt/vol) SDS, 10% glycerol, 50 m*M* DTT, and 0.1% wt/vol bromophenol blue] and scraped into a microfuge tube. Cell extract was then sonicated for 15 s, heated at 95°C for 5 min, cooled on ice, and microcentrifuged at 12,000 rpm for 5 min. Subsequently, 20 μl of the extract was loaded onto a SDS-polyacrylamide gel electrophoresis (PAGE) gel, subjected to electrophoresis, and electrotransferred to a nitrocellulose membrane. The membrane was probed for the Ser^{32}-phosphorylated form of IκBα, stripped, and reprobed for total IκBα using the PhosphoPlus IκBα (Ser32) Antibody Kit (New England Biolabs Inc., Beverly, MA, U.S.A.) as directed by the manufacturer. Density of bands was determined by densitometry.

Immunoprecipitation and in vitro kinase assay

LNCaP cells were transiently transfected, as described above, with FLAG-NIK or FLAG-

NIK(KA). After 24 h, the cells were treated with H_2O_2 (100 μM) for 30 min, followed by washing in cold PBS for immunoprecipitation and *in vitro* kinase activity as previously described (26). In brief, the cells were lysed in Nonidet P-40 lysis buffer [50 m*M* HEPES (pH 7.6), 250 m*M* NaCl, 10% glycerol, 1 m*M* EDTA, 0.1% Nonidet P-40, and Complete protease inhibitor (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.)]. Cell lysates were cleared and incubated for 4 h at 4°C with anti-FLAG M2 antibody resin (Sigma), washed three times with lysis buffer, and eluted with FLAG peptide (300 μg/ml; Sigma). The precipitated/eluted NIK and NIK(KA) were then evaluated for autophosphorylation activity using an *in vitro* kinase assay in 20 μl of kinase buffer {20 m*M* Tris-HCl (pH 7.6), 10 m*M* $MgCl_2$, 0.5 m*M* DTT, 100 μM ATP, and 5 μCi of [γ-^{32}P]ATP} at room temperature for 30 min. Samples were analyzed by 10% SDS-PAGE and autoradiography. Immunoblot to evaluate for loading was performed as described above, using rabbit polyclonal anti-FLAG and alkaline phosphatase-conjugated goat anti-rabbit secondary antibody.

Statistical analysis

Data were analyzed, as indicated in figure legends, using either Student's *t* test for paired comparisons or analysis of variance followed by Fisher's least significant difference for post-hoc analysis. Statistical significance was determined as $p \leq 0.05$.

RESULTS

H_2O_2 has minimal effect on cell viability

To ensure that the doses of H_2O_2 used in the experiments did not induce a generalized cytotoxic effect, we assessed LNCaP cell viability upon exposure to H_2O_2. Cell viability was >80% at 100 μM H_2O_2 (Fig. 1).

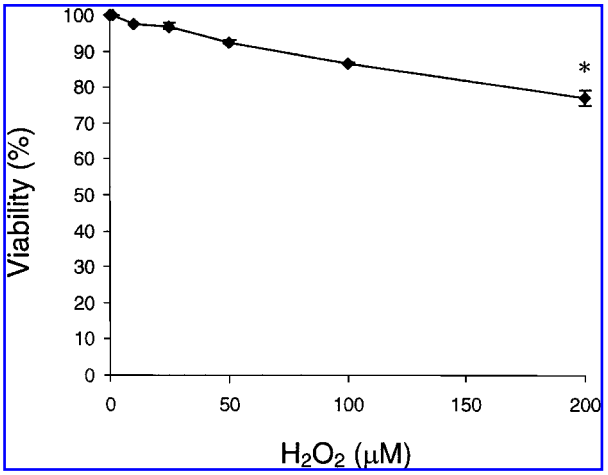

FIG. 1. Low doses of H_2O_2 do not affect viability of LNCaP and HeLa cells. LNCaP cells were plated at 1×10^6/cells/ml (2 ml) in 60-mm plates in DMEM, and then subjected to H_2O_2 at the indicated concentrations. Subsequently, cells were harvested at the indicated times and viability was assessed by trypan blue. Results are expressed as the mean percent viability of controls. *$p < 0.05$ compared with H_2O_2 at 0 m*M*.

H_2O_2 induces IL-6 protein and mRNA expression

To determine if oxidant challenge induces IL-6 expression, LNCaP cells were treated with 100 μM H_2O_2, and supernatants were collected at the indicated times after H_2O_2 treatment and evaluated for IL-6 levels. Cells were also harvested at 12 h after H_2O_2 treatment for determination of IL-6 mRNA levels. By 48 h, H_2O_2 induced a twofold increase of IL-6 protein expression from LNCaP cells (Fig. 2A). This was accompanied by increased IL-6 mRNA levels (Fig. 2B). Thus, H_2O_2 induces IL-6 protein that is accompanied by increased steady-state levels of IL-6 mRNA.

H_2O_2 activates the IL-6 promoter

The increased steady-state IL-6 mRNA levels observed in the above experiment could be due to either increased mRNA transcription or decreased mRNA degradation. To determine if altered transcription plays a role in H_2O_2-mediated increase of IL-6 mRNA levels, LNCaP cells were cotransfected with β-gal and either an IL-6 promoter-driven luciferase reporter plasmid [pGL2-IL-6p(-225)], a promoterless plasmid (pGL2-basic), or an SV40 promoter-driven control luciferase promoter plasmid (pGL2-control). The cells were then exposed to the indicated concentrations of H_2O_2 for 30 min followed by incubation with fresh medium. The cell extract was collected 24 h later for mea-

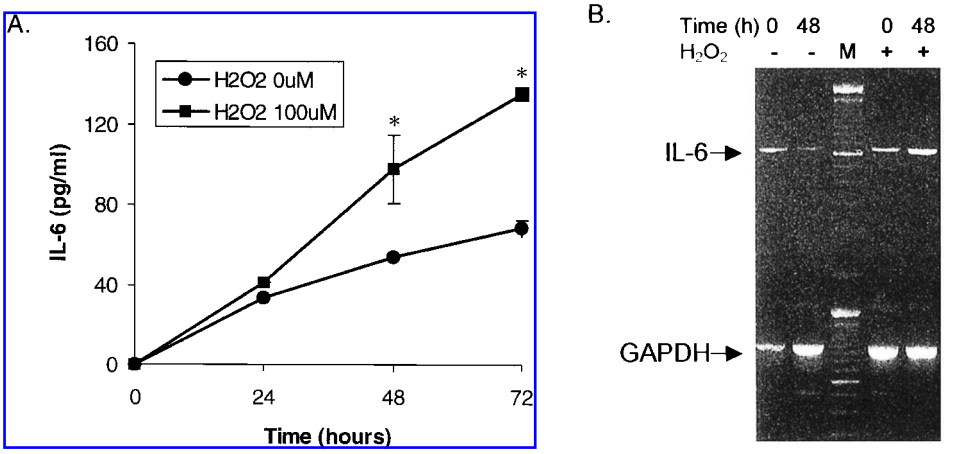

FIG. 2. H_2O_2 induces IL-6 protein and mRNA expression in LNCaP cells. LNCaP cells were plated at 1×10^5/cells/well in 96-well microtiter plates for protein evaluation or at 1×10^6 cells/plate in 60-mm plates for mRNA evaluation in DMEM. The cells were then subjected to 100 μM H_2O_2 in serum-free DMEM for 30 min, followed by replacement with 10% FBS-replete DMEM. (**A**) Supernatants were collected at the indicated times and evaluated for IL-6 protein levels by ELISA. Results are reported as mean ± SEM levels of IL-6. (**B**) Total RNA was collected and subjected to PCR analysis using primers for IL-6 and GAPDH. *$p < 0.05$ vs. H_2O_2 at 0 μM for the same time point.

surement of luciferase activity. H_2O_2 induced a dose-dependent increase of IL-6 promoter activity (Fig. 3). Furthermore, H_2O_2 did not alter luciferase levels in the cells transfected with the pGL2-control plasmid that is driven by the SV40 promoter. Taken together, these data demonstrate that H_2O_2 leads to activation of the IL-6 promoter with some specificity.

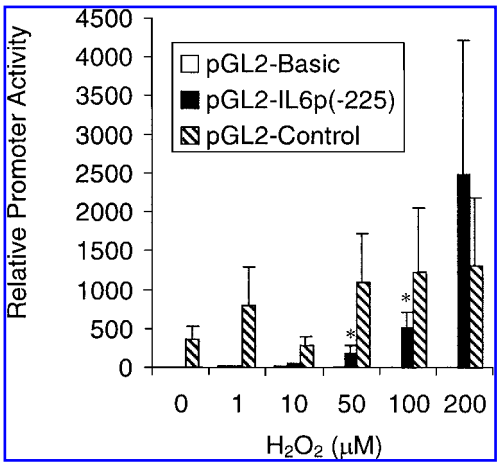

FIG. 3. H_2O_2 activates the IL-6 promoter in HeLa cells. LNCaP cells were cotransfected with β-gal and a promoterless control luciferase plasmid (basic), a constitutively active promoter plasmid (control), or a plasmid driven by the IL-6 promoter (−225). Cells (1×10^6/cells/ml) were cultured in the indicated concentrations of H_2O_2 in serum-free DMEM for 30 min, followed by 10% FBS-replete DMEM for 24 h. Cell extracts were then collected and assayed for luciferase and β-gal. Results were normalized to β-gal activity and are reported as mean ± SE levels of luciferase relative to the pGL2-IL-6p(-225) at 0 μM H_2O_2.

H_2O_2 activates NFκB

To test the possibility that H_2O_2 activates IL-6 promoter activity through NFκB, we assessed the ability of low micromolar H_2O_2 levels to activate NFκB. We identified that treatment of HeLa cells with 25 μM H_2O_2 induced NFκB nuclear translocation (Fig. 4A). The bandshift activity was competed away with 100× excess unlabeled NFκB probe.

Nuclear translocation of a transcription factor does not necessarily mean that the transcription factor will be active. Thus, to confirm that H_2O_2 induced NFκB activity, we evaluated the ability of H_2O_2 to activate a minimal promoter luciferase vector containing two tandem NFκB response element sites (NFκB2x-lux). We found that 100 μM H_2O_2 induced luciferase levels similar to those induced by tumor necrosis factor (TNF) (Fig. 4B). These data confirm that H_2O_2 can activate functional NFκB activity.

H_2O_2 activates the IL-6 promoter through activation of NFκB

The observation that H_2O_2 activates NFκB was consistent with the hypothesis that H_2O_2 activates the IL-6 promoter through NFκB. To provide direct evidence of this postulation, we

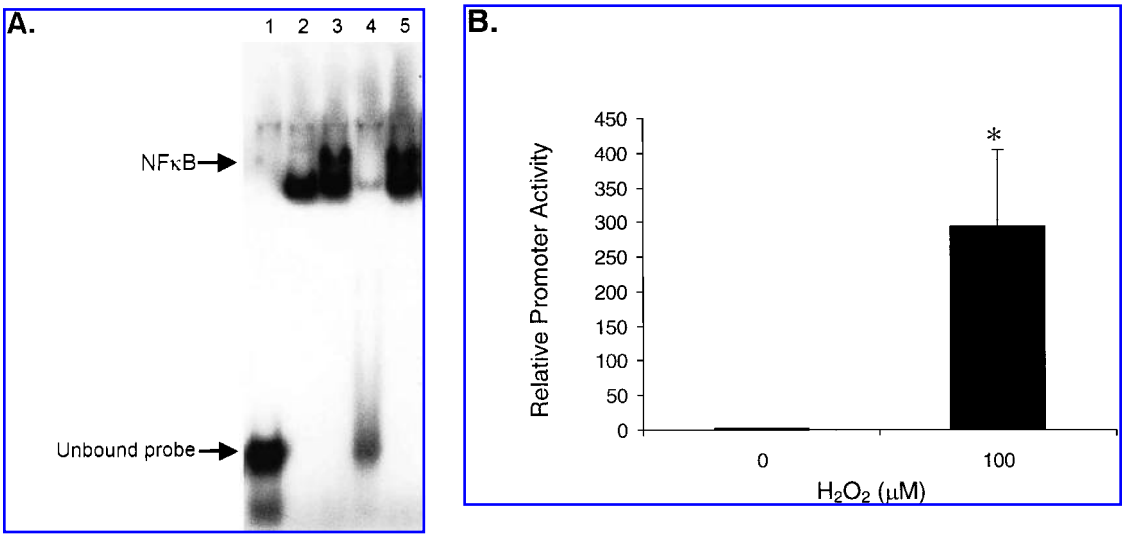

FIG. 4. H_2O_2 induces NFκB nuclear translocation and activity. (**A**) HeLa cells (1×10^6/cells/ml) were untreated or treated with H_2O_2 (25 μM) for 15 min followed by collection of nuclear extract. Ten micrograms of nuclear protein was subjected to EMSA using a ^{32}P-labeled oligomer consisting of the NFκB response element in the IL-6 promoter. The reaction was subjected to electrophoresis on a 7% nondenaturing polyacrylamide gel. The resulting gel was autoradiographed overnight at −80°C. Reactions were also performed with 100× excess unlabeled NFκB probe or nonspecific (TFIID) probe to demonstrate specificity. Lane 1, probe alone; lane 2, untreated cells; lane 3, H_2O_2-treated cells; lane 4, H_2O_2-treated cells plus 100× excess unlabeled NFκB probe; lane 5, H_2O_2-treated cells plus 100× excess unlabeled TFIID probe. (**B**) LNCaP cells were transfected with a minimal promoter luciferase vector without (Lux) or with two NFκB response elements (NFκBx2-Lux). The cells were then treated with H_2O_2 (100 μM) or TNF (3 ng/ml) for 30 min. Total cell extract was then harvested 24 h later and assayed for luciferase levels, which were normalized to β-galactosidase levels. Results are reported as means ± SEM. *$p < 0.05$ vs. H_2O_2 at 0 μM.

transfected LNCaP cells with an IL-6 promoter containing a nonfunctional NFκB site. We first demonstrated that the p65 subunit of NFκB induced the wild-type, but not the mutated, IL-6 promoter (Fig. 5), confirming that the mutated promoter lost its ability to respond to NFκB. We next observed that H_2O_2-mediated activation of the mutated construct was diminished by ~70% compared with that of the intact IL-6 promoter (Fig. 5). These results suggest that NFκB is responsible for a large component of H_2O_2-mediated activation of the IL-6 promoter. However, as the inhibition was not complete, these data also suggest that some other transcription factor contributes to H_2O_2-mediated activation of the IL-6 promoter.

H_2O_2 induces phosphorylation of IκBα

NFκB is activated secondary to phosphorylation and degradation of IκB. Thus, if oxidant challenge induces the IL-6 promoter through activation of NFκB, then IκB should be phosphorylated secondary to oxidant challenge. Accordingly, we evaluated the effect of oxidant challenge on IκBα phosphorylation. We found that H_2O_2 induced phosphorylation of IκBα (Fig. 6A) and rapidly increased the ratio of phosphorylated to unphosphorylated IκBα (Fig. 6B). We have observed similar results in

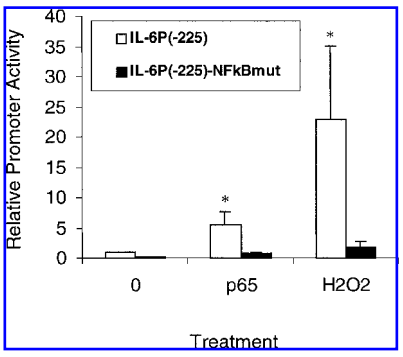

FIG. 5. H_2O_2 activates the IL-6 promoter through the promoter's *s*-acting NFκB response element. LNCaP cells (1×10^6/cells/ml) were cotransfected with pGL2-IL-6p(-225) or pGL2-IL-6p(-225)-NFκB-mut and β-gal. Some cells were also transfected with a p65 expression plasmid or treated with 100 μM H_2O_2 for 30 min, followed by addition of 10% FBS-replete DMEM. Cell extracts were collected 24 h after treatment and assayed for luciferase and β-gal. Results were normalized to β-gal activity and are reported as means ± SEM of luciferase relative to the pGL2-IL-6p(-225) with no treatment. *$p < 0.05$ vs. IL-6p(−225)-NFκB-mut for each treatment.

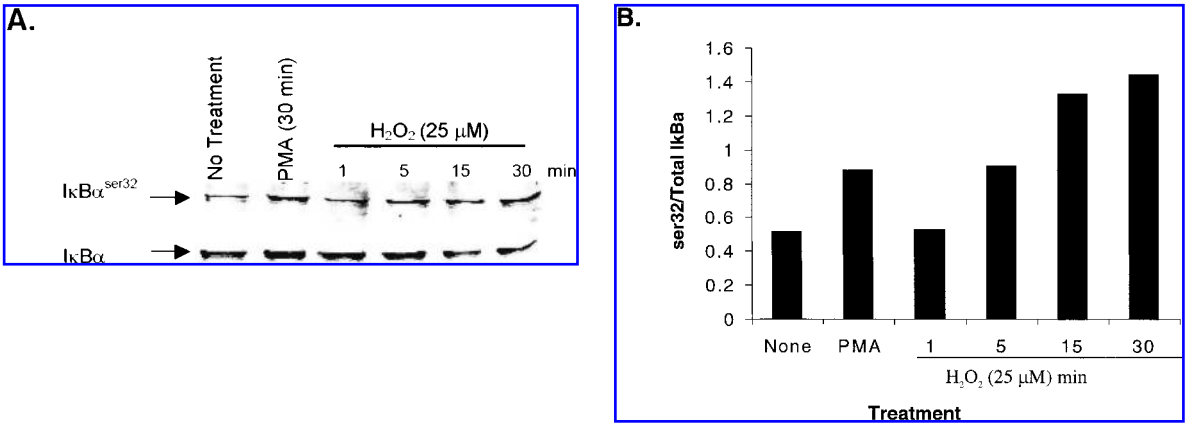

FIG. 6. H_2O_2 induces IκBα phosphorylation. HeLa cells were incubated without treatment, with phorbol myristate acetate (PMA; 160 n*M*) for 30 min, or with H_2O_2 (25 μM) for the indicated times followed by collection of total cell protein. The protein (20 μg) was subjected to immunoblot using an antibody specific for the Ser^{32} phosphorylated form of IκBα. The blot was then stripped and reprobed for total IκBα. Results were then analyzed by densitometry. (A) Blot. (**B**) Densitometry analysis.

rhesus monkey peripheral blood mononuclear cells (data not shown). To confirm these findings, we transfected LNCaP cells with either an IκBαS32G or an IκBαS36A expression vector. These vectors overexpress IκBα that is mutated at Ser^{32} or Ser^{36}. These sites are critical for phosphorylation and subsequent degradation of IκBα. Thus, these mutated proteins sequester NFκB in the cytoplasm even in the presence of stimuli that induce phosphorylation of IκBα. Expression of either mutant IκBα completely abrogated H_2O_2-mediated activation of the IL-6 promoter (Fig. 7). These findings are consistent with the postulation that H_2O_2-mediated activation of NFκB requires phosphorylation of IκBα.

H_2O_2 activates NFκB and the IL-6 promoter through NIK

NIK is upstream of the IKK, and thus it may participate in the pathway through which oxidant challenge induces IκBα phosphorylation and NFκB activation. To determine if H_2O_2 activates NFκB through NIK, we first determined if H_2O_2 promoted NIK's ability to autophosphorylate. Accordingly, we transiently expressed FLAG epitope-tagged NIK or its kinase inactive mutant [NIK(KA)], in LNCaP cells, and then exposed the cells to H_2O_2 (100 μM) for 30 min, followed by immunoprecipitation with an anti-FLAG antibody and incubation with [γ-^{32}P]ATP. NIK had basal kinase activity compared with the NIK(KA) (Fig. 8A). H_2O_2 stimulated autophosphorylation of NIK, but not NIK(KA) (Fig. 8A), which demonstrates that an oxidant challenge induces NIK activity. To confirm that H_2O_2 activates NFκB and induces IL-6 gene expression through NIK, we inhibited NIK activity in HeLa cells by overexpressing a dominant negative form of NIK. NIK alone induced >50-fold activation of the

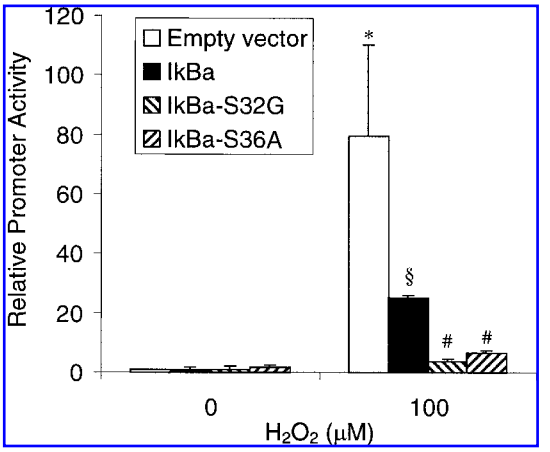

FIG. 7. H_2O_2 activates the IL-6 promoter through phosphorylation of IκBα. LNCaP cells (1×10^6/ml) were cotransfected with pGL2-IL-6p(-225), β-gal, and either empty vector, IκBα, IκBα-S32G, or IκBα-S36G expression vectors. Cells were then treated with 100 μM H_2O_2 in serum-free DMEM for 30 min, followed by replacement of medium with 10% FBS-replete DMEM. Cell extracts were collected 24 h after treatment and assayed for luciferase and β-gal. Results were normalized to β-gal activity and are reported as mean ± SE levels of luciferase relative to the pGL2-IL-6p(-225) with no treatment. $^*p < 0.001$ vs. H_2O_2 at 0 μM; $^§p < 0.01$ vs. H_2O_2 at 0 μM; $^#p < 0.01$ vs. IκBα at H_2O_2 (100 μM).

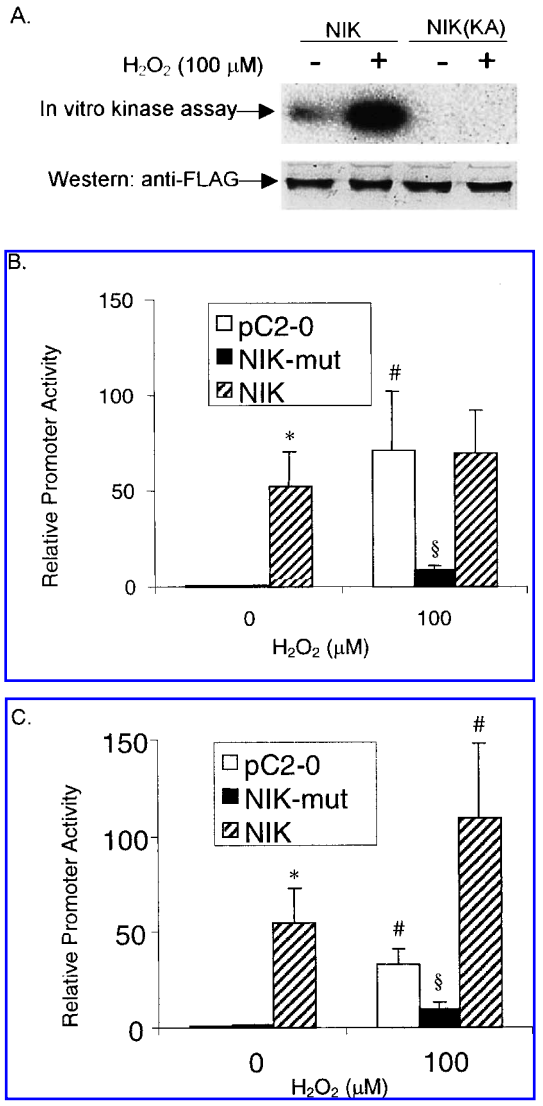

FIG. 8. H_2O_2 activates the NIK and the NFκB and the IL-6 promoter through NIK. (**A**) LNCaP cells were transfected with FLAG epitope-tagged wild-type or KA mutant of NIK. Twenty-four hours after transfection, cells were exposed to H_2O_2 (100 μM) for 30 min, and then extracts were immunoprecipitated with anti-FLAG monoclonal antibody affinity resin and eluted with FLAG peptide. Precipitated NIK was then incubated with [γ-^{32}P]ATP, resolved by SDS-PAGE, and analyzed by autoradiography. Immunoblot using anti-FLAG polyclonal antibodies was performed to determine equal loading. (**B**) NFκBx2-lux or (**C**) pGL2-IL-6p(-225) was used to cotransfect LNCaP cells with either empty vector (pC2-0) or expression vector for NIK or dominant negative NIK, and a β-gal expression vector. Cells were then treated with 100 μM H_2O_2 in serum-free DMEM for 30 min, followed by replacement of medium with 10% FBS-replete DMEM. Cell extracts were collected 24 hours after treatment and assayed for luciferase and β-gal. Results were normalized to β-gal activity and are reported as mean ± SEM levels of luciferase relative to the reporter vector activity with pC2-0 and without H_2O_2. $^*p < 0.05$ vs. pC2-0 at H_2O_2 (0 μM); $^\#p < 0.05$ vs. H_2O_2 at 0 μM; $^\ddagger p < 0.05$ vs. pC2-0 and NIK at H_2O_2 (100 μM).

NFκB2x-lux construct (Fig. 8B), confirming that NIK induces NFκB activity. H_2O_2 induced approximately a 70-fold induction of NFκB2x-luc that was repressed by ~85% in the presence of the dominant negative NIK (Fig. 8B). We next assessed if inhibition of NIK activity would diminish IL-6 promoter activation. NIK alone induced approximately a 50-fold activation of the pGL2-IL-6p(-225) construct (Fig. 8C). H_2O_2 induced approximately a 30-fold activation of pGL2-IL-6p(-225) that was inhibited by ~60% in the presence of the dominant negative NIK (Fig. 8C). Furthermore, the combination of H_2O_2 and NIK was additive, inducing approximately a 100-fold activation of pGL2-IL-6p(-225), which was twofold higher than NIK alone and threefold higher than H_2O_2 alone (Fig. 8C). This additive effect was not observed for the combination of H_2O_2 on NFκB2x-lux (Fig. 8B). These data demonstrate that H_2O_2 induces NFκB and IL-6 promoter activation through NIK.

DISCUSSION

Oxidant challenge-mediated activation of NFκB is a well recognized phenomenon. However, the level at which oxidant challenge acts in the signaling pathway leading to NFκB activation and cytokine expression has not been previously delineated. The current study demonstrates that H_2O_2 induces IL-6 protein and mRNA expression through activation of the IL-6 promoter. Furthermore, it demonstrates that oxidant challenge mediates these events through NIK-induced IκBα phosphorylation and NFκB activation.

A variety of ROS activate transcription factors, including NFκB (30, 33, 40, 45, 54). Furthermore, it has been demonstrated that ROS or prooxidant stimuli induce expression of a variety of proteins (9, 14, 57), including IL-6 (13). However, the authors believe this report to be the first documentation that connects together oxidant challenge-mediated stimulation of protein kinase activity leading to transcription factor activation and subsequent cytokine gene transcription.

NIK is the common point through which the interleukin-1 and TNF pathways mediate

NFκB activation (28). Previous reports have documented that kinase-inactive mutants of NIK suppress NFκB activation mediated by interleukin-1, TNF, and TNF receptor 1 (TNF-R1) associated proteins (28, 52). Our observation that inhibition of NIK activity diminished H_2O_2-mediated induction of NFκB activity and IL-6 promoter transcription extends these reports and has broad implications. Specifically, TNF activates NFκB through binding to its receptor (TNF-R1) and causing its trimerization (3). Subsequently, several cytoplasmic proteins, including TNF-R1-associated death domain protein (TRADD) (16), TNF-R-associated factors-2 (41) and 6 (7, 18) (TRAF2 and TRAF6), and receptor-interacting protein (RIP) (17, 53) are recruited to the TNF-R intracellular domain. NIK is recruited to this complex and activates the IKK kinases (31), which ultimately promotes degradation of IκB and NFκB activation. Intriguingly, TNF promotes ROS production through disturbance of mitochondrial electron transport (46, 47). Furthermore, TNF-mediated activation of NFκB can be blocked by antioxidants, suggesting that TNF activity is dependent on generation of oxidant challenge (15, 19, 43). However, the point at which oxidant challenge plays a role in the TNF signal transduction pathway leading to NFκB activation is currently unknown. It has been recently demonstrated that thioredoxin inhibits NFκB activation induced by overexpression of TRAF-2, TRAF-5 and TRAF 6, but not NIK (56), which suggests that ROS induce NFκB upstream of NIK. This is consistent with our observation that ROS require NIK to activate NFκB.

The observations of the current report that H_2O_2 induces NFκB DNA nuclear translocation and function are consistent with previous reports (33, 45, 59). This finding suggests that H_2O_2 can induce many other genes dependent on NFκB activity, which was not assessed in the current study. Our observation that H_2O_2 did not induce the SV40 promoter suggests that oxidant challenge has a degree of specificity for IL-6 promoter. These results are consistent with the report that H_2O_2 did not induce transcription from the TNF promoter in human T cells (27).

Although we and others have observed H_2O_2-mediated activation of NFκB in a variety of cell lines, some investigators could not identify a response in some cell lines (4, 5). This suggests that there is cell specificity in the response. One possible mechanism accounting for the cell specificity is differences among cells in intracellular calcium metabolism. Specifically, it has been shown that accumulation of proteins in the endoplasmic reticulum induces intracellular calcium release, which induces ROS formation and NFκB activation (34, 35). The current report did not evaluate intracellular calcium metabolism; thus, it is currently unknown if this affected NFκB activation in our model.

Many stimuli induce degradation of IκBα through mediating its phosphorylation at Ser^{32} and Ser^{36} (6). In many reports, oxidant challenge has been postulated to induce phosphorylation and subsequent degradation of IκBα (59). However, there are few reports that demonstrate phosphorylation by IκBα by oxidant challenge or its prevention by antioxidants. In one case, Cu^{2+} was found to inhibit TNF-mediated phosphorylation of IκBα (42). In another report, overexpression of the antioxidant glutathione peroxidase abolished TNF-mediated accumulation of phosphorylated IκBα (24). These reports were based on the observation of increased molecular weight of the IκBα complex on immunoblot, which is postulated to be due to phosphorylation of the IκBα complex. Our observation of H_2O_2-mediated IκBα phosphorylation at Ser^{32} by using an antibody specific for this moiety further documents that ability of an oxidant to phosphorylate IκBα. An antibody for the phosphorylated form of Ser^{36} is not available, and thus could not be assessed.

The observation that the dominant negative NIK inhibited H_2O_2-induced NFκB activity by 85%, but IL-6 promoter activity by 60%, suggests that H_2O_2 induces the IL-6 promoter through other mechanisms in addition to NFκB. This postulation is further supported by the observation that NIK and H_2O_2 had a synergistic effect on IL-6 promoter activation. We did not evaluate for activity of other transcription factors in the current work, but these data suggest that such explorations would be informative.

In summary, H_2O_2-mediated induction of the IL-6 promoter occurs through NIK, result-

ing in activation of the IκBα phosphorylation and subsequent activation of NFκB. Although not explored in the current study, based on the observations that ROS production increases with age in a divergent variety of species from flies (51) and rats (32, 44) to humans (21), it is plausible that this mechanism of gene activation contributes to the modulation of gene expression with age.

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ABBREVIATIONS

DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; ELISA, enzyme-linked immunoassay; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IKK, IκB kinase; IL-6, interleukin-6; NFκB, nuclear factor-κB; NIK, NFκB-inducing kinase; NIK(KA), lysine to alanine mutant of NIK; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SV40, simian virus 40; TNF, tumor necrosis factor; TNF-R1, TNF receptor 1; TRAF, TNF receptor-associated factor.

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