Cytokine-induced Activation of Nuclear Factor-κB Is Inhibited by Hydrogen Peroxide through Oxidative Inactivation of IκB Kinase

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Oxidative stress plays a role in the pathogenesis of many inflammatory diseases. For instance, diseases of the lung such as asthma or chronic obstructive pulmonary disease, are accompanied by the presence of oxidants (1–3). Reactive oxygen and nitrogen species are capable of causing oxidative damage to macromolecules including lipid peroxidation, DNA damage, and protein modifications such as oxidation of cysteine residues (4, 5). After exposure, the cellular response to oxidants depends on the induction of signaling cascades and activation of transcription factors like nuclear factor-κB (NF-κB) and activating protein-1.

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The transcription factor NF-κB is known to be responsive to oxidative stress (6, 7). NF-κB plays a pivotal role in the development of chronic inflammation, cell survival, and proliferation (8–10). In unstimulated cells, NF-κB is sequestered in the cytoplasm through interaction with IκB inhibitory proteins. Exposure to a number of stimuli (e.g. cytokines, lipopolysaccharide, and viruses) will result in the phosphorylation, ubiquitination, and degradation of IκB, liberating the NF-κB dimers (7–9). The NF-κB complex translocates into the nucleus and transcriptionally activates target genes by binding to responsive elements in the DNA called κB motifs. Phosphorylation of the inhibitory protein IκB occurs at specific serine residues (11) by IκB kinases (IKKs) (12).

IKK is present as a large (700–900 kDa) complex composed of two catalytic subunits IKKa and IKKβ and a regulatory subunit, IKKγ (13). The IKK complex serves as a point of convergence for both positive and negative NF-κB regulators (14). Upstream kinases including mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase 1 (MEKK1), NF-κB-inducing kinase, and protein kinase B (Akt) activate the IKK complex through mechanisms not fully understood but result in the phosphorylation of two serine residues in the activation loop of IKKa (serines 176 and 180) and IKKβ (serines 177 and 181) (13–15). This activation occurs rapidly and is followed by a series of autophosphorylation events of serine residues present in the carboxyl-terminal tail of the IKKβ subunit. When a critical number of serine residues are phosphorylated, the complex becomes inactive (13) as was demonstrated by replacement of 9–10 C-terminal serines residues by alanines, which resulted in a complex that was active four times longer (14). Since the IKK complex is an important mediator in the positive and negative regulation of NF-κB, it requires stringent regulation achieved through rapid activation and inactivation.

An alternative activation pathway of NF-κB exists, specifically important for activation by oxidants, including pervanadate, hypoxia/reoxygenation, and UV-C (16), in which the NF-κB complex was liberated upon tyrosine phosphorylation of IκBα (16–20). This alternative pathway involves phosphorylation of tyrosine 42 instead of serines 32 and 36 (17, 21), is in some systems (17) but not in others (21) associated with degradation of IκBα, and does not appear to involve the activation of IKK (22). The interaction between the p85 subunit of phosphatidylinositol 3-kinase (PI3K) and tyrosine-phosphorylated IκBα may serve to dissociate NF-κB from IκBα without degradation (21). Importantly oxidants, including H2O2, are known to activate the PI3K pathway (23, 24). The precise mechanism by which oxidants activate NF-κB remains to be elucidated, but it is becoming clear from the available data that oxidant-induced NF-κB activation is highly cell type-dependent (25), pointing perhaps to a lack of a uniform mechanism of activation (26).

Rapid activation of the IκB kinase (IKK) complex is considered an obligatory step in the activation of nuclear factor-κB (NF-κB) in response to diverse stimuli. Since oxidants have been implicated in the regulation of NF-κB, the focus of the present study was the activation of IKK by tumor necrosis factor α (TNFα) in the presence or absence of hydrogen peroxide (H2O2). Exposure of mouse alveolar epithelial cells to H2O2 was not sufficient to activate IκB, degrade IκBα, or activate NF-κB. In contrast, TNFα induced IKK activity rapidly and transiently resulting in IκBα degradation and NF-κB activation. Importantly, in the presence of H2O2, the ability of TNFα to induce IKK activity was markedly decreased and resulted in prevention of IκBα degradation and NF-κB activation. Neither tyrosine kinases nor phosphatidylinositol 3-kinases, known regulators of NF-κB by oxidants, were involved in IKK inhibition by H2O2. Direct addition of H2O2 to the immunoprecipitated IKK complex inhibited enzyme activity. Inhibition of IKK activity by H2O2 was associated with direct oxidation of cysteine residues present in the IKK complex and occurred only in enzymatically active IKK. In contrast to previously published observations, our findings demonstrate that the oxidant H2O2 reduces NF-κB activation by inhibiting activated IKK activity.
Redox Sensitivity of the Active IKK Complex

During pulmonary inflammatory disease states, epithelial cells are simultaneously exposed to both oxidants and inflammatory cytokines. However, most studies to date have not considered simultaneous exposure of cells to oxidants and cytokines. Furthermore, no studies have investigated the effects of oxidants on IKK activation in lung epithelial cells, an important target for inhaled or inflammatory cell-derived oxidants. Therefore, we investigated the effects of TNFα and H2O2 on IKK activation in lung epithelial cells. Our data demonstrates that in cells treated with TNFα and H2O2, H2O2 by itself does not activate NF-κB in a line of alveolar type II cells. However, H2O2 decreased the ability of TNFα to induce IKK activation, IκBα degradation, and NF-κB activation by direct oxidation of cysteine residues in the activated IKK complex. In contrast to other observations, our studies demonstrate a negative role for H2O2 in the activation of NF-κB by TNFα due to oxidative inactivation of the IKK complex.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Spontaneously transformed alveolar type II cells (C10) were kindly provided by Dr. Alvin Malkinson (27). The details of cultures of the type II cell line (C10) were propagated in high-glucose DMEM (4.5 g/l glucose) containing 10% fetal bovine serum, all from Life Technologies, Inc. Rat alveolar type II epithelial (RLE) cells were cultured as described previously (20). For the experiments, cells were plated onto 60-mm dishes and grown to 70–90% confluency. At least 1 h before adding the test agents the cells were switched to phenol-red-free Dulbecco’s modified Eagle’s medium/F12 containing 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM t-glutamine, and 10% fetal bovine serum, all from Life Technologies, Inc. Rat alveolar type II epithelial (RLE) cells were cultured as described previously (20). For the experiments, cells were plated onto 60-mm dishes and grown to 70–90% confluency. At least 1 h before adding the test agents the cells were switched to phenol-red-free Dulbecco’s modified Eagle’s medium/F12 containing 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% fetal bovine serum (Life Technologies, Inc.). Murine recombinant TNFα was purchased from Calbiochem. Herbycymycin A was obtained from Life Technologies, Inc., LY294002 and H2O2 from Sigma, and glucose oxidase (GOx) from Roche Molecular Biochemicals. N-(Biotinyl)-N’-(iodoacetyl)ethylenediamine (BIAM) was purchased from Molecular Probes (Eugene, OR). LY294002, herbycymycin A, and BIAM were dissolved in dimethyl sulfoxide (Me2SO) at a concentration of 0.1% Me2SO. IκBα, IKKγ, p65, p50, and c-Jun N-terminal kinase (JNK) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), phosphotyrosine, phospho-Akt (pAkt), and Akt antibodies were obtained from Upstate Biotechnology (Lake Placid, NY), the β-actin antibody was obtained from Sigma, and horseradish peroxidase-conjugated streptavidin was purchased from Roche Molecular Biochemicals. All experiments were performed in duplicate and repeated at least three times.

Kinase Assays—C10 cells were exposed to the test agents individually or simultaneously. At times ranging from 5 min to 24 h, cells were transferred to ice, washed once with cold phosphate-buffered saline, and lysed in lysis buffer, pH 6.5 (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 0.5 mM Na3VO4, 0.1% Nonidet P-40, 10 mM Na3VO4, 1 mM DTT, and 0.01% bromphenol blue). Samples were boiled and stored at −20 °C. Proteins were separated on a 15% polyacrylamide gel, and gels were dried and examined by autoradiography. Results were quantitated by densitometry or densitometry.

Western Blot—At selected times following exposure to test agents, cells were washed once with cold phosphate-buffered saline and lysed in 1× Laemmli sample buffer. Samples were boiled, and 50 μl was loaded on a 15% polyacrylamide gel. Proteins were transferred to nitrocellulose (Schleicher & Schuell), and membranes were subsequently blocked in 5% milk in Tris-buffered saline (TBS). pAkt and Akt were detected according to the manufacturer’s instructions. Levels of IκBα and IKKγ were detected according to the following protocol: membranes blocked overnight in TBS/milk were washed two times for 15 min in TBS containing 0.05% Tween 20 and incubated with the primary antibody against IκBα or IKKγ for 1 h at 4 °C. Membranes were washed three times with TBS, washed once with TBS containing 0.05% Tween 20 and VO4, and then incubated with the secondary antibody for 1 h at room temperature. After a 30-min wash with TBS/Tween, conjugated peroxidase was detected by chemiluminescence according to the manufacturer’s instructions (Kirkgaard and Perry Laboratories, Gaithersburg, MD).

Gel Mobility Shift Assays—To determine DNA binding activity of NF-κB, complexes binding to a radiolabeled double-stranded oligonucleotide containing a NF-κB consensus sequence were analyzed (Promega, Madison, WI). Nuclear extracts and gel shift assays were prepared as described previously (28). Four micrograms of nuclear protein was used per binding reaction, and protein-DNA complexes were resolved on a 5% polyacrylamide gel in 0.25× Tris borate–EDTA buffer at 120 V for 2 h. Gels were dried and exposed to film (X-Omat Blue XB-1, Kodak). To determine the subcomposition of the complexes, supershift reactions were performed by preincubation of the nuclear extracts with antibodies specific to the p65 or p50 subunits of NF-κB.

Construction of a C10 Cell Line Stably Expressing NF-κB Luciferase—Plasmid 6 b-tk-luc was kindly provided by Dr. Patrick Baueerle (Micromet, Martinsried, Germany). C10 cells were transfected by electroporation with the murine IkBα expression vector (30) and the expression vector p65-32P-tk-luc containing a NF-κB consensus sequence were analyzed (Promega, Madison, WI). Nuclear extracts and gel shift assays were prepared as described previously (28). Four micrograms of nuclear protein was used per binding reaction, and protein-DNA complexes were resolved on a 5% polyacrylamide gel in 0.25× Tris borate–EDTA buffer at 120 V for 2 h. Gels were dried and exposed to film (X-Omat Blue XB-1, Kodak). To determine the subcomposition of the complexes, supershift reactions were performed by preincubation of the nuclear extracts with antibodies specific to the p65 or p50 subunits of NF-κB.

RESULTS

IKK Activation in Response to TNFα and H2O2—In a number of cell lines TNFα rapidly and transiently activates the IKK

2 Y. M. W. Janssens-Heininger, unpublished observations.
complex, resulting in NF-κB activation and thus NF-κB-regulated gene transcription. In our studies, a rapid increase (2 min) of IKK activity was observed in the C10 cells in which a maximal activity was reached at 5 min that returned toward baseline in 30 min (Fig. 1A). Since H₂O₂ has been described to activate NF-κB in various cell types (6, 7), we determined whether in C10 cells H₂O₂ was able to activate IKK. As is demonstrated in Fig. 1B, H₂O₂ added as a bolus of 200 or 500 μM did not induce IKK activation in a time frame ranging from 5 to 90 min. Examination of time points up to 24 h failed to demonstrate H₂O₂-induced IKK activity.³ We next determined whether continuous production of H₂O₂ by the addition of GOx to the medium would activate IKK. GOx uses glucose in the medium to produce H₂O₂ and when adding 5 units/ml GOx to the medium for 2 h, steady state levels of H₂O₂ of ~500 μM were measured (data not shown) (30). Similar to results obtained with bolus H₂O₂, no increases in the enzymatic activity of IKK were observed in cells treated with GOx (Fig. 1C), and following 90 min of treatment with GOx, the IKK activity was decreased in comparison to the sham controls. Western blots demonstrated an equal presence of IKK indicative of the changes in kinase activity are not due to loading differences or differences in the amounts of immunoprecipitated IKK complex (data not shown). To establish that H₂O₂ acts differently on various signal transduction pathways we examined the effect of bolus H₂O₂ on JNK. In contrast to the lack of effect on IKK activity, the addition of 200 μM H₂O₂ resulted in the activation of JNK (Fig. 1D) in C10 cells, indicating that H₂O₂ acts in a specific fashion on different kinases.

During inflammatory conditions that exist in many disease states, oxidants and cytokines are present simultaneously. We therefore determined whether H₂O₂ could alter the ability of TNFα to induce IKK. While TNFα rapidly activated IKK, the presence of 200 μM H₂O₂ decreased the ability of TNFα to activate IKK (Fig. 2A). Similar results were observed when cells were exposed to TNFα and GOx (Fig. 2B). Like TNFα, interleukin-1β was able to rapidly activate IKK (Fig. 2C). The ability of interleukin-1β to activate IKK was also diminished in the presence of H₂O₂ (Fig. 2C). These results demonstrate that

³ S. H. Korn and Y. M. W. Janssen-Heininger, unpublished observations.

![Fig. 1. Effects of TNFα (10 ng/ml), H₂O₂ (200 and 500 μM), and GOx (5 units/ml) on IKK and JNK activity. A. C10 cells were exposed to TNFα for the indicated times after which cells were lysed. The IKK complex was immunoprecipitated from 200 μg of protein, and kinase assays were performed using the substrate GST-IκBα-(1–54). B. C10 cells were treated with H₂O₂ for variable times after which the kinase activity of the IKK complex was determined. C. C10 cells were exposed to GOx for variable times after which the kinase activity of the IKK complex was determined. D. C10 cells were exposed to H₂O₂ for variable times, and JNK activity was determined using GST-c-Jun as a substrate.](image)

![Fig. 2. Attenuation of TNFα- and interleukin-1β (IL1β) (10 ng/ml)-induced IKK activity by H₂O₂ (200 μM) or GOx (5 units/ml). A. Cells were exposed to TNFα and H₂O₂ simultaneously for 5 min after which proteins were collected, and an IKK assay was performed using the substrate GST-IκBα-(1–54). B. Cells were exposed to TNFα and GOx simultaneously for 5 min, and IKK activity was determined. C. Cells were exposed to interleukin-1β and H₂O₂ simultaneously for 5 min after which proteins were collected, and IKK activity was measured. D. RLE cells were exposed to TNFα and H₂O₂ separately or simultaneously for 5 min after which proteins were collected, and an IKK assay was performed.](image)
IKK activity induced by two different stimuli that activate distinct receptors was altered in a similar fashion by H₂O₂. Since in a previous study (20) an increased NF-κB luciferase activity was observed in RLE cells, IKK activity was determined in RLE cells after exposure to H₂O₂ in the absence or presence of TNFα (Fig. 2D). H₂O₂ did not activate the IKK complex in contrast to TNFα. However, when TNFα and H₂O₂ were added simultaneously, an inhibition of the TNFα-induced IKK activity was observed in the RLE cells as seen in the C10 cells. This finding clearly demonstrates that there is a discrepancy between regulation of IKK activity and NF-κB-dependent transcriptional activity in the RLE cells exposed to H₂O₂ in contrast to a coordinated regulation of NF-κB activity in C10 cells.

**IkBa Degradation, NF-κB Binding to DNA, and NF-κB-induced Luciferase Activity**—Based on the observation that H₂O₂ decreased the ability of TNFα to induce IKK activation at 5 min, we speculated that this would also be reflected in an attenuated degradation of IkBa, binding of NF-κB to DNA, and NF-κB-dependent luciferase reporter gene activity. As demonstrated in Fig. 3A, TNFα decreased the abundance of IkBa after 15 min of exposure, indicative of IkBa degradation. However, in the presence of H₂O₂, the TNFα-induced degradation of IkBa (Fig. 3A) was prevented. These results indicate that the decrease in TNFα-induced IKK activation in the presence of H₂O₂ culminated in a loss of IkBa phosphorylation and degradation. In support of the lack of effects on IKK activity, H₂O₂ by itself did not affect the levels of IkBa. A similar inhibitory effect of H₂O₂ was observed when TNFα-induced NF-κB binding to DNA was investigated. Results in Fig. 3B demonstrate decreased binding of p65/p50 and p50/p50 to the NF-κB-responsive element in cells exposed simultaneously to TNFα and H₂O₂ for 15 min compared with the binding seen with TNFα alone. Antibodies against the p65 and p50 subunits of NF-κB modified the electrophoretic mobility, indicating that the probe bound complexes consisted of p65 and p50 (Fig. 3B). No change in complex composition was observed when H₂O₂ was added to TNFα-exposed cells. To confirm that the reduced binding of the NF-κB complex to DNA that occurred in the presence of H₂O₂ and TNFα was reflected in a decreased transcriptional activation potential, we assessed C10 cells stably transfected with an NF-κB-driven reporter construct 6 h following the addition of TNFα (10 ng/ml) and/or H₂O₂.

**Redox Sensitivity of the Active IKK Complex**

![Figure 3](image)

**FIG. 3.** Attenuation of TNFα (1.0 or 10 ng/ml)-induced IkBa degradation, NF-κB binding to DNA, and NF-κB-regulated luciferase activity by H₂O₂ (200 μM). A, cells were exposed for 5 and 15 min to TNFα (10 ng/ml) and/or H₂O₂ after which a Western blot was performed for IkBa. B, nuclei were isolated from cells exposed for 15 min to TNFα (1.0 ng/ml) and H₂O₂ simultaneously for the evaluation of binding of NF-κB to its consensus DNA sequence using an electrophoretic mobility shift assay. Antibodies (Ab) directed against p65 and p50 verified their presence in the DNA binding complex. C, luciferase activity was determined in C10 cells stably transfected with an NF-κB-driven reporter construct 6 h following the addition of TNFα (10 ng/ml) and/or H₂O₂.

Involvement of the PI3K/Akt Pathway and Tyrosine Kinases—After establishing that H₂O₂ decreased the TNFα-induced IKK activity, we next determined whether the oxidant effect was mediated through a tyrosine kinase or PI3K/Akt signaling pathway, which are known to be induced by oxidants (23, 31) and important in the activation of NF-κB (31, 32). Addition of H₂O₂ to the cells resulted in the phosphorylation of Akt, the downstream kinase of PI3K, within 5 min (Fig. 4A). Preincubation of the cells (30 min) with the PI3K inhibitor LY294002 blocked the H₂O₂-induced phosphorylation of Akt, indicating that this event was regulated by PI3K. We next determined whether LY294002 could reverse the inhibitory effect of H₂O₂ on TNFα-induced IKK activity. As is demonstrated in Fig. 4B, LY294002 did not restore the TNFα-induced IKK activity in the presence of H₂O₂ but instead led to a complete inhibition of the enzymatic activity of IKK. Thus, it appears that the residual TNFα-induced IKK activity observed in the presence of H₂O₂ may be due to the activation of Akt. Although a number of investigators have demonstrated the activation of tyrosine kinases in response to oxidants (17), the addition of H₂O₂ to C10 cells for 5 min did not result in tyrosine phosphorylation of multiple proteins (Fig. 4C). Although tyrosine phosphorylation may occur at later time points following exposure to H₂O₂, these were not investigated since the inhibition of TNFα-induced IKK activity by H₂O₂ occurred as early as 5 min. In support of this observation, the tyrosine kinase inhibitor herbimycin A did not reverse the inhibitory effects of H₂O₂ on TNFα-induced IKK activity (Fig. 4D). Me₂SO (0.1%) was used as a vehicle control and did not affect IKK or NF-κB.
activation. Collectively our data demonstrate that the inhibitory effect of H₂O₂ on the TNFα-induced activation of IKK occurred independently of Akt/PI3K or tyrosine kinase activities.

**In Vitro Kinase Assay with H₂O₂**—To investigate whether the IKK complex is sensitive to oxidation, cells were exposed to TNFα for 5 min, and the active IKK was immunoprecipitated, exposed to H₂O₂, and subjected to kinase activity assays. Since the presence of 500 μM of the reducing agent DTT may buffer the effects of H₂O₂, we performed the kinase assay in the presence of varying concentrations of DTT in the presence or absence of H₂O₂. As demonstrated in Fig. 5A, DTT concentrations of 250 or 500 μM were necessary to achieve maximal kinase activity of IKK (Fig. 5A) following exposure to TNFα. When the concentration of DTT was reduced to 100 μM, a dramatic decrease in kinase activity of IKK was observed. The addition of 200 or 500 μM H₂O₂ to the immunoprecipitated IKK complex resulted in a dose-dependent reduction in kinase activity. The decrease in IKK activity induced by H₂O₂ occurred to the greatest extent in the presence of lower DTT concentrations as expected (Fig. 5A). Importantly the loss in IKK activity in the presence of H₂O₂ was not due to dissociation of IKKγ from the immunoprecipitated complex as demonstrated by the equal presence of IKKγ protein in the immunoprecipitates (Fig. 5B). Western blots for IKKα and IKKβ could not be performed since the corresponding 85- and 87-kDa bands were masked by the heavy chain of the IKKγ antibody used for immunoprecipitation. In summary, our results demonstrate that H₂O₂-induced oxidation of the IKK complex directly abolishes the kinase activity, which may account for the inhibitory effects of H₂O₂ on Akt.

**Western blot analysis examining phosphorylation** (pTyr)-containing proteins following exposure to H₂O₂ in the presence or absence of herbiycin A. β-Actin was determined as a loading control. D. IKK enzymatic activity in cells exposed for 5 min to TNFα and H₂O₂ in the presence or absence of herbiycin A. DMSO, Me₂SO.

30 min by increasing the DTT concentration from 100 to 500 μM. These findings clearly demonstrate that once IKK is oxidized within the cell by H₂O₂, subsequent manipulation of the sample in buffers containing DTT only partially reverses the oxidative inactivation of IKK within the 30-min time frame investigated here.

**Determination of Oxidant-sensitive Cysteine Residues in the IKK Complex**—After establishing that the activity of IKK was directly inhibited by H₂O₂, we determined whether cysteine oxidation occurred following treatment with H₂O₂. As BIAM selectively reacts with reduced cysteine residues, oxidation by H₂O₂ will diminish the binding of streptavidin. Since the reaction of BIAM with reduced cysteine residues is pH-dependent, two different pH levels were studied (pH = 6.5 and pH = 8.5). As demonstrated in Fig. 6A, a 5-min exposure of the immunoprecipitated IKK complex to H₂O₂ resulted in the reduced binding of streptavidin peroxidase, strongly suggesting the oxidation of cysteine residues. Importantly cysteine oxidation was only observed in IKK immunoprecipitates from TNFα-exposed cells but not sham controls. These findings demonstrate that H₂O₂ is capable of causing cysteine oxidation only in enzymatically active IKK. The difference in BIAM-streptavidin reactivity in H₂O₂ versus sham groups was not due to loading differences as demonstrated by the equal amounts of IKKγ (Fig. 6A). Since the IKK complex is sensitive to oxidation and exposure of cells to TNFα has been demonstrated to result in intracellular production of H₂O₂ by analyzing the oxidation of DCF using flow cytometry. As shown in Fig. 6B, no increases in DCF oxidation were observed in cells exposed to 10 ng/ml TNFα for 5 min compared with sham controls. As a positive control, cells exposed to a bolus of H₂O₂ displayed a marked oxidation of DCF. These results demonstrate that the concentration of intracellular H₂O₂ produced in response to TNFα may not be sufficient to cause IKK oxidation.

**DISCUSSION**

The transcription factor NF-κB is an important regulator of immune responses. It is induced by a wide variety of stresses and transcriptionally activates many cytokine and chemokine...
genes, genes important in proliferation, matrix degradation, and prevention of apoptosis. Oxidative stress has been thought to play a critical role in its activation based upon many observations that demonstrate that cytokine-induced NF-κB can be prevented following treatment with antioxidants or metal chelators. The role of oxidants per se in the activation of NF-κB has been the subject of considerable debate (7, 26). It appears that the ability of H₂O₂ to induce NF-κB depends on the cell type being investigated and may be linked to the levels of antioxidants present in those cells (7, 25, 26). However, even within one cell type marked variations in the ability of H₂O₂ to activate NF-κB are apparent. For instance, among investigated T cell lines, Wurzburg T cells but not Jurkat T lymphocytes are responsive to H₂O₂ (for a review, see Ref. 26). In support of this variability, our laboratory demonstrated in a line of RLE cells that H₂O₂ caused activation of an NF-κB-driven luciferase reporter gene (20) in contrast to our present observations obtained in the mouse counterpart, C10 cells. The differences in the response to H₂O₂ between C10 and RLE cells could be due to differences in culture conditions, antioxidant defenses, or time frames that were analyzed. The increases in NF-κB-dependent luciferase activity in RLE cells treated with H₂O₂ occurred without the preceding activation of IKK (Fig. 2D) or the degradation of IκBα (20). This observation is consistent with findings by other groups demonstrating that the PI3K/Akt pathway, induced by oxidants (23, 24), is able to activate NF-κB by dissociating tyrosine 42-phosphorylated IκBα from NF-κB without degrading IκBα (17, 18, 21, 22). Other studies have demonstrated that tyrosine phosphorylation can also prevent signal-induced degradation of IκBα and binding of NF-κB to DNA (35, 36). In contrast to the aforementioned studies, phosphorylation of tyrosine 42 of IκBα induced by H₂O₂ or pervanadate can in fact result in degradation of IκBα (22, 37), illustrating the conflicting nature of the studies published to date. In addition to the regulation of IKK and IκBα by oxidants, oxidants also affect NF-κB-dependent transcription. Phosphorylation of RelA by Akt results in transactivation of NF-κB (38, 39) and thus in NF-κB-regulated gene transcription. Variations in the activation of Akt or other kinases involved in the PI3K/Akt pathway might exist between C10 and RLE cells explaining the discrepancies observed in NF-κB-regulated luciferase activity between rat and mouse. Another explanation for this phenomenon may be the effects oxidants have on the activity of histone deacetylases. For instance, Ito et al. (40) recently demonstrated that oxidants are capable of inactivating histone deacetylase-2, thereby promoting gene transcription. Not all...
histone deacetylases might be equally sensitive to oxidants, and different histone deacetylases might be expressed in different cell lines cells.

The IKK complex is an important point of convergence used by many different stimuli to activate NF-κB. Upstream kinases in the NF-κB pathway that include MEKK1 and NF-κB-inducing kinase phosphorylate IKKα on serines 176 and 180 and IKKβ on serines 177 and 181 in their kinase domain. Mutation of these serine residues to alanines leads to inactivation of the IKK complex and inhibits the activation of NF-κB. Importantly IKK is also an important target for negative regulation of NF-κB (41–46). Of the two catalytic subunits of the IKK complex, IKKβ has the major role in responding to pro-inflammatory stimuli (5, 14) and is sensitive to inactivation by aspirin, salicylate, cyclopentenone prostaglandins, and the thiol-reactive metal arsenite (5, 26, 47). Since no information exists about the direct effects of H₂O₂ on the regulation of IKK, we investigated in the present study whether this oxidant could directly affect the enzymatic activity. In support of previous investigations demonstrating a lack of degradation of IkBα in response to oxidative stress, our present data also reveal a lack of activation of IKK in cells treated with bolus H₂O₂ or H₂O₂ generated continuously by GOx.

Despite the lack of effect of H₂O₂ itself on IKK activity, H₂O₂ affected signal-induced IKK activation by causing a marked decrease in enzyme activity. This effect was independent of Akt or tyrosine kinase activity and was also observed when H₂O₂ was added to immunoprecipitated, active IKK. These results strongly suggest that H₂O₂ is capable of directly inactivating IKK due to the redox sensitivity of the complex. This redox sensitivity was further demonstrated in experiments using BIAM (29), which detects oxidation of cysteine residues. Marked cysteine oxidation of the enzymatically active IKK complex occurred following treatment with H₂O₂. We detected a remarkable difference in cysteine oxidation between sham and TNFα-treated cells exposed to H₂O₂, indicating that only the active IKK complex is sensitive to H₂O₂-induced cysteine oxidation. This explains the lack of inhibition of H₂O₂ on baseline IKK activity as observed in this study.

At present, we do not know the cysteine site(s) of the IKK complex that may be oxidatively modified by H₂O₂. One likely candidate is cysteine 179 in the kinase domain of IKKβ. Recent studies demonstrated that the cyclopentenone prostaglandin 15-deoxy-Δ12,14-prostaglandin J₂ covalently modified cysteine 179 of IKKβ decreasing its enzymatic activity and consequently blocking NF-κB activation (41). Similarly arsenite, which is reactive toward vicinal thiols, binds cysteine 179 of IKKβ, inhibiting enzymatic activity of the IKK complex (43). Consequently a mutant of IKKβ, in which cysteine 179 is replaced with alanine, is refractory to inhibition by arsenite or 15-deoxy-Δ12,14-prostaglandin J₂. Cysteine 179 is located between serines 177 and 181 in the kinase domain. Oxidative modification of this amino acid might alter the conformation of the complex and may prevent upstream kinases from phosphorylating the serines 177 and 181, thereby preventing activation of the complex. Therefore, cysteine 179 of IKKβ also appears to be a likely candidate for oxidation and enzymatic inactivation by H₂O₂.

The decrease in TNFα-induced IKK activity observed in the presence of H₂O₂ translated into a diminished degradation of IkBα, decreased NF-κB binding to DNA, and a decrease in the NF-κB-dependent transcripational activation. These results suggest that under inflammatory conditions where H₂O₂ is formed NF-κB activation could become depressed. The ramifications of this oxidant effect are unclear. The decreased expression of pro-inflammatory mediators may be associated with a dampened inflammatory response in response to proinflammatory stimuli, which could be beneficial to disorders associated with chronic inflammation. Alternatively the decreased ability to activate NF-κB in the presence of H₂O₂ may be associated with an enhancement of apoptosis when inflammatory cytokines are present. We currently are investigating the ramifications to pulmonary epithelium following combined exposures to cytokines and oxidants.

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