It has previously been shown that hyperoxia induces non-apoptotic cell death in cultured lung epithelial cells, whereas hydrogen peroxide (H$_2$O$_2$) and paraquat cause apoptosis. To test whether pathways leading to oxidative apoptosis in epithelial cells are sensitive to molecular O$_2$, A549 cells were exposed to 95% O$_2$ prior to exposure to lethal concentrations of H$_2$O$_2$. The extent of H$_2$O$_2$-induced apoptosis was significantly reduced in cells preexposed to hyperoxia compared with room-air controls. Preexposure of the hyperoxia-resistant HaLa-80 cell line to 80% O$_2$ also inhibited oxidant-induced apoptosis, suggesting that this inhibition is not due to O$_2$ toxicity. Because hyperoxia generates reactive oxygen species and activates the redox-sensitive transcription factor nuclear factor-kB (NF-kB), the role of antioxidant enzymes and NF-kB were examined in this inhibitory process. The onset of inhibition appeared to be directly related to the degradation of Ixk and subsequent activation of NF-kB (either by hyperoxia or TNF-$\alpha$), whereas no significant up-regulation of endogenous antioxidant enzyme activities was found. In addition, suppression of NF-kB activities by transfecting A549 cells with a dominant-negative mutant construct of Ixk significantly augmented the extent of H$_2$O$_2$-induced apoptosis. These data suggest that hyperoxia inhibits oxidant-induced apoptosis and that this inhibition is mediated by NF-kB.

Pulmonary epithelial cell injury is an unfortunate consequence of therapy with supraphysiological concentrations of oxygen (hyperoxia) and a prominent feature of acute inflammatory lung injury (1, 2). Oxidant-induced cell injury and death are generally thought to occur via reactive oxygen species. Although exogenous hydrogen peroxide (H$_2$O$_2$) and paraquat induce apoptosis in cultured alveolar epithelial (A549) cells, hyperoxia kills these cells via a mode of cell death that is distinct from apoptosis both morphologically and biochemically (3–5). These observations raise the possibility that hyperoxia actually inhibits apoptosis in pulmonary epithelial cells. There are several possible ways that hyperoxia might inhibit apoptosis in pulmonary epithelial cells. First, one or more enzymes in the apoptotic pathway might be sensitive to direct oxidation by high levels of molecular O$_2$, resulting in an irreversible abrogation of the apoptotic pathway in these cells. Alternatively, hyperoxia may activate pathways that inhibit apoptosis. Several laboratories have shown that activation of the transcription factor NF-kB can prevent apoptosis induced by chemotherapeutic agents and ionizing radiation in cultured cells (6–9). In addition, activation of NF-kB may be responsible for the protective effect of low-dose amyloid $\beta$ in neuronal cell death (10) and in the survival and function of hematopoietic stem and progenitor cells (11). We have previously demonstrated that hyperoxia activates NF-kB in A549 cells (5). In this report, we examined whether preexposure of cultured epithelial cells to hyperoxia prevents subsequent oxidant-induced apoptosis and whether NF-kB activation or up-regulation of antioxidant enzymes (AOEs) mediate this inhibitory process.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human lung adenocarcinoma A549 cells (ATCC, Manassas, VA) were grown in F12K medium supplemented with 10% fetal bovine serum, 1% penicillin, 100 units/ml streptomycin (Life Technologies, Inc.) and maintained at 37 °C in 5% CO$_2$/95% room air. HeLa-20 and HeLa-80 cells were grown in Ham’s F-10 medium (Life Technologies, Inc.), supplemented with 15% fetal bovine serum, 1% penicillin, 100 units/ml streptomycin, and maintained in 2% CO$_2$/98% room air at 37 °C. In all experiments, cells were seeded at 20–40% confluence on plastic culture dishes and were allowed to adhere overnight prior to exposure. Cell cultures exposed directly to H$_2$O$_2$ were seeded slightly lower than those preexposed to hyperoxia, to reach cell densities similar to those of the preexposed cultures at the time of H$_2$O$_2$ exposure, because cell density affects the sensitivity to the cytotoxic effects of H$_2$O$_2$ (data not shown). Hyperoxic conditions were achieved by growing cells in 40, 60, or 95% O$_2$/5% CO$_2$ at 37 °C in sealed, humidified chambers for up to 48 h. Some cell cultures were treated with 500 units/ml (10 ng/ml) TNF-$\alpha$ (Phar- mingen, San Diego, CA) for 30 min, with 2–5 mM H$_2$O$_2$ or 10 mM paraquat for up to 24 h. Media and oxidants were refreshed each day when cells were cultured for more than 1 day. For this reason, and because the cultures were subconfluent, neither ATP nor glucose levels were depleted by hyperoxia.

**Assays for Antioxidant Enzyme Activities**—AOE activities were measured in A549, HeLa-20, and HeLa-80 cells as described previously (12–14). Briefly, a competitive inhibition assay was used to measure total superoxide dismutase activity spectrophotometrically through the inhibition of cytochrome c/xanthine-xanthine oxidase oxidation. Inhibition of 50% cytochrome c oxidation is defined as 1 unit of superoxide dismutase activity at 550 nm (12). Catalase activity was assayed spec-
trophometrarily by following the disappearance of H₂O₂ at 240 nm (13). Glutathione peroxidase activity was determined through the glutathione disulfide formation spectrophotometrically following the absorbance decrease of NADPH to NADP at 340 nm (14).

Western Blots—Cells were prepared according to the procedure described (2, 5). Proteins from each sample were loaded onto 10% SDS-polyacrylamide gels (Bio-Rad). After electrophoresis, proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA). For β-galactosidase detection, blots were incubated with rabbit anti-β-gal antibodies as recommended by the supplier (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Antibody binding was detected by the enhanced chemiluminescent reagent CDP-Star (Roche Molecular Biochemicals) and by exposing the filter to x-ray film.

Immunofluorescence—Detection of the p65 subunit of NF-κB was performed as described with minor modification (5). In brief, formalin-fixed cells grown on coverslips were first incubated with ice-cold 100% methanol at −20 °C and blocked with 1% bovine serum albumin (Panvera, Madison, WI). Cells were then incubated with 2.5 μg/ml (in 1% w/v bovine serum albumin) anti-NF-κB p65 antibodies (Santa Cruz Biotechnology Inc.) for 60 min and washed with three changes of 1× Tris-buffered saline (0.1 m Tris-Cl, pH 7.4, 0.15 m NaCl). Sheep anti-rabbit IgG-fluorescein isothiocyanate (Roche Molecular Biochemicals) was used as the secondary antibody. Cells were counterstained with 2 μg/ml of 4′,6-diamidine-2-phenylindole dihydrochloride (DAPI) (Roche Molecular Biochemicals) to visualize the nuclei, and the results were examined by immunofluorescence microscopy using the B-2 filter for fluorescein isothiocyanate and the UV-2A filter for DAPI (Nikon Inc., Melville, NY).

Assays for Apoptosis—The terminal transferase dUTP nick end labeling (TUNEL) assay was performed with cells seeded on coverslips and fixed in formalin as described (2, 3). The protocol utilized for TUNEL staining was as described previously except that proteinase K treatment was omitted. TUNEL reagents, including rhodamine-conjugated anti-digoxigenin Fab fragment, were obtained from Roche Molecular Biochemicals. Cells were counterstained with 2 μg/ml DAPI for 8 min at room temperature. The TUNEL assay results were examined by immunofluorescence microscopy using the G-2A filter for rhodamine and the UV-2A filter for DAPI.

The Annexin V incorporation assay was performed with cells grown in six-well dishes. After exposure to oxidants, cells were rinsed with phosphate-buffered saline, trypsinized and combined with cells detached during the oxidative exposure. Cells were pelleted (200 × g for 5 min), rinsed with ice-cold phosphate-buffered saline, and then incubated with Annexin V-fluorescein isothiocyanate according to the vendor’s instructions (R&D systems, Minneapolis, MN). The Annexin V-fluorescence intensity was measured with excitation at 488 nm and emission at 520 nm, and the cell size was determined using the forward scatter method with a Becton Dickson flow cytometer. The extent of apoptosis was assessed by fluorescence intensity or condensed cell size using Cell Quest software (Becton Dickson, Franklin Lakes, NJ). Cells with fluorescence intensity higher than 210 relative fluorescence units or cell size smaller than 345 units were scored as apoptotic. The extent of apoptosis between groups were analyzed using Kolmogorov-Smirnov analysis using Cell Quest software, and p < 0.05 is considered statistically significant (15).

Image Analysis—To quantify the extent of apoptosis by TUNEL assay, samples were illuminated with UV light to visualize either TUNEL-positive nuclei (590 nm) or total, DAPI-stained nuclei (420 nm) and analyzed by computer-aided analysis as described previously (2). To quantify the extent of nuclear condensation, nuclei were stained with DAPI. The DAPI fluorescence was examined and images were captured with a charge-coupling device video camera. Uniform camera control settings were used for image capture, and image thresholding was identical for all images. The captured images were analyzed using the Metamorph system (Universal Imaging, West Chester, PA) running on a personal computer. At least 25 fields were analyzed on each coverslip from a minimum of two independent experiments for each group. The percentage of apoptotic cells was calculated as the percentage of TUNEL-positive apoptotic nuclei divided by the total number of nuclei (detected by DAPI staining). The numbers of the nuclear sizes were analyzed by Maple distribution (Maple math software). The data were analyzed for statistical significance using the Student’s t test and analysis of variance, with p < 0.05 considered significant.

Transfection and Stable Cell Lines—A549 cells were transfected with 5 μg of lEbdn-pUSEamp, a plasmid that carries a dominant-negative construct of IκB with mutations at Ser-32 and Ser-36 (Upstate Biotechnology, Lake Placid, NY), followed by selection with 0.8 μg/ml G418 (Life Technologies, Inc.). At least three stable cell lines that express less than 50% of NF-κB activity of those expressing the pUSEamp vector alone were also established. To determine the levels of NF-κB activity in these cells, a reporter gene expression assay was performed.

Cells were transiently cotransfected with 50 ng of pCMV-SPORT-β-galactosidase, 1 μg of pBlue, and 50 ng of pNF-κB-luc, a reporter plasmid containing 5 NF-κB binding sites inserted upstream of the luciferase reporter (Stratagene, La Jolla, CA). Twenty-four hours after transfection, cells were assayed for luciferase activity, which was normalized on the basis of β-galactosidase expression according to the vendor’s instructions, and data were analyzed for statistical significance using Student’s t tests and analysis of variance.

RESULTS

Preexposure of Alveolar Epithelial Cells to Hyperoxia Inhibits Oxidant-induced Apoptosis—To determine whether hyperoxia can inhibit apoptosis, alveolar epithelial A549 cells were exposed either to 5 mM H₂O₂ to directly induce apoptosis (3) or to 95% O₂ for 24 h and then exposed to H₂O₂. Fig. 1, A–D, shows the nuclear morphology of the various treatment groups visualized with DAPI staining. The nuclei of A549 cells condensed and showed intense fluorescence with DAPI to visualize all nuclei. Control, cells were cultured in medium alone (A and B); H₂O₂, cells were cultured in 5 mM H₂O₂ (C and D). hyperoxia, cells were exposed to 95% O₂ for 24 h (B and D). Apoptotic nuclei appear condensed and brighter (C and D). Map distribution of the nuclear area (nucleus) of A549 cells exposed to H₂O₂ with (O/H₂O₂) or without (RA/H₂O₂) preexposure to hyperoxia (p < 0.001). A shift to the left indicates the nuclear condensation of these cells.

![Fig. 1. Hyperoxia inhibits H₂O₂-induced nuclear condensation in A549 cells. A–D, A549 cells treated with 5 mM H₂O₂ for 4 h with or without preexposure to 95% O₂ for 24 h and stained with DAPI to visualize all nuclei. Control, cells were cultured in medium alone (A and B); H₂O₂, cells were cultured in 5 mM H₂O₂ (C and D). hyperoxia, cells were exposed to 95% O₂ for 24 h (B and D). Apoptotic nuclei appear condensed and brighter (C and D). E, Map distribution of the nuclear area (nucleus) of A549 cells exposed to H₂O₂ with (O/H₂O₂) or without (RA/H₂O₂) preexposure to hyperoxia (p < 0.001). A shift to the left indicates the nuclear condensation of these cells.](image-url)
To determine whether hyperoxia interferes with internucleosomal cleavage, we assayed for apoptosis using the TUNEL assay. Fig. 2A shows that exposing cells to hyperoxia prior to H₂O₂ exposure reduced the number of TUNEL-positive nuclei in comparison to control cells preexposed to room air. Computer-aided image analysis revealed that the percentage of TUNEL-positive nuclei/DAPI-positive nuclei (total nuclei) decreased from 70 ± 5 to 27 ± 5% after hyperoxic preexposure (p < 0.05). Another indicator of apoptosis is the externalization of phosphatidylserine from the cytosolic surface to the extracellular surface (17). Using fluorescein isothiocyanate-conjugated Annexin V that detects phosphatidylserine translocation, we analyzed the extent of apoptosis in cells exposed to H₂O₂ with or without preexposure to hyperoxia. Fig. 2B shows that 42% of cells treated directly with H₂O₂ had high intensity for Annexin V binding, compared with 12% of cells preexposed to hyperoxia followed by H₂O₂ exposure. In addition, shrinkage of the cell size induced by exposure to H₂O₂ was reduced with hyperoxic preexposure (data not shown).

To examine whether preexposure to hyperoxia can inhibit apoptosis induced by other oxidants, we exposed A549 cells to 10 mM paraquat (an intracellular superoxide generator) for 24 h, with or without preexposure to hyperoxia. Studies from our and other laboratories have demonstrated that cytotoxicity induced by paraquat occurs via apoptosis (3, 18, 19). Fig. 3 shows that 24 h of preexposure to hyperoxia significantly in-
hibited paraquat-induced apoptosis, assessed by both the Annexin V incorporation assay (Fig. 3A) and an analysis of cell shrinkage (Fig. 3B).

The inhibition of H$_2$O$_2$-induced apoptosis was observed in A549 cells preexposed to hyperoxia (95%) for a time period as short as 30 min and as long as 48 h. However, there was no direct association between the reduction in apoptosis and the oxygen concentration or the duration of preexposure. For example, preexposure to 40, 60, or 95% O$_2$ resulted in 17, 20, or 12%, respectively, of cells undergoing apoptosis in comparison to 42% of control cells. In addition, increasing the duration of preexposure to hyperoxia did not provide additional benefits. A 40% reduction in apoptosis was observed with preexposure to 95% O$_2$ for 30 min compared to a 52% reduction in cells preexposed for 48 h.

One possible explanation for this inhibition is that hyperoxia might interfere with the activation of proapoptotic pathways as a consequence of overall oxygen toxicity suffered by the cells. A prediction of this notion is that mutant cells that are not sensitive to oxygen toxicity would not be protected by hyperoxic preexposure, as the cells would not be poisoned by hyperoxia prior to secondary oxidant insults. To test this, we repeated the above experiments in hyperoxia-resistant HeLa-80 cells, which proliferate normally in 80% O$_2$, a lethal dose to many other cell types (20, 21). The parental HeLa cells (referred to as HeLa-20) were used as controls. Both HeLa-20 and HeLa-80 cells were propagated in room air and then either preexposed to hyperoxia for 24 h prior to H$_2$O$_2$ or treated with H$_2$O$_2$ directly. Fig. 4 shows that preexposure to hyperoxia blunted subsequent H$_2$O$_2$-induced apoptosis in both cell lines. Because HeLa-80 cells are not metabolically poisoned when grown in 80% O$_2$, these results suggest that the inhibition of apoptosis by hyperoxia is derived from the activation of survival pathways rather than a general poisoning of these epithelial cells.

Antioxidant Enzymes Are Not Responsible for This Inhibition—The ability of hyperoxia to confer resistance to further oxidative injury has raised the question of how endogenous antioxidant systems respond to hyperoxic exposure. We have

FIG. 3. Hyperoxia inhibits paraquat-induced apoptosis in A549 cells. A549 cells were exposed to 95% O$_2$ for 24 h or to room air prior to the treatment with 10 mM paraquat for 24 h. Apoptosis was determined by Annexin V incorporation (A) and cell shrinkage (B) assays and analyzed by flow cytometry as described under “Experimental Procedures.” RA/Para, cells were exposed to paraquat directly; O$_2$/Para, cells were exposed to hyperoxia prior to the paraquat exposure. Apoptosis is represented by an increase in the intensity of Annexin V incorporation (a shift to the right in A) and by the cell shrinkage (a shift to the left in B). A significant reduction (p < 0.05) in apoptosis was noted in cells preexposed to hyperoxia.

FIG. 4. Inhibition of oxidant-induced apoptosis by hyperoxia is not a result of oxygen toxicity. HeLa-80 (hyperoxia-resistant cells) and HeLa-20 (control HeLa cells) were preexposed to 80%O$_2$/5%CO$_2$ or room air for 24 h and then exposed to 4 mM H$_2$O$_2$ for 4 h. RA/H$_2$O$_2$, cells were cultured in 4 mM H$_2$O$_2$ without preexposure to 95% O$_2$. O$_2$/H$_2$O$_2$, cells were cultured in 4 mM H$_2$O$_2$ with preexposure to 80% O$_2$ for 24 h. Relative fluorescence units represents the intensity of Annexin V incorporation. Apoptosis is represented by an increase in Annexin V incorporation (a shift to the right in both panels). A significant reduction (p < 0.05) in apoptosis was noted in cells preexposed to hyperoxia in both HeLa-80 and HeLa-20 cells.

FIG. 5. AOE activities are not up-regulated following hyperoxic exposure. A549 cells were grown in either room air (RA) or in 95%O$_2$/5%CO$_2$ (O$_2$) for 24 h. Total cell lysates were prepared from the trypsinized cells, and enzymatic activities of total superoxide dismutase, catalase, and glutathione peroxidase were assayed as described under “Experimental Procedures.” The y axis represents the relative enzymatic activities normalized to the total amounts of protein. U/mg protein, units of AOE activity/mg of total protein in the lysates.
focused on the classic antioxidant enzymes, including superoxide dismutase, catalase, and glutathione peroxidase, all of which may play crucial roles in responding to oxidative stress by scavenging excess reactive oxygen species. To determine whether this inhibitory effect of hyperoxia is due to increased AOE activities, we assessed the enzymatic activities of total superoxide dismutase, catalase, and glutathione peroxidase in cells with and without preexposure to hyperoxia. Fig. 5 shows that not only was there no increase in AOE activities observed during and after hyperoxic exposure, but a slight decrease was associated with the preexposure. However, the differences between the activities in room air controls and those in hyperoxic cells were not statistically significant. These data demonstrate that this inhibition of apoptosis is not related to an increase in endogenous AOE activities.

**Preexposure to TNF-α Inhibits the Extent of Oxidative Apoptosis**—Previously, we have shown that hyperoxia induces NF-κB activation in A549 cells (5). To determine whether activation of NF-κB plays a role in the protection of oxidative apoptosis, we tested whether activating NF-κB by means other than hyperoxia can also provide protection against oxidative apoptosis. A549 cells were treated with TNF-α prior to exposure to H₂O₂. Treatment of A549 cells with 500 units/ml of TNF-α induced nuclear translocation of NF-κB (Fig. 6C), indicating that NF-κB was activated in TNF-α-exposed cells. In contrast, NF-κB is sequestered in the cytoplasm in control cells that are not exposed to TNF-α (Fig. 6B). A549 cells are fairly resistant to the cytotoxic effects of TNF-α (24, 25), and no morphological evidence for apoptosis was apparent after exposure to TNF-α alone for 30 min, or even for up to 24 h (data not shown). Although H₂O₂-treated cells still underwent apoptosis, the extent of cell shrinkage was reduced from 64 to 42% (p < 0.05) in cells pretreated with TNF-α (Fig. 6A). In addition, increases in the activities of superoxide dismutase, catalase, and glutathione peroxidase were not observed in TNF-α-treated cells (data not shown). These results suggest that NF-κB activation plays an important role in the extent of oxidant-induced apoptosis.

**Activation of NF-κB via IκB Degradation Is Involved in the Inhibition of Apoptosis by Hyperoxic Preexposure**—To determine whether NF-κB activation induced by hyperoxia is via the IκB kinase (IKK) signal transduction pathway, we examined the steady-state levels of IκB (inhibitor of NF-κB). NF-κB normally remains sequestered in the cytoplasm by IκB. Upon exposure to stimuli, IκB is phosphorylated by IKK, ubiquitinated, and degraded (23). NF-κB is then released and translocates to the nucleus, regulating gene expression. Fig. 7A shows that the levels of IκBα decreased shortly after exposure to hyperoxia (i.e. 30 min) and remained lower than room air control cells for up to 48 h. Previously, we demonstrated that the p65 subunit of NF-κB translocates from the cytoplasm to the nucleus in cells exposed to hyperoxia for periods as short as 30 min and as long as 48 h (5). This time frame closely correlates with the reduction in the extent of apoptosis by hyperoxic preexposure, suggesting that NF-κB activation via activation of the IKK signal transduction pathway is involved in this inhibition. Similarly, to determine whether inhibition of apoptosis by TNF-α preexposure is also via the IKK pathway, we examined the steady-state levels of IκBα in TNF-α-exposed cells. Fig. 7B shows that TNF-α induced IκBα degradation. These results suggest that either hyperoxia or TNF-α induces NF-κB activation via activation of the IKK signal transduction pathway. This pathway may play an important role in inhibiting oxidant-induced apoptosis.

To test whether suppression of NF-κB activity via the IKK pathway alters the extent of apoptosis induced by exposure to H₂O₂, we transfected A549 cells with a construct that encodes...
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Fig. 8. Augmentation of oxidant-induced apoptosis by suppressing NF-κB activity via interfering with the IKK pathway. A549 cells were transfected with either a plasmid containing a DNM construct of IκBα or a plasmid with the vector only, and at least three stable cell lines each were established. Untransfected A549 cells (WT A549), three stable IκB DNM cell lines expressing less than 50% NF-κB activity compared to the vector clones (IκB DNM), and three stable cell lines expressing vector alone (vector) were exposed to 4 mM H₂O₂ for 4 h. Apoptosis was assessed by cell shrinkage and Annexin V incorporation assays and analyzed by flow cytometry. One representative each from the IκB DNM and vector cell lines is shown here. Apoptosis is represented by cell shrinkage (a shift to the left in A) and by an increase in the intensity of Annexin V incorporation (a shift to the right in B). A significant increase (p < 0.05) in apoptosis was noted in the DNM cells compared with either the wild-type cells or vector cells, suggesting that inhibiting NF-κB activity by inhibiting phosphorylation of IκBα enhances oxidative apoptosis.

IκB with dominant-negative mutations that prevent its degradation. Stable clones that express less than 50% (46–49%) of NF-κB activity compared with those in vector clones were established as the dominant-negative mutant (DNM) clones and exposed to H₂O₂. The extent of apoptosis induced by exposure to H₂O₂ was determined in untransfected A549 cells, in three A549 clones transfected with vector alone, and in three different DNM clones. Fig. 8 shows that the extent of apoptosis, assessed by cell shrinkage and Annexin V incorporation, was increased significantly in the DNM cell line (IκB DNM, 67%) compared to the vector cell line (25%) and the untransfected A549 cells (31%). These results suggest that NF-κB activation via the IKK signal transduction pathway is involved in regulating the extent of oxidative apoptosis.

DISCUSSION

We have shown that preexposure of epithelial cells to hyperoxia inhibits the onset of apoptosis caused by exposure to H₂O₂ or paraquat. Because this protection occurs in cells that do not suffer from oxygen toxicity, this effect is not merely a consequence of overall cell poisoning of an apoptotic pathway but appears to result from the activation of NF-κB-mediated survival pathways. That these pathways might be activated by NF-κB was demonstrated not only by the activation of NF-κB by both hyperoxia and TNF-α, which afford similar protection from oxidative apoptosis, but also by the augmentation of oxidative apoptosis in cells with suppressed NF-κB activity.

Resistance to Oxidative Injury Is Mediated by Specific Survival Pathways—Instead of Oxygen Toxicity—Exposure to sublethal levels of hyperoxia has been shown to increase resistance to further hyperoxic injury and improve survival rates in animal models (26, 27). Preexposing cultured mammalian cells to oxidants can also prime or “desensitize” the cells to further oxidative injury (22, 28). However, mechanisms for this acquired resistance have not been well delineated and, based on correlative data, may in some cases be a consequence of preinduction of antioxidant enzyme defenses (29, 30). Our observations in A549 cells (Fig. 5) indicate that resistance to oxidant-induced apoptosis is not mediated through increased activities of endogenous AOEIs. This notion is further supported by results from HeLa-80 cells preexposed to hyperoxia and TNF-α-treated A549 cells that do not up-regulate AOE activities but show resistance to oxidant-induced apoptosis.

Although prolonged exposure to hyperoxia is lethal to most cells, it does not kill pulmonary epithelial cells via apoptosis. On the other hand, oxidants such as H₂O₂ and paraquat cause these cells to undergo apoptosis (3, 4, 18, 19). The reasons for this remain unclear. It is likely that at least some of the organellar and/or macromolecular sites of O₂ damage are different from sites affected by other oxidants, because molecular O₂ diffuses throughout the cell and can target virtually all organelles and cytosolic molecules. One possible explanation for the lack of apoptosis associated with hyperoxia in these cells is that one or more steps in the oxidative pathway to apoptosis might be sensitive to direct oxidation by high levels of molecular O₂. For example, caspases may become oxidized and lose their ability to initiate and execute apoptosis (31). However, our results demonstrate that cells preexposed to hyperoxia still undergo apoptosis upon subsequent exposure to H₂O₂, suggesting that apoptotic pathways are either incompletely inhibited by molecular O₂ or circumvented by subsequent oxidant injury. Our studies further suggest that hyperoxia inhibits apoptosis by activation of prosurvival pathways, rather than via inactivating the apoptotic pathway through oxidization of the macromolecules.

Activation of NF-κB via the IKK Pathway in Hyperoxic Preexposed Cells May Be Responsible for the Inhibition of Oxidant-induced Apoptosis—NF-κB is an oxidative stress-responsive transcription factor that can be activated by a variety of agents, including cytokines and oxidants (32, 33), and has been shown to protect cells from apoptosis by regulating Bcl-2 family members and caspases (34, 35). Hyperoxia activates NF-κB in both animal models and epithelial cells (5, 36, 37). Our results
demonstrate that preexposure to hyperoxia inhibits subsequent oxidant-induced apoptosis. This inhibition was effective even with limited time (30 min) preexposure to hyperoxia. This time frame of inhibition, from 30 min to 48 h, associates closely with the activation of NF-κB (5) and IκB degradation (Fig. 7A), an indicator for the activation of the IKK signal transduction pathway. These data suggest that activation of NF-κB via the IKK pathway is involved in this inhibitory process. This hypothesis is supported by the observations that the extent of oxidative apoptosis is concomitantly affected by the levels of NF-κB activity altered either by preexposure to TNF-α (Fig. 6) or by transfection with a DNKM construct of IκB in A549 cells (Fig. 8). Furthermore, hyperoxia-induced inhibition corresponds to an increase of nuclear translocation of NF-κB in oxygen-resistant HeLa-80 cells exposed to hyperoxia (data not shown). Because hyperoxia activates NF-κB in both rat and mouse lungs (36, 37), activation of NF-κB may also be important in conferring resistance to further oxidative injury by reducing oxidant-induced apoptosis in these animal models.

The mechanism by which NF-κB may inhibit oxidant-induced apoptosis is unclear. One possibility is that activation of NF-κB may change the oxidative equilibrium in the cellular milieu to a less oxidative state. This notion is supported by the studies reported by Haddad and colleagues (38). An increase in NF-κB activation can be achieved by treating cells with an antioxidant, N-Acetyl-l-Cysteine, suggesting a correlation between NF-κB activation and increased antioxidant capacity. On the other hand, inhibiting NF-κB activation by pyrrolidine dithiocarbamate favors an oxidative equilibrium of glutathione (GSH) by lowering the ratio of GSH to its oxidized form, glutathione disulfide (38). NF-κB may also participate in the protective process by up-regulating anti-apoptotic Bcl-2 family members and/or survival and stress-response factors such as heme oxygenase-1 (HO-1). HO-1 can be markedly up-regulated in response to an increase of nuclear translocation of NF-κB (5) and IκB A549 cells from further hyperoxic injury (40). Interestingly, the 5′-upstream region of HO-1 contains some potential NF-κB binding consensus sequences (41) and over-expression of NF-κB can specifically enhance the expression of HO-1 (42). Further work on characterizing these signal transduction pathways will lead to greater understanding of how these pathways can be regulated.

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