Oxidative insults that are lethal to epithelial cells kill either via apoptosis or necrosis. Nuclear factor-κB (NF-κB) is a redox-sensitive transcription factor that is activated by oxidative insult, and NF-κB activation can protect cells from apoptosis. To test if NF-κB can protect from necrotic cell death caused by high levels of molecular O₂ (hyperoxia), we exposed human alveolar epithelial (A549) cells to hyperoxia. NF-κB was shown to be activated and was translocated to the nucleus within minutes. Nuclear translocation persisted over the course of several days, and the levels of NF-κB protein and mRNA increased as well. In hyperoxia, NF-κB regulation was independent of mitogen-activated protein kinase (MAPK). In sharp contrast, there was neither nuclear translocation of NF-κB nor any increase in expression after exposure to H₂O₂ at a concentration where this oxidant induces both MAPK and widespread apoptosis. Despite the activation and increased expression of NF-κB in hyperoxia, this oxidant remained lethal to the cells. These observations confirm the notion that apoptosis occurs in the absence of NF-κB activation but indicate that protection from cell death by NF-κB is probably limited to apoptosis.

Oxidative stress resulting from the toxic effects of reactive oxygen intermediates (ROI) is an important role in the pathogenesis of many disease states including carcinogenesis, atherosclerosis, and inflammatory disorders (1, 2). Oxidative injury can also lead to cell death, and ROI can have a role in apoptotic cell death induced by nonoxidative insults (3–8). Direct oxidative injury often occurs as a consequence of ventilatory O₂ therapy, which is used in the treatment of critically ill patients who cannot breathe efficiently. This treatment typically requires supraphysiologic concentrations of O₂ (hyperoxia), which results in an elevated level of ROI in many cell types of lung, the organ that receives the highest level of O₂ exposure (9, 10). The lung is estimated to be composed of as many as 60 cell types, which complicates the study of pathways to cell death by hyperoxia. On the other hand, cultured lung epithelial cells provide simpler models for understanding certain aspects of pulmonary biology. We have recently reported that A549 cells (derived from human type II alveolar epithelial cells) and HeLa cells succumb to hyperoxia not via apoptosis but by necrosis. In contrast, lethal doses of the oxidants H₂O₂ or superoxide kill these epithelial cells via apoptosis (11). These observations indicate that the pathways to cell death may differ, depending on the oxidant and dose used.

Very recent reports from several laboratories show that apoptotic cell death can be prevented by the expression of nuclear factor-κB (NF-κB) (12–15), a multisubunit transcription factor that rapidly activates the expression of genes involved in inflammation, infection, and stress (16). Taken together, these recent reports suggest that the induction of NF-κB may be part of a survival mechanism used to escape cell death (17). In this report, we examine the expression of NF-κB in cells exposed to lethal concentrations of hyperoxia or H₂O₂ and show that, despite the induction of NF-κB by molecular O₂, the cells do not escape death.

EXPERIMENTAL PROCEDURES

Cell Culture—Human lung adenocarcinoma A549 cells (ATCC CCL 185) were cultured and maintained in 95% room air, 5% CO₂ as described previously (11). Subconfluent cultures were used in all experiments. For hyperoxia treatment, cells were maintained in sealed humidified chambers flushed with 95% O₂, 5% CO₂. Media and gases were refreshed daily. Control cells were cultured in 95% room air, 5% CO₂. For the H₂O₂ experiment, cells were cultured in 95% room air, 5% CO₂, 5 mM H₂O₂. These cells are known to be relatively resistant to H₂O₂, and millimolar concentrations of H₂O₂ are necessary to induce apoptosis in the culture medium used (11).

Immunofluorescence—All procedures were carried out at room temperature. Cells grown on coverslips were washed once with 1× phosphate-buffered saline (PBS, Life Technologies, Inc., Gaithersburg, MD) and fixed for 10 min in 10% formalin buffered in 1× PBS. Coverslips were then rinsed with 3 changes of PBS and incubated with 1× BSA solution (Panvera Corp., Madison, WI) for 10 min. Cells were incubated with a 2.5 μg/ml (in 1% w/v BSA) anti-NF-κB p65 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 30 min and washed with 3 changes of 1× TBS (0.1 M Tris-Cl, pH 7.4, 0.15 M NaCl). Secondary antibody, sheep anti-rabbit IgG-rhodamine (Boehringer Mannheim, Indianapolis, IN) was diluted in 1× BSA to a concentration of 27 μg/ml and incubated with coverslips for 30 min. The coverslips were washed in excess water and mounted to microscope slides, and the results were examined by immunofluorescence microscopy using the UV-2A filter (Nikon Inc., Melville, NY) for rhodamine.

Western Blots—Cell lysates were prepared according to the procedures recommended by New England BioLabs (Beverly, MA). Protein from each sample was loaded onto a 10 or 12% SDS-polyacrylamide gel (Bio-Rad, Hercules, CA). After electrophoresis, proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA). For MAPK detection, blots were incubated with an antibody that recognizes only the activated (phosphorylated) MAPK protein (New England BioLabs). Antibody was detected by the enhanced chemiluminescent (ECL) reagent, CDP-Star (Boehringer Mannheim), and exposing the filter to

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‡ The abbreviations used are: ROI, reactive oxygen intermediates; NF-κB, nuclear factor-κB; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; BSA, bovine serum albumin; MOPS, 4-morpholinepropanesulfonic acid.
RESULTS

Because the lung is the primary target of O_2 toxicity, we utilized lung epithelial cells as a model to study signal transduction by injurious levels of hyperoxia and oxidants. In particular, A549 cells, which are derived from an adenocarcinoma of alveolar type II cells, have been extensively studied with respect to their responses to oxidants and other airborne insults (6, 18). In earlier studies, we have shown that cultured A549 cells exposed continuously to 95% O_2 suffer cell death via necrosis and not by apoptosis. In contrast, lethal concentrations of H_2O_2 or superoxide cause apoptosis (11).

Because recent reports suggest that NF-κB can prevent cell death, we investigated the role of NF-κB in these two distinct modes of cell death. A549 cells exposed to 95% O_2 showed evidence of cell swelling by 24 h, and the culture gradually died off over the course of one week (11). It is known that following release from the inhibitory binding protein IκB, NF-κB translocates from cytosol to the nucleus, where it regulates transcription (19). We therefore studied NF-κB activation during hyperoxia by examining its nuclear translocation. Fig. 1 shows that control cells grown in room air had weak NF-κB immunofluorescence. The signal was evident primarily in the cytoplasm although there was limited fluorescence in the nuclei of some cells. By 30 min of hyperoxia, nuclear fluorescence was more prominent, and it increased over the course of 1 day. By 24 h of hyperoxia, the cells already showed signs of swelling, and fluorescence was more intense both in the nuclei as well as in the cytoplasm of many cells. In contrast to hyperoxia, there was no nuclear translocation of NF-κB when cells were exposed to concentrations of H_2O_2 that caused apoptosis (Fig. 1, panels E-G). By 4 h of H_2O_2 treatment, the majority (~80%) of cells had undergone apoptosis (data not shown).

Fig. 1 suggests that an increase in NF-κB protein levels occurred during hyperoxia in addition to nuclear translocation. To examine this further, Western blots were performed. Fig. 2 shows that NF-κB levels were increased as soon as 30 min after exposure to 95% O_2, and peak levels were achieved by 24 h. Levels remained elevated for 3 days (Fig. 2). In sharp contrast, apoptotic concentrations of H_2O_2 caused no increased NF-κB protein, and there was even a slight decrease after 2 h (Fig. 2).

To determine if elevated NF-κB protein levels were correlated with increased mRNA abundance, Northern blot analyses were performed. Fig. 3 shows that steady-state levels of NF-κB mRNA were elevated as soon as 30 min after O_2 exposure. Message levels increased over the course of 1 day and remained elevated for 3 days. In contrast, the abundance of the message encoding the glyceraldehyde-3-phosphate dehydrogenase was unchanged (data not shown). Unlike hyperoxia, exposure of the culture to an apoptotic dose of H_2O_2 failed to induce NF-κB mRNA in these epithelial cells. Rather, a modest decrease was observed after 2 h (Fig. 3).

The differential activation and expression of NF-κB during different modes of cell death imply that different signals are transduced by hyperoxia and H_2O_2. We therefore investigated whether p42 and p44 MAPKs (mitogen-activated protein kinases) were activated. p42 and p44 MAPKs both function in a protein kinase cascade that plays a critical role in the regula-
absence of NF-κB, and chemotherapeutic agents (12–14, 17). In the apoptosis-inducing concentration of H2O2, there was a significant. In contrast, as soon as 10 min after incubation in an during exposure to hyperoxia (phosphorylated p42 is predomi-
detected in the levels of phosphorylated or activated proteins. Fig. 4 shows that there were no changes from severe oxidative insults, and it has even been suggested that apoptosis results from severe oxidative insults, and it has even been suggested that apoptosis triggered by non-oxidative insults requires lipid peroxidation in the dying cells (25). However, cellular oxidation also can activate NF-κB, which occurs through the redox-sensitive disassociation between NF-κB and its inhibitory protein IκB (26). Several groups have very recently reported that activation of NF-κB can prevent cell death induced by TNF, X-ir-
radiation, and chemotherapeutic agents (12–14, 17). In the absence of NF-κB expression or activation, these insults induce apoptosis in a variety of cell types. Taken together, these ob-
servations suggest that some cells might utilize a strategy to prevent death resulting from oxidant injury by activating NF-
κB, which presumably regulates downstream genes that sal-
ve the cell. We tested this notion in two models of oxidant-
duced cell death by exposing cells to lethal concentrations either of hyperoxia or H2O2. Surprisingly, we found that de-
spite the rapid activation of NF-κB, hyperoxia remains lethal, indicating that NF-κB is not sufficient to protect the cells from this oxidative insult. Moreover, when apoptosis was triggered in the cells by another oxidative insult, H2O2, NF-κB was not induced.

The mode of cell death induced by these two oxidative insults is different, hyperoxia caused necrosis while H2O2 caused apoptosis (11). Thus, although recent reports indicate that apoptotic cell death is avoidable by NF-κB activation, they did not address whether NF-κB could provide protection from other modes of cell death. Similarly, the time course of those studies was relatively short, and the data did not bear on slower cell death that occurs over the course of several days. Our observation that oxidative necrotic cell death can ensue despite NF-κB activation supports the notion that strategies aimed at blocking NF-κB activation might accelerate cell death (via apoptosis) but implies that with sufficient time the overall amount of cell death might be no different. Also, because necrosis in vivo is associated with inflammation and apoptosis typically is not, when radiation or chemotherapy are administered to pa-
tients, it might actually be preferable to encourage or at least permit some degree of inflammation to hasten the removal of dead tumor cells.

Interestingly, not only was NF-κB activated, but its expres-
sion was also induced at the protein and mRNA levels, and increasing amounts of NF-κB were found in cells exposed to prolonged hyperoxia. This suggests that NF-κB synthesis was also persistent. In attempting to identify the pathway leading to NF-κB activation and induction by hyperoxia, we found that the MAP kinase cascade was not induced, which is distinct from other observations (24).

Non-apoptotic epithelial cell death, which is morphologically similar to hyperoxia, occurs at lower (and perhaps more physi-
ologically relevant) levels of oxidants than are required for the induction of apoptosis (11). Interestingly, it is also known that non-apoptotic cell death in the roundworm Caenorhabditis el-
gans, can occur in mutants of a gene family that include deg-1, mec-4, and mec-10. In these cases, cell death morphologically resembles hyperoxic or low oxidant cell necrosis and is charac-
terized by swelling and lysis of a specific group of neurons (27). Likewise, germ cells in mec-3- worms undergo a necrosis, not apoptosis (28). Although mec-4, encodes a subunit of a mech-
asensory ion channel, the function of these genes are not yet fully understood. Perhaps they are steps along a pathway lead-
ing to necrosis. Taken together with our observations, it may prove valuable to explore pathways to alternate (non-apoptotic) modes of cell death.

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Fig. 2. Western blot of NF-κB. Top panel, H2O2 exposure; bottom panel, hyperoxia exposure. Cell lysates were isolated from control (C) A549 cells grown in room air and cells exposed to H2O2 or hyperoxia for the times shown. Twenty μg of protein were electrophoresed, blotted, and incubated with an anti-NF-κB antibody.

Fig. 3. Northern blot of NF-κB. Top panel, H2O2 exposure; bottom panel, hyperoxia. RNA was isolated from control (c) A549 cells grown in room air and cells exposed to H2O2 or hyperoxia for the times indicated. Ten μg (top) or twenty μg (bottom) of total RNA were electrophoresed, blotted, and hybridized with the p65 cDNA probe.

Fig. 4. Western blot of activated p42/p44 MAPK. Top panel, hyperoxia; bottom panel, H2O2 exposure. Cell lysates were isolated from control (C) A549 cells and cells exposed to hyperoxia or 5 mM H2O2 for the times indicated. Thirty μg of protein were electrophoresed, blotted, and incubated with an antibody specific for the phosphorylated forms of p42/p44 MAPK.
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