Reactive Oxygen Species Are Required for Hyperoxia-induced Bax Activation and Cell Death in Alveolar Epithelial Cells*

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Exposure of animals to hyperoxia results in respiratory failure and death within 72 h. Histologic evaluation of the lungs of these animals demonstrates epithelial apoptosis and necrosis. Although the generation of reactive oxygen species (ROS) is widely thought to be responsible for the cell death observed following exposure to hyperoxia, it is not clear whether they act upstream of activation of the cell death pathway or whether they are generated as a result of mitochondrial membrane permeabilization and caspase activation. We hypothesized that the generation of ROS was required for hyperoxia-induced cell death upstream of Bax activation. In primary rat alveolar epithelial cells, we found that exposure to hyperoxia resulted in the generation of ROS that was completely prevented by the administration of the combined superoxide dismutase/catalase mimetic EUK-134 (Eukarion, Inc., Bedford, MA). Exposure to hyperoxia resulted in the activation of Bax at the mitochondrial membrane, cytochrome c release, and cell death. The administration of EUK-134 prevented Bax activation, cytochrome c release, and cell death. In a mouse lung epithelial cell line (MLE-12), the overexpression of Bcl-XL protected cells against hyperoxia by preventing the activation of Bax at the mitochondrial membrane. We conclude that exposure to hyperoxia results in Bax activation at the mitochondrial membrane and subsequent cytochrome c release. Bax activation at the mitochondrial membrane requires the generation of ROS and can be prevented by the overexpression of Bcl-XL.

Stimuli that cause mitochondrial-dependent apoptosis utilize a variety of pathways that converge on the pro-apoptotic Bcl-2 family members Bax or Bak (8). Activation of Bax or Bak results in their oligomerization at the outer mitochondrial membrane and causes the release of a variety of pro-apoptotic molecules, including cytochrome c, from the intermembrane space into the cytosol (9). We have shown previously that hyperoxia-induced cell death requires the pro-apoptotic Bcl-2 proteins Bax or Bak and results in the release of cytochrome c, suggesting that hyperoxia acts as a mitochondrial-dependent apoptotic stimulus (10).

Several groups of investigators have observed ROS generation during exposure to hyperoxia and have hypothesized that these ROS act as upstream signaling molecules that initiate cell death (5, 11, 12). In support of this hypothesis, the generation of ROS has been demonstrated following the administration of many mitochondrial-dependent apoptotic stimuli including growth factor withdrawal, UV radiation, and the administration of some chemotherapeutic agents (13–15). Antioxidants have been shown to prevent or delay death in all of these models. In many models, however, investigators have reported that the generation of ROS during mitochondrial-dependent apoptotic processes occurs downstream of the release of cytochrome c from the intermembrane space (16, 17). Furthermore, both mitochondrial membrane proteins and components of the mitochondrial electron transport chain have been reported to be caspase substrates (14, 18). If oxidant generation occurs downstream of mitochondrial membrane permeabilization, then antioxidants are likely to delay rather than prevent cell death.

In this study, we sought to determine whether the generation of ROS acted upstream or downstream of Bax activation and cytochrome c release in primary cultures of alveolar epithelial cells. As both the superoxide anion and H₂O₂ may be required for hyperoxia-induced cell death, we chose to use the combined superoxide dismutase/catalase mimetic EUK-134. This compound has been shown to correct the mev1 genetic defect caused by a deficiency in antioxidant enzymes in the nematode Caenorhabditis elegans (19). The administration of EUK-134 concomitant with hyperoxic exposure completely prevented hyperoxia-induced depletion of reduced glutathione levels, Bax activation at the mitochondrial membrane, cytochrome c release, and cell death. In a mouse lung epithelial cell line, the overexpression of Bcl-XL prevented both Bax activation and cell death, demonstrating the requirement for Bcl-2 proteins in the apoptotic pathway initiated by hyperoxia.

EXPERIMENTAL PROCEDURES

Cell Culture and Oxygen Environment of the Cells—Rat alveolar epithelial cells were isolated from Sprague-Dawley rats weighing 200–225 g. This procedure is described in detail elsewhere (20). The cells were cultured in serum-free Dulbecco’s modified essential medium supplemented with penicillin (200 units/ml), streptomycin (200 μg/ml), kanamycin (100 μg/ml), amphotericin B (80 μg/ml), 20 mM HEPES, and...
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10% heat-inactivated fetal calf serum (37 °C, 5% CO₂). Medium was changed on Day 2 of isolation (Day 0 – day of isolation). The cells were washed twice with 2–3 ml of PBS when the medium was changed. Cells were exposed to hyperoxia (95% O₂, 5% CO₂) in Oxyburn (Bio-Sperix, Ltd., Redfield, NY) chambers or normoxia (room air) at 37 °C. Glucose concentrations were measured at the end of the hyperoxic exposure and were always in excess of 200 mg/dl.

Measurement of Lactate Dehydrogenase (LDH) Release—LDH release was measured using a commercially available assay (cytotoxicity detection kit, Roche Applied Science). After gentle agitation, 500 μl of medium was removed, and the remaining cells were lysed by adding 500 μl of 5% Triton X-100 solution. After 30 min at room temperature, each plate was gently scraped with a cell scraper, and 500 μl of the lysate was added. The samples were incubated in dark at room temperature for 10 min. LDH release was measured using a modification of a previously described method (23). The cells were fixed with 3% formaldehyde (5 min, 20 °C heating, the protein was resolved on a sodium dodecyl sulfate-15% polyacrylamide gel). The bands were visualized using enhanced chemiluminescence (PerkinElmer Life Sciences).

Immunoblotting—Immunoblotting was performed as described previously (10). Cell lysates (20 μg) were mixed with sample loading buffer (125 mM Tris base (pH 6.8), 4% β-mercaptoethanol, 20% (v/v) sodium dodecyl sulfate, 0.05% (v/v) phenol red) (Bio-Rad) and heated to 100 °C. The samples were then washed with PBS, and 5% non-fat milk in PBS (16 h, 4 °C). The cells were then washed four times in 0.1% bovine serum albumin in PBS (15 min each), incubated with tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse IgG antibody (1.5 mg/ml) (Jackson ImmunoResearch, West Grove, PA) diluted 1:100 in 0.1% bovine serum albumin in PBS (60 min, 37 °C), and washed again as above. The slides were mounted with 1,4-diazabicyclo (2,2,2)-octane (1 mg/ml) (Sigma) in gelvatol.

Fluorescence microscopy. Photos were obtained with an LSM 510 argon/helium laser confocal microscope using a ×100 Plan-Neofluar objective (Zeiss) with a pinhole of 1.0 Airy units. The excitation wavelengths for MitoTracker Red CMXRs and Alexa Fluor 488 were 543 and 488 nm, respectively. A long pass filter (585 nm) was used to measure MitoTracker emission, and a band pass filter (505–530 nm) was used to measure Alexa Fluor 488 emission.

Immunohistochemistry for the Examination of Bax Activation and Localization—Measurements of Bax activation were performed using a modification of a previously described method (24). Alveolar epithelial cells were cultered on microscope slides (Fisher Scientific, product number 12–222, 22 × 22 mm) in 35-mm tissue culture plates and placed into experimental conditions. When the cell plates were removed from experimental conditions, the medium was aspirated from each plate and replaced with MitoTracker Red CMXRs (1 μM) (Molecular Probes, Eugene, OR) in medium (30 min, 37 °C). The cells were fixed with cold triton-X-100 (1:1) (4 min), permeabilized with a 0.1% Triton X-0.05% deoxycholate solution in PBS (20 min, 20 °C), and then blocked with a 0.1% Triton, 0.3% bovine serum albumin solution in PBS (45 min, 20 °C). The cells were then incubated with mouse anti-Bax antibody (0.5 mg/ml) (PharMingen) diluted 1:100 in 0.1% bovine serum albumin in PBS (16 h, 4 °C). The cells were then washed four times in 0.1% bovine serum albumin in PBS (15 min each), incubated with Alexa Fluor 488 goat anti-mouse IgG (2 mg/ml) (Molecular Probes) diluted 1:50 in 0.1% bovine serum albumin in PBS (1 h, 37 °C) and washed again as above. The coverslips were mounted with 1,4-diazabicyclo (2,2,2)-octane (1 mg/ml) (Sigma) in gelvatol.

Measurement of Bax Activation and Localization with the Mitochondrial Membrane—Bax activation was assessed with confocal laser microscopy. Nuclei were scored as apoptotic if they demonstrated nuclear condensation and/or fragmentation and/or condensation and/or fragmentation. Single dimension confocal images (line plots) were obtained from these images by selecting arbitrary 20–μm lines in the cytoplast of multiple cells using the Zeiss LSM Image Examiner software (24).

Retroviral Transformation of MLE-12 Cells—The high capacity retroviral packaging cell line PA67 (Clontech) at 60–70% confluence was transfected in Dulbecco’s modified essential medium (supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), 20 mM HEPES, and 10% heat-inactivated fetal calf serum) with plasmid DNA encoding puromycin resistance or puromycin resistance and Bcl-XL, according to the manufacturer’s instructions (10 μg of DNA, 5 μl of Miris transfection reagent in 500 μl of serum-free medium per plate with chloroform–ethanol (1:1) solution. The cells were fixed with cold acetone (5 min, –20 °C). The cells were then washed with PBS, and 5 μl of 0.25% glutaraldehyde (1 h, 4 °C). The membrane was washed three times with PBS and subsequently incubated with mouse anti-Bax antibody (0.2 mg/ml) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:1000 overnight at 4 °C. The membrane was washed three times with PBS-T and incubated for 1.5 h at room temperature with horseradish peroxidase-conjugated secondary antibody (5 μg/ml) (Cell Signaling Technology, Inc., Beverly, MA). The membrane was washed three times with TBS-T and analyzed by enhanced chemiluminescence (PerkinElmer Life Sciences).

Statistical Analysis—One-way analysis of variance was used to test for significant differences in measured variables between groups. Statistical significance was determined using the Bonferroni correction for multiple comparisons. Statistical significance was determined at the 0.05 level.

RESULTS

ROS Are Required for Hyperoxia-induced Cell Death in Primary Alveolar Epithelial Cells—We and others have demonstrated that exposure to hyperoxia results in the generation of reactive oxygen species (5, 10, 25). We sought to determine whether the generation of ROS was required for hyperoxia-induced cell death. Primary rat alveolar epithelial cells were...
exposed to hyperoxia or normoxia for 24 h in the presence or absence of EUK-134 (20 μM). After 24 h, the administration of EUK-134 completely abolished the depletion of reduced glutathione observed in untreated cells during exposure to hyperoxia (Fig. 1A). We then sought to determine whether the generation of ROS was required for cell death in primary rat alveolar epithelial cells following exposure to hyperoxia. After 48 h, over 80% of control cells had died as determined by LDH release, whereas cells treated with EUK-134 (20 μM) had levels of death similar to normoxic controls (Fig. 1B). In cells that are susceptible to hyperoxia, the morphology of the dead cells has been reported to be either apoptotic or necrotic depending on the cell type examined (12, 26, 27). To determine whether hyperoxia-induced apoptosis required the generation of ROS, cellular morphology following exposure to hyperoxia was examined using DAPI staining. Alveolar epithelial cells exposed to hyperoxia demonstrated typical morphological changes of apoptosis with small, intensely fluorescent nuclei on DAPI staining (Fig. 1, C and D). These changes were completely prevented by treatment with EUK-134 (20 μM). Collectively, these results

addition of the combined superoxide dismutase/catalase mimetic EUK-134 (20 μM), and reduced glutathione (24 h) (A), cell death (LDH release) (48 h) (B), and nuclear morphology for apoptosis (DAPI staining, 48 h) (C and D) were assessed. Asterisks indicate apoptotic nuclei (D). *, p < 0.05, n = 3, ± S.E. (analysis of variance) (A), p = 0.002, n = 4, ± S.E. (B) and p = 0.006, n = 3, ± S.E. (C) for comparison between normoxia control and cells exposed to hyperoxia.

**Fig. 1.** Primary rat alveolar epithelial cells were exposed to normoxia (21% O₂) or hyperoxia (95% O₂) with or without the addition of the combined superoxide dismutase/catalase mimetic EUK-134 (20 μM), and reduced glutathione (24 h) (A), cell death (LDH release) (48 h) (B), and nuclear morphology for apoptosis (DAPI staining, 48 h) (C and D) were assessed. Asterisks indicate apoptotic nuclei (D). *, p < 0.05, n = 3, ± S.E. (analysis of variance) (A), p = 0.002, n = 4, ± S.E. (B) and p = 0.006, n = 3, ± S.E. (C) for comparison between normoxia control and cells exposed to hyperoxia.

**Fig. 2.** Primary rat alveolar epithelial cells were exposed to normoxia (21% O₂) or hyperoxia (95% O₂) for 32 h with or without the addition of the combined superoxide dismutase/catalase mimetic EUK-134 (20 μM), and Bax activation was determined by confocal laser microscopy. Activation of Bax at the mitochondria occurred in cells exposed to hyperoxia and was prevented in cells exposed to hyperoxia that were treated with EUK-134. Staurosporine (10 μM, 6 h)-treated cells are shown as a positive control.
Primary rat alveolar epithelial cells were exposed to normoxia (21% O₂) or hyperoxia (95% O₂) for 32 h with or without the addition of the combined superoxide dismutase/catalase mimetic EUK-134 (20 μM), and line plots of activated Bax (green) and MitoTracker Red CMXRos (red) were made through random sections of the cytoplasm with representative plots shown. In all conditions where Bax was activated, staining for activated Bax colocalized with MitoTracker Red CMXRos staining.
show that ROS are required for hyperoxia-induced cell death in primary alveolar epithelial cells.

**ROS Are Required for Bax Activation following Exposure to Hyperoxia**

Previously, we have shown that cells lacking Bax and Bak are resistant to hyperoxia-induced cell death (10). These results suggest that hyperoxic cell death occurs through a mitochondrial-dependent pathway. In many models of mitochondrial-dependent apoptosis (e.g. growth factor withdrawal, the administration of some chemotherapeutic agents), the generation of ROS occurs following mitochondrial outer membrane permeabilization (14, 16, 17). In these models, antioxidants delay cell death but fail to prevent it. In other models of mitochondrial-dependent apoptosis, for example, exposure to ionizing radiation, ROS act upstream to cause the activation of Bax or Bak (13, 14). We sought to determine whether ROS acted upstream of Bax activation in hyperoxia-induced cell death. Primary rat alveolar epithelial cells were exposed to hyperoxia (32 h) or staurosporine (10 μM) (6 h) in the presence or absence of EUK-134 (20 μM) and then stained with an antibody that recognizes an epitope on the N terminus of the Bax molecule, which is exposed only when Bax is activated. Exposure of primary alveolar epithelial cells to hyperoxia or staurosporine resulted in Bax activation. The administration of EUK-134 prevented Bax activation in response to hyperoxia but not in response to staurosporine (Fig. 2). These results suggest that EUK-134 prevents hyperoxia but not staurosporine-induced cell death upstream of the activation of Bax.

**Activated Bax Localizes to the Mitochondrial Membrane**

During mitochondrial-dependent apoptosis, activated Bax forms oligomers that permeabilize the outer mitochondrial membrane (8). To determine whether Bax activation occurred at the mitochondrial membrane, we co-stained the cells with...
MitoTracker Red following 32 h of exposure to normoxia or hyperoxia or exposure to staurosporine (10 μM) for 6 h. Line plots of activated Bax and MitoTracker Red were made through random sections of the cytoplasm, and representative plots are displayed in Fig. 3. In all conditions where Bax was activated, staining for activated Bax colocalized with MitoTracker Red staining.

**ROS Are Required for Cytochrome c Release following Exposure to Hyperoxia in Primary Alveolar Epithelial Cells**—The activation of Bax or Bak triggers apoptosis by causing the release of cytochrome c from the mitochondrial intermembrane space into the cytosol (28). If ROS are acting upstream of Bax activation to induce cell death in alveolar epithelial cells, EUK-134 would be expected to prevent mitochondrial cytochrome c release. To test this hypothesis, we examined cytochrome c staining in primary cultures of rat alveolar epithelial cells following exposure to normoxia, hyperoxia (32 h), or staurosporine (10 μM, 6 h) in the presence or absence of EUK-134 (20 μM). The administration of EUK-134 prevented hyperoxia but not staurosporine-induced cytochrome c release (Fig. 4). These results are consistent with ROS acting upstream of mitochondrial outer membrane permeabilization following exposure to hyperoxia.

**The Overexpression of Bcl-X<sub>L</sub> Prevents Cytochrome c Release from the Mitochondrial Intermembrane Space**—A major mechanism by which anti-apoptotic Bcl-2 proteins exert their protective effect is by sequestering Bax or Bak and preventing their activation in response to an apoptotic stimulus (30). To determine the mechanism by which the overexpression of Bcl-X<sub>L</sub> prevented hyperoxia-induced cell death in lung epithelial cells, we measured Bax activation following exposure to hyperoxia in mouse lung epithelial cells overexpressing puromycin resistance and Bcl-X<sub>L</sub> (Fig. 6). These results indicate that hyperoxia induces cell death through the mitochondrial-dependent pathway.

**DISCUSSION**

The effects of hyperoxia on cellular function and survival have been widely held to be secondary to the generation of ROS. Nevertheless, previous attempts to prevent hyperoxic cell death *in vitro* using antioxidants have only been partially effective (31–33). Similarly, studies using transgenic animals have demonstrated only modest protection against hyperoxia-induced lung injury in animals expressing Mn-superoxide dismutase, CuZn-superoxide dismutase, and extracellular catalase (34–36). Cells exposed to mitochondrial-dependent death stimuli generate a burst of ROS that occurs near the time of cytochrome c release from the intermembrane space (14, 16, 18). This oxidative burst is large and can be attenuated by the administration of caspase inhibitors (14, 18). Recently, Ricci et al. (18) reported that the oxidative burst occurring with the release of cytochrome c resulted from caspase-mediated cleavage of complexes I and II of the mitochondrial electron transport chain. We questioned whether ROS acted upstream in the cell death pathway following exposure to hyperoxia or whether they were generated later as a result of mitochondrial membrane permeabilization. We found that EUK-134 prevented hyperoxia-induced cell death upstream of cytochrome c release, indicating that ROS act upstream of the mitochondria in this pathway.
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For the cell to eliminate superoxide anions and other radicals, two sequential reactions are required. Superoxide anions are converted to H₂O₂ through one of the cellular superoxide dismutases, Mn-superoxide dismutase in the mitochondria and Cu/Zn-superoxide dismutase in the cytosol. H₂O₂ itself is an important pro-apoptotic molecule and must be metabolized to molecular oxygen and water by catalase (37, 38). EUK-134 is both a superoxide dismutase and a catalase mimetic and can therefore detoxify hydroxyl radicals completely. EUK-134 did not act as a nonspecific inhibitor of apoptosis as the administration of EUK-134 did not prevent Bax activation or cytochrome c release in response to staurosporine. Our results are consistent with those of Ilizarov et al. (32), who recently reported that the overexpression of catalase or superoxide dismutase alone was not as effective as the combination of the two in preventing hyperoxic cell death in a mouse lung epithelial cell line. We reported previously that the combination of a glutathione peroxidase mimetic and a superoxide dismutase mimetic failed to prevent hyperoxia-induced death in fibroblasts. Our findings are similar to those of other investigators who have demonstrated improved protection against oxidant stress with the use of EUK-134 as compared with other antioxidants (19).

Mitochondrial-dependent apoptosis is regulated by the Bcl-2 family of proteins. Members of this family are either pro- or anti-apoptotic and share homology in up to four conserved regions termed BCL-2 homology (BH) domains (1–4). The anti-apoptotic proteins, which include Bcl-2 and Bcl-XL, are all multidomain proteins that share homology throughout all four BH domains. The pro-apoptotic Bcl-2 proteins are divided into those sharing multiple BH3 domains (1–3), for example, Bax and Bak, and those sharing only the BH3 domain (8). Studies from cells derived from mice lacking the genes for Bax and Bak demonstrate that all BH3 proteins require the presence of Bax or Bak to induce apoptosis. In response to a mitochondrial-dependent stimulus, Bax or Bak become activated and translocate to the mitochondrial outer membrane. There, they oligomerize, allowing for the release of cytochrome c and other pro-apoptotic molecules into the cytosol. The activation of Bax and release of cytochrome c have been reported to occur nearly simultaneously (28). Previously, we have reported that embryonic fibroblasts from bax−/−/bak−/− mice were protected against hyperoxia-induced cell death and that fibroblasts stably overexpressing Bcl-XL were protected against hyperoxia-induced mitochondrial membrane permeabilization, cytochrome c release, caspase activation, and cell death (10). In this report, we extend those observations by demonstrating the activation of Bax in response to hyperoxia at the mitochondrial membrane in primary alveolar epithelial cells and demonstrating that ROS act upstream of Bax activation in this pathway. In accord with our previous report, we found that mouse lung epithelial cells stably overexpressing high levels of Bcl-XL were protected against hyperoxia-induced Bax activation at the mitochondrial membrane and subsequent cell death. Collectively, these findings establish the importance of the Bcl-2 family of proteins as regulators of hyperoxia-induced cell death.

Our results indicate that ROS act upstream of Bax activation in the cell death pathway initiated by hyperoxia; however, they do not allow us to draw conclusions about where in the cell ROS are generated. In whole lung homogenates and in endothelial cells, investigators have demonstrated that the mitochondrial generation of ROS increases during exposure to hyperoxia (5, 7). It is possible, however, that the ROS generation they observed was occurring after mitochondrial membrane permeabilization. We reported previously that cells lacking mitochondrial DNA (ρ0 cells) had marked attenuation of ROS generation in response to hyperoxia; however, they were not protected against hyperoxia-induced cell death (10). These results suggested that the mitochondrial generation of ROS was not required for cell death following exposure to hyperoxia. Using electron spin trapping to measure ROS, Natarajan and coworkers (25) reported that ROS generation following exposure to hyperoxia in endothelial cells was mediated primarily by the NAD(P)H oxidase system. These results are consistent with results from transgenic animals, which demonstrate only modest protection against hyperoxia-induced lung injury in animals expressing the manganese-superoxide dismutase localized primarily at the mitochondrial membrane but more marked protection in animals overexpressing the Cu/Zn-superoxide dismutase (which localizes to the cytosol) (35, 36).

Our results do not address the mechanism(s) by which the generation of ROS causes the activation of Bax. The mitogen-activated protein kinase (MAPK) pathways are activated in response to changes in the extracellular or intracellular environment and have been shown to be important in the activation of Bax in response to oxidant stress in neurons (39). Recently, two groups of investigators have demonstrated that cell death in MLE-12 cells required activation of the JNK pathway, suggesting a possible link between ROS generation and Bax activation (40, 41).

In conclusion, we demonstrate that alveolar epithelial cells undergo cell death in response to hyperoxia through a mitochondrial-dependent apoptotic pathway that requires the generation of ROS upstream of Bax activation at the mitochondrial membrane (Fig. 7). The combined superoxide dismutase/catalase mimetic EUK-134 prevents hyperoxia-induced Bax activation, cytochrome c release, and cell death. Overexpression of Bcl-XL protects cells from hyperoxia-induced cell death by preventing the activation of Bax.

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