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Hyperoxia-induced Apoptosis Does Not Require Mitochondrial Reactive Oxygen Species and Is Regulated by Bcl-2 Proteins*

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G. R. Scott Budinger‡, May Tso, David S. McClintock, David A. Dean, Jacob I. Sznajder, and Navdeep S. Chandel

From the Division of Pulmonary and Critical Care Medicine, Northwestern University, Chicago, Illinois 60611

Exposure of animals to hyperoxia results in lung injury that is characterized by apoptosis and necrosis of the alveolar epithelium and endothelium. The mechanism by which hyperoxia results in cell death, however, remains unclear. We sought to test the hypothesis that exposure to hyperoxia causes mitochondria-dependent apoptosis that requires the generation of reactive oxygen species from mitochondrial electron transport. Rat1a cells exposed to hyperoxia underwent apoptosis characterized by the release of cytochrome *c*, activation of caspase-9, and nuclear fragmentation that was prevented by the overexpression of Bcl-X_L. Murine embryonic fibroblasts from *bax*$^{-/-}$ *bak*$^{-/-}$ mice were resistant to hyperoxia-induced cell death. The administration of the antioxidants manganese (III) tetrakis (4-benzoic acid) porphyrin, ebselen, and *N*-acetylcysteine failed to prevent cell death following exposure to hyperoxia. Human fibrosarcoma cells (HT1080) lacking mitochondrial DNA (ρ^0 cells) that failed to generate reactive oxygen species during exposure to hyperoxia were not protected against cell death following exposure to hyperoxia. We conclude that exposure to hyperoxia results in apoptosis that requires Bax or Bak and can be prevented by the overexpression of Bcl-X_L. The mitochondrial generation of reactive oxygen species is not required for cell death following exposure to hyperoxia.

Exposure of normal animals to 100% oxygen for 48–72 h causes respiratory failure and death (1, 2). Examination of the lungs of animals that die following exposure to hyperoxia reveals a pattern of injury similar to that seen in patients with the acute respiratory distress syndrome with both apoptosis and necrosis of the alveolar endothelium and epithelium. Several groups of investigators have demonstrated that cultured cells undergo apoptosis following exposure to hyperoxia; however, the mechanisms by which hyperoxia induces apoptosis have not been elucidated (2, 4–6).

The intracellular production of reactive oxygen species during exposure to hyperoxia is widely held to be responsible for both the lung injury seen in intact animals and the death of cells in culture following exposure to hyperoxia. In whole lung homogenates, cells in culture, and isolated mitochondria, exposure to hyperoxia increases the intracellular production of reactive oxygen species (ROS)[1] (7–10). Most of these excess ROS are generated in the mitochondria through an increased flux of electrons through the ubisemiquinone/ubiquinone pathway at site III in the mitochondrial electron transport chain (8, 10). These ROS are thought to be responsible for the induction of necrosis and apoptosis in cultured cells and the lung injury in animals observed following exposure to hyperoxia (11).

Many stimuli that cause cell death (*e.g.* growth factor withdrawal, the chemotherapeutic agent staurosporine, and ultraviolet radiation) result in permeabilization of the mitochondrial membrane with the release of cytochrome *c* (reviewed in Ref. 12). This mitochondria-dependent apoptosis is initiated by the translocation or activation of the proapoptotic Bcl-2 family members Bax or Bak and prevented by the overexpression of anti-apoptotic molecules from the same family (Bcl-X_L or Bcl-2) (13). Permeabilization of the outer mitochondrial membrane results in release of cytochrome *c* into the cytosol, where it combines with Apaf-1 to activate caspase-9 in the apoptosome. Caspase-9 then activates the terminal caspase cascade, resulting in apoptosis (14). In animal models of hyperoxic lung injury, investigators have reported alterations in the expression of both proapoptotic and anti-apoptotic proteins of the Bcl-2 family (15, 16). It is not known, however, whether the expression of these proteins is required for or can prevent cell death following exposure to hyperoxia.

We sought to test the hypothesis that exposure to hyperoxia triggers mitochondria-dependent apoptosis that requires the generation of ROS from mitochondrial electron transport. Consistent with this hypothesis, we found that exposure to hyperoxia caused apoptosis through a mechanism that required Bax or Bak and was inhibited by Bcl-X_L. Surprisingly, however, neither the mitochondrial generation of reactive oxygen species nor a functional electron transport chain was required for cell death.

EXPERIMENTAL PROCEDURES

Cell Culture and Oxygen Environment of the Cells—Rat1a cells and HT1080 fibrosarcoma cells were cultured in serum-free 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 (37 °C, 5% CO_2). Cell lines were passaged every 2–3 days and were discarded after 65 generations. Rat1a cells constitutively overexpressing Bcl-X_L and empty plasmid encoding neomycin resistance (control cells) were a kind gift from Dr. N. Hay (17). The ρ^0 HT1080 fibrosarcoma cells were generated by incubating wild type cells in medium containing ethidium bromide (100 ng/ml), sodium pyruvate (1 mM), and uridine (100 μg/ml) for 3–5 weeks (18, 19). Murine embryonic

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‡ To whom correspondence should be addressed: Northwestern University, 303 E. Chicago Ave., Tarry 14-707, Chicago, IL 60611. Tel.: 312-908-8163; Fax: 312-908-4650; E-mail: s-buding@northwestern.edu.

[1] The abbreviations used are: ROS, reactive oxygen species; TMRE, tetramethylrhodamine ethyl ester; LDH, lactate dehydrogenase; DAPI, 4′,6′-diamidino-2-phenylindole dihydrochloride; PBS, phosphate-buffered saline; CM-H_2DCFDA, 5-(and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; FACS, fluorescence-activated cell sorting; IL, interleukin; Mn(III) TBAP, manganese(III) tetrakis(4-benzoic acid) porphyrin.

fibroblasts from wild type and $bax^{-/-}bak^{-/-}$ mice were a kind gift from Dr. Craig B. Thompson (20). These cells were immortalized with simian virus 40 (5 plaque-forming units/cell), a kind gift of Dr. Kathleen Rundell (21). Cells were exposed to hyperoxia (95% O_2, 5% CO_2) or normoxia (21% O_2, 5% CO_2, 74% N_2) in sealed, humidified 1-liter glass chambers at 37 °C. The chambers were continuously perfused with the appropriate gas mixture at a flow rate of 4 liters/min. Medium volume loss is <3% over 72 h in this system. All experiments were performed with cells at 15–20% confluence at the beginning of the experiment.

Mitochondrial Membrane Potential—After exposure to hyperoxia or normoxia for the indicated time, the cells were removed from the plate using trypsin (0.25%), which was subsequently inactivated with medium. The cells were centrifuged (200 × *g* for 5 min) and then resuspended in PBS containing tetramethyrhodamine ethyl ester (TMRE) (2 μM) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (10 μM) where indicated for 30 min. TMRE is a cationic dye that accumulates in the mitochondria in proportion to the mitochondrial membrane potential. TMRE fluorescence was measured using fluorescence-activated cell sorting analysis (FACS) (22).

Lactate Dehydrogenase (LDH) Release—LDH release was measured using a commercially available assay (Cytotoxicity Detection Kit; Roche Molecular Biochemicals). After gentle agitation, 500 μl of medium was removed, and the remaining cells were lysed by adding the same volume of 1% Triton X-100. After 30 min, 500 μl of the lysate was removed. The samples were incubated (30 min) with buffer containing NAD^+, lactate, and tetrazolium. LDH converts lactate to pyruvate generating NADH. The NADH then reduces tetrazolium (yellow) to formazan (red), which was detected by flourescence (490 mm). LDH release is expressed as the ratio of the LDH in the medium over the total LDH (lysate).

Fragmented DNA-Histone Complexes and DAPI Staining for Nuclear Morphology (Apoptosis)—DNA-histone complexes were measured using a commercially available assay (Roche Molecular Biochemicals). Cell lysates were placed in a streptavidin-coated microplate with a mixture of anti-histone-biotin and anti-DNA peroxidase-conjugated mouse monoclonal antibody and incubated for 2 h. The anti-histone biotin-labeled antibody binds to the histone component of the nucleosomes and fixes the immunocomplex to the streptavidin-coated microplate. The peroxidase-conjugated mouse monoclonal antibody binds the DNA component of the nucleosomes and uses 2,2′-azino-bis(3-ethylbenzthiazolin-6-sulfonate). After washing, nucleosome concentration is determined photometrically. Values are expressed as the -fold induction over normoxic controls. Nuclear morphology was assessed with DAPI staining as previously described (23). Briefly, floating cells were aspirated, and adherent cells were removed from the plate using phosphate-buffered saline without calcium or magnesium (PBS) containing 1 mM EDTA. Floating and adherent cells were combined and incubated with 0.15% glutaraldehyde (15 min, 25 °C), centrifuged and resuspended in 80 μl of DAPI (10 μg/ml, 15 min, 37 °C), and then placed on a slide for fluorescence microscopy. Nuclei were scored as apoptotic if they demonstrated nuclear fragmentation or condensation and reported as the percentage of total cells counted (100 cells).

Caspase-9 Activation—Caspase-9 activity was measured using a commercially available kit (R & D Systems). At the designated time, the cells were removed from the plate with 5 mM EDTA in PBS, combined with the supernatant, centrifuged, washed twice in PBS, and then lysed. The supernatant was then incubated with a caspase-9-specific peptide connected to a fluorescent reporter molecule, 7-amino-4-trifluoromethyl coumarin, and fluorescence was measured. Values are expressed as -fold induction over untreated cells.

Cell Cycle Analysis—Adherent cells were removed from the plate with calcium-free PBS containing 5 mM EDTA, washed, and incubated overnight in 95% ethanol (4 °C). The cells were then incubated in stain solution (propidium iodide (50 μg/ml), RNase, (180 units/ml), Triton X-100 (0.1%), citrate buffer (4 mM), and polyethylene glycol (3%)) at a concentration of 1×10^6 cells/ml. The solution was briefly vortexed, and an equal volume of salt solution (propidium iodide (50 μg/ml), Triton X-100 (0.1%), NaCl (4 M), and polyethylene glycol (3%)) was added (24).

Cytochrome c Release—Cells grown to 20–40% confluence were exposed to hyperoxia for the times shown. Adherent cells were removed using PBS/EDTA (1 mM), washed, and spun onto glass slides (Cytospin 3, Thermo Shandon, 200 × *g*, 5 min). The cells were fixed with a 1:1 mixture of methanol and acetone (5 min, −20 °C), blocked in 1% bovine serum albumin in PBS (30 min, 25 °C), and incubated with 1 μg of anti-cytochrome *c* antibody (clone 6H2.B4; PharMingen) per ml of 1% bovine serum albumin in PBS (120 min, 37 °C). The cells were washed four times with 1% bovine serum albumin in PBS (15 min each), incubated with 1 μg of tetramethylrhodamine isothiocyanate-conjugated anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA) (60 min, 37 °C), and washed as above. The slides were mounted with DAPI/DABCO (Molecular Probes, Inc., Eugene, OR) (25). Cytochrome *c* release was assessed with fluorescence microscopy. Fluorescent photos were obtained with a 12-bit cooled Hamamatsu CCD camera and analyzed with Openlab 3.0 software (Improvision). Appropriate filters were used to detect rhodamine and DAPI. Single fluorophore images were collected, and an overlay of the images was then created. Cells were counted as having released cytochrome *c* if there was a loss of punctuate staining. Approximately 100 cells were counted for each condition (average of 5–10 fields). Results are expressed as cells releasing cytochrome *c* as a fraction of total cells.

For immunoblotting, cells were grown to 60–70% confluence on five 100-mm plates per condition and exposed to air or hyperoxia for 60 h. Adherent and floating cells were removed (PBS/EDTA, 1 mM), washed, and resuspended in sucrose buffer (250 mM sucrose, 20 mM HEPES, pH 7.5, 2 mM $MgCl_2$, 1 mM NaEDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol) on ice for 30 min. The cells were then centrifuged and resuspended in an equal volume of sucrose buffer and transferred to a Dounce homogenizer (10 strokes). The nuclei and cell debris were removed by centrifugation (400 × *g*, 5 min, 4 °C). The supernatant was then centrifuged (20,000 × *g*, 15 min, 4 °C). The resulting supernatant (cytosolic fraction) was used for the analysis. A whole cell lysate was prepared by incubating cells in lysis buffer for 2 min followed by sonication (30 s) as a positive control. Cytosolic or mitochondrial fractions (80 μg) were mixed with sample loading buffer (125 mM Tris base (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, 200 mM dithiothreitol, 0.02% (w/v) bromphenol blue). After heating, the protein was resolved on an SDS-15% polyacrylamide gel and transferred to a Hybond-ECL nitrocellulose membrane (Amersham Biosciences). After transfer, the gel was stained with Ponceau S to verify uniform loading and transfer. Membranes were blocked with 5% (w/v) nonfat milk in TBS-T (100 mM Tris base (pH 7.5), 0.9% (w/v) NaCl, 0.1% (v/v) Tween 20) for 2 h at room temperature and subsequently incubated with 1 μg of the 7H8.2C12 anti-cytochrome *c* antibody (Pharmingen) per ml or with 0.5 μg of 20E8-C12 anti-cytochrome *c* oxidase subunit IV antibody (Molecular Probes) per ml overnight at 4 °C. The membrane was washed with TBS-T three times and incubated for 1.5 h at room temperature with horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences). The membrane was washed three times with TBS-T and analyzed by enhanced chemiluminescence (Amersham Biosciences) (23, 26, 27).

Measurement of Reactive Oxygen Species—Cells were washed with PBS and then loaded with 5-(and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) (10 μM) for 30 min in modified Eagle's medium without phenol red. The acetoxymethyl group on CM-H_2DCFDA is cleaved by nonspecific esterases within the cell, resulting in a charged molecule that does not cross the cell membrane. Intracellular generation of H_2O_2 irreversibly oxidizes the dye causing it to fluoresce (28). Cells loaded with CM-H_2DCFDA were exposed to normoxia (21% O_2), hyperoxia (95% O_2), or antimycin A (10 μg/ml) for 6 h in the presence of the CM-H_2DCFDA (10 μM) as described above. All procedures were carried out in the dark. After the exposure, the cells were washed and removed from the plate (PBS/EDTA, 1 mM), centrifuged (200 × *g* for 5 min), and resuspendend in PBS, and fluorescence was measured using FACS analysis.

Statistical Analysis—One-way analysis of variance was used to test for significant differences in measured variables between groups. Where the *F* statistic indicated a significant difference, individual differences were explored using the Bonferroni correction for multiple comparisons. Statistical significance was determined at the 0.05 level.

RESULTS

Cell Death following Exposure to Hyperoxia Occurs through a Mitochondria-dependent Pathway—Rat1a cells stably transfected with Bcl-X_L or empty plasmid (control-transfected cells) were exposed to hyperoxia for 72 h. The constitutive overexpression of Bcl-X_L prevented cell death following exposure to hyperoxia (Fig. 1*a*). Untransfected Rat1a cells die at a rate similar to control transfected cells (data not shown). To confirm that cell death following exposure to hyperoxia was apoptotic, Rat1a cells were exposed to hyperoxia for 72 h, and DNA fragmentation was assessed. Control transfected cells had similar increases in LDH release and DNA fragmentation following exposure to hyperoxia, whereas Bcl-X_L-transfected cells did not show increases in DNA fragmentation above that observed in control cells (Fig. 1*b*).

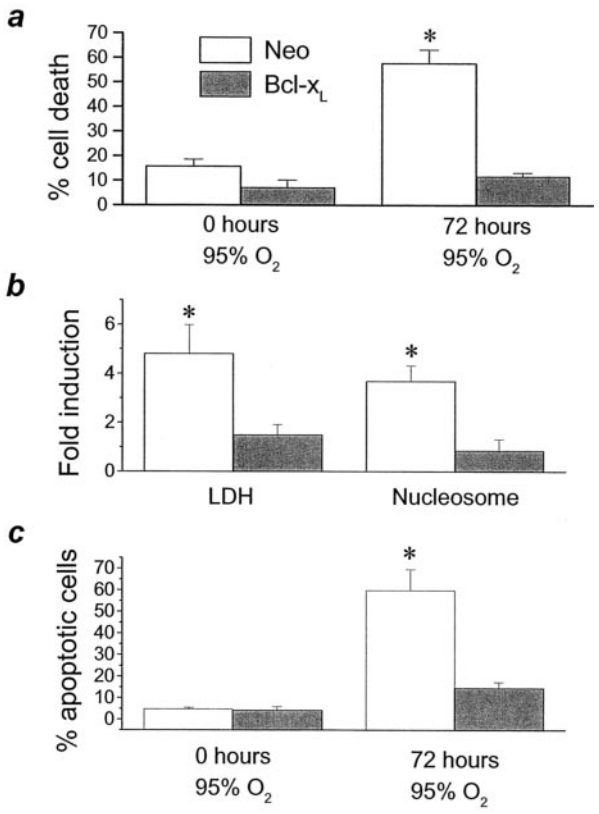

FIG. 1. **Overexpression of Bcl-X_L prevents apoptosis following exposure to hyperoxia.** *a*, Rat1a cells stably overexpressing a control vector for neomycin resistance (*neo*) (*open bars*) or Bcl-X_L (*gray bars*) were exposed to continuous hyperoxia in sealed humidified 1-liter chambers perfused with 95% O_2, 5% CO_2 or 21% O_2, 5% CO_2, 74% N_2 at 4 liters/min for 72 h, and cell death was measured by LDH release. *b* and *c*, to determine whether the observed cell death was apoptotic, control and Bcl-X_L-transfected cells were exposed to hyperoxia for 72 h, and LDH activity, DNA fragmentation, and nuclear morphology (DAPI staining) were measured. LDH and DNA fragmentation are expressed as -fold induction over normoxic control transfected cells. The results of five independent experiments are shown ± S.E. *, $p < 0.05$ compared with control transfected cells at normoxia.

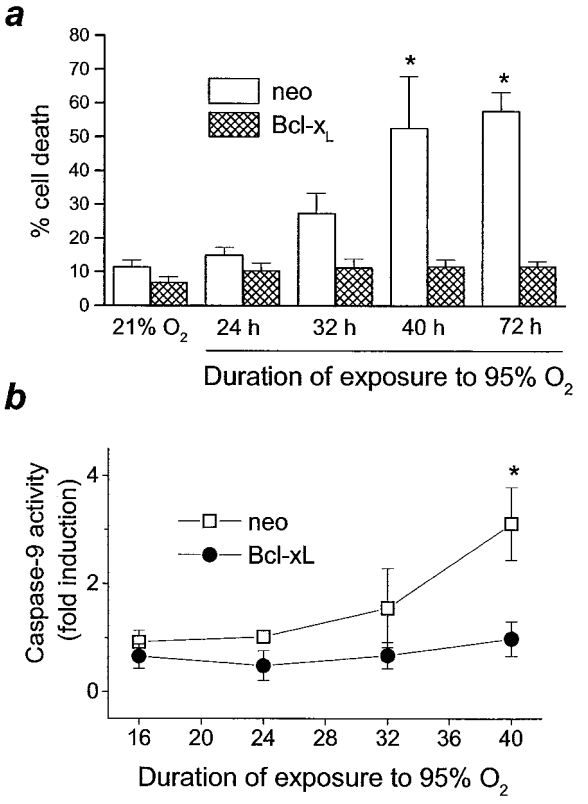

FIG. 2. **The commitment to die following exposure to hyperoxia is associated with the activation of caspase-9.** *a*, Rat1a cells stably expressing the vector for neomycin resistance (*open bars*) or Bcl-X_L (*gray bars*) were exposed to hyperoxia (95% O_2) for the times indicated, after which they were returned to normal oxygen conditions (21% O_2), and cell death was measured by LDH release after 72 h. Control cells were exposed to 21% O_2 for 72 h. The results of four independent experiments are shown. *, $p < 0.05$ for comparison between control and Bcl-X_L-transfected cells. *b*, caspase-9 activity was measured after 24, 32, or 40 h of exposure to hyperoxia. The results of five independent experiments are shown. *, $p < 0.05$ for comparison between control and Bcl-X_L-transfected cells ± S.E.

The protection conferred by overexpression of Bcl-X_L against apoptosis in response to hyperoxia suggests that hyperoxia-induced apoptosis occurs via a mitochondria-dependent pathway. To further investigate this hypothesis, we measured the activation of caspase-9 and the release of cytochrome *c* into the cytosol at the time when the cells committed to die during exposure to hyperoxia. To determine the duration of exposure to hyperoxia necessary to induce a commitment to die, Rat1a cells transfected with control vector or Bcl-X_L were exposed to hyperoxia for 24, 32, and 40 h and then brought back to 21% oxygen for 48, 40, and 32 h, respectively. Then cell death was measured. Cell death after 40 h of exposure to hyperoxia was similar to that seen after 72 h of exposure to hyperoxia, suggesting that the cells committed to die between 32–40 h of exposure to hyperoxia (Fig. 2*a*). We measured caspase-9 activation before and after the commitment to die. The timing of caspase-9 activation paralleled the timing of the commitment to die (Fig. 2*b*). In concordance with these findings, the percentage of control transfected cells that had released cytochrome *c* was increased after 40 h of exposure to hyperoxia when compared with normoxic controls (Fig. 3). Other investigators have shown that Rat1a cells are relatively resistant to CD95L-induced apoptosis (29). We found no significant activation of caspase-8 following exposure to hyperoxia for 40 h (data not shown).

Bax or Bak Are Required for Cell Death following Exposure to Hyperoxia—Stimuli that induce apoptosis through a mitochondria-dependent pathway have been shown to require the pro-apoptotic Bcl-2 family members Bax or Bak. Cells deficient in Bax and Bak have been shown to be resistant to multiple proapoptotic stimuli including the administration of chemotherapeutic drugs, growth factor withdrawal, and ultraviolet radiation but continue to undergo apoptosis upon exposure to CD95L (20). To confirm that hyperoxic cell killing required Bcl-2 proteins, we exposed immortalized murine embryonic fibroblasts isolated from mice deficient in both Bax and Bak ($bax^{-/-}bak^{-/-}$) and wild type controls to hyperoxia for 72 h and measured cell death. Cells lacking Bax and Bak were protected against exposure to hyperoxia, indicating that Bax or Bak are required for cell death following exposure to hyperoxia (Fig. 4).

Exposure to Hyperoxia Does Not Result in Early Mitochondrial Membrane Depolarization—Depolarization or hyperpolarization of the mitochondrial membrane has been observed before the commitment to die following the administration of a number of apoptotic stimuli that act through a mitochondria-dependent pathway. Therefore, we measured the mitochondrial membrane potential in control and Bcl-X_L-transfected cells before and after the commitment to die. In control transfected cells, the mitochondrial membrane potential was maintained following up to 32 h of exposure to hyperoxia after which a population of markedly depolarized cells was observed. In cells overexpressing Bcl-X_L, the mitochondrial membrane potential did not change following exposure to hyperoxia (Fig. 5). These results demonstrate that a significant fall in mitochon-

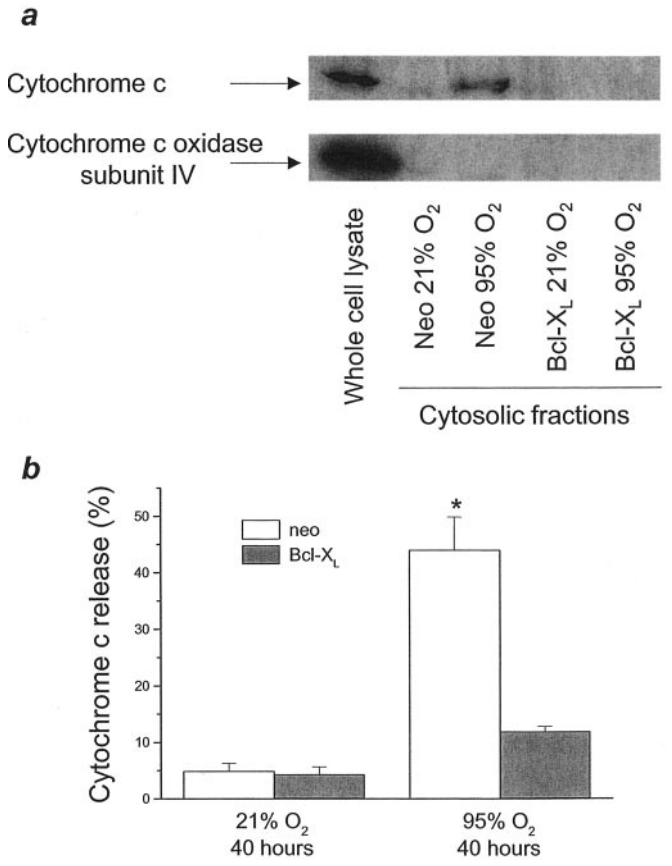

FIG. 3. **The overexpression of Bcl-XL prevents cytochrome *c* release following exposure to hyperoxia.** *a*, control and Bcl-X_L-transfected Rat1a cells were exposed to hyperoxia (95% O_2) or air (21% O_2) for 50 h, and cytochrome *c* release was determined by immunoblotting of the cytosolic fraction (*right four lanes*) and the whole cell lysate (*left lane*) for cytochrome *c*. Immunoblotting for cytochrome oxidase subunit IV was performed on the same sample to confirm appropriate cell fractionation. *b*, cytochrome *c* release in Rat1a cells exposed to hyperoxia or air was also determined by immunostaining. Results are expressed as the percentage of cells demonstrating a loss of punctuate staining. The results of three independent experiments are shown ± S.E. *, $p < 0.05$ for comparison between control and Bcl-X_L-transfected cells.

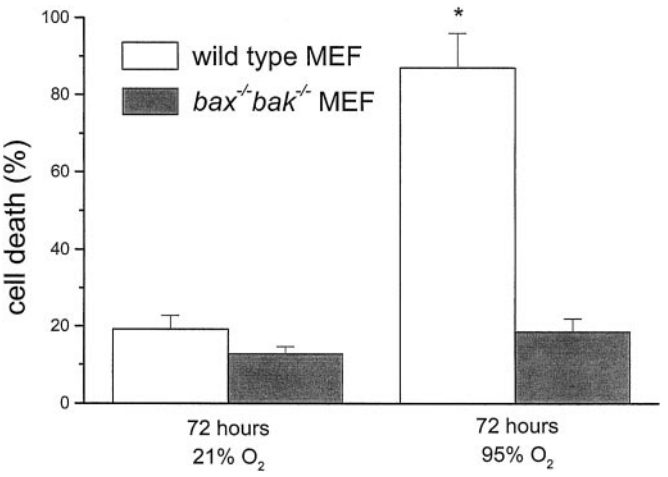

FIG. 4. **Bax or Bak are required for cell death following exposure to hyperoxia.** Murine embryonic fibroblasts from $bax^{-/-}bak^{-/-}$ mice (*gray bars*) or wild type mice (*open bars*), both immortalized with SV40, were exposed to normoxia or hyperoxia for 72 h, and cell death was measured (LDH release). The results of four independent experiments are shown. $p < 0.05$ for comparison between wild type and $bax^{-/-}bak^{-/-}$ cells.

drial membrane potential does not precede the commitment to die following exposure to hyperoxia.

The Overexpression of Bcl-X_L Does Not Prevent Cell Cycle Arrest following Exposure to Hyperoxia—Several groups of investigators have proposed that exposure to hyperoxia might result in DNA damage, causing both growth arrest and apoptosis (reviewed in Ref. 30). These investigators have demonstrated that hyperoxia results in growth arrest in both the G_0 and S phases of the cell cycle, perhaps suggesting two distinct mechanisms of hyperoxia-induced growth arrest. This growth arrest might protect or sensitize cells to hyperoxia-induced apoptosis. To determine whether Bcl-X_L prevented hyperoxia-induced cell cycle arrest, we exposed cells that overexpress Bcl-X_L to hyperoxia for 48 h (a dose of hyperoxia that is lethal to control transfected cells) and measured the percentage of cells in each stage of the cell cycle. Overexpression of Bcl- X_L did not prevent the S phase cell cycle arrest observed after 48 h of exposure to hyperoxia (Fig. 6). These results are similar to those of Strasser *et al.*, who demonstrated that apoptosis but not cell cycle arrest following exposure to ultraviolet irradiation was prevented by the overexpression of Bcl-X_L in T lymphoma cells (31).

Cell Death following Exposure to Hyperoxia Does Not Require the Mitochondrial Generation of Reactive Oxygen Species—Compared with cells exposed to 21% O_2, cells exposed to hyperoxia generate excess reactive oxygen species from the mitochondria (8, 10). These ROS have been widely hypothesized to be responsible for cell death following exposure to hyperoxia. We therefore sought to determine whether abrogation of the mitochondrial generation of reactive oxygen species prevented mitochondria-dependent apoptosis following exposure to hyperoxia. Control transfected Rat1a cells were pretreated with antioxidants for 30 min and then exposed to 72 h of continuous hyperoxia in the presence of these antioxidants (Fig. 7*a*). Neither ebselen (a glutathione peroxidase mimetic), Mn(III) TBAP (a superoxide dismutase mimetic), the combination of ebselen and Mn(III) TBAP, nor *N*-acetylcysteine prevented cell death following exposure to hyperoxia. Both the combination of Mn(III) TBAP with ebselen and with *N*-acetylcysteine prevented the increase in fluorescence of CM-H_2DCFDA seen following exposure to antimycin A (10 μg/ml) for 6 h (data not shown).

The failure of these antioxidants to prevent hyperoxia-induced cell death suggested that cell death following exposure to hyperoxia might not require the mitochondrial generation of ROS. To further test this hypothesis, we generated ρ^0 HT1080 cells and exposed them to hyperoxia. These cells lack mitochondrial DNA and are therefore unable to generate ROS from electron transport. Wild type HT1080 cells but not ρ^0 HT1080 cells demonstrated significant increases in CM-H_2DCFDA fluorescence, a measure of H_2O_2 production, following exposure to hyperoxia (6 h). Whereas ρ^0 HT1080 cells demonstrated a small increase in CM-H_2DCFDA fluorescence in response to antimycin A (10 μg/ml), this response was markedly less than that observed in wild type cells (Fig. 7). Nevertheless, cell death following exposure to hyperoxia for 72 h was similar in ρ^0 cells and wild type controls (Fig. 8). Consistent with these findings, a similar percentage of ρ^0 cells and wild type cells had released cytochrome *c* after 40 h of exposure to hyperoxia (Fig. 8). These results suggest that neither the mitochondrial generation of reactive oxygen species nor a functional electron transport chain is required for cell death following exposure to hyperoxia.

DISCUSSION

We sought to determine the mechanism by which exposure to hyperoxia results in cell death in cultured cells. We found that exposure to hyperoxia causes apoptosis through a mitochondria-

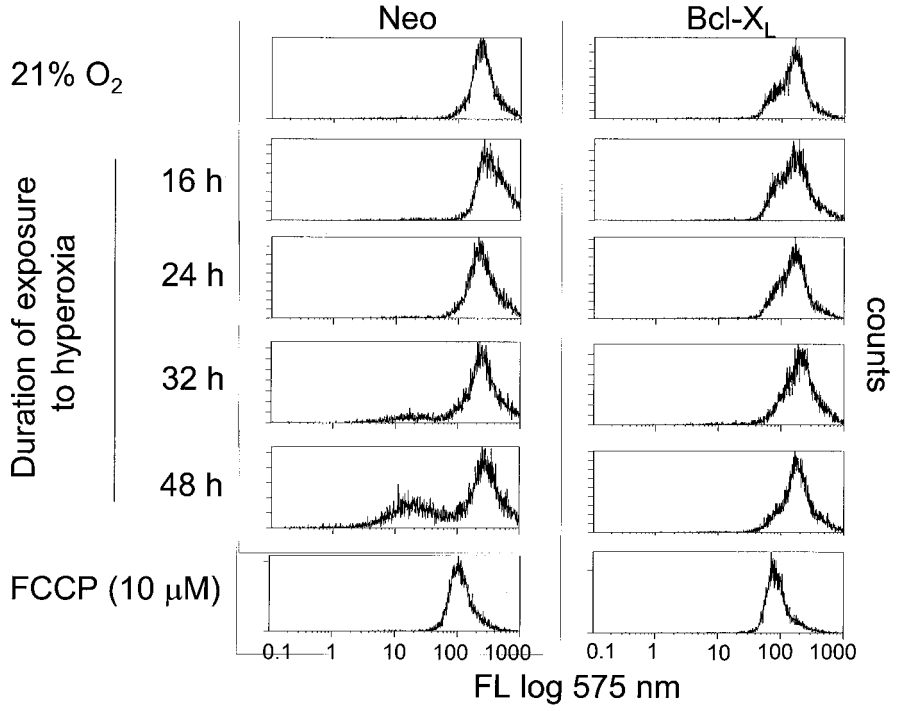

FIG. 5. **The overexpression of Bcl-X$_L$ prevents depolarization of the mitochondrial membrane potential following exposure to hyperoxia.** Neomycin control (*left panels*) and Bcl-X$_L$ (*right panels*)-transfected Rat1a cells were exposed to hyperoxia for the times indicated and then removed from the plate with trypsin (0.25%) and incubated with TMRE (2 μM) with or without carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (*FCCP*; 10 μM) for 30 min before analysis with flow cytometry. After 32 and 40 h of hyperoxia, a population of markedly depolarized cells was observed in control but not Bcl-X$_L$-transfected cells. A representative sample of five separate experiments is shown.

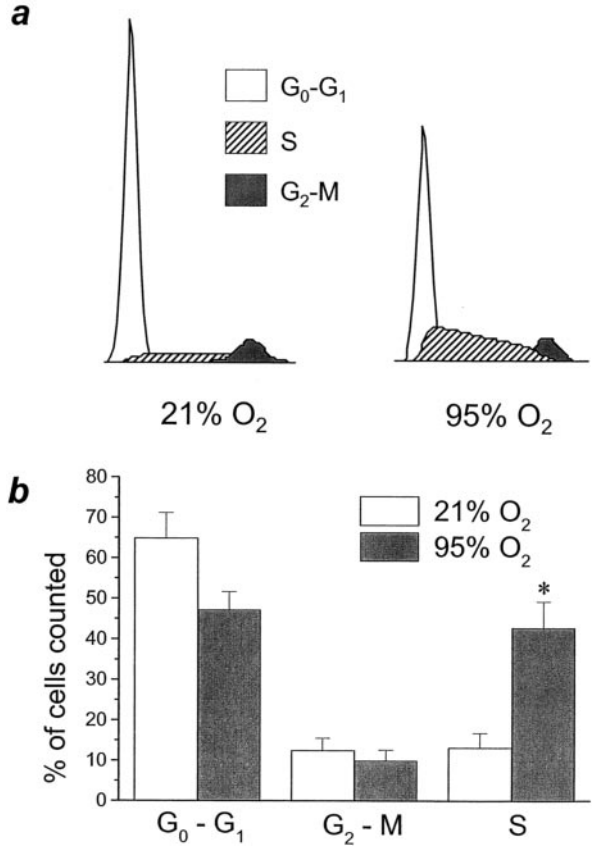

FIG. 6. **The overexpression of Bcl-X$_L$ does not prevent cell cycle arrest following exposure to hyperoxia.** Rat1a cells transfected with Bcl-X$_L$ were exposed to hyperoxia for 48 h and the percentage of cells in each cell cycle was determined using flow cytometry. *a*, representative tracing indicating the cell cycle distribution after 48 h in normoxia (21% O$_2$) (*left panel*) or hyperoxia (95% O$_2$) (*right panel*). *b*, compared with normoxic controls (*open bars*), the percentage of cells in S phase was significantly higher in cells exposed to hyperoxia (*gray bars*). The results of three independent experiments are shown ± S.E. *, $p < 0.05$ compared with normoxic controls.

dependent pathway and can be prevented by the overexpression of Bcl-X$_L$ (Fig. 9). Exposure to hyperoxia results in the release of cytochrome *c* from the intermembrane space into the cytosol accompanied by depolarization of the mitochondrial membrane potential. The release of cytochrome *c* is associated with the activation of caspase-9 and the commitment of the cells to die. Bcl-X$_L$ prevents apoptosis following exposure to hyperoxia by preventing changes in the mitochondrial membrane potential, cytochrome *c* release, and caspase-9 activation. The overexpression of Bcl-X$_L$ did not prevent growth arrest in S phase following exposure to hyperoxia.

Recently, Wei *et al.* and Lindsten *et al.* have demonstrated that multiple stimuli that trigger mitochondria-dependent apoptosis including growth factor withdrawal, UV irradiation, etoposide, staurosporine, and thapsigargan require the presence of Bax or Bak (20, 32). Lymphocytes from these animals remain sensitive to receptor-mediated (CD95L) apoptosis. Mitochondria-dependent apoptotic stimuli cause translocation of Bax and Bak from the cytosol to the mitochondria, where they cause mitochondrial membrane permeabilization. Antiapoptotic members of the Bcl-2 family (*e.g.* Bcl-2 and Bcl-X$_L$) inhibit mitochondrial permeabilization by sequestering or preventing the activation of Bax and Bak (33). Our finding that Bax or Bak are required for and Bcl-X$_L$ prevents apoptosis following exposure to hyperoxia is consistent with this hypothesis.

Our results indicating that Bcl-2 family members regulate cell death following exposure to hyperoxia might be used to explain the mechanism by which a variety of strategies protect against hyperoxia-induced lung injury. Pharmacologic pretreatment with or genetic overexpression of a number of inflammatory cytokines (tumor necrosis factor, IL-1β, IL-6, IL-11, growth factors (insulin-like growth factor and keratinocyte growth factor)), the β subunit of the Na^+K^+ ATPase, and heme oxygenase 1 have been shown to protect animals from subsequent hyperoxic injury (reviewed in Ref. 11). Some of these protective strategies may alter the expression or activity of pro- or antiapoptotic factors such as Bax, Bak, Bcl- X$_L$, or Akt. For example, Ward *et al.* (16) demonstrated that transgenic mice overexpressing the cytokine IL-6 survived hyperoxia, whereas wild type controls did not. In whole lung homogenates from

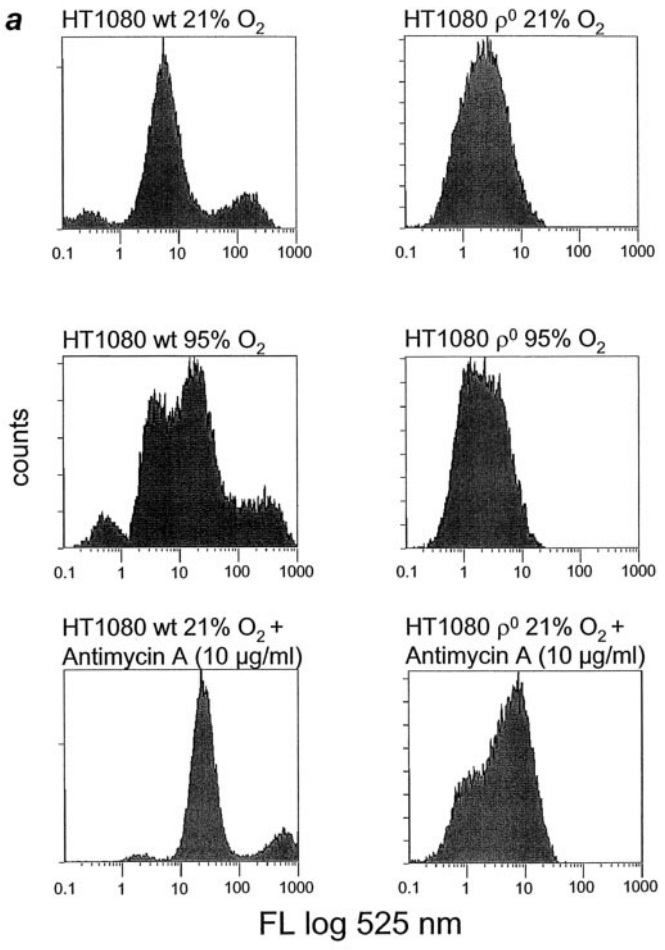

FIG. 7. **ρ^0 cells fail to generate ROS during exposure to hyperoxia.** ρ^0 HT1080 fibrosarcoma cells (*gray bars*) and wild type (*wt*) HT1080 cells (*open bars*) were exposed to normoxia (21% O_2), hyperoxia (95% O_2), or antimycin A (10 μg/ml) for 6 h, and the oxidation of the fluorescent dye CMH_2-DCFDA was measured using FACS. A representative sample (*a*) and mean fluorescence (*b*) compared with normoxic controls for five independent experiments is shown. *, $p < 0.05$ for comparison between wild type and ρ^0 cells.

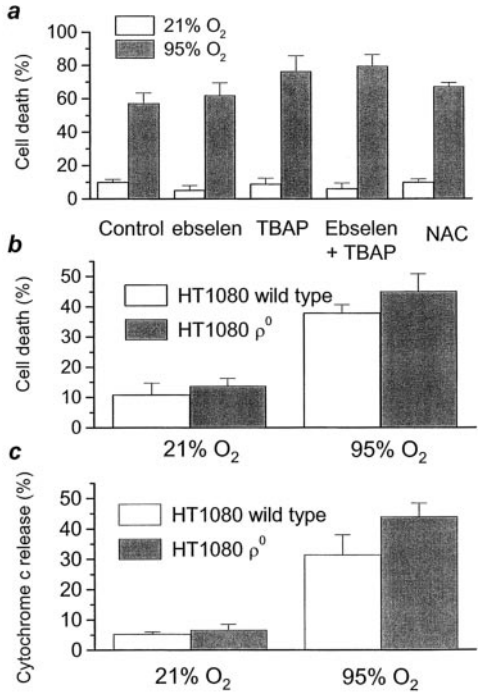

FIG. 8. **The mitochondrial generation of reactive oxygen species is not required for cell death following exposure to hyperoxia.** *a*, the administration of exogenous antioxidants failed to prevent cell death following exposure to hyperoxia. Control transfected (neomycin resistance) Rat1a cells were preincubated with the antioxidants ebselen (10 μM) (*ebselen*), Mn(III) TBAP (25 μM), ebselen (10 μM) with Mn(III) TBAP (25 μM), *N*-acetylcysteine (*NAC*) (10 mM), or vehicle (*Control*) for 30 min and then exposed to normoxia (*open bars*) or hyperoxia (*gray bars*) for 72 h, and cell death was measured (LDH release). *b*, ρ^0 HT1080 fibrosarcoma cells (*gray bars*) and wild type HT1080 cells (*open bars*) were exposed to normoxia (21% O_2) or hyperoxia (95% O_2) for 72 h, and cell death was measured (LDH release). The results of five independent experiments are shown. *p* value was not significant for comparison between ρ^0 cells and wild type cells. *c*, after 40 h of exposure to hyperoxia, ρ^0 HT1080 cells demonstrated a significant increase in the percentage of cells that had released cytochrome *c* compared with normoxic controls. The results of three independent experiments are shown. *p* value was not significant for comparison between wild type and ρ^0 cells ± S.E.

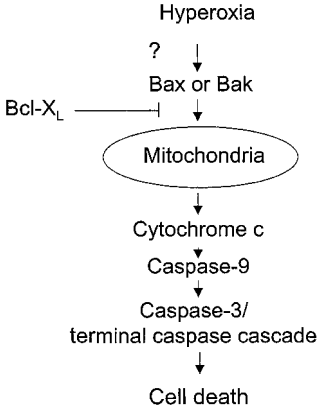

FIG. 9. **Proposed pathway of cell death following exposure to hyperoxia.** Prolonged exposure to hyperoxia resulted in the release of cytochrome *c*, activation of caspase-9, and apoptosis. These events are prevented by the overexpression of Bcl-X_L. Bax or Bak is required for this response, but the mitochondrial generation of ROS is not.

these animals, the expression of Bcl-2 was significantly increased. The antiapoptotic protein Akt attenuates mitochondria-dependent apoptosis through a mechanism that acts in part through Bcl-2 proteins (34). In rats, Lu *et al.* (35) demonstrated that adenoviral overexpression of Akt attenuated hyperoxic lung injury.

Surprisingly, we were unable to prevent cell death following exposure to hyperoxia by administering exogenous antioxidants or preventing the formation of reactive oxygen species by the mitochondria. We exposed wild type Rat1a cells to hyperoxia in the presence of several antioxidants. Mn(III) TBAP is a superoxide dismutase mimetic, and ebselen is a glutathione peroxidase mimetic. The administration of Mn(III) TBAP should accelerate the conversion of the superoxide anion to

H_2O_2, and ebselen should accelerate the conversion of H_2O_2 to water. Neither of these agents, alone or in combination, prevented cell death following exposure to hyperoxia. The antioxidant *N*-acetylcysteine also failed to prevent cell death following exposure to hyperoxia. Mitochondrial ROS generation during exposure to hyperoxia might overwhelm antioxidant defense mechanisms even in the presence of exogenously administered antioxidants. To determine whether mitochondrial ROS are required for cell death following exposure to hyperoxia, we exposed cells depleted of mitochondrial DNA to hyperoxia. The mammalian mitochondrial DNA encodes 13 polypeptides including critical catalytic subunits for complex I (NADH dehydrogenase), complex III (Bcl complex), complex IV (cytochrome *c* oxidase), and the F_1F_0-ATP synthase (19, 36). Cells lacking mitochondrial DNA contain intact mitochondria and undergo apoptosis with release of cytochrome *c* after growth factor withdrawal or the administration of staurosporine but are protected against cell death following exposure to anoxia (23, 37). While an increase in CM-H_2DCFDA fluorescence was not observed in ρ^0 cells exposed to hyperoxia, a small increase in CM-H_2DCFDA fluorescence was seen in the ρ^0 cells following exposure to antimycin A. This increase appeared to affect the majority of the cell population (FACS). While the mechanism by which this occurs is not clear, the increase in fluorescence was substantially smaller than that observed in wild type cells. Despite this marked attenuation in intracellular ROS generation observed in the ρ^0 cells following both exposure to hyperoxia and antimycin A, no reduction in cell death following exposure to hyperoxia was observed. These results, suggesting that the mitochondrial generation of reactive oxygen species is not required for cell death following exposure to hyperoxia, are consistent with those of Senturker *et al.* (38), who demonstrated that apoptosis following administration of VP-16 and cisplatin did not require ROS generation in human B lymphoma cells.

In whole lung homogenates and in endothelial cells, several groups of investigators have demonstrated that the mitochondrial generation of ROS increases during exposure to hyperoxia (8, 10). However, in 6–8-week-old mice overexpressing manganese superoxide dismutase, the primary antioxidant enzyme in the mitochondria, Wispe *et al.* (39) reported that 80% of the animals died during the hyperoxic exposure (albeit 4 days later than controls). In 6–8-week-old mice overexpressing extracellular superoxide dismutase, Folz *et al.* (40) found that several markers of lung injury were attenuated compared with wild type controls; mortality, however, was not significantly improved. White *et al.* (3) reported that death was delayed in older (5.5-month-old) transgenic mice overexpressing the copper-zinc superoxide dismutase, the major antioxidant enzyme in the cytosol. Younger transgenic mice (2.5 months old) all survived a 10-day exposure to hyperoxia (3). Some strategies that are effective at attenuating lung injury following exposure to hyperoxia (*e.g.* the overexpression of either IL-6 or heme oxygenase 1) do not effect the expression of antioxidant enzymes (6, 16). Our data demonstrate that neither the mitochondrial generation of ROS nor a functional electron transport chain is required for cell death following exposure to hyperoxia *in vitro*. The generation of ROS during exposure to hyperoxia, however, might modulate other pathways contributing to lung injury *in vivo*.

In conclusion, exposure of cultured cells to hyperoxia results in mitochondria-dependent apoptosis that is prevented by the overexpression of Bcl-X_L and requires the proapoptotic Bcl-2 family members Bax or Bak. Bcl-X_L acts by preventing the release of cytochrome *c* from the mitochondria but does not prevent cell cycle arrest during exposure to hyperoxia. The mitochondrial generation of ROS is not required for cell death following exposure to hyperoxia. Further investigation is needed to determine the mechanism(s) by which exposure to hyperoxia activates Bax or Bak, triggering the process of cell death.

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REFERENCES

1. Olivera, W. G., Ridge, K. M., and Sznajder, J. I. (1995) *Am. J. Respir. Crit. Care Med.* **152,** 1229–1234
2. Crapo, J. D., Barry, B. E., Foscue, H. A., and Shelburne, J. (1980) *Am. Rev. Respir. Dis.* **122,** 123–143
3. White, C. W., Avraham, K. B., Shanley, P. F., and Groner, Y. (1991) *J. Clin. Invest.* **87,** 2162–2168
4. Kazzaz, J. A., Xu, J., Palaia, T. A., Mantell, L., Fein, A. M., and Horowitz, S. (1996) *J. Biol. Chem.* **271,** 15182–15186
5. Barazzone, C., Horowitz, S., Donati, Y. R., Rodriguez, I., and Piguet, P. F. (1998) *Am. J. Respir. Cell Mol. Biol.* **19,** 573–581
6. Otterbein, L. E., Kolls, J. K., Mantell, L. L., Cook, J. L., Alam, J., and Choi, A. M. (1999) *J. Clin. Invest.* **103,** 1047–1054
7. Boveris, A., Oshino, N., and Chance, B. (1972) *Biochem. J.* **128,** 617–630
8. Freeman, B. A., and Crapo, J. D. (1981) *J. Biol. Chem.* **256,** 10986–10992
9. Guidot, D. M., McCord, J. M., Wright, R. M., and Repine, J. E. (1993) *J. Biol. Chem.* **268,** 26699–26703
10. Sanders, S. P., Zweier, J. L., Kuppusamy, P., Harrison, S. J., Bassett, D. J., Gabrielson, E. W., and Sylvester, J. T. (1993) *J. Clin. Invest.* **91,** 46–52
11. Barazzone, C., and White, C. W. (2000) *Am. J. Respir. Cell Mol. Biol.* **22,** 517–519
12. Kroemer, G., and Reed, J. C. (2000) *Nat. Med.* **6,** 513–519
13. Vander Heiden, M. G., and Thompson, C. B. (1999) *Nat. Cell Biol.* **1,** E209–E216
14. Green, D. R., and Reed, J. C. (1998) *Science* **281,** 1309–1312
15. O'Reilly, M. A., Staversky, R. J., Huyck, H. L., Watkins, R. H., LoMonaco, M. B., D'Angio, C. T., Baggs, R. B., Maniscalco, W. M., and Pryhuber, G. S. (2000) *Lab. Invest.* **80,** 1845–1854
16. Ward, N. S., Waxman, A. B., Homer, R. J., Mantell, L. L., Einarsson, O., Du, Y., and Elias, J. A. (2000) *Am. J. Respir. Cell Mol. Biol.* **22,** 535–542
17. Gottlob, K., Majewski, N., Kennedy, S., Kandel, E., Robey, R. B., and Hay, N. (2001) *Genes Dev.* **15,** 1406–1418
18. Chandel, N. S., Maltepe, E., Goldwasser, E., Mathieu, C. E., Simon, M. C., and Schumacker, P. T. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 11715–11720
19. King, M. P., and Attardi, G. (1989) *Science* **246,** 500–503
20. Wei, M. C., Zong, W. X., Cheng, E. H., Lindsten, T., Panoutsakopoulou, V., Ross, A. J., Roth, K. A., MacGregor, G. R., Thompson, C. B., and Korsmeyer, S. J. (2001) *Science* **292,** 727–730
21. Porras, A., Gaillard, S., and Rundell, K. (1999) *J. Virol.* **73,** 3102–3107
22. Vander Heiden, M. G., Chandel, N. S., Williamson, E. K., Schumacker, P. T., and Thompson, C. B. (1997) *Cell* **91,** 627–637
23. McClintock, D. S., Santore, M. T., Lee, V. Y., Brunelle, J., Budinger, G. R., Zong, W. X., Thompson, C. B., Hay, N., and Chandel, N. S. (2002) *Mol. Cell. Biol.* **22,** 94–104
24. Tlsty, T., Briot, A., and Poulose, B. (1995) *Methods Enzymol.* **254,** 125–133
25. Kennedy, S. G., Kandel, E. S., Cross, T. K., and Hay, N. (1999) *Mol. Cell. Biol.* **19,** 5800–5810
26. Annis, M. G., Zamzami, N., Zhu, W., Penn, L. Z., Kroemer, G., Leber, B., and Andrews, D. W. (2001) *Oncogene* **20,** 1939–1952
27. Juin, P., Hueber, A. O., Littlewood, T., and Evan, G. (1999) *Genes Dev.* **13,** 1367–1381
28. Xie, Z., Kometiani, P., Liu, J., Li, J., Shapiro, J. I., and Askari, A. (1999) *J. Biol. Chem.* **274,** 19323–19328
29. Klefstrom, J., Vastrik, I., Saksela, E., Valle, J., Eilers, M., and Alitalo, K. (1994) *EMBO J.* **13,** 5442–5450
30. O'Reilly, M. A. (2001) *Am. J. Physiol. Lung Cell Mol. Physiol.* **281,** L291–305
31. Strasser, A., Harris, A. W., Jacks, T., and Cory, S. (1994) *Cell* **79,** 329–339
32. Lindsten, T., Ross, A. J., King, A., Zong, W. X., Rathmell, J. C., Shiels, H. A., Ulrich, E., Waymire, K. G., Mahar, P., Frauwirth, K., Chen, Y., Wei, M., Eng, V. M., Adelman, D. M., Simon, M. C., Ma, A., Golden, J. A., Evan, G., Korsmeyer, S. J., MacGregor, G. R., and Thompson, C. B. (2000) *Mol. Cell* **6,** 1389–1399
33. Cheng, E. H., Wei, M. C., Weiler, S., Flavell, R. A., Mak, T. W., Lindsten, T., and Korsmeyer, S. J. (2001) *Mol. Cell* **8,** 705–711
34. del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, R., and Nunez, G. (1997) *Science* **278,** 687–689
35. Lu, Y., Parkyn, L., Otterbein, L. E., Kureishi, Y., Walsh, K., Ray, A., and Ray, P. (2001) *J. Exp. Med.* **193,** 545–549
36. Chandel, N. S., and Schumacker, P. T. (1999) *FEBS Lett.* **454,** 173–176
37. Wang, J., Silva, J. P., Gustafsson, C. M., Rustin, P., and Larsson, N. G. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 4038–4043
38. Sentürker, S., Tschirret-Guth, R., Morrow, J., Levine, R., and Shacter, E. (2002) *Arch. Biochem. Biophys.* **397,** 262–272
39. Wispe, J. R., Warner, B. B., Clark, J. C., Dey, C. R., Neuman, J., Glasser, S. W., Crapo, J. D., Chang, L. Y., and Whitsett, J. A. (1992) *J. Biol. Chem.* **267,** 23937–23941
40. Folz, R. J., Abushamaa, A. M., and Suliman, H. B. (1999) *J. Clin. Invest.* **103,** 1055–1066