Mitochondrial complex I, aconitase, and succinate dehydrogenase during hypoxia-reoxygenation: modulation of enzyme activities by MnSOD

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Powell, Charles S., and Robert M. Jackson. Mitochondrial complex I, aconitase, and succinate dehydrogenase during hypoxia-reoxygenation: modulation of enzyme activities by MnSOD. Am J Physiol Lung Cell Mol Physiol 285: L189–L198, 2003. First published March 28, 2003; 10.1152/ajplung.00253.2002.—Both NADH dehydrogenase (complex I) and aconitase are inactivated partially in vitro by superoxide (O2·-) and other oxidants that cause loss of iron from enzyme cubane (4Fe-4S) centers. We tested whether hypoxia-reoxygenation (H-R) by itself would decrease lung epithelial cell NADH dehydrogenase, aconitase, and succinate dehydrogenase (SDH) activities and whether transfection with adenoviral vectors expressing MnSOD (Ad.MnSOD) would inhibit oxidative enzyme inactivation and thus confirm a mechanism involving O2·-. Human lung carcinoma cells with alveolar epithelial cell characteristics (A549 cells) were exposed to <1% O2-5% CO2 (hypoxia) for 24 h followed by air-5% CO2 for 24 h (reoxygenation). NADH dehydrogenase activity was assayed in submitochondrial particles; aconitase and SDH activities were measured in cell lysates. H-R significantly decreased NADH dehydrogenase, aconitase, and SDH activities. Ad.MnSOD increased mitochondrial MnSOD substantially and prevented the inhibitory effects of H-R on enzyme activities. Addition of α-ketoglutarate plus aspartate, but not succinate, to medium prevented cytotoxicity due to 2,3-dimethoxy-1,4-naphthoquinone. After hypoxia, cells displayed significantly increased dihydrodihydramine fluorescence, indicating increased mitochondrial oxidant production. Inhibition of NADH dehydrogenase, aconitase, and SDH activities during reoxygenation are due to excess O2·- produced in mitochondria, because enzyme inactivation can be prevented by overexpression of MnSOD.

alveolar epithelium; mitochondria; NADH dehydrogenase; xanthine oxidase (34, 49) and organelles, especially mitochondria (7, 42). Excess production of superoxide (O2·-) and derived reactive species (e.g., ·OH) has been detected in endothelial cell cultures using electron paramagnetic resonance (49). Hypoxia renders cells more susceptible to oxidant injury during reoxygenation through a combination of decreased antioxidant defenses [especially manganese superoxide dismutase (MnSOD) and GSH] (23, 27) and increased ROS production (28).

Lung vascular endothelial and alveolar epithelial cells develop oxidant injury during reoxygenation in vitro (2). Cultured, human lung epithelial cells [H441 cells; American Type Culture Collection (ATCC), Manassas, VA] displayed increased dichlorofluorescein (DCF) fluorescence and oxidized DCF more extensively after 24 h in hypoxia, probably reflecting increased cellular peroxide levels (28). The epithelial cells demonstrated measurably increased H2O2 production for at least 24 h after reoxygenation. Lung epithelial cell death after reoxygenation is necrotic with only a small percentage of cells undergoing apoptosis. Hypoxia decreases MnSOD activity and protein content because of decreased MnSOD mRNA stability and steady-state mRNA level (23). Lung epithelial cells also have decreased total GSH and glutathione synthetic capacity, at least partly because expression of glutamate cysteine ligase and its regulatory subunit (LS) is markedly decreased after exposure to 1% oxygen for 24 h (Jackson R and Wright M, unpublished observations). Importantly, MnSOD and GSH function in concert to prevent radical chain reactions (31), so hypoxia critically impairs two of the key antioxidant defense mechanisms required in response to oxidant stress.

Both mitochondrial complexes III and I have been implicated as sources of ROS during reoxygenation (21). Some molecular oxygen (usually <1%) escapes complete reduction and forms O2·-. The reduced ubiquinone pool of complex III is probably the most important source of O2·- in mitochondria, although the contribution of ROS by complex I is increasingly evident. Inhibition of oxidative phosphorylation, due to hypoxic

A KEY COMPONENT of cellular ischemia-reperfusion (I-R) injury is sequential oxygen deprivation (hypoxia) followed by oxygen repletion (reoxygenation) (29). Central roles for reactive oxygen species (ROS) and reactive nitrogen species have been confirmed in the pathogenesis of hypoxia-reoxygenation injury (27). Important intracellular sources of ROS during reoxygenation include enzymes such as xanthine dehydrogenase/xanthine oxidase (34, 49) and organelles, especially mitochondria (7, 42). Excess production of superoxide (O2·-) and derived reactive species (e.g., ·OH) has been detected in endothelial cell cultures using electron paramagnetic resonance (49). Hypoxia renders cells more susceptible to oxidant injury during reoxygenation through a combination of decreased antioxidant defenses [especially manganese superoxide dismutase (MnSOD) and GSH] (23, 27) and increased ROS production (28).
downregulation of cytochrome oxidase activity, itself increases ROS production by the electron transport chain. MnSOD rapidly converts O$_2^.$ in mitochondria to H$_2$O$_2$, which unlike O$_2^.$ is incapable of reaction with NO to form peroxynitrite (ONOO$^-$). The minimization of ONOO$^-$ production is critical, because ONOO$^-$ irreversibly inhibits mitochondrial respiration (14). Increased production of ROS by mitochondria also precipitates apoptosis by release of cytochrome c from mitochondria (11). H$_2$O$_2$, which is produced by MnSOD from O$_2^.$, can diffuse from mitochondria and activate redox-sensitive signaling pathways in the cytosol (32). Excess O$_2^.$ is capable of reaction with iron-sulfur (4Fe-4S) centers in NADH dehydrogenase, aconitase, and other enzymes, and so O$_2^.$ may itself account for inhibition of energy production (18).

Changes in the redox state of the electron transport chain account for increased ROS production in mitochondria after I-R. For example, reperfusion after prolonged ischemia caused maximal increases in H$_2$O$_2$ production with rotenone (ROS from complex I) or antimycin (ROS from complex III) (21). These data support our observations that mitochondria isolated from reoxygenated lung epithelial cells have decreased NADH dehydrogenase activity and that reoxygengated cells released increased quantities of H$_2$O$_2$ (28).

Therefore, this study was designed to test the hypothesis that NADH dehydrogenase, aconitase, and succinate dehydrogenase (SDH) would be inhibited oxidatively during reoxygenation and that overexpression of MnSOD in mitochondria would prevent inhibition, confirming a mechanistic role for O$_2^.$. Our aims were to test effects of hypoxia-reoxygenation on NADH dehydrogenase, SDH, and aconitase activities and to test protective effects of transfection with adenoviral vectors expressing MnSOD (Ad.MnSOD). We exposed human lung epithelial cells (A549 cells) to air or hypoxia-reoxygenation and then assayed NADH dehydrogenase, aconitase, and SDH activities. In some experiments, cells were transfected with adenoviral vectors that overexpress the MnSOD protein (Ad.MnSOD) in mitochondria. Reoxygenation decreased NADH dehydrogenase and aconitase activities, and MnSOD vectors effectively prevented effects of hypoxia-reoxygenation. These results confirm a specific role for increased mitochondrial O$_2^.$ (a precursor of H$_2$O$_2$ and ONOO$^-$) production in reoxygenation of lung epithelial cells and suggest that inhibition of complex I and aconitase are mechanisms of cellular injury during reoxygenation.

**MATERIALS AND METHODS**

**Cell cultures.** A549 human papillary adenocarcinoma cells were obtained frozen at passage 78 from ATCC. A549 cells are human peripheral airway epithelial cells that demonstrate polarity, microvilli on apical surfaces, and surfactant multimembranous bodies in the apical cytoplasm. The cells contain Clara cell and alveolar epithelial type II (ATII) cell granules, surfactant protein (SP)-A protein (~170 ng/mg protein), and SP-A and SP-B mRNAs (20). A549 cells express MnSOD protein that is regulated by both TNF-$\alpha$ and oxidants (43), exactly as they are in primary cultures of ATII cells. A549 cells share many relevant morphological and functional characteristics with normal lung epithelial cells, although they do not duplicate primary ATII cell cultures.

A549 cells were thawed and split 1:4 three times before use in experiments. Cells were cultured in DMEM/F-12 (50%/50%) (MediaTech 800; Cellgro, Herndon, VA) with 10% fetal bovine serum (FBS; Hyclone, Logan, UT) and 1% penicillin-streptomycin (MediaTech). Cells were grown nearly to confluence (~80% by visual estimate) in air-5% CO$_2$ and transferred by trypsinization into 100-mm-diameter culture dishes (Falcon; Becton-Dickinson, Franklin Lakes, NJ).

For dithiothreitol (DTT) and high-intensity light experiments, A549 cells were grown at an air-liquid interface. We seeded A549 cells onto Transwell clear culture inserts (24-mm diameter, 0.4-mm pore size; Costar, Cambridge, MA). Cells were cultured under medium for 24 h in DMEM/ F-12 with 10% FBS and 1% penicillin-streptomycin. After 24 h, we created the air-liquid interface by removing the apical medium, leaving cells exposed only to the medium adjacent to the basal surface.

**Air and hypoxia exposures.** When cells reached 80% confluence, they were incubated in air-5% CO$_2$ or <1% O$_2$-5% CO$_2$ (hypoxia) for 24 h. Hypoxia exposures were done in a Plexiglas enclosure (33 × 33 × 33 cm; PlasLabs, Lansing, MI) kept at 37°C inside a tissue culture incubator (Fisher Isotemp model 230D). Medium oxygen and carbon dioxide tensions and pH were measured with a clinical blood gas instrument. Periodic inflow of 5% CO$_2$-95% N$_2$ gas controlled by a proportional-flow oxygen controller (Reming Bioinstruments, Redfield, NY) maintained hypoxia. The oxygen controller monitored percent oxygen within the chamber and kept it at the set level ± 0.1%. Cells were reoxygengated by return to air-5% CO$_2$.**

**Mitochondrial isolation.** Culture dishes (100-mm diameter) containing nearly confluent A549 cells were washed twice with 5 ml of ice-cold phosphate-buffered saline (PBS) at pH 7.4. Monolayers were scraped into 5 ml of PBS and placed in 15-ml Falcon tubes. Cells were pelleted at 500 g for 4°C for 10 min and resuspended in 2.5 ml of TES buffer (0.25 M sucrose, 1 mM EGTA, and 10 mM triethanolamine-acetate at pH 7.0). Cells were homogenized with a Dounce homogenizer with 15 strokes of the looser pestle and 30 strokes of the tighter pestle. Resulting material was transferred to a 2-ml tube and centrifuged at 1,500 g at 4°C for 10 min to obtain a mitochondria-enriched pellet (36). The pellet was washed twice with 1 ml of homogenization buffer. Protein content of the pellet was assayed (Bio-Rad Protein Assay Kit II; Bio-Rad, Hercules, CA). After isolation, the mitochondria-enriched pellet was stored at −80°C for <2 wk before assays.

**Citrate synthase assay.** We assayed citrate synthase activity by following the acetylation of 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) at 412 nm (13). Mitochondrial or cytosolic supernatant samples (1–5 µl) were added to cuvettes containing buffer [100 mM Tris, 0.1% (vol/vol) Triton X-100, pH 8.0] at 30°C, 10 µl acetyl-CoA (10 mM, 8.09 mg/ml in H$_2$O), and DTNB (20 mM, 7.9 mg/ml). We started the reaction by adding 10 µl of oxaloacetate (20 mM, 5.3 mg in 2 ml of H$_2$O), and the linear rate followed for 2–3 min. Data are expressed as umol/min per mg protein, on the basis of the molar extinction coefficient, ε = 13,600.

**NADH dehydrogenase assay.** Mitochondria were isolated as described above and sonicated to produce submitochondrial particles to prevent contamination from cytosolic dehydrogenases (36). Protein content of the submitochondrial...
particle preparations was assayed and resuspended at 1 mg/ml assay buffer. To determine $V_{\text{max}}$, we added 0.333 ml of assay buffer and various quantities of potassium ferricyanide ranging from $2.00 \times 10^{-4}$ to $1.67 \times 10^{-3}$ M to a 1-ml cuvette. NADH (16.7 $\mu$M of 9 mM) and the mitochondrial sample were then added. NADH dehydrogenase activity was measured with and without 1 mM DTT. In those experiments, 1.5 $\mu$L of 0.1 M DTT was added to 150 $\mu$L of the mitochondrial sample before assay. The change in absorbance at 420 nm was followed for 75 s at 25°C. Double reciprocal plots of change in absorbance at 420 nm and ferricyanide concentration were made, and $V_{\text{max}}$ for NADH dehydrogenase was calculated from the molar extinction coefficient for potassium ferricyanide ($\epsilon = 1,000$). Calculated $V_{\text{max}}$ is expressed in milliunits as nmol NADH minus 1 mg protein$^{-1}$ (8, 33, 40).

NADH dehydrogenase activity was measured in mitochondrial samples from cells exposed to high-intensity light (20) to 1.67 from the molar extinction coefficient for potassium ferricyanide. 

SDH assay. SDH activity was measured according to SDH assay.

Adenoviral vectors. Adenoviral constructs were prepared by the gene therapy core facility of the University of Alabama at Birmingham Cystic Fibrosis Center. Infection of lung epithelial cells with adenovirus was carried out 24 h after seeding, when monolayers remained subconfluent. A549 cells were grown to a density of $2.25 \times 10^6$ cells/10-cm plate.

Lactate dehydrogenase (LDH) was assayed in mitochondrial prep and cell supernatant (3). A549 cells were lysed with detergent to measure maximum LDH release (100%). Plates were centrifuged at 250 g for 4 min. Fifty microliters of cell culture supernatant were added to wells of an enzymatic assay plate. The assay quantitatively measures LDH, a stable cytosolic enzyme, released during cell lysis. LDH released in culture supernatant was measured by a coupled enzymatic assay, which results in the conversion of a tetrazolium salt into a red formazan product. The color is proportional to the number of lysed cells. The substrate mix (50 $\mu$L of NAD$^+$ diaphorase, lactate, and a tetrazolium salt) was added and incubated for 30 min. Reaction was stopped by addition of 1 M acetic acid. Absorbance, which is proportional to LDH activity, was read in an ELISA reader (Bio-Rad 3550 Microplate Reader). The number of cells in each well is equal, and the data are expressed as percent of total LDH released (CytoTox96 nonradioactive cytotoxicity assay; Promega, Madison, WI).

Aconitase assay. After exposure to air, hypoxia, or reoxygenation, A549 cells were rinsed with 5 ml of ice-cold PBS, scraped, and placed in a 2-ml tube in PBS. Cells were centrifuged at 1,500 g for 5 min at 4°C. The pellet was resuspended in 500 $\mu$L of lysis buffer (50 mM Tris-HCl, 0.6 mM MnCl$_2$, and 20 $\mu$L d-(+)-lactic acid). The pellet was sonicated using the microtip of a sonic oscillator in 10 1-s pulses. The sonicate was centrifuged at 14,000 g for 5 min at 4°C, and the supernatant was transferred to a 1.5-ml tube. The sonicate was diluted to 2.5 mg protein/ml lysis buffer. Reaction buffer (900 $\mu$L) consisting of 50 mM Tris-HCl, 30 mM sodium citrate, and 0.6 mM MnCl$_2$ was added to a 1-ml cuvette with 100 $\mu$L of lysis buffer. The classical supernatant (100 $\mu$L) containing 250 $\mu$L of protein was added to 0.9 ml of prewarmed reaction buffer and placed in the cuvette at 30°C. Incubation continued for 12 min, during which the sample was mixed by inversion. The change in optical density at 340 nm was measured for 3 min at 30°C. In this assay, one milliunit of aconitase was defined as enzymatic activity required to catalyze formation of 1 nmol of isocitrate/min with an extinction coefficient of 0.0062 (18, 30). An aconitase standard at 2 units/ml (Sigma Chemical) was diluted in 50 mM Tris-HCl, 0.6 mM MnCl$_2$, and 30 mM sodium citrate at pH 7.4 and assayed simultaneously. Aconitase was reactivated with 0.5 mM ferrous ammonium sulfate and 5 mM diethiothreitol buffered with 100 mM Tris-HCl, pH 7.5.

MnSOD Western blots. Mitochondrial proteins were denatured and electrophoresed in 12% SDS-polyacrylamide gels. Five micrograms of mitochondrial protein or MnSOD standard were denatured for 3 min at 95°C. Samples were centrifuged, loaded, and run at 100 V until all protein had passed into the resolving gel. The gel was run at 150 V until the dye front ran to the bottom. The gel was then equilibrated with transfer buffer for 10 min. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes using a semidry transfer apparatus (Bio-Rad). The wet PVDF membrane was placed into PBS blocking solution (0.1% Tween 20, 5% fresh nonfat dry milk, and 1% BSA) and blocked for 30 min at room temperature. Membranes were incubated with rabbit anti-human MnSOD IgG (Upstate Biotechnology, Waltham, MA) diluted 1:1,500 in blocking solution. Membranes were washed four times in PBS-0.1% Tween 20. They were incubated with a goat anti-rabbit horseradish peroxidase-coupled antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:2,000 in PBS blocking solution. The membrane was developed using KPL reagents (LumiGlo Chemiluminescent Substrate Kit; Kirkegaard and Perry Laboratories, Gaithersburg, MD) and light emission was recorded on X-ray film (Midwest Scientific, St. Louis, MO).

Lactate dehydrogenase assay. Lactate dehydrogenase activity was assayed in mitochondrial preparations and cell supernatants. A549 cells were lysed with detergent to measure maximum LDH release (100%). Plates were centrifuged at 250 g for 4 min. Fifty microliters of cell culture supernatant were added to wells of an enzymatic assay plate. The assay quantitatively measures LDH, a stable cytosolic enzyme, released during cell lysis. LDH released in culture supernatant was measured by a coupled enzymatic assay, which results in the conversion of a tetrazolium salt into a red formazan product. The color is proportional to the number of lysed cells. The substrate mix (50 $\mu$L of NAD$^+$ diaphorase, lactate, and a tetrazolium salt) was added and incubated for 30 min. Reaction was stopped by addition of 1 M acetic acid. Absorbance, which is proportional to LDH activity, was read in an ELISA reader (Bio-Rad 3550 Microplate Reader). The number of cells in each well is equal, and the data are expressed as percent of total LDH released (CytoTox96 nonradioactive cytotoxicity assay; Promega, Madison, WI).
twice with PBS, and stained overnight at 37°C with a solution containing 2.5 mM 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Boehringer Mannheim, Indianapolis, IN), 3 mM K$_2$Fe(CN)$_6$, 3 mM K$_3$Fe(CN)$_6$, 80 mM Na$_3$HPO$_4$, 20 mM NaH$_2$PO$_4$, and 1.3 mM MgCl$_2$. At least 400 cells per plate from four randomly selected fields (×320) were counted.

In some experiments, oxidant stress was imposed by addition of 25–100 μM 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) during reoxygenation. Cellular injury was assayed by release of LDH (Cytotox$^{96}$ assay) after the 24-h reoxygenation period.

**Effects of α-ketoglutarate plus aspartate and succinate on oxidant stress during reoxygenation.** Some A549 cells in 96-well plates were supplemented with α-ketoglutarate plus aspartate (both 4 mM) or succinate (5 mM) during both hypoxia and reoxygenation. Cells with or without the intermediates were exposed to hypoxia for 24 h and then reoxygenated by provision of oxygenated medium and return to air for 24 h. Additional oxidant stress was imposed by addition of 25–100 μM DMNQ during reoxygenation. Cellular injury was assayed by release of LDH (Cytotox$^{96}$ assay) after the 24-h reoxygenation period.

**Measurement of dihydrorhodamine fluorescence.** Medium containing 5,000 cells/ml was placed over microscope coverslips in six-well tissue culture plates (1 slide/well), and cells were allowed to adhere for 24 h. Medium was removed, and cells were washed with PBS. Cells were infected with Ad.null or Ad.MnSOD adenoviral vectors at an MOI of 100 in antibiotic-free DMEM/F-12 with 2% FBS. Cells were incubated for 48 h in a tissue culture incubator.

Medium was removed, and cells were washed with PBS. Fresh antibiotic-free DMEM/F-12 with 2% FBS was added. Cells were exposed to hypoxia or air for 21 h. Cells were washed with PBS. The cells were then incubated in 5 μM dihydrorhodamine (DHR) in serum-free DMEM/F-12 for 3 h at 37°C. Cells were washed with PBS, and coverslips were mounted onto microscope slides.

We obtained images with a Leica fluorescent microscope (at least 100 cells on 2–5 slides were imaged in each condition) using identical exposure settings for each slide. The excitation and emission wavelengths for DHR were 507 and 529 nm, respectively. Fluorescence intensity was measured with a cooled Hamamatsu slow-scanning personal computer-controlled camera (Hamamatsu, Hamamatsu City, Japan) and analyzed with Image Pro Plus software (MediaCybernetics, Carlsbad, CA).

**Data analysis.** Data are expressed as arithmetic means ± SE, unless indicated otherwise. Multiple comparisons were by two-way ANOVA as required (15) followed by Bonferroni’s procedure. Statistical calculations were done with SigmaStat statistical software (Jandel Scientific Software, San Rafael, CA).

**RESULTS**

**Ad.MnSOD efficiency and toxicity.** As shown in Fig. 1, hypoxia preexposure decreases A549 cell MnSOD protein content. Infecting A549 cells with 100 pfu per cell (MOI) Ad.MnSOD resulted in accumulation of MnSOD protein in mitochondria and a substantial increase in mitochondrial compartmentalized MnSOD protein and activity detected by fluorescent microscopy, Western blotting, and activity gels. These data are summarized in Fig. 2. Transfection efficiency, assessed by β-galactosidase staining after Ad.LacZ infection, was 74 ± 1% at 100 MOI. No increase in LDH release (medium LDH expressed as a percentage of total) was detected at 100 MOI.

**Mitochondrial preparations.** Citrate synthase assays of whole cell lysates and mitochondria revealed enrichment of the mitochondrial preparations with the marker enzyme. LDH assays of cytosolic supernatants and mitochondrial preparations confirmed adequate separation of mitochondria from the cytosolic fractions. These data are summarized in Table 1.

**Hypoxiareoxygenation decreases NADH dehydrogenase activity.** We assayed mitochondrial complex I in mitochondrial preparations as NADH dehydrogenase activity using ferricyanide as electron acceptor. This assay measures $V_{max}$ of the enzyme after incubation of submitochondrial particle preparations with potassium ferricyanide. NADH dehydrogenase activity in the control group averaged 1,564 ± 190 nmol K$_3$Fe(CN)$_6$·min$^{-1}$·mg protein$^{-1}$. Hypoxia did not change NADH dehydrogenase activity significantly in nontransfected cells. In contrast, reoxygenation caused a consistent and statistically significant decrease in NADH dehydrogenase activity. These data are shown in Fig. 3.

Transfection of air control cells with an empty adenoviral vector (Ad.null) had no significant effect on NADH dehydrogenase activity. In contrast to the above results, neither hypoxia (95 ± 9% of control) nor reoxygenation (91 ± 15% of control) caused a significant decrease in NADH dehydrogenase activity in cells transfected with 100 MOI Ad.MnSOD. The Ad.null vector did not prevent the decrease in enzyme activity due to reoxygenation. On fluorescent microscopy, transfected cells expressed large quantities of MnSOD protein in mitochondria, and increased MnSOD activity was evident on SOD activity gels. The Ad.MnSOD vector contains cDNA coding for the MnSOD mitochondrial leader sequence, so overexpression of the MnSOD protein occurs mainly in mitochondria. Because overexpression of MnSOD in mitochondria inhibited the decrease in NADH dehydrogenase activity due to reoxygenation, the observed inhibition of enzymatic
activity (in absence of the vector) appears due to $O_2^\cdot$ or one of its reaction products, such as $H_2O_2$ or ONOO$^-$. 

**DTT increases NADH dehydrogenase activity.** To determine whether the decrease in NADH dehydrogenase activity following reoxygenation is due to sulfhydryl oxidation, we prepared submitochondrial particles from cells exposed to air or hypoxia-reoxygenation. NADH dehydrogenase activity was assayed with or without 1 mM DTT. The control mean NADH dehydrogenase activity in air was $343.7 \pm 26.2 \text{ nmol NADH min}^{-1} \text{mg protein}^{-1}$. As above, we found a significant decrease in NADH dehydrogenase activity in samples from reoxygenated cells compared with air controls ($81 \pm 5\%$ of control, $P < 0.05$ compared with air control, buffer-treated cells). The addition of 1 mM DTT caused a significant increase in activity in samples from both reoxygenated ($122 \pm 5\%$ of reoxygenated controls, $P < 0.05$ compared with air control and reoxygenated, buffer-treated cells) and air-exposed cells ($136 \pm 7\%$ of air controls, $P < 0.05$ compared with air control, buffer-treated cells), respectively. This result suggests that NADH dehydrogenase activity is partially inactivated by sulfhydryl oxidation under both control and experimental conditions.

**High-intensity light does not reverse inhibition of NADH dehydrogenase.** Exposure of reoxygenated cells to high-intensity light did not significantly affect NADH dehydrogenase activity ($109.9 \pm 17.9 \text{ nmol NADH min}^{-1} \text{mg protein}^{-1}$) compared with cells not exposed to light ($92.5 \pm 7.6 \text{ nmol NADH min}^{-1} \text{mg protein}^{-1}$). This result suggests that nitrosylation (S-nitrosothiol formation), which would be reversed by light, is not responsible for NADH dehydrogenase inactivation.

**Hypoxia-reoxygenation decreases aconitase activity.** We also assayed total aconitase activity in whole cell lysates with and without reactivation of the enzyme by a reducing agent. Aconitase activity is inversely proportional to the amount of $O_2^\cdot$ produced during oxidative stress. Aconitase activity in the air control cells averaged $43.9 \pm 3.2 \text{ milliunits/mg protein}$. Hypoxia had no significant inhibitory effect on total aconitase activity in nontransfected cells. In contrast, reoxygenation consistently caused a significant ($-37\%$) decrease in whole cell aconitase activity. Transfection of air control cells with an empty adenoviral vector (100 MOI Ad.null) had no effect on aconitase activity and, as above, had no protective effect. Reoxygenation caused

### Table 1. Citrate synthase and lactate dehydrogenase activities in mitochondrial preparations

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<tr>
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<th>Lysate</th>
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<th>Supernatant</th>
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<tr>
<td></td>
<td>CS</td>
<td>LDH</td>
<td>CS</td>
<td>LDH</td>
<td>CS</td>
<td>LDH</td>
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<tr>
<td>Whole cell</td>
<td>0.36 ± 0.06</td>
<td>83.3 ± 3.6</td>
<td>0.13 ± 0.04</td>
<td>191.7 ± 35.0</td>
<td>0.27 ± 0.04</td>
<td>31.8 ± 3.6</td>
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<td>Low speed</td>
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<td>0.05 ± 0.00</td>
<td>132.8 ± 4.5</td>
<td>0.45 ± 0.14</td>
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<tr>
<td>High speed</td>
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<td></td>
<td>0.14 ± 0.00</td>
<td>135.6 ± 5.0</td>
<td>0.32 ± 0.05</td>
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Data for citrate synthase (CS) are means ± SE expressed as μmol·min$^{-1}$·mg protein$^{-1}$. Data for lactate dehydrogenase (LDH) are means ± SE expressed as nmol·min$^{-1}$·ml·mg$^{-1}$·protein$^{-1}$.  

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no significant decrease in aconitase activity (99 ± 10% of air control) of cells transfected with Ad.MnSOD. Overexpression of MnSOD in mitochondria eliminated the decrease in aconitase activity due to reoxygenation. These data are shown in Fig. 4.

Reactivation of aconitase samples increased activity 140% in control cells, 73% in hypoxic cells, and 205% in reoxygenated cells. These data indicate that aconitase is partially inactivated under physiological conditions, presumably because of exposure to ROS. The degree of inactivation is less in hypoxia and greater during reoxygenation.

**SDH activity.** SDH activity in the air control cells averaged 8.5 ± 1.7 milliunits/mg protein (n = 4). Hypoxia did not change SDH activity significantly (83 ± 22% of air controls, P > 0.05) in nontransfected cells. Activity after reoxygenation for 24 h decreased to 65 ± 10% of air controls (P < 0.05). These data are shown in Fig. 5.

Transfection of air control cells with an empty adenoviral vector (100 MOI Ad.null) had no effect on SDH activity and, as above, Ad.null had no protective effect. Reoxygenation did not cause a significant decrease in SDH activity in cells transfected with Ad.MnSOD. As with NADH dehydrogenase and aconitase, overexpression of MnSOD in mitochondria inhibited the decrease in SDH activity due to reoxygenation, suggesting involvement of superoxide in the mechanism of inactivation due to hypoxia.

**Effects of adenoviral vectors Ad.MnSOD and Ad.null on cytotoxicity during reoxygenation.** Neither Ad.MnSOD nor Ad.null transfection of the A549 cells prevented cytotoxicity due to 50 μM DMNQ in air control or hypoxia-preexposed cells. Both vectors decreased cytotoxicity slightly compared with nontransfected controls after hypoxia. However, in the air control cells, the vectors increased cytotoxicity slightly. These data are summarized in Table 2.
condition. Ad.MnSOD transfection increased DHR fluorescence after both air (0.74 ± 0.07 FU) and hypoxia (0.75 ± 0.08 FU) exposures (both P < 0.001 compared with nontransfected cells in the same condition), consistent with increased peroxide concentration.

**Protective effects of α-ketoglutarate plus aspartate or succinate during reoxygenation.** Addition of α-ketoglutarate plus aspartate at millimolar concentrations to lung epithelial cell medium had a significant protective effect from DMNQ-induced cytotoxicity at all concentrations of DMNQ tested. In contrast, succinate did not prevent oxidant injury due to DMNQ during reoxygenation. These data are summarized in Fig. 6, A and B.

**DISCUSSION**

Reoxygenation deenergizes mitochondria through a number of mechanisms and especially impairs utilization of NADH by complex I (22). Complex I is a multisubunit protein containing eight Fe-S centers and two flavin mononucleotide molecules as prosthetic groups (33). It catalyzes one electron reduction of oxygen, generating O$_2^-$ that can react with Fe-S and release labile, reduced ferrous iron (Fe$^{2+}$) (42).

We herein tested the hypothesis that reoxygenation of lung epithelial cells would inactivate key mitochondrial enzyme activities due to intramitochondrial O$_2^-$ production. We used lung epithelial cells exposed to hypoxia-reoxygenation to elucidate mechanisms. We detected O$_2^-$-dependent inactivation of NADH dehydrogenase, SDH, andaconitase. Increased DHR oxidation after hypoxia indicated increased mitochondrial oxidant production, resulting in accumulation of peroxide. Because abnormalities of complexes I and II were postulated to contribute to injury after hypoxia-reoxygenation, experiments also tested whether citric acid cycle intermediates (α-ketoglutarate plus aspartate) or succinate protected cells from cytotoxicity during hypoxia-reoxygenation.

We previously measured LDH release after hypoxia and reoxygenation per se. Under the conditions of those experiments, in which cells were exposed to hypoxia or reoxygenation in the presence of serum, lung epithelial H441 cells did not lyse spontaneously, nor did they undergo apoptosis during reoxygenation (28). A549 cells respond to hypoxia-reoxygenation in ways that exactly parallel H441 cells. Like H441 cells, A549 cells did not lyse spontaneously, nor did they undergo apoptosis during reoxygenation. We have found in other studies that hypoxia decreases both MnSOD expression and GSH content in A549 cells, and the increased DHR fluorescence we report here in A549 cells mirrors increased DCF fluorescence and H$_2$O$_2$ release by H441 cells after hypoxia. The decreases in complex I and II activities in A549 cells, along with decreased MnSOD and GSH, are not sufficient to cause cell death on return to normoxia, and this response is identical to

### Table 2. LDH release (percentage of maximum) from transfected A549 cells treated with 50 μM DMNQ

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Air (%)</th>
<th>Hypoxia (%)</th>
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<tr>
<td>Ad.MnSOD (100 MOI)</td>
<td>21.2 ± 3.9††</td>
<td>64.5 ± 2.3‡‡</td>
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<tr>
<td>Ad.Null (100 MOI)</td>
<td>24.9 ± 3.7††</td>
<td>59.4 ± 2.2‡‡</td>
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<tr>
<td>None</td>
<td>19.0 ± 2.7</td>
<td>77.5 ± 3.6‡‡</td>
</tr>
</tbody>
</table>

Data are means ± SE; n = 4 experiments; 6 wells averaged in each experiment. DMNQ, 2,3-dimethyl-1,4-naphthoquinone; MOI, multiplicity of infection. *P < 0.05 compared with the nontransfected air-exposed mean; †P < 0.05 compared with the nontransfected cells in the same condition.
that of H441 cells (28). Clearly, in the presence of exogenous oxidant stress, these changes in respiratory complex and aconitase activities make the cells more susceptible to oxidant injury.

Thus we demonstrate that sublethal oxidant stress is more sensitively detected as inhibition of complex I, complex II, and aconitase activities. The present DMNQ studies are physiologically relevant, because they impose an external source of O$_2^-$ and H$_2$O$_2$, testing susceptibility of previously hypoxic cells to exogenous oxidant stress like that occurring in vivo.

Reoxygenation of lung epithelial cells after 24 h of moderate hypoxia (medium Po$_2$ $<$ 30 mmHg) decreased Fe- and S-containing NADH dehydrogenase, SDH, and aconitase activities significantly. Hypoxia did not decrease enzyme activities significantly, implying that events during reoxygenation caused enzyme inactivation. Decreases in mitochondrial enzyme activities after reoxygenation were inhibited completely by transfection with Ad.MnSOD, which substantially increased mitochondrial SOD and thus confirmed O$_2^-$ production as the mechanism of inactivation.

Adenoviral transfection itself (Ad.null vector) caused no detectable change in MnSOD protein expression. Accordingly, Ad.null transfection does not prevent complex I inactivation. Ad.null transfection does, however, have an apparent protective effect against DMNQ cytotoxicity. Others have reported that adenoviral vectors induce A549 cell IL-8 (4) and the Raf/MAPK pathway, which activates transcription factors controlling the expression of cytokines (10). Induction of protective cytokines and activation of MAPK pathways could account for apparent protection from DMNQ, even though Ad.null does not increase MnSOD protein expression.

Submitochondrial particles in the presence of substrates and antimycin A generate O$_2^-$ at a rate of 4–7 nmol·min$^{-1}$·mg protein$^{-1}$ (9). Production of O$_2^-$ at the NADH dehydrogenase is about half that at the ubiquinone-cytochrome b region at pH 7.4, indicating that about one-third of O$_2^-$ produced physiologically originates from complex I. In vitro, O$_2^-$ efficiently inactivates NADH dehydrogenase, NADH oxidase, and ATPase and less efficiently inhibits SDH and succinate oxidase (48). Complex I activity in heterozygous MnSOD knockout mice appears sensitive to inactivation by O$_2^-$ (46). Complex I activity in MnSOD-overexpressing transgenic mice is protected from inactivation by adriamycin, which generates O$_2^-$ by redox cycling (47). Complex I functional defects, which occur in patients with complex I deficiencies, increase cellular production of ROS (35). Decreased MnSOD activity or protein or gene expression, as demonstrated after hypoxia (23), increases the mitochondrial steady-state concentration of O$_2^-$.

When MnSOD-depleted submitochondrial particles were inhibited with myxothiazol or stigmatellin, O$_2^-$ production was detectable by coelenterazine oxidation, suggesting that O$_2^-$ originates from the Q$_1$ semi-ubiquinone. Without MnSOD, the steady-state concentration of O$_2^-$ increases (37).

Altered thiol status also directly affects complex I activity, and diethyl maleate causes significant loss of both complex I activity and glutathione (5). N-acetyl-cysteine protects against oxidative inactivation of complex I in vitro (6). Downregulation of glutamylcysteine synthetase, the rate-limiting enzyme in GSH synthesis, results in impaired mitochondrial function, due to inhibition of complex I activity resulting from thiol oxidation (24). Consistent with this mechanism, we have found that exposure of reoxygenated cells to 1 mM DTT, but not high-intensity light, increased complex I activity. This result shows that loss of complex I activity following reoxygenation is more likely the result of sulfhydryl oxidation than nitrosylation. This occurred in association with decreased SDH and aconitase activities, likely also due to ROS production. Peroxynitrite formed from NO and O$_2^-$ can nitrate Tyr residues in complex I (14). Nitrosylation at heme or nonheme catalytic sites or the formation of nitrosyl complexes with redox-active iron would affect electron transport (12), although our results do not support this mechanism.

Aconitase and SDH are also prone to ROS attack and inactivation. Aconitase activity is inhibited by dioxygen, O$_2^-$, and peroxynitrite, presumably by oxidation of the Fe-S centers and release of Fe$^{2+}$ (19). Complex II also has structural and catalytic properties similar to quinol-fumarate oxidoreductases (1), and its Fe-S centers and flavoprotein sulfhydryls are subject to oxidative inactivation (39). 4Fe-4S clusters in these proteins can delocalize electrons and promote formation of O$_2^-$, leading to release of Fe$^{2+}$ bound to cysteinylligand.

In the present studies, we used DHR as a marker of mitochondrial ROS (presumably H$_2$O$_2$) production. We chose DHR as the indicator for reactive species production, because it is concentrated within mitochondria. DHR is oxidized by H$_2$O$_2$ and peroxynitrite, so it is not specific for O$_2^-$.

DHR is used here as a qualitative marker of cellular oxidant stress, rather than a precise indicator of rates of H$_2$O$_2$ formation. Neither DCF fluorescence nor DHR oxidation is an ideal probe of intracellular ROS production. Neither is a quantitative indicator of reactive species, because of limitations in interpretation of fluorescence as a marker for the quantitative intracellular formation of H$_2$O$_2$ (41).

As shown by increased DHR fluorescence, oxidants are produced in excess during reoxygenation after hypoxia. ROS production also increases during the reduced state that characterizes cellular hypoxia (42). The mitochondrial electron transport chain becomes reduced (i.e., the complexes harbor electrons, and the ubisemiquinone pool increases) during anoxia, potentiating increased O$_2^-$ production (16, 42). Respiring mitochondria generate increased partially reduced oxygen species (primarily O$_2^-$ and the dismutation product H$_2$O$_2$) during reoxygenation associated with decreased phosphorylation and uncoupled respiration (16). Rat pulmonary artery smooth muscle cells acutely exposed to hypoxia (~25 Torr) exhibited a marked increase in intracellular DCF fluorescence, which was attenuated by dimethylthiourea and catalase, demon...
strating increased production of peroxide(s) (25). Lung epithelial cells increase ROS production (DCF oxidation) significantly after 24 h in hypoxia (<1% O2), and they release H2O2 during reoxygenation, probably due to mitochondrial O2− production (27).

DHR is subject to oxidation by ONOO− directly and by H2O2 in the presence of heme-containing peroxidases, as is the case in epithelial cells (41). The increased oxidation of DHR observed after hypoxia is consistent with increased cellular oxidant production, especially mitochondrial H2O2. Finding increased DHR oxidation in Ad.MnSOD-transfected cells is consistent with the notion that overexpression of MnSOD will enhance mitochondrial H2O2 production, because of favorable reaction kinetics (38). Because of its positive charge, DHR is taken up by mitochondria, and, accordingly, we observed punctate distribution of DHR fluorescence consistent with organelar localization.

Therefore, these data show that complex I, complex II, andaconitase dysfunction may be prevented by providing citric acid cycle intermediates (α-ketoglutarate plus aspartate) that generate ATP anaerobically by substrate-level phosphorylation (44). For example, millimolar α-ketoglutarate plus aspartate is known to protect renal proximal tubules from reoxygenation injury (44, 45). We have confirmed a similar protective effect of α-ketoglutarate plus aspartate in previously hypoxic, reoxygenated lung cells that are exposed to DMNQ. Supplementation with the citric acid cycle metabolites prevents cytotoxicity due to O2− and H2O2 produced by DMNQ redox cycling. Such results could be useful in developing strategies to minimize lung injury after transplantation or resewicitation, where posthypoxic mitochondrial dysfunction plays a role in pathogenesis.

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