Loss of lung mitochondrial aconitase activity due to hyperoxia in bronchopulmonary dysplasia in primates.

RONALD L. MORTON, DAVID IKLÉ, AND CARL W. WHITE
National Jewish Medical and Research Center, University of Colorado Health Sciences Center, Denver, Colorado 80206; and University of Texas Health Science Center and Southwest Foundation for Biomedical Research, San Antonio, Texas 78284

Morton, Ronald L., David Iklé, and Carl W. White. Loss of lung mitochondrial aconitase activity due to hyperoxia in bronchopulmonary dysplasia in primates. Am. J. Physiol. 274 (Lung Cell. Mol. Physiol. 18): L127–L133, 1998.—The premature primate exposed to hyperoxia provides a useful model of bronchopulmonary dysplasia. A critical target in hyperoxic injury is the mitochondrial matrix enzyme aconitase. We hypothesized that this enzyme’s activity would decline in the premature baboon lung during exposure to hyperoxia. Total aconitase activity was significantly decreased in the lungs of premature baboons of 140 days gestation with exposure to 100% oxygen for 6–10 days compared with as needed [pro re nata (PRN)] oxygen exposure and fetal controls (P < 0.0001). In activity gels, lungs from 100% oxygen-exposed animals (6–10 days) showed a nearly complete loss of mitochondrial aconitase activity relative to lungs from animals exposed only to PRN oxygen. Decreased lung aconitase activity was not a nonspecific effect of hyperoxia, causing mitochondrial damage or loss, because the activity of the mitochondrial respiratory enzyme cytochrome oxidase was not different in lungs of 100% oxygen-exposed relative to PRN oxygen-exposed newborns. In 125-day-gestation premature primates (age 6–10 days), lung total aconitase activity was correlated with inspired oxygen tension (r = 0.73 for fraction of inspired oxygen > 0.35), whereas, for animals of 140 days gestation, no such correlation was found. Thus the more premature animal’s lung was more susceptible to loss of aconitase.

bronchopulmonary dysplasia (BPD) develops in the premature neonate treated with mechanical ventilation and exposed to elevated oxygen concentrations for prolonged periods of time. This high-oxygen environment increases pulmonary production of toxic oxygen metabolites (14). These products can overwhelm intrinsic antioxidant defenses, leading to structural and functional changes in the lungs of these patients (3, 5, 34).

At the cellular level, hyperoxia impairs mitochondrial function and cellular respiration in both isolated cells and intact lungs (1, 31). One critical target for hyperoxic lung damage is the mitochondrial matrix enzyme aconitase. Superoxide-mediated inactivation of aconitase was initially described in Escherichia coli and subsequently in highly purified mammalian aconitase (12, 15, 16). In addition, aconitase activity decreases rapidly in cultured human lung adenocarcinoma (A549) cells and in rat lungs exposed to hyperoxic conditions. Aconitase activity is lost more rapidly than activities of other enzymes involved in energy metabolism, which were previously described as targets sensitive to hyperoxia (18, 19). Furthermore, cellular respiration decreases in parallel with the loss of aconitase activity, and the specific aconitase inhibitor fluorocitrate causes a similar loss of respiration (18).

The potential role of aconitase and its inactivation by hyperoxia have not been explored in the premature primate model of BPD (6) or in any other specific clinical disease model for respiratory failure. Because premature newborns are deficient in multiple lung antioxidant enzymes (13, 35, 37, 41), it might be expected that they would have an exaggerated loss of lung aconitase activity upon exposure to hyperoxia. Therefore, we tested the hypothesis that aconitase inactivation is directly related to inhaled oxygen tension and inversely related to gestational age in the premature baboon during treatment of respiratory distress and the development of BPD. In these experiments, we measured aconitase activity in lungs of newborn baboons of varying gestational age that were exposed to different oxygen tensions. We found that 140-day-gestation premature primates have a profound decrease in lung aconitase activity when exposed to 100% oxygen and that the mitochondrial fraction of this activity is almost completely eliminated. This decrease in lung aconitase activity is specific and independent of mitochondrial damage or loss, as indicated by the preservation of the activity of the mitochondrial matrix enzyme cytochrome oxidase (35, 38). Our experiments demonstrate a profound, persistent inactivation of mitochondrial aconitase related to hyperoxia during the clinical evolution of BPD in primates.

MATERIALS AND METHODS

Reagents. NADP*, porcine heart isocitrate dehydrogenase, porcine heart aconitase, phenazine methosulfate, cis-aconitate, and nitro blue tetrazolium were from Sigma.

Animal care protocols. All animal care procedures were performed according to the National Research Council’s Guide for the Care and Use of Laboratory Animals. Protocols were reviewed and approved by the Animal Resources Committee of the Southwest Foundation for Biomedical Research (San Antonio, TX), where all animal studies were performed. Gestational ages were determined by timed matings as previously described (6), with confirmation by ultrasound at regular intervals during pregnancy.

The adult baboon mothers were all handled similarly throughout pregnancy, and no special treatments were given. Fetal or newborn baboons of varying gestational age (±2 days) were delivered by hysterotomy. Gestational control (fetal) animals were killed at delivery before the onset of breathing, and lungs were processed immediately for biochemical assays.

1040-0605/98 $5.00 Copyright © 1998 the American Physiological Society
Premature animals were delivered at either 140 ± 2 days (76% term) or at 125 ± 2 days (68% term) gestation and were immediately placed on positive-pressure ventilation (PPV). The normal gestational age for the baboon is 185 days. Animals of 140 days gestation were given either continuous 100% oxygen or “as needed” oxygen (pro re nata, PRN; fraction of inspired oxygen (FiO2) range 0.21–0.8) necessary to maintain the arterial partial pressure of oxygen (PaO2) at 40–50 Torr. Animals of 125 days gestation received immediate resuscitation with artificial surfactant, PPV, and PRN oxygen. All animals were supported in a state-of-the-art neonatal intensive care unit for up to 17 days. After treatment, animals were killed by administration of intravenous pentobarbital sodium. Lungs from a total of 73 baboons were studied (n = 31 for 140-day animals, n = 27 for 125-day animals, and n = 15 for 160- and 175-day animals). All newborn animals for this project were treated in a similar fashion with no special treatments given. These animals were used in the ongoing multi-investigator National Institutes of Health Collaborative Project on Bronchopulmonary Dysplasia.

Tissue samples. After death, the lungs were rapidly perfused via the pulmonary artery with phosphate-buffered saline (37°C), and the distal lung tissue was dissected free from major airways and central structures. Tissue for aconitase assay was removed first and was frozen by immediate freeze clamping under liquid nitrogen. Frozen lung was pulverized under liquid nitrogen and then homogenized at 4°C for 15 s in buffer [50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.4), 0.6 mM MnCl2, and 5 mM sodium citrate] with a polytron (Brinkman Instruments) set at maximum power. Two 200-µl samples were rapidly frozen again, stored in liquid nitrogen, and shipped for biochemical analysis within 4 wk, and held in liquid nitrogen until assay. We observed no freeze-thaw effect on aconitase activity in fresh lung tissue from adult and newborn rats after freezing one time, and, subsequently, we found no decrease in activities in these lung homogenates for up to 90 days of storage. After such processing, shipment, and storage, no decline in aconitase activities was noted in the premature baboon lung homogenate when stored in liquid nitrogen for >90 days.

A second tissue sample was prepared for cytochrome oxidase assay by homogenizing at 4°C for 15 s in buffer [50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.4), 0.6 mM MnCl2, and 5 mM sodium citrate], NADP⁺ (10 µl; 200 µM), isocitrate dehydrogenase (3 µl; 0.5 U/µl), and sample (20 µl) were added to a quartz cuvette. Aconitase activity was calculated as follows: mU aconitase activity/g tissue = absorbance rate × extinction coefficient (0.0062) × sample volume (0.02 ml). An aconitase standard (2 U/ml A5384; Sigma) diluted in 50 mM Tris-HCl, pH 7.4, 0.6 mM MnCl2, and 5 mM sodium citrate and stored at −70°C was assayed simultaneously for comparison.

Electrophoretic mitochondrial aconitase activity assay. For electrophoretic analysis, the samples were concentrated before being loaded with Microcon-50 protein concentrators (Amicon, Beverly, MA). Each sample was centrifuged (12,000 g at 4°C) until a dry pellet was formed and then was reconstituted in 3-(N-morpholino)propanesulfonic acid (MOPS, 20 mM), citric acid (3.6 mM), and sucrose (146 mM). Protein was determined by the Bradford method (see below). A 1% agarose gel (Fisher Universal Gel) was presoaked for at least 15 min in buffer containing MOPS-citrate-sucrose. Approximately 72 µg protein/well were loaded in 1-µl sequential aliquots using a Hamilton syringe. Electrophoresis (100 V, 20 min) was performed in a horizontal gel box using MOPS-citrate-sucrose buffer as the running buffer. The activity stain contained 50 mM Tris·HCl (pH 7.4), 0.6 mM MnCl2, 0.2 mM NADP⁺, 1 µM phenazine methosulfate, 0.4 mM cis-aconitate, and 1.22 mM nitro blue tetrazolium. The activity stain was prepared fresh, and the gel was soaked for 10 min in the dark without agitation. Isocitrate dehydrogenase (2 U/ml) was added after 10 min with gentle mixing, and the incubation was continued for ~10 min. Then the gel was washed in deionized water and allowed to dry in room air (adapted from Ref. 27). The mitochondrial band migrates in a cathodal direction (as opposed to the cytoplasmic band, which migrates in an anodal direction), and the mitochondrial band was the only form visualized on the gel under these running conditions. A homogenate of fetal baboon heart was run as a control, since cardiac muscle contains abundant mitochondria and demonstrates a mitochondrial aconitase band.

Protein assay. The protein concentration in the lung homogenate was determined by the Coomassie blue dye binding (Bradford) method (2) using a Spectramax 340 microtiter plate reader (Molecular Devices, Sunnyvale, CA) with a 96-well plate. Coomassie brilliant blue staining reagent (Bio-Rad) was used for protein analysis, with bovine serum albumin, fraction V (Calbiochem), as a standard. Data analysis was done with Softmax Pro 1.2 software (Molecular Devices).

Clinical course. Clinical data, including birth weight, sex, airway pressures, blood gas analysis, and vital signs, were collected sequentially and analyzed for all 125- and 140-day baboons studied. All animals were ventilated with conventional PPV, with inspiratory times ranging from 0.4 to 0.6 s and respiratory rates from 20 to 40/min. Ten animals required high-frequency ventilation at some point during their clinical course because of worsening acidosis or pulmonary complications (1 pulmonary hemorrhage, 1 bilateral pneumothoraces). The newborns treated with PPV had peak airway pressures ranging from 18 to 35 cmH2O and peak end-expiratory pressures from 2 to 4 cmH2O. The mean airway pressures during the course of ventilation ranged from 5 to 15 cmH2O (overall mean = 7 cmH2O). Three animals were bacteremic during the course of the study (2 with Candida albicans, 1 with Staphylococcus epidermidis). The inclusion of results from these septic animals did not bias the results.

Lung cytochrome oxidase activity. Bovine heart cytochrome c (100 mg/ml) was reduced with excess dithionite. Reduced cytochrome c was then separated from dithionite using gel filtration (Sephadex G-25) after equilibration of the column with 50 mM potassium phosphate buffer. The concentration of reduced cytochrome c present was calculated using an extinction coefficient of 21,000 (30). Cytochrome c (30 µM) was added to 50 mM potassium phosphate buffer, pH 7.4, for a final reaction volume of 0.5 ml. Upon addition of sample to the reaction mixture, the rate of decrease in absorbance at 550 nm was measured for 5 min at 25°C. The linear portion of the curve was used to determine the maximal velocity. One unit of enzyme activity is defined as that quantity which oxidizes 1 µmol of reduced cytochrome c per minute at 25°C.

Reactivation experiments. Attempted reconstitutions of lung homogenate aconitase activity were carried out in the pres-
ence of dithiothreitol (DTT, 5 mM) and ferrous sulfate (25 μM) or DTT, ferrous sulfate, and sodium sulfide (10 μM; see Ref. 26). The samples were incubated anaerobically under argon for 1 h at 37°C. Enzyme activities for aconitase were measured spectrophotometrically at 240 nm and 20°C after the formation of aconitase from isocitrate (modified from Ref. 26). This assay was used to avoid potential artifacts of DTT in introducing NADP+, the end point for the other spectrophotometric aconitase assay used in this study.

Statistics. Comparisons of response variables between levels of oxygenation (fetal, PRN, 100%) were made within gestational age groups by one-way analysis of variance or independent sample t-tests depending on the number of levels of oxygenation. Measures of aconitase and cytochrome oxidase activity were analyzed after obtaining the log10 of the individual measurements to accommodate the analytical assumptions of normal distributions. Data within groups were summarized using means and SE or geometric means and 95% confidence limits depending on the distributions of the variables. Grouped data are described graphically using diamond plots in which the horizontal line is the overall mean, the width of each diamond is proportional to the group sample size, the line through the center of each diamond is the group mean, the upper and lower diamond vertices are the upper and lower 95% confidence limits about the group mean, and the short horizontal lines inside the diamonds are overlap marks used to compare pairs of group means of equal size. Assessment of the association between variables was performed using simple linear regression and correlation techniques. All tests were two tailed, and an α level of 0.05 was used as the standard for assessing statistical significance. All statistical analyses were performed using JMP Version 3.1 statistical software (SAS Institute) running on a PowerPC computer (Macintosh).

RESULTS

Lung aconitase activity in the 140-day-gestation baboon. Aconitase activity decreased in the lungs of 140-day-gestation premature newborns exposed to 100% oxygen for 6–10 days (P = 0.0001) when compared with those animals receiving PRN oxygen or with fetal controls (Fig. 1A). An electrophoretic activity assay was run to evaluate specifically the mitochondrial aconitase. In 140-day-gestation animals, the lung mitochondrial aconitase band stained more intensely in the PRN oxygen-exposed group (FIO2 = 0.35–0.65) compared with the 100% oxygen exposure group. A representative sample of the findings is shown in Fig. 2. These results demonstrate the specific loss of lung mitochondrial aconitase activity at the highest concentration of oxygen.

In lungs of hyperoxia-exposed (100%) neonates, the addition of an organic reducing compound, such as DTT (5 mM) and FeSO4 or DTT, FeSO4, and sodium sulfide, failed to increase aconitase activity after incubation (37°C) under argon for 60 min (data not shown). Thus the oxidation and/or degradation of lung aconitase was not reversible by reductants plus iron.

Lung aconitase activity: relative sensitivity to hyperoxia and gestational spectrum. An inverse relationship between lung aconitase activity and FIO2 in premature baboons of 125 days gestation at 6–10 days of life was noted (r = 0.58, P = 0.014; Fig. 3A). Considerable scatter in total lung aconitase activity was noted in animals at lower FIO2 values (<0.35). At higher FIO2 values (>0.35), the inverse relationship between FIO2 and total lung aconitase activity was slightly more linear (r = 0.73, P = 0.016). At age 6–10 days, no correlation was noted between aconitase activity and FIO2 at 140 days gestation (Fig. 3B). Basal expression of total aconitase specific activity decreased across gestational ages from 125 to 175 days gestation (Fig. 4A, P = 0.003).

Clinical data. In the 140-day-gestation 100% oxygen group, the PaO2 ranged from 98 to 420 Torr at age 6–10 days. At this age, the mean FIO2 in the 140-day-gestation PRN oxygen group was 0.33 with a PaO2 ranging from 58 to 84 Torr, whereas the mean FIO2 in the 125-day-gestation PRN oxygen group was 0.42 with a PaO2 ranging from 41 to 86 Torr.

Both the alveolar-arterial oxygen gradient and oxygenation index were calculated from the available clinical data as markers of disease severity (Table 1). At 6–10 days of life, the alveolar-arterial oxygen gradient for the 140-day-gestation 100% oxygen exposure group increased significantly compared with both the 140-day-gestation PRN oxygen exposure and the 125-day-gestation PRN exposure groups (P < 0.0003).
These findings indicate greater disease severity in the 140-day 100% oxygen exposure group at 6–10 days of age. No significant differences in oxygenation index were noted between groups at this relatively late stage.

Lung cytochrome oxidase activity. Cytochrome oxidase, a mitochondrial marker enzyme, was measured to determine whether the decrease in aconitase activity was due to specific inactivation of aconitase or to a generalized damage or loss of mitochondria. Cytochrome oxidase activity (per mg lung protein) was not different in any of the oxygen exposure groups in either the 125-day-gestation or 140-day-gestation premature animals (Fig. 1B). Likewise, when cytochrome oxidase activity was compared at different gestational ages in fetal animals, there was no significant change in mean lung cytochrome oxidase activity from the most premature (125 day gestation) to the near full term baboon (175 day gestation, Fig. 4B).

DISCUSSION

This study demonstrates that persistent loss of lung aconitase activity is due to elevated FIO2 in the 140-day premature baboon. We used this model to evaluate the effects of hyperoxia on the developing primate lung in two regards, related first to total lung aconitase and then specifically to lung mitochondrial aconitase activity. First, our results demonstrate that, in the 140-day-gestation animals, there was no significant change in mean lung cytochrome oxidase activity from the most premature to the near full term baboon (175 day gestation).

Fig. 2. Effect of hyperoxia on lung mitochondrial aconitase activity in premature baboons of 140 days gestation. In lane 1, fetal heart tissue (72 µg protein) was loaded to identify the mitochondrial aconitase band. In lanes 2–4, lung homogenates of 100% oxygen-exposed animals were loaded, and in lanes 5–7, homogenates from PRN oxygen-exposed [fraction of inspired oxygen (FIO2) = 0.35–0.65] animals were loaded (72 µg protein/lane for all lung samples). Mitochondrial aconitase activity band migrates in the cathodal direction from origin.

Fig. 3. Correlation between mean FIO2 and total aconitase specific activity (mU/mg protein) in lungs of premature baboons at ages 6–10 days. A: inverse relationship between mean FIO2 and aconitase activity (squares represent 125-day animals) was noted for all FIO2, r = 0.58, P = 0.014; for FIO2, > 0.35 only; r = 0.73, P = 0.016. B: no correlation (r = 0.03) between mean FIO2 and aconitase activity in 140-day-gestation animals was noted (squares represent 140-day animals).

Fig. 4. Effect of gestational age on aconitase (A) and cytochrome oxidase (B) specific activities (mU/mg protein) in lungs of fetal baboons. Treatment of animals, preparation of lung homogenates, and assays for aconitase activities, cytochrome oxidase activities, and protein are described in MATERIALS AND METHODS. Significant increase in lung aconitase specific activity was noted at 125 days gestation compared with 160 and 175 days gestation (*P = 0.003). No significant difference was noted in cytochrome oxidase specific activities (mU/mg protein) from 125 days (69% term) through 175 days (near term) gestation. Group sizes for various gestational ages were as follows: 125 days, n = 12; 140 days, n = 7; 160 days, n = 9; and 175 days, n = 8. For explanation of diamond plot, see legend for Fig. 1.
No significant difference was noted in OI between groups.

Table 1. A-a oxygen gradient and OI for different gestational ages and oxygen exposures

<table>
<thead>
<tr>
<th>Group</th>
<th>a-a Gradient</th>
<th>n</th>
<th>Oxygenation Index</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>140-day</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRN</td>
<td>143.7 ± 30.6 (5–350)</td>
<td>11</td>
<td>3.65 ± 0.79 (1.3–10.2)</td>
<td>11</td>
</tr>
<tr>
<td>140-day</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100%</td>
<td>390 ± 29.5*</td>
<td>12</td>
<td>3.66 ± 0.76 (1.4–11.2)</td>
<td>12</td>
</tr>
<tr>
<td>125-day</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRN</td>
<td>175.5 ± 32.6 (29–361)</td>
<td>14</td>
<td>4.82 ± 1.26 (1.31–16.1)</td>
<td>14</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE; n, no. of animals (6–10 days old). Ranges are in parentheses. PRN, pro re nata oxygen. Alveolar arterial (a-a) oxygen gradient [FiO2 × (barometric pressure - 47 mmHg) - PaCO2 - PacO2] and oxygenation index [OI; (FiO2 × MAP × 100)/PaO2] are calculated for the different gestational ages and oxygen exposures. FiO2 is the fraction of inspired oxygen, PacO2 is arterial partial pressure of oxygen, PaCO2 is arterial partial pressure of CO2, and MAP is mean arterial pressure. *Significant difference between 140-day 100% compared with both 140-day PRN and 125-day PRN groups, P < 0.0003, by Kruskal-Wallis (rank sum) tests. No significant difference was noted in OI between groups.

gestation baboon, there is less total aconitase activity in lungs of animals exposed to 100% oxygen from birth compared with those given PRN oxygen only or compared with gestational control groups (Fig. 1A). The animals exposed to 100% oxygen had a marked decrease in lung total aconitase activity. In contrast, the PRN animals at this gestation still have considerable aconitase activity, with mean aconitase activities comparable to the gestational controls. Second, our results from electrophoretic experiments suggest that hyperoxia specifically affected the mitochondrial aconitase activity, which was almost completely eliminated in 100% oxygen (Fig. 2).

Aconitase inactivation after a relatively brief exposure to hyperbaric or normobaric hyperoxia has been observed in bacteria (15, 16), cultured human lung adenocarcinoma A549 cells, and rat lungs (18). The rapid inactivation of aconitase in hyperbaric oxygen has been shown to be readily reversible in both bacteria (16) and mammalian cells (20). Investigations in mammalian species done in our laboratory have shown that, after 100% oxygen exposure, aconitase inactivation occurs rapidly (within 3 h of exposure), precedes the loss of other highly oxygen-sensitive tricarboxylic acid cycle enzymes (α-ketoglutarate dehydrogenase), and parallels the loss of respiratory capacity (18, 19). The persistence of loss of aconitase activity, and particularly of mitochondrial aconitase, when animals are continuously exposed to 100% oxygen for extended periods of time was not known previously. Prior work has demonstrated clinical and histological changes consistent with BPD in the 140-day-gestation baboon exposed to 100% oxygen (6, 7, 11). These histological changes consisted of alternating areas of focal atelectasis and overexpanded saccules (7). In this model (140 day), we observed that aconitase inactivation persists for 6–10 days during continuous exposure to 100% oxygen.

Next, we asked whether the aconitase inactivation observed in the 140-day baboon after 100% oxygen exposure was due to loss of mitochondria or to selective enzyme inhibition. Other investigators have shown that, in adult rats after exposure to sublethal hyperoxia, there is progressive mitochondrial damage, with swollen, abnormal mitochondria appearing early during the first week of exposure and more bizarre, fused mitochondria appearing during the second week (8). Cytochrome oxidase has been used as a mitochondrial marker enzyme for comparison with other mitochondrial proteins (35, 38). The present study shows that the mitochondrial biochemical damage was specific. In 100% oxygen, relative to PRN oxygen, there was no decline in cytochrome oxidase activity. We observed no difference in lung cytochrome oxidase activity related to FiO2 or to gestational age (Figs. 1B and 4B).

The third question addressed was whether loss of aconitase activity was related to gestational age in the premature baboon. The 125-day-gestation baboon exposed to only PRN oxygen provides another model that is even more relevant to modern BPD than the 140-day model (32). The reasons for this similarity to the contemporary disorder are, first, the 125-day baboon’s lung developmental stage corresponds approximately to that of the 24- to 26-wk-gestation human, and, second, 100% inspired oxygen tensions are most often not sustained for the 6- to 10-day durations used with the 140-day baboon. As is the case in the management of many premature infants today, a high FiO2 concentration is required to support life in the first days after birth, but, thereafter, oxygen tension frequently can be weaned significantly. Although lung tissues were available only from animals at ages 6 days, 10 days, or greater, an inverse relationship between lung aconitase activity and FiO2 was observed, especially at oxygen concentrations >35%. This relationship was observed only in animals of 125 days gestation. Interestingly, these animals develop BPD when treated only with PRN oxygen for this duration (32), whereas 140-day gestation animals given PRN oxygen generally do not develop BPD (7).

The mechanism of aconitase inactivation in this model is unknown. Prior work done in our laboratory in mammalian cells showed that overproduction of mitochondrial manganese superoxide dismutase did not protect aconitase from inactivation when cells were exposed to normobaric hyperoxia. Thus a mechanism possibly involving direct oxidation by dioxygen or possibly other reactive species, rather than by excessive mitochondrial superoxide production, was implicated (19). These conclusions were reached in a malignant lung cell line. The lungs of premature primates or humans may be considerably more deficient in manganese superoxide dismutase or copper and zinc-containing superoxide dismutase activities (10). Hence, superoxide could still be important in the loss of aconitase activity observed in this model.

Hyperoxia increases oxygen free radical formation in (14), as well as hydrogen peroxide release from, lung mitochondria (36). Hydrogen peroxide, at higher concentrations than superoxide, also inactivate aconitase. We cannot exclude a potential role for hydrogen peroxide in aconitase inactivation. Another reactive species
that could cause aconitase inactivation in this model is peroxynitrite (4, 24), the formation of which would tend to be favored by the marked inflammation characteristic of BPD. Peroxynitrite formation is increased in stimulated rat alveolar macrophages (25), and evidence of its presence has been found in lung sections of patients with acute lung injury or adult respiratory distress syndrome and in rats exposed to lethal hyperoxia (23). Unlike superoxide, which also directly inactivates aconitase, peroxynitrite can readily cross membranes. Thus we speculate that the mechanism of aconitase inactivation may involve dioxygen, superoxide anion, hydrogen peroxide, peroxynitrite, or a combination of these.

Loss of aconitase activity also could be due in part to impaired reactivation of aconitase during its normal cyclic inactivation-reactivation. Reducing thiol, such as glutathione, which may be required for aconitase reactivation (17), may be relatively lacking in the circulation (33, 39) and lung (5) of the preterm newborn. Although iron normally would be expected to be available for reinsertion into the aconitase active site, iron-withholding conditions also could be induced during sepsis (28), a complication associated with a considerably increased incidence in BPD (21). In this clinical situation, decreased iron availability also could decrease aconitase reactivation (19). The incidence of sepsis in our study was too low to evaluate this possibility. However, the possibility that excessive iron withholding could have dire consequences in the premature neonate is not theoretical. Indeed, the cardiopulmonary and vascular collapse in hyperoxia-exposed neonatal baboons treated with even low doses of deferoxamine (9) is remarkably similar to that occurring more gradually in association with the loss of cardiac aconitase activity during normoxic exposure of manganese superoxide dismutase knockout mice (29). Thus either inactivation or reactivation of aconitase during its normal cycle could contribute to loss of lung aconitase activity. After its oxidation, aconitase, like many other proteins, also could become more susceptible to proteolysis (22). Finally, synthesis of some proteins also can be impaired in hyperoxia. Hence, in addition to increased inactivation or decreased reactivation, impaired synthesis also could contribute to loss of aconitase activity. Preservation of cytochrome oxidase activity, however, suggests that this may not have been a principal cause.

In summary, our results indicate that lung aconitase and, specifically, mitochondrial aconitase activity decrease in the 140-day-gestation baboon exposed to 100% oxygen, even after ~1 wk of age. At lesser FlO2, loss of lung aconitase activity was related to FlO2 in more premature animals (125 day gestation) at this age. During acute hyperoxic exposures, loss of aconitase activity could have adverse effects on cellular energy metabolism required for vital processes, such as surfactant synthesis and processing, ion movement, and lung growth in infants with respiratory distress. We speculate that, among the possible consequences of prolonged loss of aconitase activity could be the favored survival and proliferation of cells that are not primarily dependent on tricarboxylic acid cycle activity and/or mitochondrial respiration and that may have lower energy demands for survival and growth.

NOTE ADDED IN PROOF

Since submission of this paper, nitric oxide alone has been shown to be sufficient to inactivate aconitase under appropriate conditions (P. R. Gardner, G. Costantino, C. Szabó, and A. L. Salzman, J. Biol. Chem. 272: 25071–25076, 1997) and the structural basis for this inactivation has been partially characterized for both mitochondrial and cytosolic aconitases (M. C. Kennedy, W. E. Antholine, and H. Beinert. J. Biol. Chem. 272: 23040–23047, 1997). Therefore, nitric oxide also could be the aconitase-inactivating species in this study.

We acknowledge the superb, dedicated assistance of numerous physicians, nurses, technicians, and other personnel at the Bronchopulmonary Dysplasia (BPD) Resource Center in San Antonio, TX. We acknowledge the excellent technical assistance of J acque Guthrie in preparing the manuscript and the many helpful suggestions of Dr. Paul Gardner regarding the electrophoretic mitochondrial aconitase activity assay.

This work was supported by National Heart, Lung, and Blood Institute Grants HL-53636 (to the BPD Resource Center), HL-56263, HL-52732, HL-57144, and HL-07670.

Address for reprint requests: R. L. Morton, National Jewish Medical and Research Center, J 101, 1400 Jackson St., Denver, CO 80206.

Received 31 July 1997; accepted in final form 1 October 1997.

REFERENCES

in the lungs of human fetuses and newborn infants: development-
profile and alterations in hyaline membrane disease and
Walsh, D. J. Johnson, J. J. Coalson, T. J. Kuehl, D. M. Null, J. R.,
and J. L. Robotham. A baboon model of bronchopulmonary
1982.
inactivation of Fe-S cluster containing hydro-lyases by superox-
O2-rich environment: the antioxidant enzymes in the developing
14. Freeman, B. A., and J. D. Crapo. Hyperoxia increases oxygen
radical production in rat lungs and lung mitochondria. J. Biol.
15. Gardner, P. R., and I. Fridovich. Superoxide sensitivity of the
Escherichia coli aconitase. J. Biol. Chem. 266: 19328–19333,
16. Gardner, P. R., and I. Fridovich. Inactivation-reactivation of
aconitase in Escherichia coli: a sensitive measure of superoxide
17. Gardner, P. R., and I. Fridovich. Effect of glutathione on
aconitase in Escherichia coli. Arch. Biochem. Biophys. 301:
18. Gardner, P. R., D. H. Nguyen, and C. W. White. Aconitase is a
sensitive and critical target of oxygen poisoning in cultured
mammalian cells and in rat lungs. Proc. Natl. Acad. Sci. USA 91:
Superoxide radical and iron modulate aconitase activity in
20. Gardner, P. R., and C. W. White. Application of the aconitase
method to the assay of superoxide in the mitochondrial matricies
of cultures cells: effects of oxygen, redox-cycling agents, TNFa,
LPS and inhibitors of respiration. In: The Oxygen Paradox,
edited by K. J. A. Davies and F. Ursini. Padua, Italy: CLEUP
Claure, and E. Bancalari. Influence of infection on patent
ductus arteriosus and chronic lung disease in premature infants
Proteolysis in cultured liver epithelial cells during oxidative
stress. Role of the multicatalytic protease complex, protease-
23. Haddad, I. Y., G. Pataki, P. Hu, C. Galliani, J. S. Beckman,
and S. Matalon. Quantitation of nitrotyrosine levels in lung
sections of patients and animals with acute lung injury. J. Clin.
inactivate aconitases, but nitric oxide does not. J. Biol.
The role of iron in the activation-reactivation of aconitase. J.
27. Koen, A. L., and M. Goodman. Aconitate hydratase iso-
enzymes: subcellular localization, tissue distribution and possible
and F. M. Torti. Role for NF-kappa B in the regulation of ferritin
Olson, L. J. Noble, M. P. Yoshimura, C. Beger, P. H. Chan,
D. C. Wallace, and C. J. Epstein. Dilated cardiomyopathy and
neonatal lethality in mutant mice lacking manganese superoxide
30. Massey, V. The microstimation of succinate and the extinction
coefficient of cytochrome c. Biochim. Biophys. Acta 34: 255–256,
1959.
Klei-van Moorsel, C. J. Jakobs, and H. J. Oenene. Respiratory
failure and stimulation of glycolysis in Chinese hamster ovary
cells exposed to normoxic hyperoxia. J. Biol. Chem. 265: 11118–
11124, 1990.
32. Seldner, S., D. McCurnin, J. J. Coalson, D. Correll, M.
Leland, and R. Castro. A new model of chronic lung injury in
surfactant-treated preterm baboons delivered at very early gesta-
33. Smith, C. V., T. N. Hansen, N. E. Martin, H. W. McMicken,
and S. J. Elliott. Oxidant stress responses in premature infants
34. Sobonya, R. E., M. M. Logvinoff, L. M. Taussig, and A.
Theriault. Morphometric analysis of the lung in prolonged
enzyme maturation in the fetal and neonatal rat. I. Developmental
36. Turrens, J. F., B. A. Freeman, and J. D. Crapo. Hyperoxia
increases H2O2 release by lung mitochondria and microsomes.
Ontogeny of antioxidant enzymes in the fetal lamb lung. Exp.
fetal development. VI. Fatty acid oxidation by developing brain.
39. White, C. W., S. P. Stabler, R. H. Allen, S. Moreland, and
A. A. Rosenberg. Plasma cysteine concentrations in infants with
respiratory distress syndrome. J. Pediatr. 125: 769–777,
1994.
of the citric acid cycle and related compounds by fluorometric
41. Yam, J., L. Frank, and R. J. Roberts. Oxygen toxicity:
comparison of lung biochemical responses in neonatal and adult