Mechanical Ventilation Causes Pulmonary Mitochondrial Dysfunction and Delayed Alveolarization in Neonatal Mice

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Hyperoxia inhibits pulmonary bioenergetics, causing delayed alveolarization in mice. We hypothesized that mechanical ventilation (MV) also causes a failure of bioenergetics to support alveolarization. To test this hypothesis, neonatal mice were ventilated with room air for 8 hours (prolonged) or for 2 hours (brief) with 15 μ l/g (aggressive) tidal volume (Tv), or for 8 hours with 8 μ l/g (gentle) Tv. After 24 hours or 10 days of recovery, lung mitochondria were examined for adenosine diphosphate (ADP)-phosphorylating respiration, using complex I (C-I)-dependent, complex II (C-II)-dependent, or cytochrome C oxidase (C-IV)-dependent substrates, ATP production rate, and the activity of C-I and C-II. A separate cohort of mice was exposed to 2,4-dinitrophenol (DNP), a known uncoupler of oxidative phosphorylation. At 10 days of recovery, pulmonary alveolarization and the expression of vascular endothelial growth factor (VEGF) were assessed. Sham-operated littermates were used as control mice. At 24 hours after aggressive MV, mitochondrial ATP production rates and the activity of C-I and C-II were significantly decreased compared with control mice. However, at 10 days of recovery, only mice exposed to prolonged-aggressive MV continued to exhibit significantly depressed mitochondrial respiration. This was associated with significantly poorer alveolarization and VEGF expression. In contrast, mice exposed to brief-aggressive or prolonged-gentle MV exhibited restored mitochondrial ADP-phosphorylation, normal alveolarization and pulmonary VEGF content. Exposure to DNP fully replicated the phenotype consistent with alveolar developmental arrest. Our data suggest that the failure of bioenergetics to support normal lung development caused by aggressive and prolonged ventilation should be considered a fundamental mechanism for the development of bronchopulmonary dysplasia in premature neonates.

Keywords: mechanical ventilation; mitochondrial dysfunction; alveolarization; bioenergetics; mouse model of BPD

Bronchopulmonary dysplasia (BPD) is one of the most common complications of prematurity. BPD remains the leading cause of long-term morbidity in infants born prematurely (1–3). The exact mechanisms responsible for the development of BPD remain poorly understood. This limits the introduction of highly demanded effective therapeutic strategies against this devastating disease.

In addition to prematurity, multiple risk factors are also associated with the development of BPD, including a prolonged

CLINICAL RELEVANCE

This study investigated the link between pulmonary development and the bioenergetics of lung tissue after mechanical ventilation in neonatal mice. Our findings may pave the way toward a new therapeutic target for the treatment of bronchopulmonary dysplasia in premature neonates.

exposure to hyperoxia, the use of mechanical ventilation (MV), sepsis, fluid overload, and the presence of patent ductus arteriosus (4). Importantly, despite the diversity of these risk factors, BPD has received a unique pathological definition, namely, a failure of alveolar development. This implies the existence of a common mechanism responsible for the arrest of alveolar growth and development in premature neonates.

In premature infants, it is well-established that the use of MV and exposure to a high concentration of oxygen are associated with the development of BPD (5). During MV, several stress factors (volutrauma, barotrauma, biotrauma, and atelectotrauma) have been identified to cause ventilator-induced lung injury (VILI), the entity that in premature infants is associated with BPD (6, 7). The initial response of the lungs to ventilation-induced stress has been shown to involve a release of proinflammatory mediators. These mediators are responsible for increased pulmonary vascular permeability, the inactivation of surfactant, and the disruption of normal alveolar development (8–10). Immature lungs are especially susceptible to VILI because of surfactant deficiency, which results in poor lung compliance. VILI also makes premature lungs highly susceptible to ventilator-induced barotrauma. Pulmonary damage by hyperoxia is also a well-recognized cause of BPD. Previously, we demonstrated that in neonatal mice, prolonged hyperoxic exposure leads to a significant inhibition of oxidative phosphorylation and a decreased adenosine triphosphate (ATP) production rate by pulmonary mitochondria, and this was closely associated with delays in alveolar development (11). In addition, we have also shown that the chemical inhibition of mitochondrial Complex I (C-I), without any hyperoxia, results in a pulmonary phenotype identical to that of BPD, namely, underalveolarization. We hypothesized that not only oxygen toxicity, but also MV, results in pulmonary mitochondrial dysfunction, which in the actively developing lung causes a bioenergetics failure to support normal alveolar growth.

MATERIALS AND METHODS

Neonatal Murine VILI and Study Groups

Our experimental protocol was approved by the Institutional Animal Care and Use Committee at Columbia University.

VILI was produced in C57Bl/6J 5-day-old neonatal mice as described elsewhere (12), with minor modifications. Pups and their dams were purchased from Jackson Laboratories (Bar Harbor, ME). At this age, mice are at the end of their saccular stage of pulmonary development

(Received in original form May 11, 2012 and in final form June 5, 2013)

This work was supported by National Institutes of Health grant 5K08HL96953 (Z.V.N.).

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 49, Iss. 6, pp 943–950, Dec 2013

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DOI: 10.1165/rcmb.2012-0172OC

Internet address: www.atsjournals.org

(13). To establish airways, pups were anesthetized (100 $\mu\text{g/g}$ ketamine and 10 $\mu\text{g/g}$ xylazine, intraperitoneally), and after a midline neck incision under the dissecting microscope, the trachea was cannulated by piercing below the larynx, using an angiocatheter (24-gauge, 0.56-inch; BD Bioscience, San Jose, CA). The neck wound was closed with Vet-bond (3M Animal Care Products, St. Paul, MN) and coated with bacitracin ointment. MV was performed using MicroVent 848 (Harvard Apparatus, Holliston, MA). All mice were ventilated with room air. Ventilation rates of 100 breaths per minute (bpm) for large T_v and 150 bpm for small T_v were selected to maintain arterial blood gases at a physiological range ($\text{pH} = 7.53 \pm 0.1$, $\text{PCO}_2 = 33 \pm 5.9$ torr, $n = 4$). Samples for the blood gas analysis were obtained by left ventricular puncture, and analyzed using an i-STAT portable analyzer (Abbott Point of Care, Inc., Princeton, NJ). A temperature of 32°C and humidity at 40 to 50% were maintained using Air-Shields Isolette (Narco Scientific, Fort Washington, PA). Heart rate, oxygen saturation, and respiratory rate were recorded every 10 minutes, using a MouseOx murine pulse oximeter system (Starr Life Sciences, Oakmont, PA). A sterile mixture of 5% glucose and normal saline (1:1) was injected subcutaneously (25 $\mu\text{L/g}$ of body weight) after 4 hours of ventilation to maintain hydration and euglycemia. At the end of ventilation, all pups were taken off the ventilator and decannulated. After 4 hours of observation for any signs of respiratory distress (retractions,

irregular breathing and/or apnea, or O_2 saturations $< 90\%$), pups were returned to their dams.

All animals were divided into three experimental groups (Figure 1A): (1) a prolonged (8-h)-aggressive ($T_v = 15 \mu\text{L/g}$) MV group; (2) a brief (2-h)-aggressive ($T_v = 15 \mu\text{L/g}$) MV group, to demonstrate a “dose-dependent” effect of the duration of MV on lung development; and (3) a prolonged (8-h)-gentle ($T_v = 8 \mu\text{L/g}$) MV group, to demonstrate the “dose-dependent” effects of T_v on pulmonary development. The MV with an enlarged T_v is not uncommon strategy in neonatal respiratory care to combat intractable hypercapnia and/or severe hypoxemia. The sham littermates were subjected to an identical anesthesia and surgical procedure, but without MV. Shams ($n = 4$) demonstrated O_2 saturation and blood gases (pH , 7.44 ± 0.65 ; $\text{pCO}_2 = 43.5 \pm 6.8$ torr) comparable to those in experimental animals.

To demonstrate a causative link between lung mitochondrial bioenergetics and alveolar development, a separate cohort of pups (p5) was exposed to a well-known uncoupler of oxidative phosphorylation, 2,4-dinitrophenol (DNP; Sigma, St. Louis, MO). DNP or vehicle (10% DMSO) was injected (2 $\mu\text{g/g}$, intraperitoneally) once a day for 10 days (14, 15).

Mice were killed at 24 hours or 10 days of recovery. At both time points, pulmonary mitochondria were isolated, and their function was assessed. Pulmonary alveolarization was assessed after 10 days

Figure 1. (A) Experimental design and timeline. (B) Kaplan-Meier survival graphs. (C) Animal body weights at 10 days of recovery from mechanical ventilation (MV) or sham operation. (D) Absolute amounts of activated neutrophils (lymphocyte antigen 6 complex, locus G/CD11b $^+$) in a single-cell lung suspension at 24 hours after MV or sham operation with representative FACS dot-plot graphs (activated neutrophils are in the right-upper quadrant). (E) Bronchoalveolar lavage (BAL) protein concentrations ($n = 4$ for all groups). Open bars, 8-hour sham-operated control mice; checkered bars, 2-hour sham-operated control mice for brief MV; solid bars, mice subjected to prolonged-aggressive MV; shaded bars, mice subjected to brief-aggressive MV; hatched bars, mice subjected to prolonged-gentle MV; lattice bars, vehicle-treated mice; brick bars, 2,4-dinitrophenol (DNP)-treated mice. Data represent means $\pm$ SEMs. Two-way ANOVA, $^*P < 0.03$ and $^{**}P < 0.001$, between ventilated and sham-operated groups. One-way ANOVA, $^{\#}P < 0.001$, compared with the prolonged-gentle MV group, $^{\Psi}P < 0.001$, compared with both aggressive MV groups, $^{\S}P = 0.04$, compared with 8-hour sham-operated mice, and $^{\alpha}P = 0.02$, compared with vehicle-treated mice. χ^2 test, $^{\beta}P = 0.001$, compared with brief-aggressive MV and 8-hour sham-operated mice.

of recovery (i.e., the age when alveolarization in rodents is complete) (13).

All other methods are more fully described in the online supplement.

RESULTS

MV Alters Survival and Growth

Aggressive MV for 8 hours significantly decreased 10-day survival, (56.1%) compared with their littermates that were also ventilated aggressively, but only for 2 hours (82.91%, $\chi^2 P = 0.003$), and compared with their shams (96.4%; $\chi^2, P = 0.0001$) (Figure 1B). Brief-aggressive and prolonged-gentle MV did not significantly ($\chi^2, P = 0.4$, and $\chi^2, P = 0.56$, respectively) increase mortality, compared with sham control mice. We observed a 100% survival rate in the DNP-treated and vehicle-treated groups.

Initial body weights were comparable among all animal study groups (data not shown). At the end of the experiments (p15), mice subjected to aggressive MV of any duration demonstrated significantly ($P < 0.001$) lower body weights compared with their sham-control counterparts (Figure 1C). However, compared with brief-aggressive MV, prolonged-aggressive MV resulted in a significantly ($P = 0.005$) poorer rate of weight gain (0.27 ± 0.19 g/d versus 0.4 ± 0.1 g/d, respectively). At 2 weeks of life, animals subjected to prolonged MV with physiological Tv demonstrated body weights similar to those of corresponding sham-control mice. Mice subjected to DNP exhibited significantly ($P = 0.02$) lower body weights, compared with vehicle-treated littermates (Figure 1C).

MV Induces Lung Injury

Flow cytometry analysis revealed that MV did not change the amount of CD45-positive cells in the lungs, compared with that in sham littermates (data not shown). However, at 24 hours after aggressive MV of any duration, the amount of pulmonary lymphocyte antigen 6 complex, locus G/CD11b-positive (activated neutrophils) cells was significantly ($P = 0.019$) increased, compared with that in sham-control mice (Figure 1D). No difference was evident in the amount of activated granulocytes in the lungs after prolonged-gentle MV with physiological Tv, compared with sham-operated littermates.

At 24 hours after aggressive MV, the BAL protein concentration was significantly ($P < 0.001$) higher compared with that in shams (Figure 1E). Mice subjected to a prolonged-aggressive MV also demonstrated a significantly ($P < 0.001$) higher BAL protein concentration, compared with the gentle ventilation group (Figure 1E). Animals ventilated for 8 hours, using the gentle strategy, also exhibited a significantly ($P = 0.04$) higher BAL protein concentration compared with the control animals, but it was substantially lower compared with both aggressively ventilated groups.

MV Affects Pulmonary Alveolarization

At 2 weeks after MV, an analysis of lung histopathology revealed significantly poorer alveolarization only in mice exposed to prolonged-aggressive MV (Figure 2A). The alveolarization pattern in these p14 mice was markedly closer to that in p3 naive mice (Figure 2A). In contrast, all other groups exhibited well-advanced alveolarization compared with the p3 mice. A morphometric analysis revealed that aggressive MV significantly inhibited alveolar growth, compared with that in sham-control mice (Figures 2B and C). However, only prolonged-aggressive MV greatly and significantly delayed alveolar development, compared with the sham procedure, brief-aggressive MV, and prolonged-gentle MV (Figures 2A–2C). No difference was evident in alveolarization after brief-aggressive and prolonged-

gentle MV compared with the corresponding shams. Importantly, mice treated with DNP, the agent that decreases the generation of ATP, also demonstrated a robust ($P < 0.01$) alveolar developmental delay, compared with vehicle-treated littermates (Figures 2A–2C).

Mitochondrial Function Is Depressed by MV

Short-term changes. At 24 hours after MV, compared with control animals, pulmonary mitochondria from animals subjected to a large Tv ventilation (prolonged and brief) exhibited a severe inhibition of their respiration, supported by either C-I-dependent or C-II-dependent substrates. In these groups of ventilated mice, phosphorylating (State 3) respiration rates were significantly decreased compared with their sham-operated littermates (Figures 3A and 3C). However, mitochondria isolated from mice subjected to prolonged MV, using physiologic Tv, demonstrated State 3 respiration rates comparable to those of sham-control mice, and these respiration rates were significantly higher than those in mice after prolonged but aggressive MV (Figures 3A and 3C). This depression of mitochondrial phosphorylating activity in both aggressively ventilated groups was coupled with a significant inhibition of the enzymatic activities of Complexes I and II (Figures 3A and 3B). However, when the same pulmonary mitochondria were supported by a C-IV-dependent substrate, phosphorylating respiration rates were similar between experimental animal groups and corresponding sham-control mice (Figure 3G). As predicted by the analysis of mitochondrial respiration, the rate of mitochondrial ATP production in both groups of aggressively ventilated animals was significantly ($P < 0.05$) decreased, compared with that in sham-operated animals (Figure 4C). The mitochondrial respiratory control ratio (RCR) of a nicotinamide adenine dinucleotide (NAD^+)-linked substrate was significantly ($P = 0.03$) decreased in mice subjected to aggressive MV, compared with that in control mice (Figure 3B). However, the mitochondrial RCR in mice subjected to gentle MV was similar to that of their control counterparts, and was significantly higher than in animals after aggressive MV of the same duration (Figure 3B). No changes were evident in resting respiration rates (State 4, data not shown) between experimental and control animal groups.

Long-term changes. At 10 days after aggressive MV, compared with control animals, mice exhibited almost a full recovery of C-I-dependent and C-II-dependent mitochondrial adenosine diphosphate-phosphorylating respiration rates (Figures 3A–3F). At this age, these groups of animals also demonstrated a normalization of their C-I and C-II activities, compared with control littermates (Figures 4A and 4B). However, mice subjected to prolonged-aggressive MV continued to exhibit significantly decreased mitochondrial C-I-dependent and C-II-dependent State 3 respiration rates, and a significantly depressed activity of C-I and C-II complexes compared with that in the mice subjected only to brief-aggressive MV. At 10 days after prolonged MV, animals ventilated using physiological Tv demonstrated pulmonary mitochondrial phosphorylation rates supported by both substrates, comparable to those in corresponding sham control mice, and these rates were significantly higher compared with those of littermates subjected to prolonged MV with large Tv (Figures 3A and 3B). Similar to the results obtained at 24 hours after MV, at 10 days after the initial MV stress, pulmonary mitochondrial respiration rates supported by a C-IV-dependent substrate showed no difference among all experimental and control groups (Figures 3G). Consistent with mitochondrial phosphorylating dysfunction, the ATP production rate was significantly ($P < 0.04$) decreased in 2-week-old animals after aggressive MV during neonatal age compared

Figure 2. (A) Representative images of hematoxylin-and-eosin-stained lung sections with corresponding radial alveolar count (B) and mean linear intercept (C) analyses at 2 weeks of life. Scale bar = 500 μ m. Open bars, 8-hour sham-operated control mice; checkered bars, 2-hour sham-operated control mice for brief MV; solid bars, mice subjected to prolonged-aggressive MV; shaded bars, mice subjected to brief-aggressive MV; hatched bars, mice subjected to prolonged-gentle MV; lattice bars, vehicle treated mice; brick bars, 2,4-dinitrophenol (DNP)-treated mice. Data represent means $\pm$ SEMs. Two-way ANOVA, * P < 0.03 and ** P < 0.001, between ventilated and sham-operated groups, and $^{\$}P$ < 0.02, compared with the brief-aggressive MV group. One-way ANOVA, $^{\#}P$ < 0.05 compared with the prolonged-gentle MV group, and $^{\beta}P$ < 0.01, compared with vehicle-treated mice. Tv, tidal volume.

with sham-control mice, because of a persistent, significantly ($P = 0.01$) reduced rate of ATP production in animals subjected to prolonged and aggressive MV (Figure 4C). At this time point, no difference was found in rates of pulmonary mitochondrial ATP production between sham-operated control mice and mice subjected to brief-aggressive MV.

As expected, the exposure of lung mitochondria to DNP resulted in a significant reduction in ATP production rate compared with vehicle-treated animals (553 ± 22 nmol/min/mg protein versus 295 ± 38 nmol/min/mg protein, respectively; $P < 0.001$).

MV Affects Pulmonary Vascular Endothelial Growth Factor Expression

At 2 weeks of age, MV with large Tv during the neonatal period was associated with a significantly ($P = 0.028$) decreased pulmonary expression of vascular endothelial growth factor (VEGF)-A compared with shams (Figure 4D). Furthermore, mice subjected to prolonged-aggressive ventilation exhibited a significantly lower expression of VEGF-A in their lungs, compared not only with their control mice but also with mice subjected to brief-aggressive MV. The expression of pulmonary VEGF-A after prolonged-gentle MV was similar to that in control littermates. Mouse pups exposed to DNP also exhibited a significantly ($P < 0.001$) decreased level of pulmonary expression for VEGF-A protein, compared with their control counterparts (Figure 4D).

DISCUSSION

This study is the first to demonstrate that in neonatal mice, prolonged normoxic MV with large tidal volumes results in persistent mitochondrial dysfunction. Mitochondrial dysfunction is defined as a significant inhibition of mitochondrial phosphorylating respiration, leading to a decreased rate of ATP production. Importantly, this persistent mitochondrial dysfunction was closely associated with alveolar developmental delay, the hallmark of BPD in premature neonates. Neither brief ventilation with a large tidal volume nor prolonged ventilation with a physiological tidal volume caused persistent mitochondrial depression, and alveolar development was preserved. These data strongly suggest that, mechanistically, developmental alveolarization failure is caused by insufficiency of cellular bioenergetics to support normal growth and development. If this hypothesis is correct, then the experimental alteration of mitochondrial ATP generation should also result in alveolar developmental delay. Indeed, when the generation of ATP was reduced by the uncoupling of oxidative phosphorylation, mice exhibited severe under-alveolarization (i.e., the pulmonary phenotype identical to that in their ventilated counterparts). This strongly suggests that a failure of bioenergetics may represent a fundamental mechanism for alveolar underdevelopment in the developing subject. The fundamental nature of the proposed mechanism implies that diverse pulmonary stresses execute their detrimental effects via this mechanism. Earlier, we reported that prolonged hyperoxia, without MV-induced stress, also leads to mitochondrial

Figure 3. Pulmonary mitochondrial oxidative phosphorylation respiration rates (State 3) were supported by C-I-dependent (A) and C-II-dependent (C) substrates, with corresponding respiratory control ratios (RCR) (B and D) after 24 hours and 10 days of recovery after MV or sham procedure. Graphs represent recordings of mitochondrial respiration, using C-I-dependent (E) and C-II-dependent (F) substrates at two different time points of the experiment. Time points when mitochondria (mito), adenosine diphosphate (ADP), and DNP were added are indicated. (G) Lung mitochondrial rate of oxidative phosphorylation initiated by C-IV-dependent substrate, after the two points of recovery already mentioned. All data represent means $\pm$ SEMs. *Open bars*, 8-hour sham-operated control mice; *checkered bars*, 2-hour sham-operated control mice for brief MV; *solid bars*, mice subjected to prolonged-aggressive MV; *shaded bars*, mice subjected to brief-aggressive MV; *hatched bars*, mice subjected to prolonged-gentle MV. Two-way ANOVA, * $P < 0.03$, between ventilated and sham-operated groups, and $^{\S}P < 0.03$, compared with the brief-aggressive MV group. One-way ANOVA, $^{\#}P \leq 0.05$, compared with the prolonged-gentle MV group, and $^{\Psi}P < 0.01$, compared with sham-operated littermates.

depression and significant underalveolarization (11). Importantly, in that work, alveolar developmental arrest was also fully replicated by a pharmacological inhibition of oxidative phosphorylation. Thus, despite the different means of effecting insults to the lungs (i.e., hyperoxia or ventilation), the developing tissue responds similarly, with an inhibition of mitochondrial respiratory rates and failing alveolarization. Taken together, our experiments indicate that, regardless of the nature of the initial pulmonary stress, the failure of bioenergetics during active pulmonary development is responsible for the BPD phenotype in immature mice.

A decreased intracellular level of ATP constitutes a well-recognized cause for poor cellular proliferation and decreased protein synthesis (19–21). It is well-established that the appropriate production of VEGF is critical for normal alveolar development (23). Bland and colleagues reported reduced lung VEGF gene expression immediately after 8 hours of MV with

40% O₂ in p4–6 mice (12). Our study extends this observation, and demonstrates that at 10 days of recovery from prolonged-aggressive MV, a significantly decreased ATP production rate was associated with the significantly decreased expression of VEGF protein in the lungs. Whether this is the result of a decreased energy supply for protein synthesis or a bioenergetics-independent event remains to be determined. Of note, however, protein synthesis consumes approximately two thirds of the total energy produced by rapidly growing cells (22). The depletion of ATP has also been shown to affect VEGF synthesis adversely in cultured pneumocytes (24).

The generation of ATP in mitochondria depends on several conditions: (1) the activity of the respiratory chain and the availability of substrates, (2) the function of the adenine-nucleotide transporter, (3) the availability of NAD and flavin adenine dinucleotide and of inorganic phosphorus, (4) the activity of ATP-synthase, and (5) the coupling of oxidative phosphorylation with

Figure 4. Activity of isolated pulmonary mitochondrial C-I (A) and C-II (B) at 24 hours and 10 days after MV. (C) ATP production rate by isolated pulmonary mitochondria at recovery for 24 hours and 10 days after MV. (D) Representative Western blot scan for the expression of lung vascular endothelial growth factor (VEGF)-A protein and corresponding semiquantitative analysis of this protein expression at age 2 weeks ($n = 4$ for all groups). All data represent means $\pm$ SEMs. OD = optical density. Open bars, 8-hour sham-operated control mice; checkered bars, 2-hour sham-operated control mice for brief MV; solid bars, mice subjected to prolonged-aggressive MV; shaded bars, mice subjected to brief-aggressive MV; hatched bars, mice subjected to prolonged-gentle MV; lattice bars, vehicle-treated mice; brick bars, DNP-treated mice. Two-way ANOVA, $*P < 0.05$, between ventilated and sham-operated groups, and $^{\S}P < 0.03$, compared with the brief-aggressive MV group. One-way ANOVA, $^{\#}P \leq 0.001$, compared with the prolonged-gentle MV group, $^{\Psi}P < 0.05$, compared with sham-operated littermates, and $^{\beta}P < 0.001$, compared with vehicle-treated mice.

the consumption of oxygen. In our experiments, mitochondria were tested in the presence of saturating concentrations of inorganic phosphorus and substrates. Because the MV-induced inhibition of phosphorylating (State 3) respiration was coupled with the inhibition of a maximal, uncoupled respiration (data not shown), our interpretation states that MV inhibits the respiratory chain. Indeed, the decreased enzymatic activities of C-I and C-II confirmed that the MV-induced depression of mitochondrial respiration was secondary to an inhibition in the proximal respiratory chain. Notably, testing the same mitochondria on a C-IV-dependent substrate revealed no functional alteration in the distal respiratory chain. Thus, our study suggests that MV primarily affects the proximal respiratory chain. Therefore, the functional preservation of C-I and C-II activities represents

a potential therapeutic strategy for the alleviation of mitochondrial dysfunction initiated by prolonged MV. Our work contains a limitation, insofar as we did not test the activity of Complex III (C-III). Theoretically, the inhibition of C-III may also contribute to the decreased ATP production in pulmonary mitochondria. However, the magnitude and relative contributions of different complexes to the failure of ATP production after MV-induced stress deserve a separate study.

Our data show that if the MV stress is limited by the duration (2 h) or the severity of lung-stretching (gentler Tv), then no persistent mitochondrial dysfunction is found, and alveolar development is preserved. This implies that the severity of MV stress defines the duration of mitochondrial dysfunction, and if mitochondrial dysfunction coincides with

a period of active pulmonary development, then alveolar growth is affected.

The present study is also the first to report data on a long-term pulmonary outcome of MV in neonatal mice. Earlier, Bland and colleagues showed only an immediate response of the lungs to ventilation-induced stress in neonatal mice (12). Therefore, a potentially deleterious long-term outcome of MV was inferred from acute changes in the expression of the proteins (VEGF, transforming growth factor- β , and tropoelastin) known to be important for lung development (12, 16). Because we used a survival model of MV-induced lung injury, this work demonstrates that in neonatal mice, 8 hours of normoxemic MV with a large T_v result in significantly delayed pulmonary alveolarization, detected at 2 weeks of life. Similar results were obtained using VILI models in neonatal lambs and nonhuman primates (10, 17, 18). In those studies, however, animals were exposed to additional potential pulmonary stressors, including hyperoxia, parenteral nutrition, prolonged sedation and muscle paralysis, the surgical ligation of patent ductus arteriosus, and the administration of systemic antibiotics. Furthermore, full-term animals, free of all these interventions, were used as controls. This experimental design complicates the identification of the primary mechanism responsible for the long-term effects of ventilation on lung development. Because all sham-control animals were treated identically to their experimental littermates in our study, except for the exposure to MV, we conclude that in neonatal mice, 8 hours of aggressive MV cause delays in alveolar development.

MV causes global stress. Clinical studies have shown a strong correlation between the duration of MV and the development of BPD, poor weight gain, and increased mortality (19, 25, 26). In our study, mouse pups subjected to prolonged MV exhibited significantly greater mortality and poorer weight gain, compared with pups ventilated briefly. Systemic inflammation has been suggested as one of the potential mechanisms responsible for the poor weight gain in human neonates and animals after prolonged MV (27, 28). Given that systemic inflammation is associated with mitochondrial dysfunction in the skeletal muscles and in circulating mononuclear cells (29), MV-induced pulmonary inflammation may be linked mechanistically to the mitochondrial dysfunction observed in our study. The release of various proinflammatory cytokines in response to repeated cyclic stretch-stress during MV is viewed as the mechanism of ventilation-induced lung injury (30, 31). Rowlands and colleagues have shown that an increased level of TNF- α in the lung was associated with persistent elevations of intramitochondrial Ca^{2+} concentrations (the event that results in the dissipation of the proton motive force for ATP generation), and with the generation of mitochondrial reactive oxygen species in the pulmonary endothelium (32). Our study also demonstrates a significantly increased population of activated granulocytes and other signs of pulmonary injury, as well as increased protein content in the BAL, in the lungs after aggressive and prolonged ventilation. Of note, the extent of granulocyte activation was comparable in all groups of ventilated mice. However, delayed alveolar development was detected only after prolonged and aggressive MV, and paralleled the extent of mitochondrial dysfunction. This suggests that that (1) mitochondrial depression is not directly caused by inflammation, and (2) the mechanisms of alveolar developmental arrest are not defined by the severity of the initial inflammatory response to the lung injury.

In conclusion, our work demonstrates that pulmonary mitochondrial ATP production is inhibited by prolonged and aggressive normoxic ventilation. This depression of oxidative phosphorylation persists throughout the period of active alveolarization, and eventuates in the delay of alveolar development in neonatal mice.

Thus, this study provides another set of evidence in support of the mechanistic role for the failure of mitochondrial bioenergetics in the development of BPD among premature infants.

Author disclosures are available with the text of this article at www.atsjournals.org.

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