Vascular Endothelial Mitochondrial Function Predicts Death or Pulmonary Outcomes in Preterm Infants

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Abstract

Rationale: Vascular endothelial mitochondrial dysfunction contributes to the pathogenesis of several oxidant stress-associated disorders. Oxidant stress is a major contributor to the pathogenesis of bronchopulmonary dysplasia (BPD), a chronic lung disease of prematurity that often leads to sequelae in adult survivors.

Objectives: This study was conducted to identify whether differences in mitochondrial bioenergetic function and oxidant generation in human umbilical vein endothelial cells (HUVECs) obtained from extremely preterm infants were associated with risk for BPD or death before 36 weeks postmenstrual age.

Methods: HUVEC oxygen consumption and superoxide and hydrogen peroxide generation were measured in 69 infants.

Measurements and Main Results: Compared with HUVECs from infants who survived without BPD, HUVECs obtained from infants who developed BPD or died had a lower

maximal oxygen consumption rate (mean $\pm$ SEM, 107 ± 8 vs. 235 ± 22 pmol/min/30,000 cells; $P < 0.001$), produced more superoxide after exposure to hyperoxia (mean $\pm$ SEM, $89,807 \pm 16,616$ vs. $162,706 \pm 25,321$ MitoSOX Red fluorescence units; $P < 0.05$), and released more hydrogen peroxide into the supernatant after hyperoxia exposure (mean $\pm$ SEM, $1,879 \pm 278$ vs. 842 ± 119 resorufin arbitrary fluorescence units; $P < 0.001$).

Conclusions: Our results indicating that endothelial cells of premature infants who later develop BPD or die have impaired mitochondrial bioenergetic capacity and produce more oxidants at birth suggest that the vascular endothelial mitochondrial dysfunction seen at birth in these infants persists through their postnatal life and contributes to adverse pulmonary outcomes and increased early mortality.

Keywords: energy metabolism; bronchopulmonary dysplasia; endothelium

Preterm birth has emerged as the most important cause of worldwide childhood mortality (1). Bronchopulmonary dysplasia (BPD), characterized by impaired alveolar and lung vascular development, affects 25 to 50% of extremely low birth weight (birth weight [BW], <1 kg) premature infants (2). Abnormalities in lung function seen in such infants persist into childhood and are second only to asthma in terms of

childhood respiratory disease healthcare costs (3, 4). Preterm birth exposes lungs that normally develop in a hypoxic intrauterine environment to a relatively hyperoxic environment, leading to increased reactive oxygen species (ROS) generation and to oxidant stress, which is an important pathogenetic factor for BPD. Biomarkers of oxidant stress, such as serum malondialdehyde, and polymorphisms

involving antioxidant response genes correlate strongly with abnormal lung function in preterm infants (5–7). Mitochondria have been recognized as an important ROS source and as a key intracellular buffer that protects against such oxidant stress (8). Hyperoxia is known to inhibit pulmonary mitochondrial bioenergetic function and impair alveolar development in animal models of lung

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At a Glance Commentary

Scientific Knowledge on the

Subject: Endothelial cell bioenergetics and mitochondrial oxidant production are known to be involved in the pathogenesis of disorders such as diabetes and hypertension. Their role in the pathogenesis of developmental lung disorders such as bronchopulmonary dysplasia is currently unknown.

What This Study Adds to the

Field: This study provides the first evidence obtained from human-derived vascular endothelial cells suggesting that mitochondrial dysfunction in these cells is a strong predictor for risk of poor pulmonary outcomes in infants who are born prematurely. Further investigations in this novel area could lead to development of therapeutics that could be used specifically for preterm infants at high risk for mitochondrial dysfunction-associated lung disease.

injury (9, 10). Mitochondrial DNA (mtDNA) is exquisitely sensitive to oxidant injury, and hyperoxia-induced mtDNA damage has been shown to inhibit branching morphogenesis in rat lung explants, leading to abnormal alveolar maturation (11, 12).

Abnormal lung vascular development is also a characteristic pathophysiologic feature of BPD (13), and higher concentrations of endostatin (an angiogenesis inhibitor) have been found in tracheal aspirates of infants with BPD (14, 15). Animal studies show that hyperoxia decreases endothelial mitochondrial antioxidant defenses and disrupts pulmonary endothelial tight junctions, leading to arrested pulmonary development (16–20). Furthermore, hyperoxia-induced pulmonary capillary endothelial ROS generation increase is blocked by inhibition of the mitochondrial electron transport chain (ETC), suggesting that endothelial mitochondrial reactive oxygen species (mtROS) generated by hyperoxia contribute to lung injury (21).

Taken together, this evidence suggests that vascular endothelial mitochondrial bioenergetic dysfunction, disordered ROS

generation, and mtDNA damage may disrupt lung development in prematurely born infants. Human umbilical vein endothelial cells (HUVECs) have been used as model systems to study endothelial function in diseases such as hypertension (22). Therefore, in this study, we hypothesized that impaired mitochondrial bioenergetics, increased ROS generation, and greater severity of mtDNA damage in HUVECs obtained from preterm infants at their birth would be associated with higher risk of BPD or death before 36 weeks postmenstrual age (PMA).

Methods

Study Design and HUVEC Isolation

This prospective cohort study was approved by the University of Alabama at Birmingham Institutional Review Board. Informed consent was obtained from pregnant women at less than 32 weeks of gestation. Infants with congenital anomalies were excluded. BPD or death before 36 weeks PMA was used as the primary outcome. HUVECs were isolated from umbilical cords of enrolled infants and grown in culture. HUVEC growth rates were determined to ensure study feasibility. Passage-matched HUVECs from each infant were used for these experiments to ensure comparability between infants who died or developed BPD (BPD susceptible) and infants who survived without BPD (BPD resistant).

Bioenergetics

Second-passage HUVECs were used to measure intact cell bioenergetics using a Seahorse XFe/XF24 extracellular flux analyzer (Agilent Technologies, Santa Clara, CA). Briefly, basal oxygen consumption rate (OCR) and extracellular acidification rate were measured, followed by sequential measurements after addition of oligomycin to block ATP synthase (complex V of the ETC), carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) to stimulate mitochondrial oxygen consumption to maximal levels, and antimycin A/rotenone to inhibit substrate-derived electron transfer through ETC complexes III and I.

ROS Generation

HUVECs in their fourth passage were used. Fluorescence probes were purchased from

Thermo Fisher Scientific (Waltham, MA). HUVEC mitochondrial superoxide ion ($O_2^{\cdot -}$) measurements were obtained in normoxia and after hyperoxia exposure using MitoSOX Red fluorescence (Thermo Fisher Scientific). Extracellular hydrogen peroxide (H_2O_2) release was measured in supernatants of hyperoxia-exposed HUVEC cultures using Amplex Red reagent (ARR; Thermo Fisher Scientific) and horseradish peroxidase; catalase inhibition of ARR fluorescence was used to confirm H_2O_2 generation. ROS fluorescence values were normalized with sulforhodamine B protein absorbance. Hyperoxia-exposed HUVECs from 16 infants were labeled with dihydrorhodamine 123 (DHR123) and MitoTracker Red (Thermo Fisher Scientific) to obtain live-cell images of global mtROS generation.

mtDNA Damage and Copy Number

Following exposure to hyperoxia, genomic DNA (gDNA) from fourth-passage HUVECs from 16 infants was extracted using a Genomic-tip and Genomic DNA Buffer set (QIAGEN, Valencia, CA) and quantified using Quant-iT PicoGreen fluorescence (Thermo Fisher Scientific). Long and short polymerase chain reactions for mtDNA and a short polymerase chain reaction for nuclear DNA were performed using similar quantities of gDNA from HUVECs of each infant to obtain relative mtDNA lesion frequency using term infant samples as controls. Relative mtDNA copy number per diploid nuclear genome was calculated to examine mitochondrial number differences between HUVECs from BPD-resistant infants and BPD-susceptible infants.

Statistical Analysis

Fisher's exact test was used for between-group comparisons of categorical variables, and Student's *t* test, the Mann-Whitney *U* test, and analysis of variance were used for continuous variables. Logistic regression models and a classification tree to predict BPD or death were developed using R software packages (R Foundation for Statistical Computing, Vienna, Austria) and a set of 11 independent variables. More details regarding all the experimental methods described above are provided in the online supplement.

Results

Clinical Characteristics Associated with BPD or Death

Of the 69 infants in the study, 35 (51%) developed BPD or died, 24 of whom survived with BPD and 11 of whom died (median age, 3 d) before their BPD status could be assessed at 36 weeks PMA. As would be expected for a disease of prematurity, gestational age (GA) and BW were lower for BPD-susceptible infants than for BPD-resistant survivors; infants who died had the lowest GA and BW compared with the infants who survived with BPD and infants who survived without BPD (mean $\pm$ SD GA, 23 ± 1 vs. 26 ± 2 vs. 27 ± 2 wk, respectively; mean $\pm$ SD BW, 443 ± 187 vs. 770 ± 276 vs. $1,057 \pm 412$ g, respectively; $P < 0.05$ for both comparisons). Clinical differences noted between BPD-resistant and BPD-susceptible infants are shown in Table 1. The online supplement contains additional comparisons of study variables between infants who survived without BPD, those who survived with BPD, and those who died before they could be assessed for BPD (see Table E1 in the online supplement).

Decreased HUVEC Oxygen

Consumption in BPD-Susceptible Infants

HUVEC basal mitochondrial OCR was lower for BPD-susceptible infants than for BPD-resistant infants ($n = 35$ vs. $n = 34$;

mean $\pm$ SEM, 66 ± 6 vs. 121 ± 11 pmol/min/ 3×10^4 cells; $P < 0.001$). Similarly, maximal mitochondrial HUVEC oxygen consumption, measured after the addition of FCCP, was lower in BPD-susceptible infants than in BPD-resistant infants (mean $\pm$ SEM, 107 ± 8 vs. 235 ± 22 pmol/min/ 3×10^4 cells; $P < 0.001$), as was reserve mitochondrial capacity for oxygen consumption (mean $\pm$ SEM, 40 ± 7 vs. 114 ± 14 pmol/min/ 3×10^4 cells; $P < 0.001$), indicating decreased HUVEC mitochondrial bioenergetic function (Figure 1). HUVEC response to inhibited mitochondrial respiration (increased anaerobic glycolysis) was measured as extracellular acidification rate, but no differences were noted between HUVECs from BPD-susceptible infants and BPD-resistant infants, either at baseline or after mitochondrial effector injections (mean $\pm$ SEM basal, 17 ± 2 vs. 18 ± 4 milli-pH [mPH]/min/ 3×10^4 cells; oligomycin, 25 ± 2 vs. 24 ± 3 mPH/min/ 3×10^4 cells; FCCP, 25 ± 3 vs. 22 ± 3 mPH/min/ 3×10^4 cells; and antimycin A/rotenone, 25 ± 3 vs. 22 ± 3 mPH/min/ 3×10^4 cells; $P > 0.05$ for all four comparisons).

Increased HUVEC Proton Leak in BPD-Susceptible Infants

Baseline intact cellular oxygen uptake (Figure E2B) is consumed by mitochondrial ATP synthase to generate ATP (ATP-linked

OCR) in cycling of protons across a “leaky” inner mitochondrial membrane (proton leak) and for nonmitochondrial oxidative processes (e.g., nicotinamide adenine dinucleotide phosphate oxidases). ATP-linked OCR was lower for HUVECs from BPD-susceptible infants than for BPD-resistant infants ($n = 35$ vs. $n = 34$, respectively; mean $\pm$ SEM, 44 ± 4 vs. 89 ± 9 pmol/min/ 3×10^4 cells; $P < 0.001$). HUVECs obtained from BPD-susceptible infants and BPD-resistant infants did not differ in the percentages of baseline cellular oxygen uptake used for ATP synthesis (mean, 47 vs. 51%; $P > 0.05$) or for nonmitochondrial consumption (mean, 27 vs. 30%; $P > 0.05$). However, HUVECs from BPD-susceptible infants had a higher proportion of their baseline oxygen uptake consumed by proton leak than those obtained from BPD-resistant infants (mean, 26 vs. 19%; $P < 0.05$), suggesting increased uncoupling of mitochondrial ATP generation from oxygen consumption (Figure 2).

Increased HUVEC mtROS Generation in BPD-Susceptible Infants

In a normoxic environment, HUVEC mitochondria from BPD-susceptible infants generated more O_2^- than those from BPD-resistant infants ($n = 35$ vs. $n = 34$; mean $\pm$ SEM, $130,250 \pm 19,062$ vs. $73,526 \pm 12,031$ MitoSOX fluorescence units; $P < 0.05$).

Table 1. Clinical Characteristics of Enrolled Infants

Variable	Overall	Survival without BPD	BPD and/or Death	P Value*
n	69	34	35	
Birth weight, g, mean $\pm$ SD	860 $\pm$ 375	1,058 $\pm$ 349	667 $\pm$ 292	<0.001*
Gestational age, wk, mean $\pm$ SD	26 $\pm$ 3	28 $\pm$ 2	25 $\pm$ 2	<0.001*
White race, n (%)	32 (46)	16 (47)	16 (45)	0.91
Male sex, n (%)	36 (52)	18 (53)	18 (51)	1
Antenatal steroid use, n (%)	62 (90)	33 (97)	29 (83)	0.13
Histologic chorioamnionitis, n (%)	29 (42)	12 (35)	17 (49)	0.38
Maternal antibiotic use, n (%)	31 (45)	13 (38)	18 (51)	0.39
5-min Apgar score, median (IQR)	7 (4–8)	7 (6–8)	6 (4–7)	0.01*
Intubated at delivery, n (%)	31 (45)	10 (29)	21 (60)	0.04*
Early use of antibiotics <72 h, n (%)	59 (86)	26 (77)	33 (94)	0.03*
Late-onset sepsis, n (%)	14 (21)	2 (6)	12 (35)	0.01*
F _{IO₂} at 36 wk PMA, mean $\pm$ SD	0.3 $\pm$ 0.2	0.21 $\pm$ 0	0.4 $\pm$ 0.3	<0.001*
Oxygen supplementation duration by 36 wk PMA, d, mean $\pm$ SD	29 $\pm$ 28	14 $\pm$ 16	49 $\pm$ 30	<0.001*
Invasive ventilation duration by 36 wk PMA, d, mean $\pm$ SD	8 $\pm$ 19	2 $\pm$ 4	16 $\pm$ 27	0.003*

Definition of abbreviations: BPD = bronchopulmonary dysplasia; IQR = interquartile range; PMA = premenstrual age.

* $P < 0.05$. P values were calculated using Fisher's exact test for categorical variables, the Mann-Whitney U test for nonparametric continuous variables, and Student's t test for parametric continuous variables.

Figure 1. Oxygen consumption measured on the basis of extracellular flux. Absolute values of different oxygen consumption rates (OCRs) that make up total human umbilical vein endothelial cell oxygen consumption of infants who survived without bronchopulmonary dysplasia (BPD; the “no BPD” group) compared with infants who died or developed BPD (the “BPD or died” group) are shown. Bars indicate means; error bars represent SEM. Student's *t* test was used to compare groups. **P* < 0.001.

This increase in $O_2^{\cdot -}$ generation persisted after exposure to hyperoxia (mean $\pm$ SEM, $162,706 \pm 25,321$ vs. $89,807 \pm 16,616$ MitoSOX fluorescence units; *P* < 0.05). Because superoxide dismutase rapidly dismutates $O_2^{\cdot -}$ to H_2O_2 that diffuses across membranes, H_2O_2 content in supernatants of HUVECs exposed to hyperoxia was measured. We found that HUVECs obtained from BPD-susceptible infants released more H_2O_2 into their supernatants than those obtained from BPD-resistant infants (mean $\pm$ SEM, $1,879 \pm 278$ vs. 842 ± 119 resorufin fluorescence units; *P* < 0.005). Average (mean $\pm$ SEM) catalase inhibition of resorufin fluorescence was $88 \pm 7\%$ for the BPD-susceptible group and $76 \pm 5\%$ for the BPD-resistant group, confirming that H_2O_2 contributed significantly to ARR oxidation to resorufin in these experiments (Figure 3).

Fluorescence quantification in images of DHR123-labeled HUVECs from eight infants in each group indicated increased global ROS generation in HUVECs derived from BPD-susceptible infants compared with those from BPD-resistant infants (Figure 4). Finally, colocalization of corresponding DHR123- and MitoTracker Red-labeled HUVEC images showed increased rhodamine fluorescence in mitochondria of HUVECs from BPD-susceptible infants, providing more evidence to suggest that these infants have increased endothelial mtROS generation than BPD-resistant infants (see representative images in Figure E3).

Increased HUVEC mtDNA Damage in BPD-Susceptible Infants

Because the findings of decreased mitochondrial bioenergetics and increased

ROS generation suggested increased oxidant load in HUVECs from BPD-susceptible infants, we next measured mtDNA lesion frequency. HUVEC mtDNA damage (Figure 5) was higher in BPD-susceptible infants than in BPD-resistant infants (*n* = 8 for each group; mean $\pm$ SEM, 2.5 ± 0.7 vs. 0.4 ± 0.2 DNA lesions per kb; *P* < 0.05). We also observed that mtDNA copy number normalized to gDNA was similar between the two groups (mean $\pm$ SEM, 2.8 ± 0.2 vs. 2.5 ± 0.3 relative mtDNA copy number; *P* > 0.05). This indicates similar mitochondrial content in HUVECs obtained from BPD-susceptible infants compared with BPD-resistant infants, but more precise estimates using other mitochondrial markers such as citrate synthase activity in a larger sample size are required to accurately determine mitochondrial content in these cells.

Figure 2. Oxygen consumption rate (OCR) components as percentage of baseline cellular OCR. Human umbilical vein endothelial cell oxygen use of infants who survived without bronchopulmonary dysplasia (BPD; the “no BPD” group) compared with that of infants who died or developed BPD is shown. Baseline cellular oxygen consumption is divided into ATP-linked OCR and non-ATP OCR, which is made up of nonmitochondrial and proton leak components. Bars indicate means; error bars represent SEM. Student's *t* test was used to compare groups. **P* < 0.05.

Figure 3. Reactive oxygen species generation. Human umbilical vein endothelial cell (HUVEC) mitochondrial superoxide ion ($O_2^{\cdot-}$) generation (MitoSOX Red fluorescence) and hydrogen peroxide (H_2O_2) release (resorufin fluorescence) compared between infants who survived without bronchopulmonary dysplasia (BPD; the “no BPD” group) and those who died or developed BPD. All fluorescence values are normalized to sulforhodamine B protein absorbance. (A) $O_2^{\cdot-}$ generation at baseline (normoxia). (B) $O_2^{\cdot-}$ generation after HUVEC exposure to hyperoxia. (C) H_2O_2 in supernatants of HUVECs exposed to hyperoxia. Catalase addition, which breaks down H_2O_2 , strongly inhibited supernatant resorufin fluorescence signaling in both groups (light gray bars). Bars represent means; error bars represent SEM. Student's *t* test was used to compare groups. **P* < 0.05. HRP = horseradish peroxidase.

Clinical Factors Associated with HUVEC Biological Differences

Oxygen supplementation requirement indicates magnitude of lung disease in early postnatal life. Duration of oxygen supplementation for infants during their first week correlated negatively with their basal and maximal HUVEC oxygen consumption ($n = 69$; $r = -0.58$ and $r = -0.70$, respectively; $P < 0.001$ for both comparisons). Neither GA ($r = 0.20$ and $r = 0.24$, respectively) nor BW ($r = 0.15$ and $r = 0.16$, respectively) correlated well with HUVEC mitochondrial basal or maximal OCR ($P > 0.05$ for all comparisons). GA also correlated poorly with normoxic

HUVEC mitochondrial $O_2^{\cdot-}$ generation ($r = -0.16$), hyperoxia-induced mitochondrial $O_2^{\cdot-}$ generation ($r = -0.20$), and hyperoxia-induced H_2O_2 release ($r = -0.18$) ($P > 0.05$ for all comparisons). Thus, physiologic immaturity alone did not account for the mitochondrial bioenergetic and redox dysfunction noted in the smaller and younger (lower GA) BPD-susceptible infants.

Several clinical variables were examined for their effects on HUVEC bioenergetic and redox function. HUVECs from infants exposed to maternal hypertension or preeclampsia had similar basal and maximal mitochondrial OCR

compared with those from infants without such exposure ($n = 31$ and $n = 38$; mean $\pm$ SEM, 139 ± 11 vs. 115 ± 12 and 195 ± 20 vs. 150 ± 19 pmol/min/ 3×10^4 cells, respectively; $P > 0.05$ for both comparisons). However, HUVECs from infants exposed to chorioamnionitis (infection and inflammation of the fetal membranes and placental layers) had lower basal and maximal mitochondrial OCR than those from infants without such exposure ($n = 29$ and $n = 40$; mean $\pm$ SEM, 68 ± 9 vs. 112 ± 9 and 113 ± 15 vs. 212 ± 19 pmol/min/ 3×10^4 cells, respectively; $P < 0.001$ for both comparisons), and they had higher H_2O_2 content in their supernatants (mean $\pm$ SEM, $2,015 \pm 306$ vs. 900 ± 139 resorufin fluorescence units, respectively; $P < 0.001$). HUVEC oxygen consumption also varied on the basis of race. African American (AA) infants had lower basal and maximal oxygen consumption than white infants ($n = 32$ per group; mean $\pm$ SEM, 75 ± 14 vs. 115 ± 10 and 113 ± 16 vs. 212 ± 19 pmol/min/ 3×10^4 cells, respectively; $P < 0.05$ for both comparisons) and higher H_2O_2 content in their supernatants (mean $\pm$ SEM, $1,892 \pm 299$ vs. 945 ± 145 resorufin fluorescence units, respectively; $P < 0.001$), as shown in Figure 6.

HUVEC Maximal OCR Identified as a Significant Death/BPD Predictor

In addition to individual pairwise comparisons, we developed logistic regression models for the outcome of death or BPD using 11 predictor variables in a training cohort of 35 infants randomly selected from among the 69 infants in our study. Analysis of the relative importance of all 11 variables averaged across all possible logistic regression models to predict death or BPD revealed maximal OCR to be a highly significant predictor (Table 2). Maximal HUVEC OCR and hyperoxia-induced HUVEC H_2O_2 release (Table 3) were also shown to be the most significant predictors for death or BPD in the final model. The McFadden pseudo R^2 score, a measure of logistic regression goodness of fit, was found to be 0.78 for this model. When applied to the testing cohort of 34 infants, its accuracy in predicting death or BPD status was 89% (95% confidence interval, 84–94%; sensitivity, 90%; specificity, 94%). Robustness was estimated

Figure 4. Live cell imaging of global human umbilical vein endothelial cell (HUVEC) mitochondrial reactive oxygen species generation. (A) Representative images, obtained using a confocal laser-scanning microscope, of dihydrorhodamine 123 (DHR123)-labeled HUVECs that show the typical fluorescence intensity and pattern seen after hyperoxia exposure. Images are of cells obtained from three infants in each group (infants who survived without bronchopulmonary dysplasia [BPD; the “no BPD” group] in the upper panels; BPD group in the lower panels) are shown. (B) HUVEC DHR123 fluorescence quantified in arbitrary units (a.u.) from all images obtained for infants with and without BPD. Bars represent means; error bars represent SEM. Student’s *t* test was used to compare groups. ****P* < 0.005.

using the leave-one-out cross-validation method and showed that the model had a 13% error rate in predicting death or BPD status for the entire cohort. Receiver operating characteristic curve analysis revealed an area under the curve of 0.89 (Figure 7A). In addition, we also derived a classification tree model for BPD or death using a classification and regression tree algorithm using all 11 variables as predictors. Maximal HUVEC oxygen

consumption less than 200 pmol/min/3 × 10⁴ cells, GA less than 28 weeks, and BW less than 850 g were found to be highly predictive of death before discharge or of BPD for prematurely born infants (Figure 7B).

HUVEC Growth Rates and mtDNA Copy Number

To determine if HUVEC growth rate differences due to GA or BPD susceptibility affected our experimental results, we tested for possible associations between these factors. We observed poor correlation for HUVEC proliferation and apoptosis rates with GA from 23 to 32 weeks (*n* = 35; *r* = 0.20; *P* > 0.05; and *n* = 26; *r* = −0.17; *P* > 0.05, respectively) (Figure E1). In addition, growth rates of HUVECs between BPD-susceptible infants and BPD-resistant infants (quantified as gDNA content obtained from HUVEC cultures that were grown using similar cell-seeding densities for 1 wk in similar conditions) also did not show significant differences (*n* = 8 in each group; mean ± SEM, 1.5 ± 0.1 vs. 1.3 ± 0.1 ng/μL, respectively; *P* > 0.05). HUVEC bioenergetics, ROS generation, and mtDNA damage comparisons between discrete outcomes (infants who survived without BPD vs. those who survived with BPD and those who died before they could be assessed for BPD) are available in the online supplement (Table E1).

Discussion

Our objective in the present study was to determine whether impaired vascular endothelial mitochondrial function is a predictor of risk for early death or BPD. Compared with HUVECs from BPD-resistant infants, HUVECs from infants who died or developed chronic lung disease had lower basal and maximal mitochondrial OCR and a lower reserve capacity for oxidative phosphorylation. Compared with HUVECs from BPD-resistant survivors, HUVECs from infants who died or developed chronic lung disease also produced more ROS, including mitochondrial-derived O₂^{•−}; released more H₂O₂ into their environment; and had increased mtDNA damage with hyperoxia exposure. Regression analysis identified maximal HUVEC OCR as the most significant variable associated with BPD or death. In addition to decreased absolute oxygen consumption (indicating poor mitochondrial health), these HUVECs also had a higher proportion of their baseline OCR lost to proton leak, implying that their mitochondria had increased futile oxygen consumption that was uncoupled from ATP generation (23).

Endothelial ATP depletion can induce premature cellular aging and limit endothelial cell survival during hyperoxia (24). Pulmonary angiogenesis is a key component of alveolar development.

Figure 5. Mitochondrial DNA (mtDNA) damage. Relative human umbilical vein endothelial cell mtDNA damage after hyperoxia exposure is normalized to mitochondrial copy number and represented as lesions per 10-kb DNA fragment size. Bars represent means; error bars represent SEM. Student’s *t* test was used to compare groups. **P* < 0.05. BDP = bronchopulmonary dysplasia.

Figure 6. Bioenergetics and hydrogen peroxide release versus clinical differences. (A) Basal and maximal oxygen consumption rates (OCRs) of human umbilical vein endothelial cells (HUVECs) from infants exposed to chorioamnionitis versus infants who were not. $*P < 0.001$. (B) Basal and maximal OCRs of HUVECs from white and African American infants. $*P < 0.05$. (C) Hydrogen peroxide content in the supernatants of HUVECs exposed to hyperoxia. $*P < 0.001$. Grouped bars represent means; error bars represent SEM. Student's *t* test was used to compare groups.

Stimulation of vascularization with proangiogenic factors such as fibroblast growth factor 2 and angiopoietin 1 augments pulmonary alveolar growth, and

endothelial–epithelial interactions are necessary for normal morphogenesis of branching distal airways, especially in the saccular stage of lung development, during

which most premature infants who will develop BPD are born (25–27). Endothelial bioenergetic dysfunction could thus lead to dysfunctional interaction between a depleted vascular endothelium and the developing alveolar epithelium as well as to functionally inadequate lung architecture. Hyperoxic endothelial bioenergetic failure can also lead to pulmonary edema and inflammation, as observed in the newborn rat lung (28). Therefore, increased uncoupling and decreased HUVEC mitochondrial function seen in BPD-susceptible infants indicate that similar bioenergetic dysfunction in the pulmonary vascular endothelium could accelerate pulmonary endothelial senescence, decrease alveolarization, and increase inflammation in the developing lung.

Another important mitochondrial function is generation of mtROS at low levels for signaling and cell function regulation. H_2O_2 , a membrane diffusible ROS, is involved in several elements of vascular endothelial function, such as modulation of hypoxia-inducible factor-1 α activity and endothelial proliferation (29–31). However, mitochondrial uncoupling, which was observed in BPD-susceptible HUVECs, often generates supraphysiologic amounts of mtROS that decrease endothelial cell viability and diffuse into surrounding cells to cause vascular smooth muscle hypertrophy or initiate apoptotic cascades to inhibit alveolar epithelial wound repair (32–34). mtROS also amplify inflammatory responses in pulmonary venular capillaries exposed to hyperoxia (35). Our finding of

Table 2. Average Predictor Variable Performance

Variable	Estimate	Unconditional Variance	Number of Models Included	Importance
Maternal antibiotic exposure (yes)	0.001	0.0001	15	0.1
Leak OCR/basal OCR	0.0002	0	17	0.12
Basal OCR	−0.0001	0	19	0.14
Antenatal steroid exposure (yes)	−0.03	0.004	27	0.21
Gestational age	−0.01	0.0002	26	0.25
Number of surfactant doses	0.01	0.0003	30	0.25
Birth weight	−0.0001	0	41	0.42
O ₂ requirement at 24 h	0.18	0.05	48	0.51
Chorioamnionitis exposure (no)	−0.09	0.01	50	0.55
Hyperoxia-induced H_2O_2 in HUVEC supernatant	0.0001	0	94	0.97
Maximal OCR	−0.002	0	100	1

Definition of abbreviations: H_2O_2 = hydrogen peroxide; HUVEC = human umbilical vein endothelial cell; OCR = oxygen consumption rate.

Estimates for all 11 variables chosen for logistic regression model building were averaged across all fitted models using the “glmulti” package in R. On the basis of their relative importance averaged across all models, maximal HUVEC OCR and HUVEC supernatant H_2O_2 content were found to be the most significant predictors of risk for death or bronchopulmonary dysplasia in premature infants in our study.

Table 3. Logistic Regression Model for Prediction of Death or Bronchopulmonary Dysplasia

Variable	Crude OR (95% CI)	Adjusted OR (95% CI)	P Value (Wald's test)
Chorioamnionitis exposure (yes)	1.7 (0.7–4.6)	0.04 (0–2.7)	0.14
Days on supplemental oxygen in first week of life	1.6 (1.3–2.1)	2.1 (1–4.3)	0.05
Maximal OCR	0.89 (0.78–0.94)	0.90 (0.83–0.99)	0.003*
H ₂ O ₂ content in HUVEC supernatant	1.001 (1.0002–1.0013)	1.002 (1.0001–1.003)	0.04*

Definition of abbreviations: CI = confidence interval; H₂O₂ = hydrogen peroxide; HUVEC = human umbilical vein endothelial cell; OCR = oxygen consumption rate; OR = odds ratio.
Log likelihood = 11; number of observations = 69; Akaike information criterion value = 35.1. ORs for the variables in the final logistic regression model to predict bronchopulmonary dysplasia or death are shown.
*P < 0.05.

increased mtDNA damage in HUVECs from BPD-susceptible infants that produced increased amounts of mtROS also suggests that mitochondrial injury is exacerbated by hyperoxia-induced mtROS in vascular endothelial cells in these infants.

Thus, both increased signaling through mtROS indicative of increased oxidant stress and the more deleterious effects of increased ROS generation may be active in the pulmonary vasculature of BPD-susceptible infants.

Race and exposure to perinatal inflammation were identified as factors affecting HUVEC bioenergetic function. Chorioamnionitis exposure, as noted in our study, is not consistently associated with increased BPD, but it is known to amplify

Figure 7. Statistical model performance in predicting bronchopulmonary dysplasia (BPD) or death. (A) Receiver operating characteristic curve generated for the performance of the final logistic regression model to predict death or BPD in our study cohort. (B) Classification and regression tree algorithm showing variables identified as predictors of BPD or death in our infant cohort. Maximal oxygen consumption rate less than 200 pmol/min/ 3×10^4 cells, gestational age (GA) less than 28 weeks, and birth weight (BW) less than 850 g were identified as significant prognosticators that distinguished BPD-susceptible infants from survivors without BPD. AUC = area under the receiver operating characteristic curve.

inflammatory responses to other stimuli that cause lung injury (36, 37). Our findings of increased mtROS generation in HUVECs from AA infants are supported by our own findings of enhanced oxidative stress in HUVECs from healthy term AA infants and by findings in other studies (38, 39). Therefore, the endothelial bioenergetic and redox dysfunction seen in these infant subgroups indicates potential mechanisms through which BPD risk could be amplified in specific infants.

Methodologic strengths of this study include quantitative measurements of ROS generation and mtDNA damage in human-derived cells from a unique patient population for an exploratory study of this nature. We used intact cells to measure bioenergetic function in endothelial cells to eliminate artifacts associated with mitochondrial isolation (40). We also ascertained that potential differences in HUVEC growth rates in culture due to developmental differences (GA effects) or due to varying susceptibility to BPD in these infants that might affect our experimental results did not exist. Finally, we used robust and cross-validated statistical models to verify our primary hypothesis that HUVEC bioenergetics is an

important predictor of BPD in premature infants.

Our study is also subject to certain limitations. Extrapolation of HUVEC biology to other vascular endothelial beds that often have heterogeneous responses to oxidant damage may be of limited value (41). However, HUVEC monolayer models for the study of microvascular pathophysiology have several merits, and we have used them successfully in previous studies (42). Additionally, fluorescence-based methods for measurement of ROS such as $O_2^{\cdot-}$ and H_2O_2 are prone to issues such as mitotoxicity from these dyes and the relative nonspecificity of their oxidation by multiple oxidants (43). To mitigate such concerns, we used modified experimental methods such as the use of $O_2^{\cdot-}$ -specific excitation ranges for the MitoSOX Red experiments (44). Finally, endothelial cells are primarily glycolytic, and the relative contribution of oxidative phosphorylation and glycolysis in early-passage HUVECs that we used for this study remains unknown (45). We observed similar HUVEC glycolytic rates in infants with different BPD susceptibility, but we acknowledge that more precise

characterization using chemical inhibitors of glycolysis needs to be obtained.

In summary, vascular endothelial mitochondrial function at birth is a potential biomarker for BPD susceptibility in preterm infants. Evaluation of HUVEC mitochondrial biology could be valuable for studying pathology related to prematurity in general and for studying endothelial and lung biology. Recent advances in high-throughput technology have permitted bioenergetic analyses in a variety of biological structures, and researchers have proposed novel biomarkers, such as a personalized “bioenergetic health index,” to quantify an individual’s bioenergetic health (46). Such individualized bioenergetic measures derived from readily accessible cells such as HUVECs could help modify therapeutic strategies to decrease risk for adverse pulmonary outcomes in susceptible infants. ■

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