Fatty Acid Oxidation Protects against Hyperoxia-induced Endothelial Cell Apoptosis and Lung Injury in Neonatal Mice

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Abstract

In neonates, hyperoxia or positive pressure ventilation causes continued lung injury characterized by simplified vascularization and alveolarization, which are the hallmarks of bronchopulmonary dysplasia. Although endothelial cells (ECs) have metabolic flexibility to maintain cell function under stress, it is unknown whether hyperoxia causes metabolic dysregulation in ECs, leading to lung injury. We hypothesized that hyperoxia alters EC metabolism, which causes EC dysfunction and lung injury. To test this hypothesis, we exposed lung ECs to hyperoxia (95% O₂/5% CO₂) followed by air recovery (O₂/rec). We found that O₂/rec reduced mitochondrial oxidative phosphorylation without affecting mitochondrial DNA copy number or mitochondrial mass and that it specifically decreased fatty acid oxidation (FAO) in ECs. This was associated with increased ceramide synthesis and apoptosis. Genetic deletion of carnitine palmitoyltransferase 1a (Cpt1a), a rate-limiting enzyme for carnitine shuttle, further

augmented O₂/rec-induced apoptosis. O₂/rec-induced ceramide synthesis and apoptosis were attenuated when the FAO was enhanced by L-carnitine. Newborn mice were exposed to hyperoxia (>95% O₂) between Postnatal Days 1 and 4 and were administered L-carnitine (150 and 300 mg/kg, i.p.) or etomoxir, a specific Cpt1 inhibitor (30 mg/kg, i.p.), daily between Postnatal Days 10 and 14. Etomoxir aggravated O₂/rec-induced apoptosis and simplified alveolarization and vascularization in mouse lungs. Similarly, arrested alveolarization and reduced vessel numbers were further augmented in EC-specific *Cpt1a*-knockout mice compared with wild-type littermates in response to O₂/rec. Treatment with L-carnitine (300 mg/kg) attenuated O₂/rec-induced lung injury, including simplified alveolarization and decreased vessel numbers. Altogether, enhancing FAO protects against hyperoxia-induced EC apoptosis and lung injury in neonates.

Keywords: bronchopulmonary dysplasia; mitochondrial fuel use; ceramide; lung alveolarization; vascularization

Bronchopulmonary dysplasia (BPD) is a chronic lung disease that affects 10,000–15,000 premature infants annually in the United States (1). This disease is characterized by alveolar dysplasia and impaired vascularization and is defined clinically by continued dependency on supplemental oxygen beyond 36 weeks corrected gestation in premature infants (2). However, oxygen supplementation can

blunt normal lung development and the growth of pulmonary microvasculature (3, 4). Although most BPD survivors eventually can be weaned from supplemental oxygen, there can be residual pulmonary dysfunction and cardiovascular sequelae in adolescence and adulthood (5–8). The molecular mechanisms underlying hyperoxia-induced continued lung injury are not fully understood.

Endothelial cells (ECs) are highly glycolytic and generate more than 80% of their ATP through this pathway (9, 10). Nevertheless, both glycolysis and mitochondrial fatty acid oxidation (FAO) are essential for EC proliferation and vessel sprouting (9, 11), and blood vessel growth is tightly linked to metabolic status in ECs. It has been shown that human umbilical venous ECs from patients with BPD had

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lower mitochondrial respiration than ECs from infants who survived without BPD (12). However, it is not known whether supplemental oxygen in patients with BPD alters mitochondrial oxidative phosphorylation in lung ECs, leading to impaired alveolar formation. Hyperoxia followed by air recovery (O_2 /rec) is a clinically relevant scenario of lung injury and repair in patients with BPD because patients wean from oxygen over time (13). Therefore, we hypothesized that O_2 /rec causes metabolic reprogramming in lung ECs, leading to EC dysfunction and lung injury. To test this hypothesis, we evaluated mitochondrial respiration and fuel use in lung ECs, as well as their roles in hyperoxia-induced EC apoptosis and subsequent lung injury. Some of these results were presented at the American Thoracic Society International Conference in May 2017 and were previously published in abstract form (14).

Methods

Hyperoxia Exposure

Cells at 70–80% confluence were exposed to hyperoxia (95% O_2 and 5% CO_2) or air (21% O_2 and 5% CO_2) for 24 hours with cell viability (>80%). Cells were then cultured in normoxia for 1, 3, or 7 days after exposure. Culture media were changed every 24 hours. Newborn C57BL/6J and EC-specific carnitine palmitoyltransferase 1a (*Cpt1a*)-knockout (KO) mice (<12 h old), together with their mothers, were exposed to room air or hyperoxia (>95% O_2) for 72 hours in an A-Chamber (BioSpherix) (15). The dams were switched every 24 hours between room air and hyperoxia to avoid injury. The pups were allowed to recover in air for up to 11 days after the hyperoxia exposure. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Brown University.

Generation of EC-Specific *Cpt1a*-KO Mice

Cpt1a^{lox/lox} mice (C57BL/6J) were obtained from Dr. Peter Carmeliet (University of Leuven, Belgium) (11). EC-specific *Cpt1a*-KO mice were generated by crossing *Cpt1a*^{lox/lox} mice with mice expressing the Cre recombinase transgene under control of the VE-cadherin promoter [B6;129-

Tg(Cdh5-cre)1Spe/J, stock no. 017968; The Jackson Laboratory] that were then backcrossed five generations to a C57BL/6J background. PCR analysis of genomic DNA from EC-specific *Cpt1a*-KO pups, showing the appearance of a 300-bp band for flox and a 345-bp band for Cre.

Measurement of Mitochondrial Respiration

After optimization, we seeded ECs (30,000 cells/well) in Seahorse XF24 microplates (Agilent Technologies) overnight before measuring the oxygen consumption rate (OCR) (16). Detailed information is provided in the data supplement.

Direct FAO Measurement Using Isotope-labeled Palmitic Acid

FAO was measured by determining the release of 3H_2O from the oxidation of [9,10- $^3H(N)$]-palmitic acid. Detailed information is provided in the data supplement.

Administration of Etomoxir and L-Carnitine in Mice

Mouse lung alveolarization starts at Postnatal Day (pnd) 4 and peaks between pnd10 and pnd14 (17, 18). Therefore, we administered etomoxir (30 mg/kg) and L-carnitine (150 and 300 mg/kg) daily by intraperitoneal injection to mice from pnd10 to pnd14 after they were exposed to hyperoxia for 3 days as neonates. The doses of etomoxir and L-carnitine were chosen on the basis of previous reports without affecting body weight in neonatal rodents (11, 19). At 15 days of age, mice were killed, and their lungs were used for further analysis.

Lung Morphometry

Mean linear intercept (Lm) and radial alveolar counts (RAC) were measured in mouse lung slides as we previously described (20–22). Detailed information is provided in the data supplement.

Statistical Analysis

Statistical analyses were performed using Prism 6 software (GraphPad Software). The results were expressed as mean $\pm$ SEM. One-way ANOVA with Bonferroni *post hoc* multiple comparisons test was performed to measure the effect of one factor. The statistical significance of the differences between groups was evaluated by using two-way ANOVA for overall significance,

followed by the Tukey-Kramer test. Statistical significance was considered present when $P < 0.05$.

Results

O_2 /Rec Decreases Mitochondrial Respiration in Lung ECs

Although maximal OCR in human umbilical venous ECs was identified as a significant predictor of BPD (12), it is not known whether hyperoxia exposure alters mitochondrial respiration in lung ECs. First, we employed the primary mouse lung microvascular endothelial cells (LMVECs) and exposed them to hyperoxia for 24 hours followed by O_2 /rec for 24 hours. Primary LMVECs are characterized by positive staining of acetylated low-density lipoprotein and VE-cadherin (see Figure E1A in the data supplement). Exposure to O_2 /rec significantly reduced OCR in primary LMVECs, which was reflected by the decreased basal, proton leak-dependent, ATP-linked, and maximal respiration compared with the air group (Figure E1B). Similarly, O_2 /rec decreased OCR in a mouse fetal lung EC line (MFLM-91U) (Figure E1B).

To determine whether O_2 /rec alters mitochondrial DNA (mtDNA) copy number, we quantified mtDNA/nuclear DNA ratio by qPCR. As shown in Figure E2A, the ratio of 16S/HK2 or ND1 (NADH-ubiquinone oxidoreductase chain 1)/HK2 (hexokinase 2) DNA was not altered in cells exposed to O_2 /rec. The concentrations of Tom20, a mitochondrial outer membrane protein, were not changed in cells exposed to O_2 /rec (Figure E2B). Similarly, mitochondrial mass measured by MitoTracker Green (Molecular Probes) using flow cytometry was not significantly altered in cells exposed to O_2 /rec compared with the air group (Figure E2C). These results demonstrate that O_2 /rec reduces oxidative phosphorylation in lung ECs without affecting mtDNA copy number or mitochondrial mass. This suggests that O_2 /rec results in mitochondrial dysfunction rather than a reduction in mitochondrial numbers.

O_2 /Rec Reduces FAO in Lung ECs

ECs have metabolic flexibility to maintain cell function under stress (23). Thus, we measured mitochondrial use of fuel substrates (glucose, glutamine, and

long-chain fatty acid [FA]) according to the Seahorse XF Mito Fuel Flex Test protocol (Agilent Technologies). The percentage of FAO was reduced in primary LMVECs exposed to O₂/rec (Figure 1A). The percentage of glucose or glutamine oxidation was not changed in primary LMVECs exposed to O₂/rec. We further performed direct FAO assay using ³H-labeled palmitic acid and quantified the amount of ³H₂O that was released by its metabolism. Using this sensitive assay, ³H₂O release was significantly reduced in MFLM-91U cells exposed to O₂/rec compared with the air group (Figure 1B).

To determine whether hyperoxia exposure alters FAO in lung ECs *in vivo*, we exposed neonatal mice (<12 h old) to room air or hyperoxia (95% O₂) for 3 days and allowed them to recover in air for 11 days. Primary LMVECs were isolated from these mice to determine the changes in FAO. As shown in Figure 1C, O₂/rec decreased FAO in LMVECs. Similarly, the oxidation of exogenous palmitate (175 μM) was also reduced in LMVECs isolated from hyperoxia-exposed mice followed by O₂/rec as compared with air control cells (Figure 1D). Altogether, these results demonstrate that FAO is reduced in ECs exposed to O₂/rec.

Cpt1a Concentrations Are Reduced in Lung ECs Exposed to O₂/Rec

Cpt1 is a rate-limiting enzyme for the carnitine shuttle, which catalyzes the transfer of the acyl group of long-chain FAs from coenzyme A to carnitine to form acylcarnitine for mitochondrial transport. Thus, we determined the mRNA concentrations of Cpt1 isoforms. As shown in Figure 2A, mRNA concentrations of *Cpt1a* were significantly reduced in both primary LMVECs and MFLM-91U cells exposed to O₂/rec. This was in agreement with reduction of Cpt1a protein concentrations after normalization to β-actin or Tom20 protein in lung ECs exposed to O₂/rec (Figures 2B and 2C). The isoforms of *Cpt1b* or *Cpt1c* mRNA were not changed in lung ECs exposed to O₂/rec. There was a decreasing trend in *Cpt1a*, *Cpt1b*, and *Cpt1c* mRNA in mouse lungs exposed to O₂/rec (Figure 2A). These results suggest that hyperoxia-induced FAO reduction is associated with the decreased concentrations of Cpt1a.

O₂/Rec Does Not Affect Intracellular Free FAs, Neutral Lipids, or Lipid Peroxidation in Lung ECs

To determine whether O₂/rec alters FA transport, we first determined gene expression of fatty acid transport proteins (FATP, FATP1, FATP3, and FATP5), which are commonly expressed in lungs. We found that *FATP5* mRNA and protein concentrations were significantly increased in lung ECs exposed to O₂/rec (Figures E3A and E3B). The mRNA concentrations of *FATP1* or *FATP3* were not changed in lung ECs exposed to O₂/rec (Figure E3A). These results suggest that hyperoxia may enhance FA transport. We next asked whether hyperoxia exposure affects intracellular free FAs in lung ECs under the condition of reduced FAO. As shown in Figure E3C, the concentrations of free FAs were not altered in cells exposed O₂/rec. Neutral lipid storage is enhanced upon increased uptake of FAs. Hence, we measured neutral lipids using fluorescent dye BODIPY 493/503 (Molecular Probes). As shown in Figure E3D, O₂/rec had no effects on neutral lipids in lung ECs exposed to hyperoxia. Finally, we measured the product of lipid peroxidation, 4-hydroxynonenal (4-HNE), and found that there were no changes in

Figure 1. Hyperoxia (95% O₂/5% CO₂) followed by air recovery reduced fatty acid oxidation (FAO) in lung endothelial cells (ECs). (A) Primary lung microvascular endothelial cells (LMVECs) were exposed to air or hyperoxia for 24 hours and then cultured in normoxia for 24 hours (O₂). Fuel use was immediately measured in cells using the Seahorse XF24 Analyzer when the following pathway inhibitors were injected: etomoxir (4 μM), bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide (BPTES; 3 μM), and UK5099 (2 μM). The oxidation of fatty acid, glutamine, and glucose was calculated as the difference of oxygen consumption rate (OCR) between etomoxir, BPTES, or UK5099 and vehicle treatments, respectively. Data are expressed as the percentage of specific fuel oxidation normalized to total fuel oxidation, which did not need the normalization of raw OCR data by cell numbers. (B) FAO was quantified using a radioactive assay in which ³H-labeled palmitic acid was provided in the medium, and its oxidation by MFLM-91U cells was measured by the release of ³H₂O. (C) Primary LMVECs were isolated from mice exposed to hyperoxia for 3 days and then kept in air for 11 days. FAO was measured using the Seahorse XF24 Analyzer. (D) Primary LMVECs were exposed to air or hyperoxia for 24 hours followed by air recovery for 24 hours. Cells were incubated with substrate-free base medium containing glucose (2.5 mM) and carnitine (0.5 mM) overnight, and OCR was measured immediately when palmitate-BSA (175 μM) was added in the presence or absence of etomoxir (40 μM for 15 min). FAO was calculated and normalized to total fuel oxidation in terms of maximal respiration after normalization to the number of live cells. Data are expressed as mean ± SEM (n = 6–8). *P < 0.05, **P < 0.01, and ***P < 0.001 versus air; †††P < 0.001 versus air/BSA using ANOVA followed by Bonferroni *post hoc* test.

Figure 2. Hyperoxia (95% O₂/5% CO₂) followed by air recovery reduced carnitine palmitoyltransferase 1a (Cpt1a) concentrations. Primary LMVECs and MFLM-91U cells were exposed to hyperoxia for 24 hours followed by air recovery for 24 hours (O₂). C57BL/6J mice (<12 h old) were exposed to air or hyperoxia for 3 days and then kept in air for 11 days. (A) RNA was extracted, and gene expression was measured by qRT-PCR. (B) Cpt1a protein concentrations were measured by Western blotting. (C) Normalization of Cpt1a protein concentrations from B was to Tom20 protein concentrations from Figure E2C in MFLM-91 cells exposed to hyperoxia (95% O₂/5% CO₂) followed by air recovery. Data are expressed as mean ± SEM (n = 6). *P < 0.05, **P < 0.01, and ***P < 0.001 versus air using ANOVA followed by Bonferroni *post hoc* test.

4-HNE in lung ECs exposed to O₂/rec (Figure E3E). Altogether, these results indicate that O₂/rec did not cause FA accumulation or lipid peroxidation in lung ECs.

O₂/Rec Increases Ceramide Concentrations in Lung ECs

It has been shown that increased FAs can be used for ceramide synthesis when FAO is inhibited (24, 25) (Figure 3A). Serine palmitoyltransferase long-chain base subunit 1 (Sptlc1) is a rate-limiting enzyme for the *de novo* synthesis of ceramide. Thus, we determined *Sptlc1* mRNA in lung ECs exposed to O₂/rec. We found that gene expression of *Sptlc1* was increased when cells were exposed to O₂/rec (Figure 3B). Furthermore, the concentrations of dihydroceramides, precursors for *de novo*

synthesis of ceramide, were significantly increased in lung ECs exposed to O₂/rec (Figure 3C). Finally, O₂/rec increased ceramide concentrations in lung ECs (Figure 3D). Altogether, these results suggest that O₂/rec reduces FAO to favor increased *de novo* synthesis of ceramides in lung ECs.

O₂/Rec Augments Apoptosis

Hyperoxia exposure has been shown to cause apoptosis in lung ECs (26, 27). However, it is not known whether O₂/rec affects apoptosis in lung ECs. To answer this question, we exposed lung ECs for 24 hours and allowed them to recover in air for 1, 3, and 7 days and then determined apoptosis using the Annexin V Apoptosis Detection Kit (Thermo Fisher Scientific). As expected, hyperoxia exposure for

24 hours significantly increased the number of apoptotic cells. Interestingly, the percentage of annexin V⁺ cells remained increased when cells were recovered in air for 1, 3, and 7 days compared with the air group (Figures E4A and E4B). This was in agreement with increased caspase 3 activity in cells exposed to O₂/rec (Figure E4C). These results demonstrate that O₂/rec further increases apoptosis in lung ECs.

Enhancing FAO by L-Carnitine Attenuates Hyperoxia-induced Apoptosis via Cpt1a

Although hyperoxia reduced FAO but increased apoptosis, it is not known whether FAO modulates hyperoxia-induced apoptosis in lung ECs. L-carnitine, as a substrate of Cpt1, functions as a carrier of

Figure 3. Hyperoxia (95% O₂/5% CO₂) followed by air recovery (O₂/rec) increased dihydroceramide (DHceramide) and ceramide concentrations. (A) Simplified schematic of palmitoyl-coenzyme A (CoA) for ceramide synthesis or the transfer of the acyl group of palmitoyl CoA from CoA to L-carnitine to form acylcarnitine by Cpt1. Myriocin is a specific inhibitor of serine palmitoyltransferase (Spt), which is a rate-limiting enzyme of ceramide synthesis. (B) Primary lung microvascular endothelial cells were exposed to O₂/rec. The mRNA concentrations of serine palmitoyltransferase long-chain base subunit 1 (Sptlc1) were measured by qRT-PCR. (C and D) Dihydroceramide and ceramide were measured by mass spectrometry in primary lung microvascular endothelial cells exposed to O₂/rec. During the air recovery phase, cells were treated with L-carnitine (0.25 and 0.5 mM) for 12 hours. (E) MFLM-91U cells were treated with myriocin (100 nM) for 12 hours during the air recovery phase after hyperoxia exposure, and FAO was measured using the Seahorse XF24 Analyzer. Data are expressed as mean ± SEM (n = 5–6). *P < 0.05 and **P < 0.01 versus air or air/vehicle (Veh) group; †P < 0.05 versus O₂/vehicle group; ###P < 0.001 versus air/myriocin using ANOVA followed by Bonferroni *post hoc* or Tukey-Kramer test.

activated long-chain FAs across the inner mitochondrial membrane for β -oxidation (28). Thus, we exposed primary LMVECs and MFLM-91U cells to hyperoxia for 24 hours, and cells were treated with L-carnitine (0.25 and 0.5 mM) for 12 hours during O₂/rec. As shown in Figure 4A, L-carnitine (0.5 mM) treatment significantly increased FAO in MFLM-91U cells. L-Carnitine at 0.25 and 0.5 mM significantly attenuated caspase 3 activity in response to O₂/rec in a concentration-dependent manner (Figure 4B). Similarly, L-carnitine at 0.5 mM reduced cleaved caspase 3 concentrations and annexin V⁺ cells in response to O₂/rec (Figures 4C and 4D).

To determine whether L-carnitine protects against hyperoxia-induced

apoptosis via Cpt1a, we isolated LMVECs from EC-specific *Cpt1a*-KO mice and wild-type (WT) littermates. Genotyping was performed to confirm the Cre transgene in *Cpt1a*-KO mice (Figure 4E). Western blot analysis also confirmed the deficiency of Cpt1a protein in LMVECs isolated from *Cpt1a*-KO mice (Figure 4F). We exposed *Cpt1a*-KO and WT cells to O₂/rec and treated them with L-carnitine (0.5 mM) during O₂/rec. *Cpt1a* gene disruption increased annexin V⁺ cells compared with WT cells when they were exposed to O₂/rec (Figure 4G). L-Carnitine treatment (0.5 mM) significantly attenuated hyperoxia-induced apoptosis in WT cells but not in *Cpt1a*-KO cells (Figure 4G). These results demonstrate that Cpt1a is

required for L-carnitine's protection against hyperoxia-induced apoptosis in lung ECs.

L-Carnitine Attenuates O₂/Rec-induced Increase in Ceramide, Thereby Reducing Apoptosis in ECs

Because hyperoxia increased ceramide synthesis, we treated cells with an inhibitor of *de novo* ceramide synthesis (100 nM myriocin for 12 h) or L-carnitine (0.5 mM for 12 h) in the presence of exogenous palmitate/BSA (50 and 175 μ M for both 0.5 and 12 h) during the O₂/rec phase after hyperoxia exposure. As shown in Figure E5A, treatment with palmitate (50 μ M for 12 h) significantly increased apoptosis as reflected by annexin V⁺ cells, whereas palmitate (175 μ M for 0.5 h) did not. Treatments with myriocin or L-carnitine

Figure 4. Enhancing FAO by L-carnitine reduced hyperoxia-induced apoptosis via Cpt1a in lung endothelial cells. (A–D) MFLM-91U cells were treated with L-carnitine (L-carn; 12 h) during air recovery after hyperoxia exposure. (A) FAO was measured using the Seahorse XF24 Analyzer when cells were treated with L-carnitine (0.5 mM). (B) Caspase-3 activity and (C) cleaved caspase 3 were measured when L-carnitine was added at 0.25 and 0.5 mM, and (D) annexin V⁺ cells were measured when cells were treated with L-carnitine at 0.5 mM. PI = propidium iodide. (E) PCR analysis was performed to determine the Cre and flox expression in tails from Cpt1a-knockout (KO) mice and wild-type (WT) littermates. (F) Cpt1a protein was measured by Western blotting in primary lung microvascular endothelial cells isolated from Cpt1a-KO mice and WT littermates. (G) Primary lung microvascular endothelial cells isolated from Cpt1a-KO mice and WT littermates were exposed to hyperoxia (95% O₂/5% CO₂) followed by air recovery, and these cells were then treated with L-carn (0.5 mM) for 12 hours during the air recovery phase. Apoptosis was measured using the FITC Annexin V Detection Kit with the BD FACSAria cell sorter (BD Biosciences). Data are expressed as mean ± SEM (*n* = 5–6). ***P* < 0.01 and ****P* < 0.001 versus air or air/vehicle; †*P* < 0.05, ††*P* < 0.01, and †††*P* < 0.001 versus O₂/vehicle; ‡*P* < 0.01 versus WT group using ANOVA followed by Bonferroni *post hoc* or Tukey-Kramer test.

during the air recovery phase significantly reduced palmitate (50 μM)-induced apoptosis in the cells exposed to hyperoxia and recovered in room air (Figure E5B). In addition,

L-carnitine treatment reduced concentrations of ceramide and its precursors (i.e., dihydroceramide) in lung ECs exposed to O₂/rec (Figures 3C and 3D). Interestingly,

treatment with myriocin (100 nM for 12 h) significantly increased the FAO in the air group, and these effects were attenuated in lung ECs exposed to O₂/rec (Figure 3E).

It has been shown that exogenous C8-ceramide stimulates *de novo* ceramide synthesis in ECs (29). Thus, we treated cells with C8-ceramide (10 μ M) together with L-carnitine (0.5 mM) for 12 hours. We found that treatment with C8-ceramide significantly increased annexin V⁺ cells, cleaved caspase 3 concentrations, and caspase 3 activity in lung ECs (Figures E5C-E5E). This was attenuated by L-carnitine treatment. These results suggest that L-carnitine decreases hyperoxia-induced ceramide synthesis, thereby leading to reduced apoptosis.

L-Carnitine Attenuates Hyperoxia-induced Apoptosis in Mouse Lungs

To determine the role of FAO in hyperoxia-induced apoptosis in mouse lungs, we

exposed newborn mice to hyperoxia as shown in Figure 1C and first measured the concentrations of cleaved caspase 3 in mouse lungs. As shown in Figure 5A, O₂/rec increased the concentrations of cleaved caspase 3 in mouse lungs. This was confirmed by the caspase 3 activity and TUNEL staining mouse lungs (Figures 5B and 5C). We then administered L-carnitine (300 mg/kg i.p.) or etomoxir (30 mg/kg i.p.) daily to the aforementioned hyperoxia-exposed mice from pnd10 to pnd15. Administration of L-carnitine significantly attenuated the numbers of TUNEL-positive cells (Figure 5C). Pharmacological inhibition Cpt1 by etomoxir further increased hyperoxia-induced caspase 3 activity and TUNEL-positive cells in mouse lungs (Figures 5B and 5C). Similarly,

genetic disruption of *Cpt1a* in ECs enhanced hyperoxia-induced increases in caspase 3 activity in mouse lungs (Figure 6A). These results demonstrate that enhancing FAO protects against hyperoxia-induced apoptosis in mouse lungs.

Hyperoxia-induced Lung Injury Is Aggravated When Cpt1 Is Inhibited or Disrupted

The lung pathology of BPD can be mimicked in rodents exposed to hyperoxia as neonates (3, 30, 31). To further determine the role of Cpt1 in hyperoxia-induced lung injury, we generated EC-specific Cpt1a-KO mice, exposed them to hyperoxia for 3 days, and allowed them room air for 11 days. There was no difference in body weight between WT and

Figure 5. Enhancing FAO reduced hyperoxia-induced apoptosis in mouse lungs. C57BL/6J neonatal mice (<12 h old) were exposed to air or hyperoxia (95% O₂) for 3 days and then kept in air for 11 days. (A) Lung tissue was harvested for detecting concentrations of cleaved caspase 3 by Western blotting. Calnexin was used as the loading control. Densitometry of cleaved caspase 3 was normalized to calnexin. *n* = 4. (B and C) Etomoxir (Eto; 30 mg/kg) and L-carnitine (300 mg/kg) were administered daily through i.p. injection in the aforementioned mice from Postnatal Day 10 to Postnatal Day 14. At 15 days of life, mice were killed, and (B) caspase 3 activity was measured in lung homogenates, or (C) lungs were fixed for TUNEL (red) and von Willebrand factor (green) staining. The insets show the positive expression of TUNEL and von Willebrand factor. The arrows indicate TUNEL-positive cells. Scale bars: 100 μ m (upper panels) and 50 μ m (lower panels). *n* = 8. ****P* < 0.001 versus air; †*P* < 0.05, ††*P* < 0.01, and †††*P* < 0.001 versus hyperoxia/vehicle using ANOVA followed by Bonferroni *post hoc* test. HPF = high-power field.

Figure 6. Hyperoxia-induced apoptosis and lung injury were aggravated in EC-specific *Cpt1a* KO mice. EC-specific *Cpt1a* KO mice and WT littermates (<12 h old) were exposed to air or hyperoxia (95% O₂) for 3 days and then kept in air for 11 days. (A) Caspase 3 activity was measured in lung homogenates. (B) Body weight was measured in mice at 15 days of life. (C) Mouse lungs were fixed for von Willebrand factor immunofluorescence to enable counting of the vessel number per HPF. Lungs were fixed for hematoxylin and eosin staining to measure (D) mean linear intercept (Lm) using MetaMorph software (Molecular Devices) and (E) radial alveolar counts. Scale bars: 200 μ m. Data are expressed as mean $\pm$ SEM ($n = 7-8$). ** $P < 0.01$ and *** $P < 0.001$ versus air; † $P < 0.05$ and †† $P < 0.01$ versus WT group using ANOVA followed by Tukey-Kramer test.

Cpt1a-KO mice when they were exposed to hyperoxia (Figure 6B). The number of vessels, as detected by von Willebrand factor staining, was further reduced in *Cpt1a*-KO mice compared with their WT littermates in response to O₂/rec (Figure 6C). In addition, genetic disruption of *Cpt1a* in ECs further aggravated hyperoxia-induced increases in Lm and reduction in RAC (Figures 6D and 6E). We then used the same exposure and treatment protocol as shown in Figure 5B to determine the role of Cpt1 inhibition by etomoxir in hyperoxia-induced simplified alveolarization. Hyperoxia exposure impaired lung alveolarization, as reflected by increased Lm and reduced RAC, which was aggravated by etomoxir treatment (Figures 7A and 7B). Etomoxir treatment further decreased hyperoxia-induced reduction in vessel numbers as determined by von Willebrand factor staining (Figure 7C). These findings demonstrate that hyperoxic lung injury is enhanced when Cpt1 is pharmacologically inhibited or genetically disrupted.

L-Carnitine Protects Against Hyperoxia-induced Impaired Alveolarization and Vascularization in Mouse Lungs

To determine whether enhancing FAO protects against hyperoxic lung injury, we administered L-carnitine (150 and 300 mg/kg) as shown in the protocol for Figure 5B. We found that L-carnitine (300 mg/kg) significantly attenuated hyperoxia-induced increase in Lm in mouse lungs (Figure 7A). Hyperoxia-induced reduction in RAC was also ameliorated by L-carnitine treatment (300 mg/kg) (Figure 7B). Last, L-carnitine (300 mg/kg) treatment ameliorated hyperoxia-induced reduction of vessel number in mouse lung (Figure 7C). These results demonstrate that L-carnitine protects against hyperoxia-induced arrest in alveolarization and vascularization.

Discussion

Although ECs are metabolically flexible under stress, the effects of hyperoxia

exposure on mitochondrial respiration and fuel use in lung ECs are not known. In the present study, we showed that hyperoxia exposure reduced mitochondrial respiration in lung ECs, which is corroborated by the findings in lung epithelial cells (32). We also showed that O₂/rec specifically decreased FAO but increased ceramide synthesis, leading to apoptosis. Enhancing FAO by L-carnitine reduced, whereas inhibiting FAO by etomoxir or genetic disruption of *Cpt1a* further augmented, hyperoxia-induced alveolarization and vascularization. Hence, in neonatal mice, hyperoxia exposure has long-term effects on lung EC apoptosis via FAO, and enhancing FAO protects against hyperoxia-induced lung injury. Our study also provides novel mechanistic insights into the role of FAO in preventing hyperoxia-induced EC injury and subsequent alveolar simplification.

We observed an increase in glycolysis in lung ECs exposed to O₂/rec (data not shown). These cells may attempt to restore glycolysis as a compensatory mechanism against reduced mitochondrial respiration and cellular dysfunction. O₂/rec-induced reduction in mitochondrial oxidative phosphorylation was attributed to mitochondrial dysfunction in a mouse model of BPD (33, 34). Interestingly, O₂/rec did not cause any changes in mtDNA copy number, Tom20 protein, or MitoTracker Green fluorescence intensity in lung ECs, suggesting that the reduced mitochondrial respiration was not due to decreased mitochondrial number or mass. Further research will be needed to elucidate whether O₂/rec alters mitochondrial dynamics, including fission/fusion and mitophagy, leading to reduced mitochondrial respiration.

Long-chain FAs are the most abundant FA species in mammalian cells. The carnitine shuttle is responsible for transporting long-chain FAs into mitochondria for β -oxidation and further oxidative phosphorylation (28). As a rate-limiting step in the carnitine shuttle, Cpt1 has three isoforms: Cpt1a, Cpt1b, and Cpt1c. Both Cpt1a (in all cells except for skeletal muscle cells) and Cpt1b (the muscle mitochondrial isoform of Cpt1) localize to mitochondria and have acyltransferase activity, whereas Cpt1c localizes to the endoplasmic reticulum and does not have acyltransferase activity (35). We found that Cpt1a concentrations were significantly reduced in lung ECs recovered

Figure 7. Enhancing FAO protected against hyperoxia-induced arrested alveolarization in mouse lungs. C57BL/6J neonatal mice (<12 h old) were exposed to air or hyperoxia (95% O₂) for 3 days and then allowed to recover in room air for 11 days. Etomoxir (30 mg/kg) and L-carnitine (150 and 300 mg/kg) were administered daily through i.p. injection in mice from Postnatal Day 10 to Postnatal Day 14. At 15 days of life, mice were killed, and their lungs were fixed for hematoxylin and eosin staining to measure (A) Lm using MetaMorph software (Molecular Devices) and (B) radial alveolar counts. (C) These mouse lungs were also fixed for von Willebrand factor immunofluorescence to count the vessel number per HPF. (D) Schematic showing that hyperoxia exposure increased ceramide synthesis in the setting of insufficient FAO, leading to EC apoptosis and subsequent lung injury in neonates. Scale bars: 100 μ m. Data are expressed as mean $\pm$ SEM ($n = 6-7$). *** $P < 0.001$ versus air; $^{*}P < 0.01$ and $^{***}P < 0.001$ versus hyperoxia/vehicle using ANOVA followed by Bonferroni *post hoc* test.

in air after hyperoxia exposure. This may be one of the reasons for the reduction of FAO in lung ECs exposed to O₂/rec. One of the known molecular mechanisms resulting in changes in FA metabolism is activation of peroxisome proliferator-activated receptor (PPAR), leading to increased expression of genes involved in FA metabolism (36). A genome-wide microarray analysis showed increased activation of the PPAR pathway during alveolarization in rat pups subjected to intrauterine growth restriction (18). Further study is required to determine whether the PPAR pathway is responsible for Cpt1a gene expression in response to hyperoxia exposure (37). Oxidative stress has been shown to cause Cpt1 oxidation leading to reduced enzymatic activity in HeLa cells (38). It may be that hyperoxia exposure causes a redox modification of Cpt1 isoforms resulting in FAO reduction in lung ECs. This will need to be explored.

Hyperoxia exposure causes lung EC apoptosis (26, 27). This is likely due to increased ceramide synthesis in the setting of insufficient oxidation of FA. Dihydroceramides have no double bond in sphingoid base and are incorporated by desaturase to form ceramide during the *de novo* synthesis of ceramide. We found that O₂/rec increased ceramide and its precursor (i.e., dihydroceramide) concentrations in lung ECs. This is in agreement with the findings that hyperoxia exposure increased ceramide concentrations in lungs, this increase was maintained during O₂/rec phase (39, 40). In addition to FA transporters, FATPs also function as acyl-CoA synthetases (41). Thus, increased FATP5 expression by hyperoxia exposure may generate fatty acyl-CoA, which is condensed with serine by the Spt for the *de novo* synthesis of ceramide. This also may explain the lack of increase in intracellular FAs or neutral lipid storage in response to

O₂/rec. Altogether, O₂/rec increases the *de novo* synthesis of ceramide in lung ECs. Whether O₂/rec also affects sphingolipid degradation leading to ceramide synthesis remains to be investigated.

Inhibition of FAO increases the *de novo* synthesis of ceramide, leading to apoptosis (24), whereas augmenting FAO protects against palmitate-induced apoptosis (25). This is corroborated by our findings that hyperoxia-induced apoptosis was aggravated when the FAO was inhibited by etomoxir or Cpt1a gene disruption. Enhancing FAO by L-carnitine protects against hyperoxia-induced increase in (dihydro)ceramide and apoptosis. In addition to apoptosis, hyperoxia exposure causes necroptosis in neonatal lungs (42). We did not determine whether decreased FAO seen in O₂/rec results in necroptosis in lung ECs.

Mouse lungs at birth are structurally similar to those of human neonates born at

24–28 weeks gestation. We exposed neonatal mice to hyperoxia (>95% O₂) to serve as a model of human severe BPD and treated them with L-carnitine or etomoxir to manipulate FAO. We noted that L-carnitine treatment also reduced hyperoxia-induced apoptosis in other cells, including epithelial cells, in mouse lungs. This may be due to the paracrine effects of ceramide secreted from injured ECs. L-Carnitine is able to scavenge hydrogen peroxide and superoxide radical as well as to protect the endogenous antioxidant defense system from peroxidative damage (43, 44). Further study is required to determine the role of L-carnitine in protecting from oxidative damage during hyperoxic lung injury, despite Cpt1a being required for L-carnitine's protection against hyperoxia-induced apoptosis in lung ECs.

Enhancing FAO by L-carnitine attenuated, whereas FAO inhibition by etomoxir or Cpt1a gene disruption further

augmented, hyperoxia-induced arrested alveolarization and vascularization in neonatal mice. This is in agreement with the findings that L-carnitine (200 mg/kg) protects against lung injury in neonatal rats exposed to hyperoxia without O₂/rec (19). Premature infants have lower tissue carnitine stores than term infants, and these concentrations drop quickly within the first 2 weeks of life (45–47). This may be due to the increased carnitine esterification for β -oxidation, as we showed increased expression of the *Cpt1a* gene in the lungs of ventilated premature infants who died within a few days of life (data not shown). A clinical trial of L-carnitine supplementation in premature infants with respiratory distress syndrome helps to decrease the duration of mechanical ventilation and surfactant requirement (48). This is supportive of our finding that L-carnitine supplementation improves alveolarization in neonatal mice.

In conclusion, hyperoxia exposure followed by O₂/rec increased ceramide synthesis but reduced FAO, leading to apoptosis in lung ECs. Enhancing FAO attenuates hyperoxia-induced apoptosis in lung ECs, simplified alveolarization, and vascularization in mouse lungs (Figure 7D). Therefore, dietary L-carnitine may serve as an adjunct to prevent BPD. This remains to be tested rigorously in humans. ■

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References

- Voytsov JA. "New" bronchopulmonary dysplasia and chronic lung disease. *Paediatr Respir Rev* 2017;24:17–18.
- Shennan AT, Dunn MS, Ohlsson A, Lennox K, Hoskins EM. Abnormal pulmonary outcomes in premature infants: prediction from oxygen requirement in the neonatal period. *Pediatrics* 1988;82:527–532.
- Yee M, White RJ, Awad HA, Bates WA, McGrath-Morrow SA, O'Reilly MA. Neonatal hyperoxia causes pulmonary vascular disease and shortens life span in aging mice. *Am J Pathol* 2011;178:2601–2610.
- Elberson VD, Nielsen LC, Wang H, Kumar HS. Effects of intermittent hypoxia and hyperoxia on angiogenesis and lung development in newborn mice. *J Neonatal Perinatal Med* 2015;8:313–322.
- Caskey S, Gough A, Rowan S, Gillespie S, Clarke J, Riley M, et al. Structural and functional lung impairment in adult survivors of bronchopulmonary dysplasia. *Ann Am Thorac Soc* 2016;13:1262–1270.
- Islam JY, Keller RL, Aschner JL, Hartert TV, Moore PE. Understanding the short- and long-term respiratory outcomes of prematurity and bronchopulmonary dysplasia. *Am J Respir Crit Care Med* 2015;192:134–156.
- Gough A, Linden M, Spence D, Patterson CC, Halliday HL, McGarvey LP. Impaired lung function and health status in adult survivors of bronchopulmonary dysplasia. *Eur Respir J* 2014;43:808–816.
- Check J, Gotteiner N, Liu X, Su E, Porta N, Steinhorn R, et al. Fetal growth restriction and pulmonary hypertension in premature infants with bronchopulmonary dysplasia. *J Perinatol* 2013;33:553–557.
- De Bock K, Georgiadou M, Schoors S, Kuchnio A, Wong BW, Cantelmo AR, et al. Role of PFKFB3-driven glycolysis in vessel sprouting. *Cell* 2013;154:651–663.
- Culic O, Gruwel ML, Schrader J. Energy turnover of vascular endothelial cells. *Am J Physiol* 1997;273:C205–C213.
- Schoors S, Bruning U, Missiaen R, Queiroz KC, Borgers G, Elia I, et al. Fatty acid carbon is essential for dNTP synthesis in endothelial cells. *Nature* 2015;520:192–197.
- Kandasamy J, Olave N, Ballinger SW, Ambalavanan N. Vascular endothelial mitochondrial function predicts death or pulmonary outcomes in preterm infants. *Am J Respir Crit Care Med* 2017;196:1040–1049.
- Jensen EA, Schmidt B. Epidemiology of bronchopulmonary dysplasia. *Birth Defects Res A Clin Mol Teratol* 2014;100:145–157.
- Yao H, Carr JF, Peterson AL, Dennery PA. Hyperoxic exposure reduces glycolysis and mitochondrial respiration but increases mitochondrial utilization of fatty acid in lung endothelial cells [abstract]. *Am J Respir Crit Care Med* 2017;195:A6921.
- Ito M, Nagano N, Arai Y, Ogawa R, Kobayashi S, Motojima Y, et al. Genetic ablation of Bach1 gene enhances recovery from hyperoxic lung injury in newborn mice via transient upregulation of inflammatory genes. *Pediatr Res* 2017;81:926–931.
- Lu Q, Mundy M, Chambers E, Lange T, Newton J, Borgas D, et al. Alda-1 protects against acrolein-induced acute lung injury and endothelial barrier dysfunction. *Am J Respir Cell Mol Biol* 2017;57:662–673.
- Mund SI, Stipanovich M, Schittny JC. Developmental alveolarization of the mouse lung. *Dev Dyn* 2008;237:2108–2116.
- Zana-Taieb E, Pham H, Franco-Montoya ML, Jacques S, Letourneur F, Baud O, et al. Impaired alveolarization and intra-uterine growth restriction in rats: a postnatal genome-wide analysis. *J Pathol* 2015;235:420–430.
- Caner I, Tastekin A, Saruhan F, Hacimuftuoglu A, Cayir A, Unal D, et al. Efficacy of L-carnitine administration on lungs of neonatal rats exposed to hyperoxia. *J Anim Vet Adv* 2011;10:1080–1088.
- Yao H, Chung S, Hwang JW, Rajendrasozhan S, Sundar IK, Dean DA, et al. SIRT1 protects against emphysema via FOXO3-mediated reduction of premature senescence in mice. *J Clin Invest* 2012;122:2032–2045.
- Cooney TP, Thurlbeck WM. The radial alveolar count method of Emery and Mithal: a reappraisal 1—postnatal lung growth. *Thorax* 1982;37:572–579.
- Cooney TP, Thurlbeck WM. The radial alveolar count method of Emery and Mithal: a reappraisal 2—intrauterine and early postnatal lung growth. *Thorax* 1982;37:580–583.
- Vallerie SN, Bornfeldt KE. Metabolic flexibility and dysfunction in cardiovascular cells. *Arterioscler Thromb Vasc Biol* 2015;35:e37–e42.
- Paumen MB, Ishida Y, Muramatsu M, Yamamoto M, Honjo T. Inhibition of carnitine palmitoyltransferase I augments sphingolipid synthesis and palmitate-induced apoptosis. *J Biol Chem* 1997;272:3324–3329.
- Henique C, Mansouri A, Fumey G, Lenoir V, Girard J, Bouillaud F, et al. Increased mitochondrial fatty acid oxidation is sufficient to protect skeletal muscle cells from palmitate-induced apoptosis. *J Biol Chem* 2010;285:36818–36827.

26. Audi SH, Jacobs ER, Zhao M, Roerig DL, Haworth ST, Clough AV. In vivo detection of hyperoxia-induced pulmonary endothelial cell death using ^{99m}Tc -duramycin. *Nucl Med Biol* 2015;42:46–52.
27. Zhang Y, Jiang G, Sauler M, Lee PJ. Lung endothelial HO-1 targeting in vivo using lentiviral miRNA regulates apoptosis and autophagy during oxidant injury. *FASEB J* 2013;27:4041–4058.
28. Zhao H, Dennery PA, Yao H. Metabolic reprogramming in the pathogenesis of chronic lung diseases, including BPD, COPD, and pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2018;314:L544–L554.
29. Medler TR, Petrusca DN, Lee PJ, Hubbard WC, Berdyshev EV, Skirball J, et al. Apoptotic sphingolipid signaling by ceramides in lung endothelial cells. *Am J Respir Cell Mol Biol* 2008;38:639–646.
30. Warner BB, Stuart LA, Papes RA, Wispe JR. Functional and pathological effects of prolonged hyperoxia in neonatal mice. *Am J Physiol* 1998;275:L110–L117.
31. Yee M, Vitiello PF, Roper JM, Staversky RJ, Wright TW, McGrath-Morrow SA, et al. Type II epithelial cells are critical target for hyperoxia-mediated impairment of postnatal lung development. *Am J Physiol Lung Cell Mol Physiol* 2006;291:L1101–L1111.
32. Das KC. Hyperoxia decreases glycolytic capacity, glycolytic reserve and oxidative phosphorylation in MLE-12 cells and inhibits complex I and II function, but not complex IV in isolated mouse lung mitochondria. *PLoS One* 2013;8:e73358.
33. Ratner V, Starkov A, Matsiukevich D, Polin RA, Ten VS. Mitochondrial dysfunction contributes to alveolar developmental arrest in hyperoxia-exposed mice. *Am J Respir Cell Mol Biol* 2009;40:511–518.
34. Ratner V, Sosunov SA, Niatsetskeya ZV, Utkina-Sosunova IV, Ten VS. Mechanical ventilation causes pulmonary mitochondrial dysfunction and delayed alveolarization in neonatal mice. *Am J Respir Cell Mol Biol* 2013;49:943–950.
35. Casals N, Zammit V, Herrero L, Fado R, Rodriguez-Rodriguez R, Serra D. Carnitine palmitoyltransferase 1C: from cognition to cancer. *Prog Lipid Res* 2016;61:134–148.
36. Marion-Letellier R, Savoye G, Ghosh S. Fatty acids, eicosanoids and PPAR γ . *Eur J Pharmacol* 2016;785:44–49.
37. Song S, Attia RR, Connaughton S, Niesen MI, Ness GC, Elam MB, et al. Peroxisome proliferator activated receptor α (PPAR α) and PPAR γ coactivator (PGC-1 α) induce carnitine palmitoyltransferase 1A (CPT-1A) via independent gene elements. *Mol Cell Endocrinol* 2010;325:54–63.
38. Setoyama D, Fujimura Y, Miura D. Metabolomics reveals that carnitine palmitoyltransferase-1 is a novel target for oxidative inactivation in human cells. *Genes Cells* 2013;18:1107–1119.
39. Tibboel J, Joza S, Reiss I, de Jongste JC, Post M. Amelioration of hyperoxia-induced lung injury using a sphingolipid-based intervention. *Eur Respir J* 2013;42:776–784.
40. Husari AW, Dbaibo GS, Bitar H, Khayat A, Panjarian S, Nasser M, et al. Apoptosis and the activity of ceramide, Bax and Bcl-2 in the lungs of neonatal rats exposed to limited and prolonged hyperoxia. *Respir Res* 2006;7:100.
41. Jia Z, Pei Z, Maiguel D, Toomer CJ, Watkins PA. The fatty acid transport protein (FATP) family: very long chain acyl-CoA synthetases or solute carriers? *J Mol Neurosci* 2007;33:25–31.
42. Syed MA, Shah D, Das P, Andersson S, Pryhuber G, Bhandari V. TREM-1 attenuates RIPK3 mediated necroptosis in hyperoxia induced lung injury in neonatal mice. *Am J Respir Cell Mol Biol* [online ahead of print] 3 Oct 2018; DOI: 10.1165/rcmb.2018-0219OC.
43. Gülçin İ. Antioxidant and antiradical activities of L-carnitine. *Life Sci* 2006;78:803–811.
44. Boyacioglu M, Turgut H, Akgulu C, Eryilmaz U, Kum C, Onbasili OA. The effect of L-carnitine on oxidative stress responses of experimental contrast-induced nephropathy in rats. *J Vet Med Sci* 2014;76:1–8.
45. Van Aerde JE. In preterm infants, does the supplementation of carnitine to parenteral nutrition improve the following clinical outcomes: growth, lipid metabolism and apneic spells? Part B: clinical commentary. *Paediatr Child Health* 2004;9:573.
46. Van Aerde T. In preterm infants, does the supplementation of carnitine to parenteral nutrition improve the following clinical outcomes: growth, lipid metabolism and apneic spells? Part A: evidence-based answer and summary. *Paediatr Child Health* 2004;9:571–572.
47. Clark RH, Chace DH, Spitzer AR. Impact of L-carnitine supplementation on metabolic profiles in premature infants. *J Perinatol* 2017;37: 566–571.
48. Ozturk MA, Kardas Z, Kardas F, Gunes T, Kurtoglu S. Effects of L-carnitine supplementation on respiratory distress syndrome development and prognosis in premature infants: a single blind randomized controlled trial. *Exp Ther Med* 2016;11:1123–1127.