Amelioration of hyperoxia-induced lung injury using a sphingolipid-based intervention

Jeroen Tibboel¹,3, Stephen Joza¹,2, Irwin Reiss³, Johan C. de Jongste³, Martin Post¹,2
¹Dept. of Physiology and Experimental Medicine, Hospital for Sick Children, Toronto, Canada. ²Dept. of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Canada. ³Dept. of Pediatrics, Erasmus University Medical Center – Sophia Children’s Hospital, Rotterdam, the Netherlands

Corresponding Author:
Martin Post, PhD
The Hospital for Sick Children
555 University Avenue, McMaster Building
Toronto, ON M5G 1X8
Tel: (416) 813-6772
Fax: (416) 813-5002
e-mail: martin.post@sickkids.ca

Copyright 2012 by the European Respiratory Society.
Abstract

Aim: To characterize lung function and broncho-alveolar lavage sphingolipid profile of newborn mice during hyperoxia exposure and recovery in room air, and to examine the effect of D-sphingosine supplementation during recovery.

Methods: Newborn mice were exposed to 80% O₂ for 4 weeks and allowed to recover in room air for another 4 weeks. Lung function measurements and morphometrical analysis of lung tissue were performed and BAL fluid was collected during hyperoxia and recovery with and without D-sphingosine supplementation.

Results: Hyperoxia exposure altered lung function, which partially recovered in room air. Lungs had fewer and enlarged alveoli which persisted during recovery. Multiple sphingolipids were significantly increased after hyperoxia. Ceramides were increased after 2 weeks of recovery, but normalized to control values after 4 weeks. Addition of D-sphingosine during the first 5 days of recovery accelerated the normalization of ceramide levels at 2 weeks and partially reversed the hyperoxia-induced increase in alveolar size and arrest in alveolarization at 4 weeks.

Conclusion: Exposure of newborn mice to hyperoxia caused restrictive and obstructive lung function changes that partially recovered in room air, while alveolar morphology remained abnormal. Hyperoxia increased ceramide levels, with normalization after recovery. D-sphingosine addition during recovery reduced ceramide levels and ameliorated hyperoxia-induced alveolar arrest.
Introduction

Respiratory Distress Syndrome (RDS) in preterm infants is often managed by mechanical ventilation and supplemental oxygen. This occurs in the setting of an immature, surfactant-deficient lung, devoid of anti-oxidant defenses. A subset of survivors develop Bronchopulmonary Dysplasia (BPD), originally characterized by parenchymal fibrosis, edema, vascular changes and persistent inflammation. Advances in neonatal care, including exogenous surfactant, prenatal steroids, better nutritional management and new modes of ventilation have improved survival of infants with very low birthweight (VLBW). However, these VLBW infants are prone to develop “new BPD” with fewer and larger alveoli as a result of interrupted septation and abnormal vascular organization. Even though progress has been made in the management of infants with BPD, current treatment remains symptomatic.

Studies looking at the hyperoxia-induced model of BPD are numerous, but to this date no studies have looked at the influence of sphingolipids in this model. Sphingolipids are important structure-bearing constituents of the cell membrane which also function as regulatory molecules of cell proliferation and cell death, endothelial barrier function, angiogenesis, and immune response. Altered sphingolipid levels have been found in a variety of diseases, such as atherosclerosis, chronic heart failure, asthma, diabetes, sepsis, cystic fibrosis and COPD. Two sphingolipids, ceramide and sphingosine-1-phosphate determine the pro- and anti-apoptotic balance. In this rheostat, ceramide stimulates apoptosis and cell cycle arrest, and sphingosine-1-phosphate stimulates cell survival and proliferation. Increased apoptosis has been found in epithelial cells of BPD patients and in animal models of BPD and total lung ceramide levels are upregulated in
hyperoxia-exposed neonatal rats\textsuperscript{10}. To date, no detailed analysis of the sphingolipid metabolome in the hyperoxia-induced BPD model\textsuperscript{11} has been reported. Furthermore, little is known about the evolution of lung injury in murine models of BPD. Mice exposed to \textgreater 60\% oxygen during the first days of life showed lung function abnormalities that persisted until 67 weeks of age\textsuperscript{12}, but the precise evolution of lung function during the acute phase of hyperoxic injury, followed by recovery in room air, has not been examined. The objective of our study was to determine the evolution of lung function and sphingolipids in hyperoxia-exposed newborn mice during both the acute hyperoxic phase and recovery in room-air. In addition, we studied the effect of pharmacological intervention on hyperoxia-induced lung damage by giving D-sphingosine supplementation during the recovery phase.

**Materials and Methods**

**Animals:** C57-Bl6 mice were obtained from Charles River (St. Constant, Quebec, Canada) and animal studies were conducted according to criteria established by the Canadian Council for Animal Care and approved by the Animal Care and Use Committee of the Hospital for Sick Children, Toronto, ON, Canada.

**Hyperoxia-induced BPD:** We used a modified hyperoxia model as described by Warner et al.\textsuperscript{13}. Two pathogen-free timed pregnant C57/BL6 mice gave birth in room air. At postnatal day 1, the mothers and their pups were placed in paired Oxycycler exposure chambers (Biospherix Ltd, Lacona, NY). Litters were exposed to hyperoxia (80\% O\textsubscript{2}) or room air for 28 days. Litter sizes were kept at 6 pups in both the hyperoxia and room air groups. Dams were switched daily. Oxygen exposure was maintained for 28 days, after which the mice
had gained enough weight to perform lung function measurements. Hyperoxia-treated litters were then exposed to room air for 7, 14 or 28 days. Room air control litters were treated in the same manner. D-sphingosine (Sigma-Aldrich) was administered (1.25 µg per g bodyweight, ip) daily to a subset of hyperoxia-treated litters during the first 5 days of recovery in room air. Control animals were treated with D-sphingosine in the same manner. At various times during these conditions, lung function was assessed, bronchoalveolar lavage (BAL) was performed, and tissues processed for histology (Fig. E1 of the online supplement).

*Lung function measurements:* Twenty-eight days after the start of hyperoxia, the Flexivent rodent ventilator (Scireq, Montreal, Canada) was used to assess lung function as described in detail in the online supplement. For the long-term follow-up experiments of hyperoxic mice in room air, mice were subjected to lung function measurements at postnatal day 35, 42 and 56.

*Bronchoalveolar lavage:* Lungs were infused through the endotracheal tube with 600 µl sterile saline, followed by withdrawal and re-infusion two more times\textsuperscript{14,15}. The collected fluid was centrifuged at 1400g for 8 min. The supernatant was collected in a siliconized eppendorf tube and stored at -80°C for mass spectrometry analysis.

*Measurement of sphingolipids:* Sphingolipid levels in BAL were measured as described in the online supplement.
Histology and morphometry: Following lung function measurements, the lungs were pressure inflated and processed for histology and morphometry as described in the online supplement.

Statistics: All values are presented as mean ± standard error of the mean, assuming a normal distribution (Sigmaplot version 11 for Windows). Differences were assessed by Student’s t test or, for comparison of three or more groups, by one-way analysis of variance followed by Tukey HSD comparison. Significance was inferred as p<0.05.

Results

Lung function measurements: In newborn mice exposed to 28 days of hyperoxia, Flexivent measurements revealed a significant increase in resistance and reduced compliance, total lung capacity, and forced vital capacity compared to controls (Fig. 1A, Table E1 of the online supplement). Long term follow-up of hyperoxia-exposed mice showed recovery of lung function parameters to control levels after 1 week of recovery in room air. Total lung capacity, static and dynamic compliance took 2 weeks to normalize (Fig. 1B). However, forced expirations remained abnormal during the 4 weeks of recovery in room air. Flow-volume curves at 0 and 4 weeks of recovery showed persistent obstructive abnormalities with improvement of volume (Fig. E2 and Table E1 of the online supplement). Addition of D-sphingosine during recovery in room air did not significantly improve lung function.

Sphingolipid measurements: Sphingolipid levels in BAL by LC-MS/MS are shown in Tables E2-E4 of the online supplement. A significant increase in multiple sphingomyelin and ceramide species was found after 2 and 4 weeks of hyperoxia exposure (Fig. 2, Table E2
of the online supplement). In particular, long chain ceramides (Cer16:0 and Cer18:0) and very long chain ceramides (Cer24:0 and Cer24:1) were greatly elevated (Fig. 2, Table E2 of the online supplement). Although small, significant increases were also found in sphinganine and a few dihydroceramides, both precursors of ceramides formed by the de novo pathway. All four major sphingomyelins (SM16:0, SM18:0, SM24:0, SM24:1) showed 2-4 fold increases after hyperoxia exposure. Following 2 weeks of recovery in room air, all sphingomyelin species in the BAL of the hyperoxia-treated group returned to control levels, but many ceramide species were still significantly elevated compared to controls (Fig. 2, Tables E3 and E4 of the online supplement). Treatment with D-sphingosine accelerated the normalization of ceramides in room-air (Fig. 3, Tables E3 and E4 of the online supplement).

**Histological analysis:** Compared to room air controls (Fig. 4A), histological sections of hyperoxia-exposed lungs (Fig. 4B) showed a homogeneous pattern of decreased alveolar septation, compared to room air-exposed newborn mice. Morphometry demonstrated a significant increase in mean linear intercept, a reduction in radial alveolar count and in the number of alveoli. Lung sections of mice exposed to 4 weeks of hyperoxia and allowed to recover in room air (Fig. 4C) showed fewer and enlarged alveoli when compared to room air controls. No significant difference in mean linear intercept and radial alveolar count was noted at 1, 2 and 4 (Fig. 4E) weeks of recovery in room air. Interestingly, when compared to hyperoxia-exposed non-treated mice (Fig. 5A), mice treated with D-sphingosine for 5 days immediately after hyperoxia exposure (Fig. 5B) had a significantly lower mean linear intercept and higher radial alveolar count (Fig. 5C) at 2 weeks of recovery in room air.
Mean linear intercept and radial alveolar counts of hyperoxia-exposed and D-sphingosine-treated mice did not return to control levels.

**Discussion**

In this study we examined BAL sphingolipid levels, lung function and histology in neonatal mice during hyperoxia exposure and after recovery in normoxia. Our findings showed that 4 weeks of exposure to hyperoxia causes alveolar damage and obstructive lung function abnormalities, together with increased sphingolipid levels, including ceramides. We demonstrated normalization of ceramides and partial improvement of lung function, but found no improvement in histological abnormalities within 4 weeks of recovery in room air. D-sphingosine supplementation during normoxic recovery accelerated ceramide normalization and improved hyperoxia-induced alveolar arrest, but did not affect lung function.

In our hyperoxia model, the increased airway resistance, decreased mean forced expiratory flow and forced expiratory volume could be caused by airway remodelling as a consequence of ongoing inflammation. The observed changes in lung function are similar to those reported for newborn mice after 14 days of 85% oxygen exposure. Another possible explanation for the lung function changes is a reduced number of alveolar attachments leading to decreased radial traction, which would result in airway narrowing and earlier airway collapse during expiration. Prolonged exposure to hyperoxia in newborn mice caused an arrest in alveolarization, thereby delaying the formation of secondary septa, reducing alveolar number and increasing alveolar wall thickness. In the present study, this decrease in alveolarization was reflected in the radial alveolar counts.
At the end of 4 weeks hyperoxia exposure, we observed a combined obstructive and restrictive pattern that shifted to an obstructive pattern at the end of 4 weeks recovery in room air. The persistent abnormal forced expiration measurements, as well as the abnormal morphology after 4 weeks in room air indicate incomplete recovery and a permanently altered lung structure. Velten et al. studied newborn mice exposed to 85% O₂ for 14 days, which recovered for 14 days in room air, and these newborns developed increased resistance at 14 days, followed by an increased compliance of the lungs at 28 days16. Yee et al. noted a reduced compliance after 4 days of 100% O₂ exposure in newborn mice, and an increase in compliance at 67 weeks of age12. They speculated that the transient decrease in compliance during the acute phase of hyperoxic injury was due to pulmonary edema, impaired surfactant protein-C production and pro-inflammatory free oxygen radicals. Both studies seemed to indicate that the response of the newborn lung to hyperoxia can be divided into two stages: acute lung injury characterized by a less compliant lung, and chronic lung injury, characterized by a more compliant lung. Our measurements after 4 weeks of recovery could reflect an intermediary phase, between acute and long term hyperoxia-induced lung injury, in which edema and inflammation decreases, and the lung becomes more compliant. We observed partial recovery of lung function, but no apparent recovery in morphology after 4 weeks of recovery in room air. This emphasizes the importance of obtaining both structural and physiological data when tracking changes in disease state or monitoring the effects of an intervention. The persistence of morphological abnormalities in our mouse model is in line with the abnormalities seen in adult survivors of BPD18,19, and the persistent abnormal lung function parameters are consistent with results of lung function measurements performed in human
survivors of BPD\textsuperscript{20,21}, making this model a good choice for intervention studies aimed at repairing hyperoxia-induced lung damage.

Ceramides play an important role in apoptosis and lung inflammation\textsuperscript{22,23}. Kolliputti et al. recently showed that ceramides regulate endothelial permeability and the inflammatory process caused by hyperoxia in adult mice\textsuperscript{24}. Ceramides have long been known to mediate acute lung injury by increasing alveolar permeability and proinflammatory cytokine production\textsuperscript{25}, but no studies have reported detailed ceramide profiles in neonatal hyperoxia-induced lung damage and during recovery in room air. Our study shows that multiple ceramide and sphingomyelin species are increased during exposure of neonatal pups to hyperoxia. Long chain ceramides (Cer16:0, Cer18:0, Cer20:0) have anti-proliferative and pro-apoptotic effects\textsuperscript{26}, whereas very long-chain ceramides (Cer22:0, Cer24:0, Cer24:1) promote cell proliferation\textsuperscript{27}. We observed an 8-fold increase in long chain Cer16:0 during hyperoxia, suggesting a role for increased apoptosis via ceramide signalling in this hyperoxia model. Indeed, increased apoptosis has been observed in the lungs of mice and rats exposed to hyperoxia\textsuperscript{28–30}, similar to that noted in autopsy lungs of BPD patients\textsuperscript{31}. Very long chain(24:0, 24:1) ceramides demonstrated a 2-3 fold increase in our model, suggesting a role for proliferation, possibly of smooth muscle cells, which have been shown to proliferate in rat and mouse models of hyperoxia-induced BPD\textsuperscript{12,29,32}. Both ceramides and sphingomyelin levels were increased, suggesting that the increase of ceramides is not due to breakdown of sphingomyelin via sphingomyelinase. The increased sphingosine levels (Table E2 of the online supplement) may indicate increased ceramide formation via the salvage pathway, although the general increase in most sphingolipids argued against activation of that pathway. Based on the observation that both sphinganine...
and dihydroceramides were increased (Table E2 of the online supplement), we hypothesize that hyperoxia activates the pulmonary *de novo* pathway of ceramide synthesis in the newborn. Further studies focusing on the activity of the rate-limiting enzyme serine palmitoyltransferase would be needed to confirm the increase in the de novo synthesis.

Long chain ceramides have been shown to increase the permeability of endothelium\(^{33}\), which causes endothelial leakage\(^ {34}\) which might contribute to the reduced compliance of the lungs seen in our hyperoxia model. We postulate that reduced forced expiratory flows might be related to structural changes, such as a reduced number of alveolar attachments, which remained low during air recovery. In contrast, resistance and compliance may be determined by reversible factors in the lung, such as inflammation and endothelial leakage. Our study shows that sphingolipid levels, including ceramides, returned to normal within 4 weeks of recovery in room air, corresponding to the time course of lung function changes. It may well be that when the primary stress factor (hyperoxia) disappears during recovery in room air, this leads to a decrease in sphingolipids and also in lung inflammation and edema, resulting in partial recovery of lung function. However, no additional effect of D-sphingosine supplementation on partial lung function recovery was observed. Note that sphingolipid levels were measured in BAL, providing a good estimate of the sphingolipid metabolism in the broncho-alveolar compartment, but they may not represent sphingolipid metabolism in the interstitial and vascular compartment. Further research into the complexity of the interaction between these different compartments is warranted.
Treatment with D-sphingosine significantly decreased ceramide levels after 1 and 2 weeks of recovery in room air. Lung morphometry showed an improvement of mean linear intercept and radial alveolar count after 4 weeks of recovery in the D-sphingosine-treated animals compared to untreated control mice. These findings suggest that D-sphingosine supplementation had a beneficial effect on the alveolar injury caused by hyperoxia exposure, and that lower ceramide levels might be responsible for this improvement in alveolar histology. A reduction in the amount of apoptosis is the most likely mechanism by which D-sphingosine acts, but further experiments are needed to confirm this hypothesis.

No obvious beneficial effects on lung function were found, which could well be due to imperfect agreement between measures of lung structure and function. The incomplete recovery of histology, sphingolipid levels and lung function after D-sphingosine treatment and the absence of a significant difference in these parameters between hyperoxic mice with and without D-sphingosine might be due to dose and/or timing of the supplementation, or might have been missed due to relatively small numbers of observations. Our D-sphingosine dose was based on a study by Diab et al. who showed that adult mice injected with D-sphingosine at 20µg per mouse for 5 consecutive days had significantly increased sphingosine-1-phosphate (a pro-survival sphingolipid) levels in homogenized lung tissue at day 5 after the start of administration. The administration of D-sphingosine in our model started after hyperoxia exposure and lasted for 5 days. It is possible that earlier or longer might yield better results.

One could argue that prevention of damage by adding D-sphingosine at an earlier time point would be preferable. We decided for another approach, as currently there are no reliable predictors to indicate which infant will develop BPD, making it difficult to target
preventative treatment to those who might benefit. Therefore, we supplemented D-sphingosine only during the recovery phase to see if this might stimulate repair of hyperoxia-induced damage.

In summary, we demonstrated that hyperoxia in the newborn mouse lung caused a transient increase of sphingolipids, including ceramides, which returned to normal during recovery in room air, while histology remained abnormal. The time course of sphingolipid reduction during recovery matched the normalization of airway resistance and compliance. Importantly, D-sphingosine supplementation during recovery accelerated the normalisation of ceramides, and significantly improved hyperoxia-induced lung damage. We propose that D-sphingosine and other inhibitors of bioactive sphingolipids should be studied further for their potential to reduce lung damage in human preterm infants who require neonatal oxygen treatment. The ultimate goal would be to develop a new strategy to prevent or correct bronchopulmonary dysplasia.

Acknowledgements

This work was supported by an operating grant (MOP-86472) from the Canadian Institute of Health Research and infrastructure grants (CCURE, CSCCD) from the Canadian Foundation for Innovation. Martin Post is the holder of a Canadian Research Chair in Fetal, Neonatal and Maternal Health.
References


Figure legends

Figure 1: Lung function measurements after 4 weeks of hyperoxia-exposure (A) and during recovery in room air (B) of hyperoxia-exposed mice and room air controls (n=12 per group for the 28 days' timepoint except for MEF and PEF where n=8. n=6 for the 35, 42 and 56 days' timepoints). Data are expressed as mean ± SEM. * = p<0.05, ** = p<0.001.

Figure 2: Representative sphingolipid measurements performed during hyperoxia treatment and recovery in room air. Results represent a total of 12 mice per group for the 14 and 28 days' time-points in hyperoxia and 3 mice per group for the 35, 42 and 56 days' time-points of recovery in room-air Cer: ceramide, SM: sphingomyelin. * = p<0.05, ** = p<0.001, when compared to room air treated mice at the same time-point.
Figure 3: Representative sphingolipid measurements after 1 and 2 weeks recovery in room-air with and without D-sphingosine (DS) treatment results represent a total of 12 mice per group for 28 days’ time-point in hyperoxia and 3 mice per group for the 35, 42 and 56 days’ time-points of recovery in room-air and the D-sphingosine supplementation experiments. Cer: ceramide, SM: sphingomyelin. Grey * represent a significant change in hyperoxia treated mice when compared to normoxia treated mice at the same time-point. Black * represent a significant change in hyperoxia + DS treated mice when compared to normoxia + DS treated mice at the same time-point. * = p<0.05, ** = p<0.001.
Figure 4: Effect of hyperoxia and subsequent room air recovery on lung histology.

Representative histological sections of room air-exposed newborn mice (A), hyperoxia-treated newborn mice after 4 weeks of exposure (B) and hyperoxia-treated newborn mice that were allowed to recover for another 4 weeks in room-air (C) are shown. Sections were stained with hematoxylin and eosin. Morphometry results (D) are expressed as mean ± SEM for both room air and hyperoxia groups for mean linear intercept (n=8), radial alveolar counts (n=8), and alveolar number (n=4). Morphometry results for 4 weeks of recovery in room-air (E) are expressed as mean ± SEM for mean linear intercept (n=4) and radial alveolar count (n=3). * = p<0.05 ** = p<0.001.
Figure 5: Effect of D-sphingosine supplementation on lung histology. Representative histological sections are shown of lungs after 4 weeks of hyperoxia exposure followed by 2 weeks of recovery in room air (A), and 4 weeks of hyperoxia exposure followed by 2 weeks of recovery in room air with D-sphingosine supplementation (B). Sections were stained with hematoxylin and eosin and high power inserts were taken at 200x magnification. Morphometry results (C) represent a total of 3 mice per group and are expressed as mean ± SEM for all groups. * = p<0.05.