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Hyperoxia induces paracellular leak and alters claudin expression by neonatal alveolar epithelial cells

Shilpa Vyas-Read^{1,2}, Rachel J. Vance³, Wenyi Wang¹, Jennifer Colvocoresses-Dodds¹, Lou Ann S. Brown^{1,2}, and Michael Koval^{2,3,4}

¹Emory University School of Medicine, Department of Pediatrics, Division of Neonatology, Atlanta, GA

²Emory+Children's Center for Cystic Fibrosis and Airways Disease Research, Emory University School of Medicine and Children's Healthcare of Atlanta, Atlanta, GA

³Emory University School of Medicine, Department of Medicine, Division of Pulmonary, Allergy, Critical Care and Sleep Medicine, Atlanta, GA

⁴Emory University School of Medicine, Department of Cell Biology, Atlanta, GA

Abstract

Background—Premature neonates frequently require oxygen supplementation as a therapeutic intervention that, while necessary, also exposes the lung to significant oxidant stress. We hypothesized that hyperoxia has a deleterious effect on alveolar epithelial barrier function rendering the neonatal lung susceptible to injury and/or bronchopulmonary dysplasia (BPD).

Materials and Methods—We examined the effects of exposure to 85% oxygen on neonatal rat alveolar barrier function *in vitro* and *in vivo*. Whole lung was measured using wet-to-dry weight ratios and bronchoalveolar lavage protein content and cultured primary neonatal alveolar epithelial cells (AECs) were measured using transepithelial electrical resistance (TEER) and paracellular flux measurements. Expression of claudin-family tight junction proteins, E-cadherin and the Snail transcription factor SNAI1 were measured by Q-PCR, immunoblot and confocal immunofluorescence microscopy.

Results—Cultured neonatal AECs exposed to 85% oxygen showed impaired barrier function. This oxygen-induced increase in paracellular leak was associated with altered claudin expression, where claudin-3 and claudin-18 were downregulated at both the mRNA and protein level. Claudin-4 and claudin-5 mRNA were also decreased, although protein expression of these claudins was largely maintained. Lung alveolarization and barrier function *in vivo* were impaired in response to hyperoxia. Oxygen exposure also significantly decreased E-cadherin expression and induced expression of the SNAI1 transcription factor *in vivo* and *in vitro*.

Corresponding Author: Michael Koval, PhD, Emory University School of Medicine, Division of Pulmonary, Allergy, Critical Care and Sleep Medicine, 205 Whitehead Building, 615 Michael Street, Atlanta, GA 30322, mhkoval@emory.edu.

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Conclusions—These data support a model in which hyperoxia has a direct impact on alveolar tight and adherens junctions to impair barrier function. Strategies to antagonize the effects of high oxygen on alveolar junctions may potentially reverse this deleterious effect.

Keywords

Bronchopulmonary Dysplasia (BPD); Respiratory Distress Syndrome & ARDS; Airway & Lung Cell Biology; Tight Junction; Lung Development; Alveolar Epithelium

Introduction

Despite improvements in healthcare for extremely premature infants, bronchopulmonary dysplasia continues to affect 25–42% of infants born at extremely low birth weights ¹. Exposure to high oxygen concentrations in the course of clinical care is a life-saving measure for extremely premature infants, but it contributes to pulmonary edema and interstitial fluid accumulation that ultimately impairs gas exchange ². Pulmonary edema can result from impaired microvascular filtration and endothelial leak, disruptions in the alveolar epithelial barrier, deficiencies in lung surfactant, and limitations in normal mechanisms of fluid clearance ³. At the level of the alveolar epithelial cell, multiple mechanisms regulate barrier integrity and prevent the leakage of fluid and molecules into the alveolar space. Along with ion-mediated and endocytic transport mechanisms ⁴, tight junctional proteins are key regulators of alveolar epithelial barrier function ⁵.

Located at type I-type I cell interfaces and type I-type II cell interfaces, tight junctions regulate paracellular fluid permeability due to the expression and function of a tetraspan transmembrane family of proteins known as claudins ^{6,7}. The predominant claudins expressed by alveolar epithelial cells are claudin-3, -4, and -18, with claudin-5 detected at lower levels ⁸. The role of each of these claudins in the regulation of paracellular permeability depends on its expression, localization, and interaction with underlying scaffolding proteins. Claudin-3 is largely expressed by type II cells, and increases in its abundance promote alveolar “leak” and edema ^{9,10}. In contrast, overexpression of claudin-4 in alveolar epithelial cells improves transepithelial resistance and barrier integrity, and pathologic specimens from human lungs have shown claudin-4 to be positively associated with alveolar fluid clearance ^{9,10}. Similar to claudin-4, claudin-18 is also “pore-sealing” and serves to limit barrier permeability. By contrast alveolar epithelial expression of claudin-5 is associated with impaired alveolar barrier function, most notably in alcoholic lung syndrome ^{11,12}.

The expression of claudins and their role in pulmonary edema in the developing lung has been characterized in some investigations in human specimens and neonatal animal models. In culture, human fetal lung cells transitioning to a type II cell phenotype have been shown to alter the expression and localization of multiple claudins, a process that enhances tight junction integrity ¹³. In pathological specimens, transcript and expression of claudin-3 and -4 have been shown to peak during the cannicular stages of lung development, whereas alveolar epithelial cell claudin-5 expression, detected in early embryological stages,

decreases by 28 weeks gestation ¹⁴. Similarly, claudin-18 has recently been shown to play a role in postnatal alveolar development in animal models ¹⁵.

Hyperoxia contributes to pulmonary edema and alveolar arrest in the lungs of premature infants ¹⁶. In adult mice, exposure to over 95% oxygen increases bronchoalveolar cellular counts, lung wet/dry ratio, and claudin-4 levels, although claudin-18 mRNA is decreased ^{17,18}. In other epithelial cell types, oxidative stress is associated with increased claudin-3 and permeability, and altered claudin-4 localization through a MAP kinase-dependent mechanism ^{19,20}. Although the ontogeny of claudin expression is being investigated, hyperoxia has been shown recently to impair the tight junctional integrity of cultured neonatal alveolar epithelial cells ²¹. In that study, claudin-4, ZO-1 and occludin were found to be downregulated after 72 hours of hyperoxia and barrier function was simultaneously impaired. Whether other claudins are affected by hyperoxia was not determined, nor was the effect of hyperoxia on neonatal lung function or morphology measured.

In this study, we examined the effects of hyperoxia on paracellular permeability in cultured primary neonatal alveolar epithelial cells and in the neonatal lung *in vivo*. Hyperoxia significantly impaired alveolar barrier function and was associated with significant alteration of alveolar epithelial claudins at the mRNA and protein level.

Methods

Hyperoxia exposure

All animal procedures were performed with the approval of the Institutional Animal Care and Use Committee at Emory University and performed in accordance with NIH guidelines. Two groups of neonatal Sprague-Dawley rat pups were housed in identical Plexiglass chambers with either normal atmospheric oxygen (20.8%, referred to as control conditions) or 85% hyperoxia (H85) conditions starting on day of life 3 (Proox model 110; Biospherix, Redfield, NY). A continuous oxygen monitor was used to assess oxygen concentrations and animals were exposed to control conditions or hyperoxia for up to 14 days. For the duration of the treatment, a lactating mother was housed with the control and hyperoxia-exposed animals, and every 24 hours, the mothers were switched between room air and hyperoxia to avoid toxicity to the mothers and to allow continuation of lactation. All animals had 12-hour light/dark cycles and free access to water and a standard diet.

At the end of the treatment protocol, neonatal rat litters from each group were deeply sedated with ketamine prior to sternotomy and tracheal cannulation. Cannulated lungs were lavaged with 1 mL sterile PBS 3 times to remove cellular debris and macrophages from the airspace. The pulmonary artery was then threaded with a 30- gauge catheter, and gently perfused with PBS to remove red blood cells. Following perfusion, the lungs were removed as described below and animals were euthanized by aortic transection and exsanguination.

Measurement of lung leak *in vivo*

Bronchoalveolar protein—At the conclusion of the experimental exposure, the lungs were removed *en bloc*, and a lung lavage was performed as described above using routine procedures in our laboratory ²². Aspirated fluid from the first lavage was centrifuged at

1,500 g for 10 min, and the supernatant was stored at -80°C for subsequent protein analysis. Protein levels in the lung lavage fluid were determined using the Bradford reagent assay and compared to a bovine serum albumin standard curve (Bio-Rad, DC protein assay). Total protein was calculated as the product of the concentration of protein and the amount of fluid recovered.

Lung wet/dry—Immediately following lung perfusion, the right mainstem bronchus was tied with silk suture and removed. The removed right lung was then measured (wet lung weight) prior to placement in an 80°C oven for 72 hours for drying. The dried lung weight was determined and a ratio between the wet and dry lung weights was recorded relative to animal weight at the time of sacrifice.

Lung immunohistochemistry—Neonatal Sprague-Dawley rat pups, treated with normal atmospheric oxygen or hyperoxia for 7 days, had their tracheas cannulated, and their lungs were lavaged with 3 ml of PBS to remove cells in the airways and alveolar space. The lungs were then perfused through right ventricular cannulation, and instilled with 2–3 mL optimal cutting temperature compound (OCT) fixative. Animal lungs were snap-frozen in liquid nitrogen and stored in -80°C . Before use, 10- μm sections of lungs were made and stained with H&E. Images were acquired using Olympus BX41 microscopy with a UPlan FLN $\times 10/0.3$ lens. Radial alveolar count and mean linear intercept were measured from H & E stained sections as previously described ²³.

***In vitro* alveolar epithelial evaluation**

Neonatal alveolar epithelial cell isolation—One litter of neonatal Sprague-Dawley rats were sedated, the trachea was cannulated, and type II AECs were isolated largely following previously determined techniques that are utilized in our laboratory ^{24,25}. Following lung removal and disaggregation of the lungs in dispase (1unit/mL), the cellular suspension was filtered through a 100 micron filter twice, a 40 micron filter twice, a 20 micron filter once, and a 10 micron filter once. The resultant cellular suspension was then incubated with biotinylated antibodies for vimentin and CD 16/32 and passed over streptavidin-coated beads for removal of macrophages and fibroblasts (CELLection Biotin Binder Kit, Life Technologies). Purified neonatal AECs were maintained on permeable Transwell membranes (Corning, Tewksbury, MA) for 48 hours with *cis*-hydroxyproline (100 $\mu\text{g/mL}$) without changing media to allow for cellular adherence and removal of fibroblasts. Following 2 days, AECs were treated with DMEM/F12 Nutrient Mix media containing 0.25% BSA, 10 nM hydrocortisone, 50 μL Primocin antibiotic, and 0.5 $\mu\text{L/mL}$ insulin-transferrin-sodium-selenite supplementation, and media was changed daily.

Transepithelial electrical resistance (TEER)—Following isolation, neonatal type II AECs were cultured on permeable Transwell membranes. TEER was measured at the cellular surface every 24 hours using a sterilized epithelial voltohmmeter (World Precision Instruments, Sarasota, FL). As tight junctions between cells formed, a rapid increase and stabilization in TEER measurements was seen between 48 and 72 hours of culture, as previously described ¹³ (6). On the day of TEER stabilization, AEC cultures were treated with either 5% CO_2 -95% normal atmospheric oxygen, or hyperoxia (5% CO_2 -85% O_2 - 10%

normal atmospheric oxygen) for 3 days. An oxygen analyzer (Pacifitech Industries, Los Angeles, CA) was used to confirm the O₂ concentration in the incubator daily. Culture dishes were covered with a sterile, gas-permeable membrane (Diversified Biotech, Boston, MA) to allow equilibration of oxygen at the cellular surface. Media in control and hyperoxia-treated cells were changed every 24–48 h.

Solute permeability—Paracellular permeability was evaluated by quantification of fluorophore diffusion across a cell monolayer²⁶. The relative abundance of carboxyfluorescein (0.37 kDa) and Texas Red Dextran (10 kDa) in the media of the bottom chamber was determined every 24 hours using a Biotek Multimode microplate reader (Biotek, Winooski, VT).

Claudin expression—Following treatment, control and hyperoxia-treated, cultured neonatal AECs had mRNA extracted using a commercially available kit (RNAEasy; Qiagen, Valencia, CA). *TaqMan* gene expression assays were used to evaluate genes of interest claudin-3 (Rn00581751_s1), claudin-4 (Rn01196224_s1), claudin-5 (Rn01753146_s1), claudin-18 (Rn01447445_m1), E-cadherin (Rn00580109_m1) and SNAI1 (Rn00441533_g1) (Applied Biosystems, Grand Island, NY). All cycle threshold results were normalized to the average of three housekeeping genes: β -microglobulin (Rn00560865_m1), β -actin (Rn00667869_m1), and ribosomal protein Lg (Rn00821065_g1). Fold change was calculated by determination of the 2^{-CT} between control and hyperoxia-exposed cells.

Another group of control and hyperoxia-treated cultured neonatal AECs had protein lysate concentrations determined using the DC protein assay (Bio-Rad, Hercules, CA). Equal amounts of protein from control and treated samples were resolved on a 4%–20% Mini-Protean TGX precast gels (Bio-Rad) and electrophoretically transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked for 2 h at room temperature with 5% nonfat dry milk in TBS with 0.2% Tween 20 and incubated with primary antibodies overnight at 4°C. Primary antibodies for immunoblot experiments include the following: rabbit polyclonal claudin-3, rabbit polyclonal claudin-4, rabbit polyclonal claudin-5, and mouse monoclonal claudin-18 (all from ThermoFisher). Additionally, a mouse monoclonal E-cadherin antibody and a mouse monoclonal SNAI1 antibody was used (BD Biosciences, San Jose, CA; Cell Signaling Technology, Danvers, MA, respectively). Secondary anti-mouse or anti-rabbit IgG antibodies conjugated to horseradish peroxidase were applied for 1 h, and antibody complexes were visualized using enhanced chemiluminescence (ECL substrate; ThermoFisher, Rockford, IL). GAPDH was measured as a loading control, densitometric analyses were performed, and protein abundance was normalized to GAPDH expression.

For evaluation of claudin expression following *in vivo* treatment, immunoblots were performed on whole lung cellular extracts from neonatal animals receiving room air or hyperoxia treatment for 7 days. Primary antibodies utilized were the following: rabbit anti-claudin-3, rabbit anti-claudin-4, rabbit anti-claudin-5, rabbit anti-claudin-18, and mouse anti-actin (Thermo Fisher). The secondary antibodies used were goat anti-rabbit IgG IRDye 800CW and goat anti-mouse IRDye 680RD IgG (LI-COR). For band detection, fluorescence

imaging was used with the Odyssey Classic Imager (LI-COR). Band quantification was done using Image Studio and was calculated relative to an actin loading control ¹¹.

Immunofluorescence—Following experimental treatment, cultured neonatal AECs were fixed in an ice-cold acetone/methanol solution, washed, permeabilized with 0.2% Triton X-100, and blocked with antibody-reducing diluent for 20 min (Dako, Carpinteria, CA). Cells were then incubated with primary antibodies overnight at 4°C followed by 1-h incubation in secondary antibodies. Primary antibodies used for immunofluorescence were then same as for immunoblot experiments (above). Additionally, mouse monoclonal lamellar body protein and mouse monoclonal SNAIL antibodies were utilized (Abcam and Cell Signaling Technology, respectively). Secondary antibodies were anti-mouse/rabbit IgG conjugated to Alexa 488 or Alexa 594 (ThermoFisher). Filters with cultured AECs were then transferred to glass slides and mounted using Prolong Gold with DAPI (ThermoFisher).

Statistical Analyses

Comparisons between continuous outcomes for 2 experimental groups were performed using two-tailed paired t-test or Mann-Whitney tests. ANOVA was used for comparisons of outcome between 3 or more groups. All statistical analyses were performed using GraphPad Prism software. The statistical significance level was set at 0.05.

Results

In vitro hyperoxia exposure impaired alveolar epithelial junctional integrity

The effect of 85% hyperoxia (H85) on cells cultured on Transwell permeable supports was assessed using type II alveolar epithelial cells (AECs) isolated from neonatal 3-day old Sprague-Dawley rats. The purity of neonatal AECs following isolation exceeded 95%, as assessed by lamellar body protein immunofluorescence (Figure 1A). Immunoblots of lysates from cultured primary cells demonstrated the expression of thyroid transcription factor-1 (TTF-1) and pro-surfactant C (pro-SPC), confirming that the isolated AECs had type II characteristics (Figure 1B). Transepithelial electrical resistance (TEER) was measured daily across cells on Transwells cultured in room air for 3–5 days until it stabilized at ~400 Ohms/cm². Some cells were then switched to an H85 incubator. At the initiation of the experiment, AECs to be treated under control or H85 conditions had comparable TEER measurements, averaging 441 ± 97 Ohms/cm² in aggregate. After two days of additional incubation, control AECs retained barrier function with TEER of 427.6 ± 98.4 Ohms/cm², but H85-treated cells had a 50% decrease in TEER (213.2 ± 45 Ohms/cm²), indicating a deleterious effect of hyperoxia on AEC barrier function (Figure 1C).

To determine if changes in TEER induced by hyperoxia were accompanied by paracellular leakage, neonatal AECs cultured under normal ambient room air conditions or H85 were labeled on the apical surface with fluorescent tracers and the percentage of tracer transferred to the basolateral aspect of AECs was determined. There was increased detection of both carboxyfluorescein (0.37 kDa) and Texas-Red Dextran (10 kDa) on the basolateral aspect of neonatal AECs indicating increased paracellular flux in response to hyperoxia. On day 2 of treatment, H85 increased carboxyfluorescein flux 3.08 ± 0.57 -fold over control cells

($p=0.002$, Figure 1E). Large molecule flux was significantly increased over controls cells as well, as evidenced by a 5.9 ± 2.6 fold increase in Texas-Red Dextran leakage in AECs exposed to H85 ($p=0.03$, Figure 1F).

Hyperoxia exposure alters claudin expression

To determine if alterations in TEER and paracellular flux were accompanied by changes in tight junctional proteins, we evaluated the effects of hyperoxia on the mRNA and protein expression of claudin-3, -4, -5 and -18 that are predominantly expressed in adult AECs (Figure 2A). Neonatal AECs cultured under control conditions expressed mRNA for all four of these claudins and they were all significantly decreased in response to hyperoxia. H85 exposure decreased claudin-3 transcript levels by 45% ($p<0.05$), decreased claudin-4 mRNA by 39% ($p=0.01$), decreased claudin-5 mRNA by 30% ($p=0.007$) and decreased claudin-18 by 38% relative to control values ($p=0.04$).

In addition, at the protein level, hyperoxia significantly decreased claudin-3 to nearly half of control values ($p=0.03$, Figure 2B,C) and decreased claudin-18 protein 29% ($p=0.008$). By contrast, neonatal AEC claudin-4 protein was not significantly changed by H85 treatment ($p=0.5$). Claudin-5 protein expression also was unchanged by H85 exposure in neonatal AECs ($p=0.31$). These data suggest that hyperoxia has an impact on claudin expression at the transcriptional and translational level.

The intracellular distribution of claudins in neonatal AECs under normal ambient room air conditions and hyperoxia conditions was then investigated using immunofluorescence and confocal microscopy (Figure 2D). Similar to the immunoblots for claudin protein, a differential effect of hyperoxia exposure was detected in neonatal AECs depending on which claudin was being evaluated. Junction-associated claudin-3 was present in abundance in control neonatal AECs, but H85 treatment nearly eliminated claudin-3 expression at cellular junctions (Figure 2D, left most panel). Interestingly, claudin-4 was barely visible under normal ambient room air conditions, and was slightly more abundant at cellular junctions following H85 exposure (Figure 2D, second column from the left). A modest amount of claudin-5 was present in neonatal AECs at cellular junctions under control conditions, and its expression did not seem significantly altered by H85 (Figure 2D, second column from the right). Similar to claudin-3, claudin-18 was expressed in abundance by control neonatal cells, but its presence at cellular junctions was diminished following H85 exposure (Figure 2D, right most panel). Taken together, hyperoxia had mainly a deleterious effect on claudin localization in neonatal AECs.

To determine whether hyperoxia had effects on other classes of junctional proteins, we examined E-cadherin, an adherens junction protein, that is known to be altered by oxidants and other stresses in adult alveolar epithelial cells²⁷ and lung endothelial cells²⁸. Following 2 days of hyperoxia, E-cadherin mRNA decreased 38% from control levels (Figure 3A). Moreover, using immunofluorescence confocal microscopy, we found that neonatal AECs exposed to H85 expressed dramatically less E-cadherin when compared with cells that remained in normal ambient room air (Figure 3B). The transcription factor, SNAI1, modulates E-cadherin transcription by binding to its promoter²⁹, and its induction is associated with pulmonary fibrosis and other diseases of lung remodeling^{30,31}. We found

that neonatal AECs treated with hyperoxia had 2.9 ± 0.6 (n=3) fold more SNAIL mRNA expression (Figure 3C). By confocal microscopy, SNAIL co-localized with DAPI in hyperoxia-treated primary AECs, demonstrating its nuclear translocation (Figure 3D).

***In vivo* hyperoxia exposure alters lung morphology and increases edema**

We then examined the effects of hyperoxia on the intact neonatal lung. Figure 4A–D demonstrate representative images of H & E stained lungs from control and H85-exposed neonatal rats. Whereas control animals had thinned interstitial tissue and numerous small alveoli, H85-treated pups had fewer large alveoli with mildly thickened interstitial spaces, suggesting a defect in lung maturation (Figure 4C, D). Quantitatively, radial alveolar count decreased and mean linear intercept increased, consistent with impaired alveolarization in response to hyperoxia (Figure 4E, F).

To determine whether hyperoxia induced pulmonary edema in neonatal lungs, 3-day old neonatal Sprague-Dawley rats were continuously exposed to H85 or normal ambient room air for 7 to 14 days and lung/wet dry ratios were determined. Figure 4G shows that animal weights between control and H85-treated animals were not significantly different on day 7 or 10 of treatment (mean difference day 7 3.07, 95% CI –0.42, 6.57, day 10 1.224, 95% CI –2.98, 5.38). By day 14 of treatment, control animals had a mean weight of 41.8 grams whereas H85-treated animals had a mean weight of 33.4 grams (mean difference 8.4 grams, 95% CI 4.25, 12.6, $p < 0.05$). Both control and H85-treated animals had significant weight gain over the 7 days period, with control animals gaining 22.8 grams on average, and H85-treated animals gaining 17.5 grams on average. Although animal weights did not differ on day 7 of treatment, lung wet/dry ratio relative to animal weight was increased in H85-treated animals 25% over control animals (Figure 4H, 0.59 vs. 0.74, $p < 0.05$). Similarly, on day 14 of treatment lung wet/dry weight was increased in H85-treated animals 38% over normal ambient room air controls (0.26 vs. 0.36, $p < 0.05$).

We then examined whether changes in lung wet/dry ratios corresponded with alterations in bronchoalveolar protein concentration. We measured total protein from neonatal rat pups on exposure days ranging from 7 to 14 (Figure 4I). Protein concentrations between groups were not different between control and H85 groups on day 7 and day 10 of treatment (data not shown). On day 14 of treatment, control animals had a bronchoalveolar lavage total protein concentration of 0.2 ± 0.08 mg/mL compared with H85-treated animals that had concentrations of 0.84 ± 0.18 mg/mL ($p = 0.016$). This increase in lung lavage protein corresponded to a 4.2 fold increase in H85-treated animals relative to controls, consistent with impaired lung barrier function in response to hyperoxia.

Since hyperoxia had effects on gross lung morphology and barrier function, we examined total lung junction proteins by immunoblot and confocal microscopy (Figure 5). Figure 5A shows immunoblot analyses of cellular lysates from the lungs of neonatal animals exposed to room air (control) or hyperoxia (85%) for 7 days. As shown in Figure 5B, there were no significant differences in claudin protein expression in response to hyperoxia-exposed neonatal animals, although the trends were consistent with results obtained from hyperoxia exposed AECs *in vitro* (Figure 2C). At the morphological level, hyperoxia had a modest effect on claudin-3, which qualitatively appeared to be distributed more throughout the

alveoli in response to hyperoxia (Figure 5C). By contrast, claudin-4 localization in control vs. hyperoxia exposed lungs was roughly equivalent (Figure 5D). Similarly, localization of claudin-18 did not appear significantly altered by hyperoxia treatment (Figure 5E).

At the total lung level, E-cadherin mRNA expression was significantly decreased after 2 days of hyperoxia (Figure 6A), although changes were not detectable by immunofluorescence (Figure 6B). We previously demonstrated that hyperoxia treatment of cultured adult AECs induces an epithelial-to-mesenchymal (EMT) phenotype²⁵. Since upregulation of the transcription factor SNAIL is a hallmark of EMT³² and represses claudin expression³³, we examined the effects of hyperoxia on SNAIL expression and localization. As shown in Figure 6C, hyperoxia significantly upregulated SNAIL at the mRNA level. Moreover, SNAIL showed increased nuclear localization in the lungs of neonatal animals exposed to hyperoxia as compared with control treated lungs (Figure 6D, arrows). This suggests activation of SNAIL in response to hyperoxia that is likely to have a negative impact on alveolar barrier function.

Discussion

Investigations in infants younger than 28 weeks gestation at birth have shown that 44% of neonates who require mechanical ventilation for over one week had “severe leaky lung syndrome,” as defined by excessive tracheal aspirate fluid, opacities on chest X-ray, and increasing ventilatory demands³⁴. Interestingly, even brief exposures to 100% oxygen results in increased lung/wet dry ratio, bronchoalveolar protein concentrations, and perivascular edema in animals models of lung injury, and antioxidant deficiency exaggerates this effect, suggesting that oxidative balance is critical in the prevention of pulmonary edema formation^{35,36}. Neonatal animals have been shown to have a greater increase in antioxidant availability, particularly AECs, in response to a hyperoxia challenge relative to adult animals^{37,38}. However, over time, when the neonatal protection against oxygen diminishes, antioxidant responses, such as Nrf-2 activity and the availability of enzymes such as superoxide dismutase, glutathione reductase and catalase, may be impaired^{35,39–41}.

Limited information is available about the effects of hyperoxia on claudin localization and expression. Recently, Xu et al.²¹ used cultured neonatal AECs to demonstrate that hyperoxia-induced increased paracellular leak was associated with decreased ZO-1, occludin and claudin-4 expression. Here we expanded this analysis by examining other alveolar claudins, notably claudin-18, as well as demonstrate that neonatal lung barrier function *in situ* is impaired in response to hyperoxia.

In AECs isolated from neonatal lungs, we showed a significant down-regulation of claudin-3 and claudin-18 expression with hyperoxia exposure, but no alteration in claudin-5 protein abundance. Claudin-3 is affected by oxidative stress, and studies in knockout animals have shown a critical role for claudin-18 in the regulation of alveolar fluid clearance, transepithelial resistance, and solute permeability^{15,42}. There also was a trend towards decreased claudin-4, and also a significant decrease in claudin-18 expression following oxygen exposure to neonatal AECs, the combination of which is likely to have an additive deleterious effect on alveolar barrier function. The effects of hyperoxia on these proteins in

the intact lung were comparable, although muted in that they only showed trends towards decreased claudin protein as opposed to significant differences. This could reflect the diversity of alveolar epithelial cell phenotype in the intact lung or heterogeneity in extent of injury. Nonetheless, hyperoxia exposure caused an increase in neonatal lung permeability similar to that measured for AECs *in vitro*.

We previously demonstrated that increasing claudin-3 expression caused an increase in paracellular permeability of adult AECs⁹. Although this is in apparent contrast to the results obtained here where decreased claudin-3 was associated with a leakier neonatal alveolar barrier, it is important to note that the role of any particular claudin in regulating tight junction permeability is context dependent. In neonatal AECs exposed to hyperoxia, changes in claudin-3 expression were accompanied by changes in expression of other claudins. This is distinct from adult AECs where claudin-3 was specifically and individually manipulated⁹.

Similarly, both claudin-5 and 18 have been shown to be decreased in animal models of lung injury and fibrosis; however, in models of lung injury involving oxidative stress from alcohol exposure, claudin-5 is increased in type II alveolar epithelial cells¹². Recently, we have found that increased claudin-5 in the context of alcoholic lung syndrome destabilizes the ability of claudin-18 to form functional tight junctions and increases alveolar leak¹¹. Moreover, a claudin-5 mimetic peptide restored AEC barrier function¹¹. In neonatal cells exposed to hyperoxia, claudin-5 protein was unchanged, but claudin-18 decreased, effectively enriching claudin-5 content in tight junctions which is known to impair alveolar barrier function¹¹. Whether claudin-5 can be targeted to promote neonatal alveolar barrier function remains to be determined.

Adolescent mice, exposed to 3 days of 90% hyperoxia, express significantly less claudin-18 mRNA than non-exposed control animals¹⁸. In one recent study, claudin-4 transcripts were upregulated in the adult mouse lung following hyperoxia exposure, and was essential for resolution of acute lung injury and prevention of pulmonary edema¹⁷. Similar to those findings, we found that hyperoxia promoted lung water, bronchoalveolar protein and morphologic changes in neonatal rats relative to controls. Additionally, although we found a decrease in claudin-4 mRNA in neonatal type II alveolar cell isolates exposed to hyperoxia, this did not cause a significant decrease in claudin-4 protein. The effects of hyperoxia on claudin-4 may differ between these^{17,21} and our study due to differences in the type of model, the timing and concentration of oxygen exposure, and the cell type examined, but both investigations implicate changes in claudin abundance in hyperoxia-induced pulmonary edema. In keeping with this concept, in animal models of ventilator-induced lung injury and fibrosis, claudin-4 transcript and protein levels have been shown to be increased in an a likely mechanism to regulate paracellular permeability and contain lung damage^{17,43–45}.

Certainly, endothelial barrier dysfunction contributes to lung water accumulation and lack of fluid clearance. Several investigators have demonstrated impaired endothelial barrier function with hyperoxia exposure due to nitric oxide-dependent signaling, apoptosis, and antioxidant availability^{35,46–48}. We recognize that a proportion of the lung edema and protein transit across the lung barrier in our *in vivo* hyperoxia animals may be the result of increased microvascular leak. However, our investigations using cultured neonatal AECs

demonstrates that paracellular leak of small and large molecules increases with exposure to sustained oxidative stress, and that alveolar epithelial barrier dysfunction due to claudin alterations may contribute to pulmonary edema in neonatal animals.

In addition to barrier deficits, claudin-18 knockout animals have been shown to have normal lung structure at birth, but have defective alveolar development by postnatal week four and are susceptible to asthma as adults^{15,49}. Our observation that claudin-18 expression and localization in neonatal AECs is altered and markers of alveolarization are diminished in the lung following hyperoxic injury supports this role for claudin-18 in development. Further, we have shown an upregulation of SNAI1 in neonatal AECs and in the whole lung following hyperoxia treatment. SNAI1 has been shown to repress the promoters of claudin-3 and claudin-4 in other epithelial cell types, and it is upregulated in normal lung development and in epithelial-to-mesenchymal transition, promoting a migratory epithelial phenotype^{50,51}. Future work will further define the relationship between hyperoxia exposure, SNAI1, and junctional proteins in the setting of the developing lung.

In summary, the findings in this study show that oxygen exposure modulates claudin expression, increases paracellular leak by neonatal AECs, and contributes to pulmonary edema in the developing lung. Given that exposure to high oxygen concentrations is essential to the management of critically ill premature infants, and that pulmonary edema is a frequent clinical complication of bronchopulmonary dysplasia, a better mechanistic understanding of alveolar barrier function in the developing lung is essential and likely to provide an opportunity for novel therapeutic interventions in the face of oxygen therapy.

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Figure 1. Hyperoxia decreases barrier function of neonatal AECs

A. AECs from neonatal Sprague-Dawley rats were isolated and immunostained for lamellar body protein expression by immunofluorescence and DAPI nuclear stain. Merged images are shown in the third panel. Bar = 10 micron. B. AECs expressed TTF-1 and pro-SPC as assessed by immunoblot, consistent with a type II cell phenotype. C. Neonatal AECs cultured for 3 days show higher Transepithelial resistance (TEER) measurements (Ohms × cm²) under normal ambient room air oxygen (21%, Control) as compared with cells cultured in hyperoxia (85%), n = 7, *p<0.05. D,E. Paracellular flux of carboxyfluorescein (D) and Texas Red Dextran (10 kDa; E) across neonatal AEC monolayers cultured in hyperoxia was significantly higher than for cells in control normoxia, n = 3, *p<0.05.

Figure 2. Hyperoxia decreased claudin mRNA and protein expression

Neonatal AECs were isolated from Sprague-Dawley pups and cultured on an air-liquid interface in the presence of normal ambient room air (21%, control) or hyperoxia (85%). A. RT-PCR was performed for claudin-3, claudin-4, claudin-5, and claudin-18 mRNA in hyperoxia-treated cells (black bar) and controls (white bar), $n=3$ for each gene, $*p < 0.05$. B. Immunoblot analyses of claudin-3, claudin-4, claudin-5, and claudin-18 relative to GAPDH were performed. C. Protein was assessed by densitometry for control (white bar) vs. hyperoxia-treated (black bar) cells normalized to GAPDH for claudin-3, claudin-4, claudin-5, and claudin-18. Bar graphs represent the mean of 3–8 independent experiments where $*p < 0.05$ relative to controls. D. Confocal microscopy imaging claudin-3, claudin-4, claudin-5, or claudin-18 demonstrated specific tight junction morphological changes to AECs in response to hyperoxia as compared with cells in normoxia (control). Photomicrographs are representative of at least 3 independent experiments. Hyperoxia had a particularly strong effect on localization of claudin-3 and claudin-18. Bar = 10 micron.

Figure 3. Hyperoxia decreased E-cadherin and increased SNAI1 in neonatal AECs

A. RT-PCR was performed for E-cadherin mRNA from neonatal AECs cultured with normal ambient room air (control 21%, white bar) or hyperoxia (85%, black bar). B. Confocal microscopy was used to evaluate E-cadherin expression and localization in neonatal AECs cultured with room air or hyperoxia. C. RT-PCR was used to determine SNAI1 mRNA in control and hyperoxia-treated primary AECs. D. Photomicrographs of primary neonatal AECs cultured in control or hyperoxia conditions for 2 days to evaluate SNAI1 expression and localization. E-cadherin and SNAI1 were imaged using Alexa 488 secondary antibodies, and nuclei were DAPI-labeled. Merged images are in the far right panels. Arrows indicate enlarged nuclei containing high levels of SNAI1. A, C. * $p < 0.05$ relative to controls. B, D. Bar = 50 micron. Photomicrographs represent 3 independent experiments.

Figure 4. Hyperoxia decreased alveolarization in neonatal rats and impaired lung barrier function in vivo

Sprague-Dawley rat pups were housed in plexiglass chambers and subjected continuously to normal ambient room air (con) or hyperoxia (85%, hyp) from day 3 of life to day 14. A–D. H&E staining of the lungs of animals exposed to control (A,C) or hyperoxia (B,D) conditions. A,B. bar = 500 micron, C,D. bar = 100 micron. Photomicrographs represent one of at least 3 independent experiments with 5–6 fields per experiment. E, F. Changes in lung morphology were quantified by examining radial alveolar count (E) and mean linear intercept (F). Hyperoxia resulted in a decrease in alveoli and increase in mean linear intercept consistent with impaired alveolarization. G. Animal weights for con and H85 animals on days 7, 10 and 14 (* $p < 0.05$ con vs hyp animals on day 14, and # $p < 0.05$ con animals on day 7 relative to hyp day 14). H. Lung wet/dry ratios relative to each animal's weight on the day of harvest are demonstrated in the bar graphs for control (white bars) and hyperoxia-treated animals (black bars), * $p < 0.05$ control vs. hyperoxia (days 7,14). A litter of 6–8 neonatal animals in each experiment group comprised one independent experiment, and $n = 3$ experiments were performed. I. Bronchoalveolar protein (mg/mL) was quantified in the lungs of control and hyperoxia treated animals, $n = 5$, * $p < 0.05$ relative to control. There was

a 7.4 ± 3.2 fold increase (n=5) in BAL protein induced by hyperoxia, consistent with an increase in lung permeability.

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Figure 5. Claudin expression in the lungs of neonatal animals exposed to normoxia or hyperoxia Sprague-Dawley rat pups were housed in plexiglass chambers and subjected continuously to normal ambient room air or hyperoxia (hyp) from birth until day 7 of life. **A.** Neonatal animals on day 7 had lungs resected and cellular lysates were evaluated for claudin expression relative to actin by immunoblot. **B.** Densitometric analysis of claudin expression relative to actin, $n = 3$ control, $n = 6$ hyp. **C–E.** Localization of claudin-3 (**C**), claudin-4 (**D**) and claudin-18 (**E**) was evaluated in frozen lung sections from control and hyperoxia-treated animals by confocal immunofluorescence microscopy. Nuclei were labeled with DAPI and merged images are in the far right panels. Photomicrographs representative of 3 independent experiments. Bar = 50 micron.

Figure 6. E-cadherin and SNAIL expression in neonatal animals exposed to hyperoxia Lungs from control (21%) or hyperoxia (85%) exposed neonatal rats were removed for mRNA quantification using the RT² Profiler Assay (A,C) and for frozen sections and confocal microscopy (B,C). **A.** E-cadherin mRNA normalized to housekeeping genes in whole lung from control (white bar) or hyperoxia-treated animals (black bar) on day 2 and 5 of exposure showed decreased expression in response to hyperoxia * $p < 0.05$, $n = 3$. **B.** Confocal photomicrographs of lung from control and hyperoxia-treated animals stained for E-cadherin and DAPI nuclear stain. Merged images are in the far right panels. There was little effect of hyperoxia on E-cadherin localization. Bar = 50 micron. **C.** SNAIL mRNA normalized to housekeeping genes in whole lung from control (white bar) or hyperoxia-treated animals (black bar) on day 2 and 5 of exposure showed increased expression in response to hyperoxia * $p < 0.05$, $n = 3$. **D.** Confocal photomicrographs of lung from control and hyperoxia-treated animals stained for SNAIL and DAPI. Merged images are in the far right panels. Hyperoxia induced nuclear localization of SNAIL (arrows), consistent with activation of this transcription factor. Bar = 50 micron. Photomicrographs are representative of 3 independent experiments.