Alterations of the Thioredoxin System by Hyperoxia

Implications for Alveolar Development

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Alterations in vascular endothelial growth factor (VEGF) contribute to alveolar simplification seen in animal models of bronchopulmonary dysplasia, and VEGF expression is redox regulated by thioredoxin (Trx)-1 in other diseases. The present studies tested the hypothesis that exposure to 85% O₂ negatively impacts the Trx1 system and VEGF expression in the lungs of newborn mice. There was no effect of fraction of inspired oxygen on lung Trx1 or Trx reductase-1 protein levels; however, lung Trx1 protein was predominantly oxidized in the lungs of newborn mice exposed to 85% O₂ by 24 hours of exposure. In room air (RA), lung Trx interacting protein (Txnip) levels decreased developmentally through Day 7 (1.0 ± 0.06 [Day 1] vs. 0.49 ± 0.10 [Day 3] vs. 0.29 ± 0.03 [Day 7]; $P < 0.01$), whereas VEGF expression increased (1.25 ± 0.16 [Day 1] vs. 4.35 ± 1.51 [Day 3] vs. 13.23 ± 0.37 [Day 7]; $P < 0.01$). Newborn mice exposed to 85% O₂ had no developmental decrease in Txnip protein levels and a delayed increase in VEGF protein levels. Lung Txnip and VEGF protein levels were different than in corresponding RA controls at Day 3, before the detection of lung morphologic abnormalities in our model. Txnip and VEGF protein levels were inversely correlated in both the RA and hyperoxia-exposed groups ($n = 18$; $R = -0.66$; $P = 0.003$). In conclusion, oxidation of Trx1 and sustained Txnip expression in the lungs of newborn mice exposed to 85% oxygen is likely to severely attenuate normal Trx1 function. The inverse correlation of Txnip with VEGF expression suggests that decreased Trx1 function contributes to the observed lung developmental abnormalities.

Keywords: hyperoxia; thioredoxin; thioredoxin interacting protein; vascular endothelial growth factor; bronchopulmonary dysplasia

Preterm delivery occurs in approximately 12% of births and causes more than 85% of perinatal illness and death (1). Prematurely born infants, especially those born at 24 to 28 weeks of gestation, are at high risk for the development of bronchopulmonary dysplasia (BPD), a chronic lung disease of infancy characterized by a histologic pattern of "alveolar simplification" (fewer and larger alveoli with loss of septation), loss of small pulmonary arteries, and decreased capillary density (2). BPD represents the impact of injury, including oxygen toxicity, volutrauma, barotrauma, and infection, on the immature developing lung (3). The signal transduction mechanisms responsible for normal alveolar development are poorly understood, and thus represent a significant barrier to discovering meaningful therapies directed at the prevention of BPD.

Prolonged exposure of mice, rats, rabbits, lambs, and baboons to hyperoxia causes structural abnormalities in the developing lung that mimic the findings observed in human

CLINICAL RELEVANCE

Premature birth into a hyperoxic environment relative to the *in utero* environment negatively impacts lung development and contributes to the development of bronchopulmonary dysplasia (BPD) in human infants. The present studies demonstrate that hyperoxia-induced alterations of the lung thioredoxin (Trx) system are inversely correlated with changes in vascular endothelial growth factor protein expression and precede observed alterations in alveolar development in a newborn mouse model of BPD. Similar effects of preterm birth on the Trx system in human infants could contribute to altered lung development that is characteristic of BPD.

patients with BPD (4–12). Major effects of postnatal hyperoxic exposure on the developing murine lung are the reduction of secondary septation and the lack of subdivision of airspaces resulting in fewer and larger alveoli (5, 10, 11, 13). Vascular endothelial growth factor (VEGF), a potent endothelial cell mitogen produced by type-2 alveolar epithelial cells (AECs), is significantly involved in the formation of secondary septae and subsequent alveolar development (14–16). Numerous studies in newborn animal models have demonstrated the importance of normal VEGF signaling to lung alveolar development (2, 9, 17–21).

Thioredoxin (Trx)-1 is a ubiquitously expressed 12 kD redox protein with a conserved -Cys³²-Gly-Pro-Cys³⁵- active site that, along with Trx reductase (TrxR)-1, constitutes the cytosolic Trx system (22, 23). TrxR1 reduces the active site of oxidized Trx1 (Trx-S₂) from the inactive disulfide form to the biologically active dithiol form (Trx(SH)₂). Functionally, Trx1 modulates VEGF production in a variety of cell types and tissues (24). If the Trx1 active site is inactivated via oxidation or direct inhibition by endogenous or exogenous Trx1 inhibitors, then Trx1 biological functions are lost (25). Trx interacting protein (Txnip), also called vitamin D-up-regulated protein 1 and Trx1 binding protein 2, is an endogenous inhibitor of Trx1 biological function (26, 27). *Txnip* gene expression is induced by H₂O₂, hypoxia, persistent hyperglycemia, dexamethasone, heat shock factor, anticancer agents, and antiproliferative agents (28–34).

The overall working hypothesis for our ongoing research is that premature infants, born into a hyperoxic environment relative to the *in utero* environment, have impaired Trx1-mediated biological functions in the lung due to effects of elevated O₂ tension on Trx1 oxidation and Txnip protein levels, and that this decrease in Trx1 function negatively impacts VEGF-mediated angiogenesis, contributing to the lung vascular and alveolar deficits that are characteristic of BPD. Although premature infants that develop BPD are often exposed to minimal amounts of supplemental oxygen, they exhibit marked evidence of oxidant stress (35). Newborn mice have more mature antioxidant defenses compared with prematurely born human infants with similarly developed lungs, so that the effect

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of 85% O₂ on the developing murine lung may be similar to the effects of supplemental oxygen on the developing preterm infant lung. Newborn mice exposed to 85% hyperoxia exhibit evidence of arrested lung development by 7 days of exposure, suggesting that the molecular mechanisms responsible for these hyperoxia-induced alterations occur early in the time course of exposure (5, 11, 13).

The objective of the present studies was to test the hypothesis that exposure to 85% hyperoxia negatively impacts the Trx1 system in the lungs of newborn mice through effects on Trx1 and TrxR1 protein levels, Trx1 redox state, and Txnip protein levels, and that these hyperoxia-induced alterations would precede the detection of lung developmental alterations that occur as early as Day 7 of exposure in our model (13). Our findings indicate that the biological functions of the Trx1 system in the lungs of newborn mice exposed to 85% O₂ are likely to be dramatically altered through effects of hyperoxia on Trx1 redox state, evident by 1 day of exposure, and Txnip protein expression, evident by 3 days of exposure. We conclude that the negative impact of 85% hyperoxia on Trx1 function in the lungs of newborn mice is most reasonably correlated with Txnip protein expression, and our data indicate a significant, inverse correlation between lung Txnip and VEGF protein levels in the lungs of newborn mice exposed to room air (RA) or 85% O₂.

MATERIALS AND METHODS

Animal Model

Animal study protocols were approved by the Institutional Animal Care and Use Committee at The Research Institute at Nationwide Children's Hospital. C3H/HeN mice were bred, and pregnant dams were dated as to time of delivery. To be included in the study, at least two dams were required to deliver within 12 hours. Once born, the pups were randomized and equally distributed between the two dams. One dam and litter was placed in a plexiglass chamber containing a 10 liter/min flow of 85% O₂ while the corresponding dam and litter were placed in RA. The dams were switched every 24 hours to prevent oxygen toxicity. On 1, 3, or 7 days of life, the pups were killed by intraperitoneal injections of 200 mg/kg of sodium pentobarbital. Lung tissues were either snap frozen in liquid N₂ and stored at -80°C for further analyses, or were inflation fixed with formalin at 20 cm H₂O pressure for immunohistochemical studies.

Western Blot Analyses

Frozen lung tissues were homogenized in lysis buffer (20 mM Hepes, pH 7.4; 50 mM β -glycerol phosphate; 2 mM EGTA; 1 mM DTT; 10 mM NaF, 1 mM Na orthovanadate; 1% Triton-100; 10% glycerol; 20 μ l saturated PMSF; 20 μ l 100 μ M leupeptin; 20 μ l 10 mg/ml aprotinin; 200 μ l of 10 nM okadaic acid in 20 ml total volume buffer; 20 μ l of 0.5 ng/ml Microcystin-LR; 200 μ l of 10 nM Tautomycin), and protein contents were measured using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Proteins (25–50 μ g) were separated by 4–12% SDS-PAGE (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes (iBlot; Invitrogen). Trx1 and TrxR1 contents were assessed by Western analyses using rabbit anti-human Trx1 and rabbit anti-human TrxR1 (1:2,000; Abcam, Cambridge, MA) IgG primary antibodies, which cross react with murine Trx1 and TrxR1 proteins, respectively, and horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:12,000; Bio-Rad). VEGF and Txnip contents were assessed by Western analyses using mouse anti-human VEGF (1:500; Abcam) and mouse anti-human Txnip/VDUP-1 (1:1,000, clone JY-2; MJB Research, Woburn, MA) primary antibodies, which cross react with their respective murine equivalent proteins and horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:10,000; Bio-Rad). The membranes were developed with enhanced chemiluminescence reagent (GE Healthcare, Buckinghamshire, UK), and films were scanned with band quantitation using ImageQuant software version 5.0 (Molecular Dynamics/GE Healthcare, Piscataway, NJ). To control for loading differences, the density of the band for the protein of

interest was normalized to the density of β -actin protein (mouse anti-human, 1:5000; Abcam; goat anti-mouse, 1:10,000; Bio-Rad) for each sample. Exposure times were fixed for each individual antibody, and all data were reported as ratios of the density of the band of interest to the density of β -actin.

Redox Western Blot Analyses

For Trx1 redox analyses, a portion of frozen lung tissue was sonicated in ice-cold trichloroacetic acid (10%). Samples were centrifuged at $12,000 \times g$ for 10 minutes, resuspended in 100% acetone, and incubated at 4°C for 30 minutes. After centrifugation at $12,000 \times g$ for 10 minutes, acetone was removed, and protein pellets were dissolved in lysis/derivatization buffer (20 mM Tris/HCl [pH 8], 15 mM 4-acetoamido-4'-maleimidylstilbene-2,2'-disulphonic acid) (Molecular Probes/Invitrogen) and incubated at room temperature for 3 hours to alkylate the free thiol groups of proteins in the tissue sample. After alkylation, Trx1 redox forms were separated on an SDS/15% polyacrylamide gel in the presence of nonreducing loading buffer, resulting in two distinct bands, which correlate to the oxidized and reduced forms of Trx1. Shifts that were detected in our samples were very typical, and were identical to the 4-acetoamido-4'-maleimidylstilbene-2,2'-disulphonic acid-derivatized oxidized and reduced purified protein. The gel was electroblotted onto a nitrocellulose membrane and probed with a goat anti-human Trx1 antibody (American Diagnostica, Stamford, CT), which cross reacts with murine Trx1 protein and with an Alexa-Fluor-680-conjugated anti-goat secondary antibody (Molecular Probes/Invitrogen). Bands corresponding to Trx1 were visualized using the Odyssey scanner (LI-COR, Lincoln, NE), and were quantified using the Odyssey version 1.1 software.

Statistical Analysis

Data collected from analyses were tested for homogeneity of variances and were log transformed where indicated. Data were then analyzed by two-way ANOVA with days of exposure and FiO₂ (fraction of inspired oxygen) as independent variables. When a two-way ANOVA indicated a significant difference, individual differences were detected with one-way ANOVA and *post hoc* testing using least significant differences. Differences were noted at *P* values less than 0.05. All statistical analyses were performed with SPSS Windows version 14.0 (SPSS Inc., Chicago, IL).

RESULTS

Trx1 and TrxR1 protein levels were measured in whole-lung homogenates from newborn C3H/HeN mice exposed to RA or 85% oxygen for 1, 3, or 7 days. Western blot analyses indicated no effect of FiO₂ or days of oxygen exposure on lung Trx1 protein levels (Figure 1A). Furthermore, lung Trx1 protein levels in newborn pups exposed to 85% hyperoxia were not different than those in RA control pups at any timepoint tested. Similar to Trx1 protein levels, we observed no effect of FiO₂ or days of oxygen exposure on TrxR1 protein levels in the lungs of newborn mice exposed to RA or 85% hyperoxia (Figure 1B). Lung TrxR1 protein levels in newborn mice exposed to 85% hyperoxia were not different from those in RA control mice at any timepoint tested. Our data indicate that lung Trx1 and TrxR1 protein levels in newborn mice are not affected by exposure to 85% hyperoxia.

The biological function of Trx1 is dependent upon the availability of its active site to act as an electron donor to oxidized thiol groups. Consequently, redox Western blot analyses of lung homogenates from newborn mice exposed to RA or 85% hyperoxia for 1, 3, or 7 days (Figure 2) were performed. In pups raised in RA, our data indicate that the active site of Trx1 is predominantly reduced. In contrast, the active site of Trx1 in lung homogenates from newborn mice exposed to 85% O₂ is predominantly oxidized. The effect of 85% hyperoxia exposure on lung Trx1 active site thiol/disulfide status was seen as early

Figure 1. Lung thioredoxin (Trx)-1 (A) and Trx reductase (TrxR1) (B) contents in newborn C3H/HeN mice exposed to room air (RA) or 85% O_2 for 1, 3, or 7 days. Western blots were quantified by densitometry and normalized to β -actin. Analyses of density data, expressed as means ($\pm$ SEM) ($n = 5-6$), indicated no effect of days of exposure or FiO_2 . Total Trx1 and TrxR1 protein levels were not different between mice exposed to hyperoxia or RA.

as Day 1, and similar levels of Trx1 oxidation were apparent in the lungs of mice exposed to 85% hyperoxia for 3 and 7 days.

We hypothesized that neonatal mice exposed to 85% oxygen would exhibit alterations in pulmonary Txnip protein expression that would precede (3 d or less) lung growth arrest that we have observed at 7 days of exposure in our previous studies (13). Western blot analyses were performed on lung homogenates obtained from newborn C3H/HeN mice exposed to RA or 85% O_2 for 1, 3, or 7 days (Figure 3). Results indicated independent effects of days of exposure and FiO_2 on lung Txnip contents;

however, no interaction was detected. Our data indicate that pulmonary Txnip expression decreased developmentally over the first 7 days of life in newborn mice raised in RA. On Day 3, lung Txnip levels were 50% less than in Day 1 RA control mice, and were 70% less than Day 1 RA control mice on Day 7. Conversely, exposure to 85% O_2 attenuated the developmental decrease in lung Txnip expression seen in RA control mice. In the lungs of hyperoxia-exposed pups, Txnip protein levels were not different than RA control pups on Day 1, and were more than twice the levels in RA-exposed pups on Day 3. By Day 7, Txnip protein levels were less than those in pups exposed to

Figure 2. Lung Trx1 redox state in newborn C3H/HeN mice exposed to RA or 85% O_2 for 1, 3, or 7 days. Redox Western blot analyses of whole-lung homogenates were performed as described in MATERIALS AND METHODS. The active site of Trx1 is predominantly reduced in the lungs of mice raised in RA. In contrast, the active site of Trx1 is predominantly oxidized in newborn mice exposed to 85% O_2 , with similar levels of Trx1 oxidation apparent in the lungs of mice exposed to 85% hyperoxia for 3 and 7 days.

Figure 3. Lung Trx interacting protein (Txnip) contents in newborn C3H/HeN mice exposed to RA or 85% O₂ for 1, 3, or 7 days. Western blots were quantified by densitometry and normalized to β -actin. Analyses of density data, expressed as means ($\pm$ SEM) ($n = 6$), indicated an effect of days of exposure and of FiO₂. Groups sharing common lower case letters are different from each other. Pulmonary Txnip expression decreased developmentally over the first 7 days of life in RA control mice, whereas the developmental decrease was attenuated in the lungs of mice exposed to 85% O₂.

hyperoxia for 3 days, but were still more than twice the level detected in the lungs of RA-exposed pups at Day 7. Western blot analyses of lung homogenates obtained from C3H/HeN mouse pups exposed to RA or 85% hyperoxia for 1, 3, or 7 days were performed, and indicated independent effects of days of exposure and FiO₂ on VEGF protein levels (Figure 4). We observed a developmental increase in VEGF protein levels over the first 7 days of life in the lungs of newborn C3H/HeN mice. On Day 3, VEGF levels were more than twice those in the lungs of Day 1 mice, and were 10 times greater than Day 1 levels by Day 7. In the lungs of pups exposed to 85% oxygen, VEGF

protein levels were not different than RA control pups on Day 1, and were less than half the levels in RA-exposed pups on Day 3. By Day 7, VEGF protein levels in the lungs of hyperoxia-exposed pups were greater than Day 1 and Day 3 levels, but were still less than 50% of the levels in the lungs of Day 7 RA-exposed animals. To investigate the relationship between lung VEGF and Txnip protein levels in the lungs of newborn C3H/HeN mice, relative densitometry values for lung VEGF expression were plotted against the relative densitometry values for Txnip that were obtained from Western blot analyses (Figure 5). A significant inverse correlation between lung VEGF and Txnip

Figure 4. Lung vascular endothelial growth factor (VEGF) contents in newborn C3H/HeN mice exposed to RA or 85% O₂ for 1, 3, or 7 days. Western blots were quantified by densitometry and normalized to β -actin. Analyses of density data, expressed as means ($\pm$ SEM) ($n = 3$), indicated an effect of days of exposure and of FiO₂. Groups sharing common lower case letters are different from each other. The observed developmental increase in VEGF protein levels in the lungs of newborn C3H/HeN mice over the first 7 days of life was significantly attenuated in the lungs of pups exposed to 85% oxygen.

Figure 5. Regression analysis of lung Txnip and VEGF contents in newborn C3H/HeN mice exposed to RA or 85% O₂ for 1, 3, or 7 days. Normalized lung VEGF relative density values were plotted against normalized lung Txnip values, and a linear regression analysis was performed ($n = 18$) that indicated a significant inverse correlation between lung VEGF and Txnip protein levels.

protein levels was detected in newborn mice exposed to RA or hyperoxia ($R = -0.66$; $P = 0.003$; $n = 18$). Our data indicate that decreases in lung Txnip protein levels and increases in lung VEGF levels are associated with normal lung development in newborn mice. Conversely, increased lung Txnip and decreased lung VEGF protein levels are associated with alterations in lung development previously characterized by our group (13).

DISCUSSION

The results of the present studies provide fundamental insights into the effects of 85% hyperoxic exposure on the Trx1 system in a newborn mouse model of arrested alveolarization, and represent three important findings that have not been previously reported in this model. First, our data indicate no effect of 85% hyperoxia on Trx1 and TrxR1 protein levels in the lungs of newborn mice. Second, the redox state of Trx1 is affected by exposure to 85% hyperoxia, as Trx1 is oxidized to a similar extent in the lungs of newborn mice exposed to 85% O₂ for up to 7 days, whereas Trx1 is predominantly reduced in the lungs of control mice. Finally, Txnip protein levels decrease developmentally in the lungs of newborn mice raised in RA, but Txnip protein levels remain elevated in the lungs of newborn mice raised in 85% O₂. Although the oxidation of Trx1 would be likely to seriously impair Trx1 function, we conclude that the net effect of exposure to 85% oxygen on Trx1-dependent biological functions in the newborn mouse lung is most reasonably correlated with Txnip protein expression, and our data indicate a significant inverse correlation between lung Txnip and VEGF protein levels. Our data indicate that Txnip is present in type-2 AECs (data not shown), the cell type primarily responsible for VEGF production, although the inverse relationship between lung Txnip and VEGF protein levels does not prove causality, nor does it preclude the existence of a similar relationship in other cell types in the lung. Nevertheless, our data are consistent with the overall working hypothesis

that increased O₂ tension negatively impacts Trx1 function, which likely contributes to alterations in VEGF-mediated lung vascular and alveolar development in the developing mouse lung.

The fact that pulmonary Trx1 and TrxR1 protein levels in the lungs of mice exposed to sublethal hyperoxia were not different than in RA control mice after exposure to hyperoxia (FiO₂ = 0.85) for up to 7 days is in contrast with the data presented by Das and colleagues (36) in premature baboons exposed to hyperoxia (FiO₂ = 1.0). Variable increases in levels of Trx1 and TrxR1 gene expression were reported in the lungs of preterm baboons that were either continuously exposed to 100% O₂ and mechanical ventilation, or received O₂ as needed to normalize arterial oxygenation combined with mechanical ventilation for 6 or 10 days in these studies. Continuous exposure to 100% O₂ for 6 or 10 days resulted in increases in Trx1 protein levels *in vivo*, but the effect on TrxR1 protein levels was not reported. Although both models are designed to investigate the effects of hyperoxic exposure on developmentally immature lungs, the apparent discrepancy between our data and those of Das and colleagues is most reasonably explained by differences in species (baboon and murine), mode of ventilation (mechanical ventilation vs. spontaneous breathing), and oxygen concentration (100 vs. 85%) in the animal models of BPD used in each study. We have previously reported distinct morphometric and inflammatory differences in the lungs of newborn mice continuously exposed to 85 or 95% O₂, which lead us to most reasonably conclude that pulmonary response(s) of the Trx system to hyperoxia may be both species specific and oxygen concentration dependent (13).

Investigations into the effect of hyperoxia exposure on the Trx1 redox system, and the subsequent effect on the pathogenesis of alveolar simplification in this newborn model of BPD, cannot be determined by measurements of protein levels under denatured and reduced conditions alone. The reduced form of Trx1 is responsible for the majority of biological functions of the protein. Therefore, investigations into the role of Trx1 in physiological events require that the effect of a given exposure on the redox state of the Trx1 active site is measured. Our data indicate that, although the reduced form of Trx1 protein is present in the lungs of newborn mice exposed to 85% oxygen for up to 7 days, the majority of Trx1 in these lungs is oxidized (Figure 2). These findings are also consistent with those of Das and colleagues (36), who also observed significant Trx1 oxidation in the lungs and lung explants taken from hyperoxia-exposed newborn baboons. Thus, our data suggest significant impairment of Trx1-dependent functions in the lungs of hyperoxia-exposed newborn mice at as early as 1 day of exposure, preceding the appearance of lung morphological abnormalities (13).

Exposure of adult mice to 100% O₂ results in increased production of endogenous glucocorticoids by 72 hours of exposure, and Txnip is well described as a glucocorticoid-inducible gene product (32, 37, 38). As previously discussed, oxygen concentration-dependent differences in response(s) to hyperoxia are characterized by differences in lung injury and infiltration of inflammatory cells (13). The adult hyperoxia model is much harsher than the neonatal model in that there is a profound inflammatory response and a marked loss of weight. In the present model, the inflammatory response is less profound, and there is minimal effect on postnatal weight gain, strongly suggesting that newborn mice exposed to 85% O₂ are far less stressed and are less likely to have increased glucocorticoid expression than adult mice exposed to 100% O₂ (13). Thus, although our findings lead us to conclude that alterations in lung Txnip protein levels are directly mediated by alterations in oxygen exposure, we cannot exclude the contribution of alterations in endogenous glucocorticoid production to this process.

In addition to oxidation/reduction of the active site, Trx1 function is regulated by Txnip, which abolishes the biological activities of Trx1 through the formation of a mixed disulfide bond between cysteine 247 of Txnip and cysteine 32 of the Trx1 active site (39). Txnip is implicated as a critical regulator of Trx1 function in a variety of disease models, including human cancers, cardiac dysfunction, metabolic syndrome, and glucose homeostasis (40–46). Txnip levels are inversely correlated with cellular growth and *Txnip* gene expression, and has been shown to be induced by H₂O₂, hyperglycemia, and hypoxia (23, 28, 29, 32, 34). Our data indicate that lung Txnip protein expression decreases over the first 7 days of life in newborn mice raised in RA, which would promote Trx1-mediated processes. Exposure to 85% oxygen severely attenuated the developmental decrease in lung Txnip protein expression, with Txnip protein levels remaining persistently elevated in the lungs of newborn mice raised in 85% hyperoxia compared with those of newborn mice raised in RA. Whether the effect of hyperoxia on Txnip protein levels in the lungs of newborn mice is mediated through transcriptional or post-translational events is the subject of ongoing studies.

Assessment of Trx1 activity can be measured using the insulin disulfide reduction assay originally described by Holmgren and Björnstedt (47). This assay, which uses insulin as a substrate for disulfide reduction, uses saturating amounts of reduced nicotinamide adenine dinucleotide and TrxR, and contains an initial “activation step” with the disulfide reducing agent, DTT. Incubation of samples with DTT not only reduces the active-site disulfide of oxidized Trx1, but is also likely to disrupt the interaction of Txnip with the Trx1 active site through reduction of the disulfide bond between Txnip and Trx1. Although our data suggest that Trx1 function is likely to be severely attenuated in the lungs of newborn mice exposed to 85% hyperoxia through both oxidation of the Trx1 active site and direct inhibition of Trx1 by Txnip, such effects are unlikely to be reliably detected by the Trx1 activity assay (48).

Exposure to high concentrations of oxygen leads to impaired lung vascular and alveolar development in animal models of BPD, mediated, at least in part, by perturbations in VEGF signaling (2, 9, 49). Trx1-dependent modulation of VEGF signaling has been implicated as a mechanism that contributes significantly to angiogenesis in human cancers, although the precise mechanisms are incompletely understood. The transcription factor p53, which represses VEGF gene transcription, represents a potential link between Trx1 and VEGF production in the lung. p53 and p21, a downstream target of p53, are induced in the distal lung epithelium of ventilated, hyperoxia-exposed preterm baboons (50). Furthermore, Trx1-dependent regulation of p53-mediated p21 activation has been demonstrated *in vitro*, and Trx1 has been demonstrated to translocate to the nucleus under conditions of oxidative stress (51, 52). Neither the effect of 85% hyperoxic exposure on cellular localization of Trx1 nor its effect on p53 activation in the lungs of newborn mice is presently known. The species-specific and O₂ concentration-specific differences in responses to hyperoxic exposure in the lungs of baboons and newborn mice preclude the extrapolation of data between the two animal models, although a potential role for Trx1-mediated, p53-dependent VEGF production cannot be excluded by the present studies.

Our data indicate that the developmental increase in VEGF protein levels in the lungs of newborn mice over the first 7 days of life, and the developmental decrease in lung Txnip protein levels, correlate with lung alveolar development. Conversely, hyperoxic exposure severely attenuates both the developmental increase in lung VEGF protein levels and the decrease in lung Txnip protein levels in newborn mice, and these changes

precede the appearance of lung morphologic abnormalities previously characterized in our model (13). These VEGF and lung developmental data are consistent with findings in other animal models of BPD (2, 9, 49). In the present studies, Trx1 is oxidized to a similar degree in the lungs of newborn mice exposed to 85% hyperoxia for up to 7 days. The effect of exposure to hyperoxia on lung Txnip protein expression in the newborn mice, however, is time dependent, as lung Txnip protein levels on Day 7 are 46% lower than Day 1 values. Although the increase in oxidized Trx1 that occurs with exposure to hyperoxia would be likely to negatively impact Trx1-mediated processes, the net effect of exposure to hyperoxia on pulmonary Trx1 function is more likely to be correlated with Txnip protein levels than with Trx1 oxidation. The significant inverse correlation between lung Txnip and lung VEGF protein levels in newborn mice exposed to either RA or hyperoxia for up to 7 days leads us to speculate that oxygen concentration-dependent perturbations in Txnip expression potentially contribute to the deficits in alveolar development observed in our model through the regulation of VEGF production by type-2 AECs. Studies designed to determine the precise relationship between VEGF and Txnip, including potential mechanism(s) by which Txnip regulates VEGF production by type-2 AECs, are currently ongoing.

In human microvascular endothelial cells, treatment with VEGF induces migration, with a concomitant decrease in Txnip expression, whereas silencing Txnip through RNA interference strongly induces human microvascular endothelial cell migration (53). These results suggest a role for Txnip in mediating VEGF-induced endothelial cell migration, a key component of angiogenesis. The significant inverse correlation between lung Txnip and VEGF protein levels in whole-lung homogenates in our studies support the possibility that a similar relationship between VEGF and Txnip could also exist in our model. The complexities of such cell-specific effects and interactions between Txnip and VEGF demonstrate the need for well designed mechanistic studies, not only in type-2 AECs, but also in other cell types, including endothelial cells.

Our data were obtained from whole-lung homogenates taken from newborn mouse pups, and thus provided no information on cell-specific localization of Txnip. Filby and colleagues (54) demonstrated that Txnip is highly expressed in type-1 and type-2 AECs, and in the distal airways in the developing ovine lung. Given the possibility that both Txnip-dependent regulation of VEGF production and the converse may occur in a cell-specific fashion, careful histologic studies that address changes in cell-specific expression of both proteins over time during hyperoxic exposure are needed.

In conclusion, we report important findings regarding the effect of hyperoxic exposure on the Trx1 system in a newborn mouse model of arrested alveolarization that have potential implications for alveolar development. Our data indicate that Trx1 function is likely to be severely attenuated in the lungs of newborn mice exposed to 85% hyperoxia through oxidation of the Trx1 active site at as early as 1 day of exposure, and sustained elevation of Txnip protein seen by 3 days of exposure, both before the detection of hyperoxia-induced lung morphological alterations evident by 7 days of exposure (13). We speculate that the inhibition of Trx1-mediated biological functions by hyperoxia, predominantly mediated by effects on Txnip protein levels, contributes to alterations in alveolar development through effects on lung VEGF protein expression in type-2 AECs; however, a reciprocal relationship between VEGF and Txnip in other cell types, including endothelial cells, is also supported by our data, and cannot be excluded by the present studies. Similar effects of increased oxidant stress on the Trx1

system in premature human infants could contribute to altered lung development that is characteristic of BPD.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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