ORIGINAL RESEARCH

Thioredoxin Reductase Inhibition Attenuates Neonatal Hyperoxic Lung Injury and Enhances Nuclear Factor E2–Related Factor 2 Activation

Qian Li[1,2], Stephanie B. Wall[1,2], Changchun Ren[2], Markus Velten[3], Cynthia L. Hill[4], Morgan L. Locy[5], Lynette K. Rogers[4], and Trent E. Tipple[1,2]

[1]Neonatal Redox Biology Laboratory, [2]Division of Neonatology, and [5]Medical Scientist Training Program, University of Alabama at Birmingham, Birmingham, Alabama; [3]Department of Anesthesiology and Intensive Care Medicine, Rheinische Friedrich-Wilhelms University, University Medical Center, Bonn, Germany; and [4]Center for Perinatal Research, The Research Institute at Nationwide Children's Hospital, Columbus, Ohio

Abstract

Oxygen toxicity and antioxidant deficiencies contribute to the development of bronchopulmonary dysplasia. Aurothioglucose (ATG) and auranofin potently inhibit thioredoxin reductase-1 (TrxR1), and TrxR1 disruption activates nuclear factor E2–related factor 2 (Nrf2), a regulator of endogenous antioxidant responses. We have shown previously that ATG safely and effectively prevents lung injury in adult murine models, likely via Nrf2-dependent mechanisms. The current studies tested the hypothesis that ATG would attenuate hyperoxia-induced lung developmental deficits in newborn mice. Newborn C3H/HeN mice were treated with a single dose of ATG or saline within 12 hours of birth and were exposed to either room air or hyperoxia (85% O_2). In hyperoxia, ATG potently inhibited TrxR1 activity in newborn murine lungs, attenuated decreases in body weight, increased the transcription of Nrf2-regulated genes nicotinamide adenine dinucleotide phosphate reduced quinone oxidoreductase-1 (NQO1) and heme oxygenase 1, and attenuated alterations in alveolar development. To determine the impact of TrxR1 inhibition on Nrf2 activation *in vitro*, murine alveolar epithelial-12 cells were treated with auranofin, which inhibited TrxR1 activity, enhanced Nrf2 nuclear levels, and increased NQO1 and heme oxygenase 1 transcription. Our novel data indicate that a single injection of the TrxR1 inhibitor ATG attenuates hyperoxia-induced alterations in alveolar development in newborn mice. Furthermore, our data support a model in which the effects of ATG treatment likely involve Nrf2 activation, which is consistent with our findings in other lung injury models. We conclude that TrxR1 represents a novel therapeutic target to prevent oxygen-mediated neonatal lung injury.

Keywords: hyperoxia; thioredoxin reductase; nuclear factor E2–related factor 2; bronchopulmonary dysplasia

Clinical Relevance

Our novel data indicate that a single injection of the Food and Drug Administration–approved thioredoxin reductase-1 inhibitor ATG attenuates hyperoxia-induced alterations in alveolar development in a newborn model of bronchopulmonary dysplasia, likely via a mechanism that involves nuclear factor E2–related factor 2 activation. We conclude that thioredoxin reductase-1 represents a possible novel therapeutic target to prevent neonatal lung injury in preterm infants.

(Received in original form July 10, 2015; accepted in final form April 4, 2016)

This work was supported by grants from the American Thoracic Society Foundation Research Program (T.E.T.) and by National Institutes of Health grants K08HL093365 (T.E.T.) and R01HL119280 (T.E.T.).

Author Contributions: Q.L., L.K.R., and T.E.T. conceived the ideas and designed and wrote the paper; L.K.R. and T.E.T. oversaw and analyzed studies; and Q.L., S.B.W., C.R., M.V., C.L.H., and M.L.L. performed and analyzed the studies.

Correspondence and requests for reprints should be addressed to Trent E. Tipple, M.D., Division of Neonatology, University of Alabama at Birmingham, 176F Suite 9380, 619 South 19th Street, Birmingham, AL 35249-7335. E-mail: ttipple@peds.uab.edu

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 55, Iss 3, pp 419–428, Sep 2016

Originally Published in Press as DOI: 10.1165/rcmb.2015-0228OC on April 8, 2016
Internet address: www.atsjournals.org

Despite changes in clinical care that have reduced cumulative oxygen exposure, bronchopulmonary dysplasia (BPD) remains a significant cause of morbidity in preterm infants, partly because of antioxidant deficiencies. Past research efforts have been directed logically at the use of exogenously administered antioxidants to prevent BPD; however, these strategies have failed. Nuclear factor E2–related factor 2 (Nrf2) is a critical regulator of endogenous antioxidant responses. Approaches that enhance multiple antioxidant pathways are an attractive alternative to lessen the impact of hyperoxia on the preterm lung (1).

Fetal lung development takes place in a hypoxic environment (~3% O_2); therefore, even room air (RA) (21% O_2) is hyperoxic for the premature lung (2, 3). Antioxidant systems, including superoxide dismutases, thioredoxin (Trx) and glutathione (GSH) systems, heme oxygenases, and small molecular weight antioxidants, protect against and repair oxygen-mediated injury (1, 2). Preterm neonates are more susceptible to the effects of oxygen because of the developmental deficits in antioxidant defenses and an impaired ability to mount rapid antioxidant responses to hyperoxia (4–8). Nrf2 activation increases the transcription of many classical antioxidant genes that contain antioxidant response elements in their promoter regions and enhances survival in lung injury models of chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, and oxidant exposure (9–14). A dual role for Nrf2 in both normal lung development and in protection from hyperoxia-mediated neonatal lung injury suggests that therapies that enhance Nrf2 activation could mitigate the effects of hyperoxic exposure on lung development (15, 16).

Trx reductase-1 (TrxR1) is an nicotinamide adenine dinucleotide phosphate reduced–dependent oxidoreductase enzyme best known for reducing oxidized Trx1. Genetic and pharmacologic TrxR1 disruption enhances Nrf2 activation, suggesting that TrxR1 inhibition may be a common feature of Nrf2 agonists (17–23). Aurothioglucose (ATG) and auranofin (AFN) are Food and Drug Administration–approved antirheumatic drugs that potently inhibit TrxR1 (24, 25). We have shown previously *in vivo* that ATG inhibits TrxR1, induces Nrf2-dependent gene transcription, and prevents lung injury in adult murine models (26, 27). Moreover, *in vitro* data from our laboratory suggest that the protective effects of TrxR1 inhibition are mediated by Nrf2 activation (28).

The use of TrxR1 inhibition to prevent oxygen-mediated neonatal lung injury has not been tested. The current studies tested the hypothesis that TrxR1 inhibition using the TrxR1 inhibitor ATG would attenuate the effects of hyperoxia on neonatal lung development. Our novel data reveal that a single injection of ATG given shortly after birth attenuates hyperoxia-induced alterations in alveolar development. Complimentary studies in murine lung alveolar epithelial cells demonstrated that TrxR1 inhibition enhanced nuclear Nrf2 protein levels. TrxR1 inhibition *in vivo* and *in vitro* also increased the transcription of Nrf2-regulated genes. Collectively, our novel data support a conceptual model in which the protective effects of TrxR1 inhibitors are mediated by Nrf2 activation.

Materials and Methods

Animal Model

Animal protocols were approved by the Institutional Animal Care and Use Committee at the Research Institute at Nationwide Children's Hospital. Mice were handled in accordance with the National Institutes of Health guidelines. C3H/HeN mice were bred, and at least two dams were required to deliver within 12 hours. Once born, pups were injected intraperitoneally with saline or 25 mg/kg ATG (Research Diagnostics, Flanders, NJ) in saline. Pups were randomly assigned and equally distributed between two dams. Hyperoxic exposure was performed as described (29). At 1 day and 3 days, pups were killed with ketamine/xylazine (200/20 mg/kg, i.p.).

Morphometric Assessments

Lungs were fixed, and morphometric analyses for alveolar number and alveolar area were performed as published previously (29, 30).

Cell Culture

Murine lung epithelial (MLE)-12 cells (gift of Dr. Jeffrey Whitsett) were maintained in HITES media as described (31). For supplementation studies, the cells were cultured in selenium-free media, HITES media (29 nM sodium selenite [Se]), or supplemented media (100 or 200 nM Se). Cells cultured in HITES media with 100 nM Se for subsequent studies were plated at equal densities, allowed to adhere overnight, and treated with 1 μM AFN (Sigma, St. Louis, MO) in DMSO (Fisher, Walther, MA) or an equal volume of DMSO for 1 hour. The cells were washed three times with Dulbecco's phosphate-buffered saline and collected, or cultured for an additional 2 hours in AFN-free media.

Sample Preparation

Frozen lungs were homogenized (on ice, Dounce homogenizer), and cells were lysed in buffer (10 mM Tris buffer, pH 7.4, containing 0.1% Triton-X-100, 100 μM diethylenetriamine penta-acetic acid, with protease and phosphatase inhibitors [Thermo Scientific]). Supernatant (obtained by centrifugation at 20,000 × *g* for 10 min) protein concentrations were determined by bicinchoninic acid assay. Nuclear fractions were prepared using EpiQuik Kit (Epigentek, Farmingdale, NY).

TrxR1 Activity

TrxR1 activity was determined by insulin disulfide reduction assay as described previously (32–34).

Western Blot

Samples were loaded onto 4–15% Criterion or Mini-PROTEAN TGX gels (Bio-Rad, Hercules, CA), transferred to polyvinylidene fluoride membranes (Trans-Blot; Bio-Rad), blocked with 5% milk in Tris-buffered saline containing 0.05% Tween-20, and probed with anti–heme oxygenase 1 (HO-1) (ADI-SPA-894; Enzo Life Sciences, Farmingdale, NY; 1:1,000), anti–nicotinamide adenine dinucleotide phosphate reduced: quinone oxidoreductase-1 (NQO1) (ab34173; Abcam, Cambridge, MA; 1:1,000), or anti-Nrf2 antibody (gift from Dr. Edward Schmidt), followed by goat antirabbit IgG-HRP secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 1:5,000). Membranes were developed using Clarity ECL Substrate (Bio-Rad) and imaged using a ChemiDoc System (Bio-Rad). For loading control, the membranes were reprobed with anti-Actin (sc-1615; Santa Cruz; 1:5,000) or antinucleolin antibody (ab22758; Abcam; 0.2 μg/mL).

Quantitative Real-Time Polymerase Chain Reaction

RNA was isolated from frozen lungs using RNeasy (QIAGEN, Valencia, CA) and QIAcube (QIAGEN). cDNA was

synthesized using PrimeScript RT Master Mix (Takara/Clontech, Mountain View, CA). Quantitative real-time polymerase chain reaction (PCR) was performed using Premix Ex Taq Probe (Takara/Clontech) and Rotor-Gene Q instrument (QIAGEN). Primers for 18S (Hs99999901_s1), murine HO-1 (Mm00516005_m1), and NQO1 (01253561_m1) were obtained from Applied Biosystems/Thermo Fisher (Foster City, CA). Cycle threshold (CT) values were normalized to 18S (ΔCT). Fold changes relative to control were calculated using $2^{-\Delta CT}$.

Statistics

Data (mean ± SEM) were analyzed using GraphPad Prism 6.0 (La Jolla, CA) by unpaired *t* test or two-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison tests. Significance was accepted at $P < 0.05$.

Results

ATG Treatment Improves Alveolarization in Hyperoxia-Exposed Newborn Mice

Treatment of adult mice with a single dose of 25 mg/kg ATG is well tolerated and prevents lung injury in adult models (24, 26, 27). To determine the effect of ATG administration on hyperoxia-induced lung developmental deficits in newborn C3H/HeN mice, pups were treated with a single dose of ATG (25 mg/kg administered i.p.) or saline within 12 hours of birth and were exposed in either RA (21% O_2) or hyperoxia (85% O_2) for up to 14 days (Figure 1). Gross inspection of lung sections from 14-days-old mice revealed the well-described "alveolar simplification" phenotype caused by neonatal hyperoxic exposure. Conversely, lung morphology in ATG-treated hyperoxia-exposed mice was less abnormal (Figure 2A). Morphometric analyses for alveolar number and individual alveolar area was performed to assess the effects of hyperoxia and ATG on lung development. Our analyses indicated independent effects of and an interaction between ATG and hyperoxia on both parameters (Figures 2B and 2C). In saline-treated control mice, hyperoxic exposure decreased the alveolar number by 60% (Figure 2B). ATG attenuated the effect of hyperoxia on the alveolar number by 44%. In ATG-treated, RA-exposed control mice, the alveolar number was 20% greater than in saline-treated mice. The area of individual alveoli was not different between RA-exposed saline–treated or ATG-treated mice (Figure 2C). Compared with that in saline-treated RA control mice, the individual alveolar area was 2.4 times greater in saline-treated hyperoxia-exposed mice. ATG treatment attenuated the effect of hyperoxia on the individual alveolar area by 48%. Taken together, our data demonstrate that hyperoxic exposure resulted in altered lung architecture characterized by fewer and larger alveoli, whereas ATG treatment significantly attenuated this effect.

ATG Treatment Attenuates Hyperoxia-Induced Decreases in Body Weight in Newborn Mice

We next determined the effects of hyperoxic exposure and ATG treatment on lung and body weights at 7 days of life. An independent effect of ATG on lung weight was detected. Lung weight was 25% lower in ATG-treated hyperoxia-exposed mice when compared with saline-treated RA control mice (Figure 3A). Neonatal hyperoxic exposure impairs weight gain in newborn mice (35). We detected independent effects of and an interaction between ATG and hyperoxia on body weights at 7 days (Figure 3B). Body weights in saline-treated hyperoxia-exposed mice were 25% lower than in corresponding RA control mice. Body weights in ATG-treated mice exposed to hyperoxia were not different than in saline-treated RA mice. As an index of injury, lung/body weight ratios were calculated (Figure 3C). Independent effects of and an interaction between ATG and hyperoxia were detected. Compared with saline-treated RA control mice, lung/body weight ratios were 40% greater in saline-treated hyperoxia-exposed mice. Conversely, lung/body weight ratios in ATG-treated mice were not different than in RA control mice.

AFN Treatment Inhibits TrxR1 Activity and Increases Nuclear Nrf2 Levels and Nrf2-Regulated Gene Expression in MLE-12 Cells

Previous data from our group indicated that 1 μM AFN significantly inhibits TrxR1 activity in murine airway epithelial cells (28). We used AFN to pharmacologically inhibit TrxR1 *in vitro* because it more easily transfers across cell membranes, is more stable in solution, and requires lower concentrations for complete inhibition (36). The selenocysteine residue in the TrxR1 C-terminal active site directly contributes to its catalytic function; therefore, adequate selenium saturation promotes optimal function (20). To ensure adequate selenium saturation for TrxR1 synthesis, MLE-12 cells were supplemented with increasing concentrations of selenium (as sodium selenite). Dose-dependent increases in TrxR1 activity in supplemented MLE-12 cells were detected (*see* Figure E1 in the online supplement). TrxR1 activity was nearly twofold greater in cells cultured in standard HITES media (29 nM selenium) than in cells cultured in selenium-free media. TrxR1 activity was 40% greater in MLE-12 cells cultured in media containing 100 nM sodium selenite than in cells cultured in standard HITES media. Selenium supplementation at concentrations above 100 nM did not further enhance TrxR1 activity in MLE-12 cells. Media was therefore supplemented with 100 nM sodium selenite in all subsequent experiments.

To determine the impact of AFN on TrxR1 activity in murine alveolar epithelial (MLE) cells, MLE-12 cells were treated

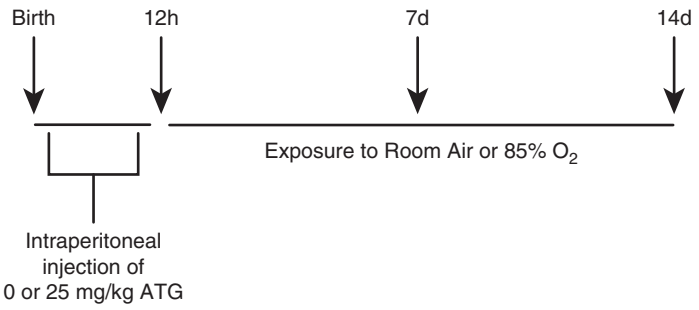

Figure 1. Experimental design for experiments *in vivo*. ATG, aurothioglucose.

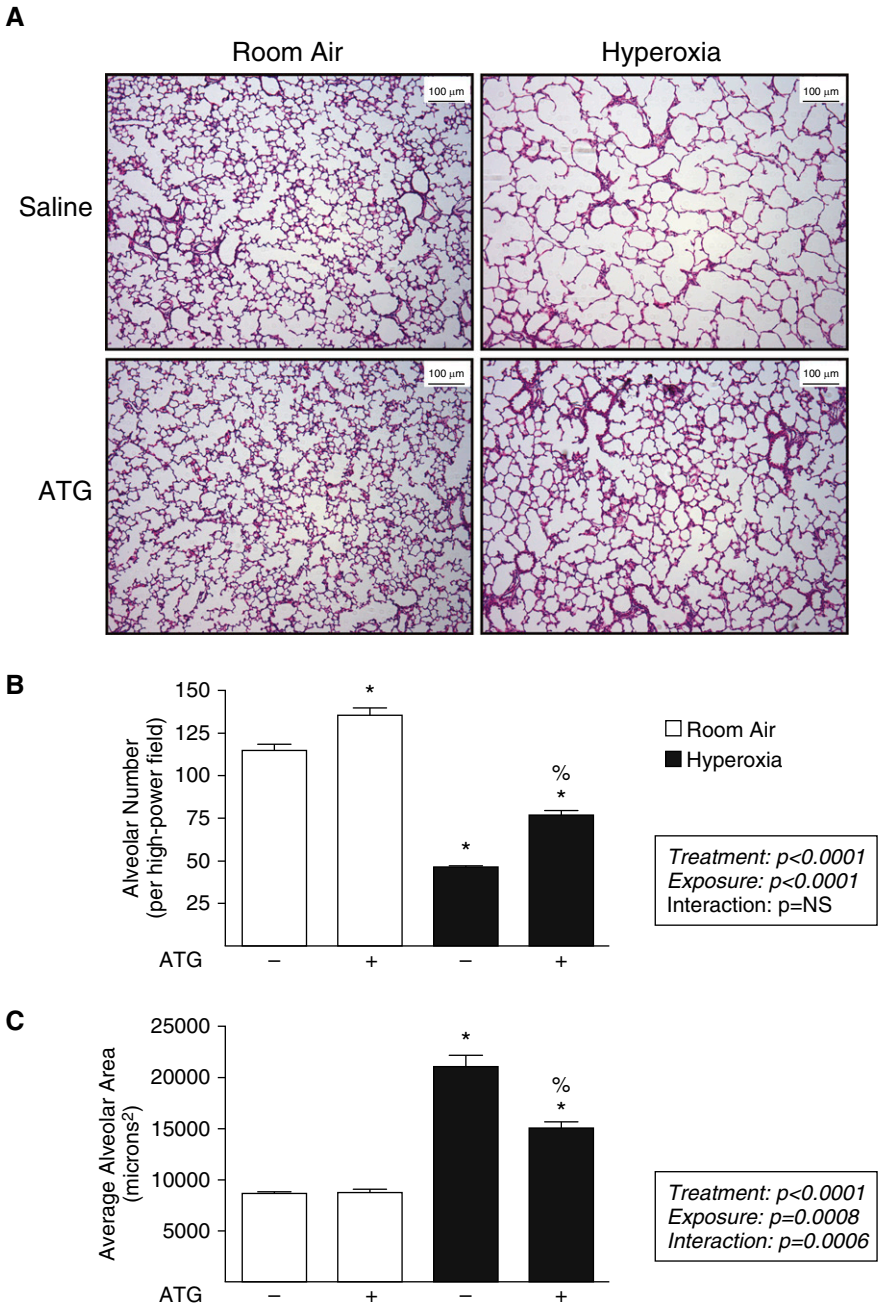

Figure 2. Lung histology and morphometry at 14 days of life. Newborn C3H/HeN mice were treated with a single intraperitoneal dose of 25 mg/kg ATG or saline and were exposed to room air or hyperoxia (85% O_2) for 14 days. (*A*) Representative hematoxylin and eosin–stained lung sections, (*B*) alveolar number, and (*C*) individual alveolar area after 14 days of exposure. Data (mean ± SEM; five images/lung; $n = 4$–8 mice/group) were analyzed by two-way ANOVA followed by Tukey's *post hoc* analysis; *versus saline + room air; %versus saline + hyperoxia; $P < 0.001$. *Scale bars*: 100 μm. NS, not significant.

for 1 hour with either 1 μM AFN in DMSO or an equivalent volume of DMSO alone. Cells were then harvested or were washed and incubated in AFN-free media for an additional 2 hours as outlined in Figure 4A. After 1 hour of treatment, TrxR1 activity was 90% lower in AFN-treated cells than in DMSO-treated control cells. In cells harvested 3 hours after initiation of AFN treatment, TrxR1 activity was 54% lower than in cells that had been treated with DMSO alone (Figure 4B).

TrxR1 inhibition enhances Nrf2 nuclear localization in murine airway epithelial cells (28). To determine the effect of TrxR inhibition on Nrf2 localization in alveolar epithelial cells, Western blot analyses for nuclear Nrf2 were performed on nuclear fractions isolated from MLE-12 cells treated for 1 hour with either 1 μM AFN in DMSO or DMSO alone. Nuclear Nrf2 protein levels were 4.5 times greater in AFN-treated cells than in cells treated with DMSO alone (Figure 4C).

TrxR1 inhibition enhances the transcription of Nrf2-driven genes in murine airway epithelial cells; however, the effect on alveolar epithelia is unknown (28). Therefore, quantitative reverse transcriptase-mediated PCR was used to assess mRNA levels of contain antioxidant response elements–regulated genes in alveolar epithelial cells after AFN treatment. MLE-12 cells treated for 1 hour with 1 μM AFN in DMSO or DMSO alone were either harvested immediately or washed, incubated in AFN-free media for an additional 2 hours, and harvested 3 hours after the initiation of AFN treatment. Compared with control-treated cells, HO-1 expression was increased threefold after 1 hour of AFN treatment and sixfold 3 hours after AFN treatment (Figure 5A). Expression of NQO1 expression was not different at 1 hour but was increased twofold at 3 hours when compared with control-treated cells (Figure 5B).

ATG Inhibits TrxR1 in the Lungs of Newborn Mice

We have demonstrated previously that a single 25 mg/kg injection of ATG efficiently inhibits TrxR1 activity in the lungs of adult mice (26). Therefore, we next determined the effect of ATG on TrxR1 activity in lung homogenates from 1- and 3-days-old newborn mice treated as outlined in Figure 1. Two-way ANOVA indicated an independent effect of ATG treatment at both time points (Figure 6). At 1 day, TrxR1 activity was 70% lower in the lungs of ATG-treated newborn mice than in vehicle-treated control mice. In contrast, at 3 days of life, lung TrxR1 activity was 43% lower in ATG-treated RA-exposed mice and was 55% lower in hyperoxia-exposed mice. Among ATG-treated mice, TrxR1 activity was 41% greater at 3 days when

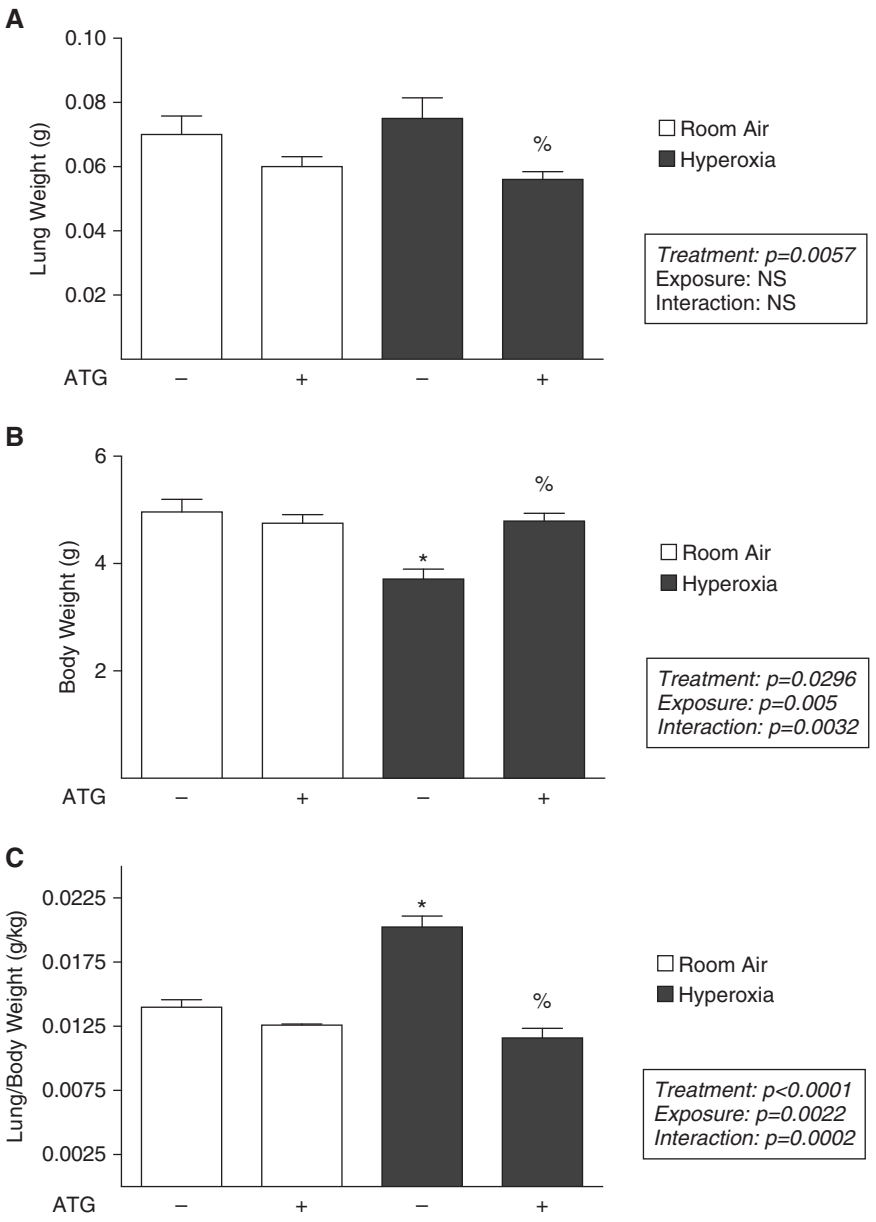

Figure 3. Lung, body, and lung/body weight ratios at 7 days of life. Newborn C3H/HeN mice were treated with a single intraperitoneal dose of 25 mg/kg ATG or saline and were exposed room air (RA) or hyperoxia (HO) (85% O_2) for 7 days. Data (mean ± SEM, $n = 3$–5) were analyzed by two-way ANOVA followed by Tukey's *post hoc* analysis. (*A*) Lung weight; *versus ATG + HO, $P = 0.0312$. (*B*) Body weight; *versus saline + RA, $P = 0.0028$; %versus saline + HO, $P = 0.0031$; and (*C*) Lung/body weight ratio; *versus saline + RA, $P = 0.0003$; %versus saline + HO, $P < 0.0001$.

compared with their respective 1-day-old RA and hyperoxia exposure groups.

ATG Enhances Nrf2 Activation in the Lungs of Hyperoxia-Exposed Newborn Mice

NQO1 is a prototypical gene used to determine Nrf2 activation, whereas HO-1 is the most strongly and consistently induced Nrf2-regulated gene after TrxR1 inhibition *in vitro*. We therefore assessed the effects of ATG treatment on Nrf2 activation *in vivo* by analyzing NQO1 and HO-1 mRNA and protein expression in whole lung samples from 1- and 3-days-old newborn C3H/HeN mice treated as outlined in Figure 1 (Figure 7).

Two-way ANOVA indicated an independent effect of hyperoxic exposure on NQO1 and HO-1 at 1 day (Figures 7A and 7B). HO-1 transcripts were increased twofold in lungs from ATG/hyperoxia mice when compared with saline/RA control mice and was also significantly greater than in ATG/RA mice (Figure 7B). Neither NQO1 nor HO-1 levels were significantly increased at 1 day in ATG/RA mice when compared with saline/RA control mice.

To compare the effects of ATG and hyperoxia on NQO1 and HO-1 protein expression with transcript levels, Western blot analyses were performed using lung homogenates from 1-day-old mice. At 1 day, an independent effect of exposure was detected for NQO1 (Figure 7C). NQO1 levels were 2.3 times greater in both hyperoxia-exposed groups when compared with saline/RA mice and were two times greater in the lungs of ATG/hyperoxia-exposed mice than in the ATG/RA group. No effects of treatment or exposure were detected for HO-1 (Figure 7D).

At 3 days, two-way ANOVA indicated independent effects of ATG and hyperoxia on NQO1 mRNA expression (Figure 7E). NQO1 transcripts were 2.3-fold greater in ATG/hyperoxia than in saline/RA mice. NQO1 expression was also significantly increased lungs from both the ATG/RA and the saline/hyperoxia group. Independent effects of ATG treatment and hyperoxic exposure were also detected for HO-1 (Figure 7F). Compared with saline/RA, HO-1 expression was increased 2.9-fold and 4.5-fold, respectively, in the saline/hyperoxia and ATG/hyperoxia group.

Western blot analyses of lung homogenates were performed to determine NQO1 and HO-1 expression at 3 days. Similar to the 1-day findings, an independent effect of hyperoxia was detected for NQO1 (Figure 7G). NQO1 levels were two times higher in the saline/hyperoxia and ATG/hyperoxia groups when compared with the saline/RA group. Similarly, an independent effect of hyperoxic exposure was detected for HO-1 at 3 days. As in the mRNA data, the most robust changes in protein expression were detected for HO-1. Compared with saline/RA, HO-1 expression was 9 times greater in the ATG/hyperoxia group and 10 times greater in the saline/hyperoxia group. When compared with ATG/RA, HO-1 levels were four times greater in the ATG/hyperoxia group.

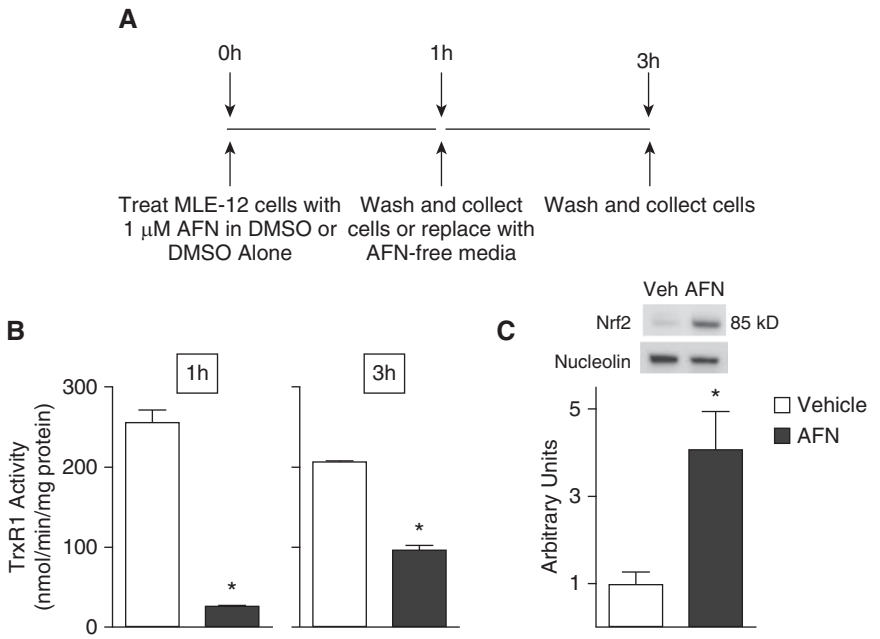

Figure 4. (*A*) Experimental design for experiments *in vitro* in Figures 4 and 5. (*B*) Thioredoxin reductase-1 (TrxR1) activity in murine lung epithelial (MLE)-12 cell lysates after auranofin (AFN) treatment. Data (mean ± SEM, $n = 3$) were analyzed by unpaired *t* test; *different from vehicle, $P < 0.0002$. (*C*) Nuclear factor E2–related factor 2 (Nrf2) nuclear abundance in MLE-12 cell lysates after AFN treatment. Data (mean ± SEM, $n = 4$) were normalized to nucleolin and analyzed by unpaired *t* test; *different from vehicle, $P = 0.0171$. Veh, vehicle.

Discussion

The results of the current studies support our primary hypothesis that a single dose of the TrxR1 inhibitor ATG given shortly after birth attenuates hyperoxia-induced alterations in alveolar development. Additional findings indicate that (*1*) in hyperoxia, ATG potently inhibits TrxR1 activity in newborn murine lungs, attenuates decreases in body weight, and increases the transcription of Nrf2-regulated genes, and that (*2*) AFN inhibits TrxR1 activity, enhances Nrf2 nuclear levels, and increases Nrf2-regulated gene transcription in alveolar epithelial cells *in vitro*. To the best of our knowledge, our studies are the first to demonstrate that TrxR1 inhibitors attenuate hyperoxia-induced lung developmental deficits in newborn mice. Our data are consistent with a protective mechanism involving enhanced Nrf2 activation by TrxR1 inhibitors in a murine BPD model.

Nrf2 significantly contributes to normal lung maturation and protection from neonatal hyperoxic lung injury (15, 16). Newborn $Nrf2^{-/-}$ mice exhibit decreased survival and greater hyperoxia-induced alveolar growth arrest than do wild-type mice (15, 16). In hyperoxia, newborn $Nrf2^{-/-}$ mice also exhibit increased inflammation, protein oxidation, and DNA base lesions when compared with similarly exposed wild-type mice (15). We have shown previously that TrxR1 disruption increases Nrf2 activation and Nrf2-mediated antioxidant responses in airway epithelial cells *in vitro*. TrxR1 inhibition also induces pulmonary Nrf2 activation and protection against hyperoxic and inflammatory lung injury in adult mice *in vivo*, suggesting the possible clinical usefulness of TrxR1 inhibitors in conditions such as acute respiratory distress syndrome (26–28). By demonstrating that TrxR1 inhibitors increase Nrf2 activation in alveolar epithelial cells *in vitro* and in the lungs of hyperoxia-exposed neonatal mice *in vivo*, the current studies extend our previous findings and support the possible clinical use of TrxR1 inhibition to prevent and/or treat BPD.

The lungs of premature infants are especially susceptible to the effects of hyperoxia because of developmental deficits in antioxidant defenses and impaired induction of antioxidant responses on exposure to hyperoxia (4–8). Our data indicated an effect of ATG treatment on Nrf2 activation, as assessed by NQO1 and HO-1 transcript levels, in the lungs of 3-days-old neonatal mice (Figures 7E and 7F). An independent effect of hyperoxic exposure on NQO1 and HO-1 transcripts *in vivo* at 1 day and 3 days (Figures 7A, 7B, 7E, and 7F) suggests that the neonatal murine lung is capable of mounting an antioxidant response to hyperoxia. Unfortunately, the hyperoxia-induced endogenous antioxidant response is insufficient to prevent alterations in lung development (Figure 2). Our finding of enhanced HO-1 and NQO1 mRNA levels at 3 days in ATG/hyperoxia-exposed mice when compared with mice exposed to hyperoxia alone (Figure 7E) suggests a synergistic effect of ATG and hyperoxia on Nrf2 activation. It is noteworthy that this finding is seen immediately before the start of alveolarization, which begins at ~ 4 days in newborn mice. At 7 days, we detected an independent effect of hyperoxic exposure and no effect of ATG treatment on NOQ1 and HO-1 mRNA levels (data not shown). Collectively, we interpret our data to indicate that ATG pretreatment enhances the ability of the newborn lung to mount an endogenous antioxidant response in hyperoxia, which preserves the signaling pathways that guide alveolar development.

Although we were perplexed initially by the lack of Nrf2 gene induction in RA-exposed ATG-treated mice (Figure 7), especially in light of equivalent TrxR1 inhibition in the lungs of ATG-treated mice (Figure 6), differential expression of the transcription factor Bach1 in RA and hyperoxia in the newborn lung may explain these findings. Bach1 was shown recently to be a key modulator of Nrf2-dependent responses in settings of altered O_2 tension *in vitro*. Alterations in Bach 1 expression differentially affected NQO1/HO-1 and GSH system gene transcription such that NQO1 and HO-1 but not GSH-related gene transcription was blocked in settings of physiologic normoxia, whereas NQO1-, HO-1–, and GSH–related gene transcription was enhanced in relative hyperoxia (37). Altered Bach1 expression may also explain observed differences in NQO1 induction between saline and ATG treatment in hyperoxia-exposed mice (Figure 7E). Although beyond the scope of this article, the effects of ATG and

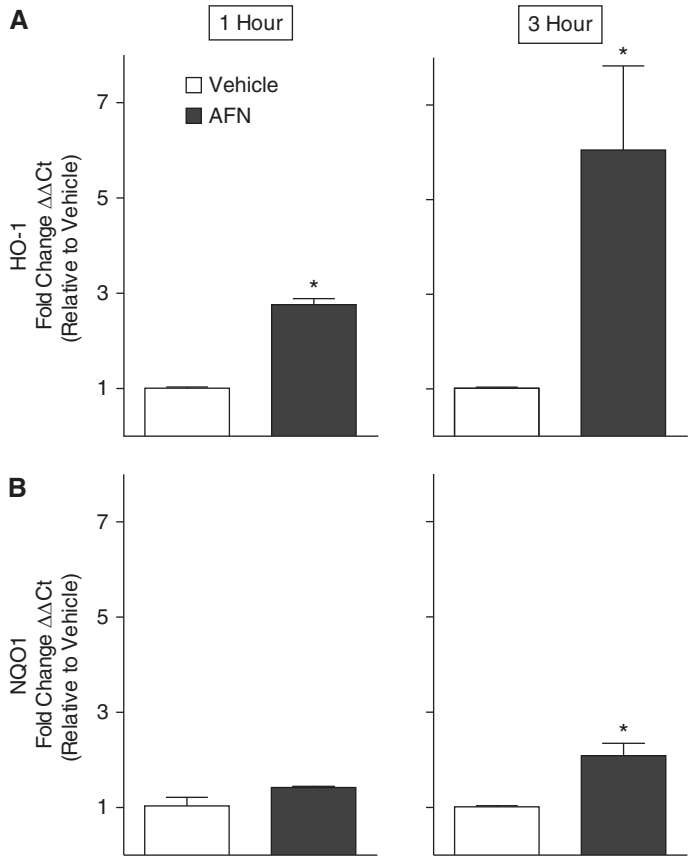

Figure 5. mRNA levels of (*A*) heme oxygenase 1 (HO-1) and (*B*) nicotinamide adenine dinucleotide phosphate reduced:quinone oxidoreductase-1 (NQO1) in MLE-12 cell lysates after AFN treatment. Cells were cultured as outlined in Figure 4A. ΔCt values (mean ± SEM) were analyzed by unpaired *t* test. Quantitative RT-PCR analyses at 1 hour; *different from vehicle, $P < 0.01$, $n = 3$, and (*B*) 3 hours; *different from vehicle, $P < 0.04$, $n = 3$–6.

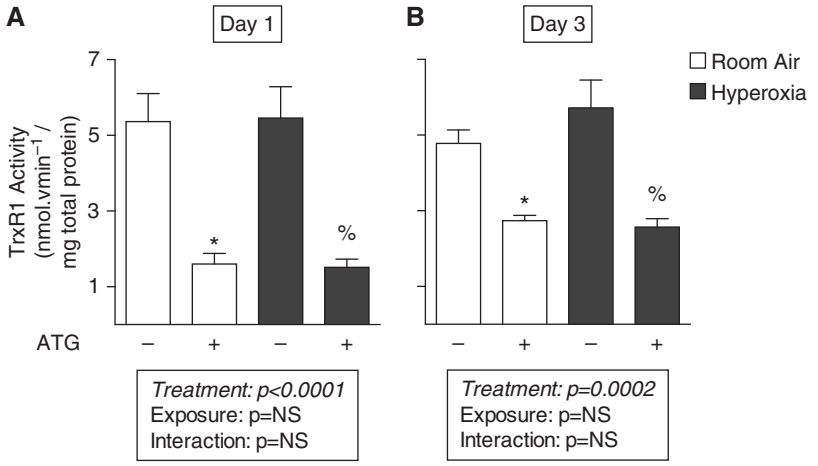

Figure 6. TrxR1 activity in whole-lung homogenates from neonatal mice treated with ATG or saline and exposed to RA or HO (85% O_2) for 1 day or 3 days as outlined in Figure 1. Data were analyzed by two-way ANOVA followed by Tukey's *post hoc* analysis. (*A*) TrxR1 activities at 1 day; *versus saline + RA, $P = 0.0009$; %versus saline + HO, $P = 0.0101$, $n = 3$–6 and (*B*) 3 days; *versus saline + RA, $P = 0.0267$; %versus saline + HO, $P = 0.0029$, $n = 3$–4.

hyperoxia on Bach1 expression in the lung will be a focus of future studies.

Differences were observed between Nrf2 target gene induction and Nrf2 target protein levels in the current studies (Figure 7). Effects of hyperoxic exposure on NQO1 and HO-1 protein expression were detected (Figures 7C and 7G) in the absence of significant increases in gene transcripts (Figures 7A and 7E). We interpret these data to suggest the influence of post-transcriptional and/or post-translational events on NQO1 and HO-1 expression in our model. It is also probable that these inconsistencies are reflective of the differences in the sensitivity of quantitative reverse transcriptase-mediated PCR and Western blot, especially when using whole lung samples. Although we observed significant decreases in TrxR1 activity in ATG-treated mice at 1 day and 3 days, the consequence of this inhibition on the magnitude of Nrf2 activation and subsequent downstream effects are likely to be different in various cell compartments. TrxR1 is highly expressed in lung epithelia, and its inhibition consistently elicits Nrf2 activation in past and current studies (28). Future studies will use epithelial-specific TrxR1 knockout mice to discern the effects of altered epithelial TrxR1 expression on pulmonary responses to hyperoxia in our BPD model. In addition, we will also use TrxR inhibitors *in vivo* and *in vitro* in settings of Nrf2 deficiency to identify the contribution(s) of Nrf2 signaling toward attenuation of neonatal hyperoxic lung injury.

Enhancement of Nrf2-driven gene expression is unlikely to account completely for the attenuation of hyperoxic lung injury seen in the current studies. Although our studies are consistent with a role for Nrf2-mediated protective effects of TrxR1 inhibitors, many pathways are likely to be influenced by TrxR1 inhibition and hyperoxic exposure both singularly and in combination. Peroxisome proliferator-activated receptor γ (PPAR-γ) is an Nrf2-regulated gene that is critically important in the protection from hyperoxic lung injury in adult mice (38). Importantly, the Nrf2 inducer curcumin, a potent PPAR-γ agonist, significantly attenuates hyperoxic lung injury in a newborn rat model of BPD (39, 40). Although the effect of curcumin on Nrf2 activation was not studied in the newborn rat model, it is likely that the protective effect of curcumin is at least

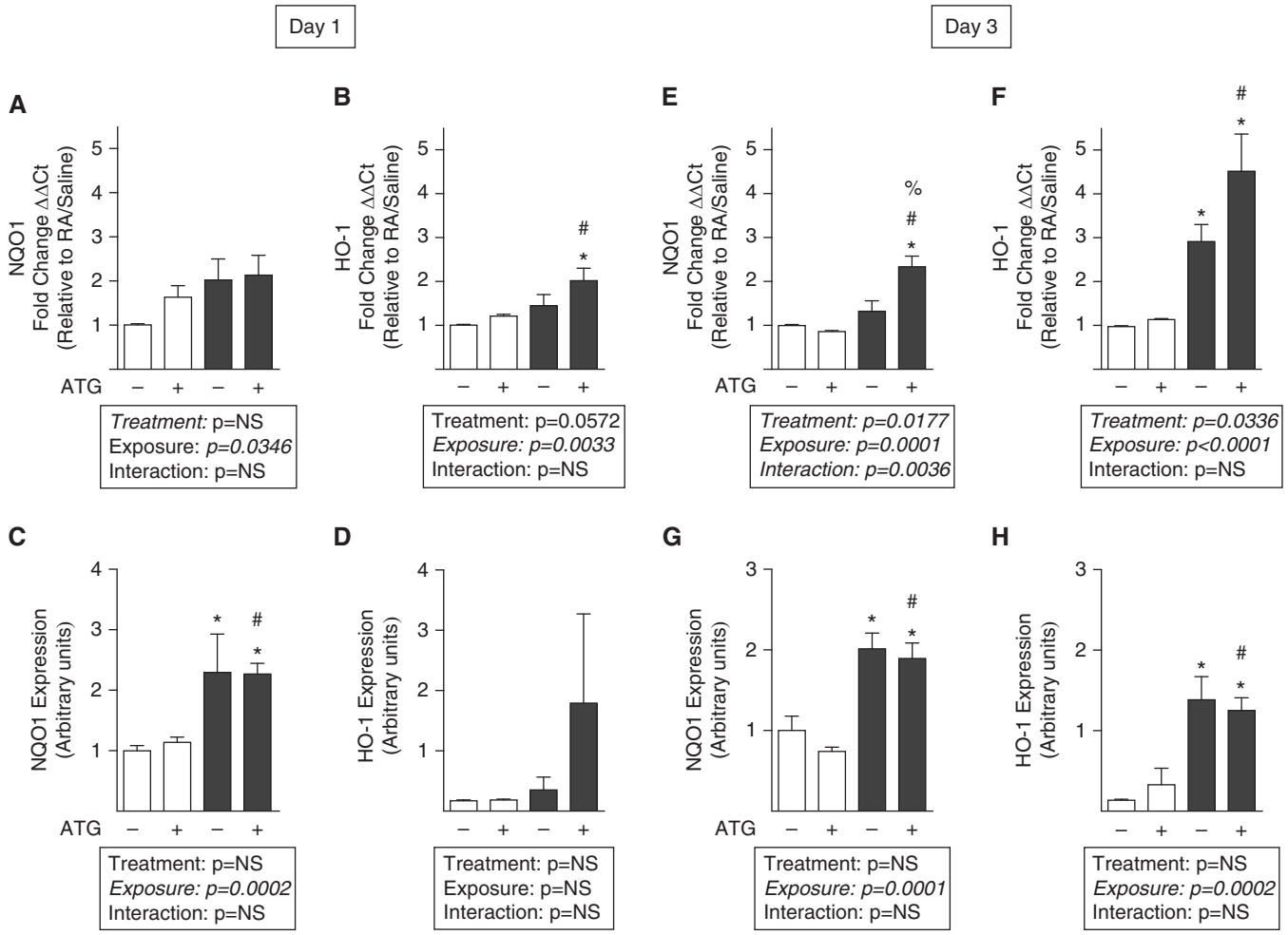

Figure 7. mRNA and protein levels of NQO1 and HO-1 in whole-lung homogenates from neonatal mice treated with ATG or saline and exposed to RA or HO (85% O_2) for 1 day (*A–D*) or 3 days (*E–H*) as outlined in Figure 1. Data were analyzed by two-way ANOVA followed by Tukey's *post hoc* analysis. ΔCt or relative densitometry values (mean ± SEM) were analyzed by two-way ANOVA followed by Tukey's *post hoc* analysis. For Day 1: quantitative RT-PCR analyses ($n = 7–8$) for (*B*) HO-1; *versus saline + RA, $P = 0.0055$; #versus ATG + RA, $P = 0.0319$; and Western blot analyses ($n = 3–5$). For (*C*) NQO1; *versus saline + RA, $P < 0.015$; #versus ATG + RA, $P = 0.0234$. For Day 3: quantitative RT-PCR analyses ($n = 3–6$) for (*E*) NQO1; *versus saline + RA, $P = 0.0004$; #versus ATG + RA, $P = 0.0002$; %versus saline + HO, $P = 0.0055$. For (*F*) HO-1; *versus saline + RA, $P < 0.009$; #versus ATG + RA, $P = 0.0003$; and Western blot analyses ($n = 3–4$). For (*G*) NQO1; *versus saline + RA, $P < 0.02$; #versus ATG + RA, $P = 0.0058$. For (*H*) HO-1; *versus saline + RA, $P < 0.006$; #versus ATG + RA, $P = 0.0248$ (*see* Figure E2 for Western blot images).

partially mediated by Nrf2 activation. Similarly, we speculate that PPAR-γ activation by ATG in the current studies could also contribute to attenuated lung injury in our model.

The phosphatidylinositol-3′-kinase (PI3K/AKT) signaling pathway is required for Nrf2 activation in lung epithelial cells and modulates hyperoxic lung injury in adult mice (41). We have shown previously that the Trx system regulates PI3K/AKT activation in settings of altered oxygen tension *in vitro* (42). It is probable that hyperoxia-mediated Trx1 oxidation in the lungs of newborn mice, which was previously described by our group, modulates PI3K/AKT signaling and Nrf2 activation in response to ATG and hyperoxia in the current studies (43). Furthermore, alterations in Trx1 function via hyperoxic exposure and TrxR1 inhibition are also likely to influence cellular signaling events in the lung, as suggested by Floen and colleagues (44).

Studies investigating the effects of Nrf2 induction in settings of newborn hyperoxic exposure are limited. Administration of the well-characterized Nrf2-inducing agent sulforaphane (SFN) to pregnant mice did not alter postnatal hyperoxic sensitivity in the offspring (45). Unfortunately, the authors of this study provided no evidence of Nrf2 activation in the lungs of offspring born to SFN-supplemented dams, so it is unclear if *in utero* SFN exposure at the doses tested induced pulmonary Nrf2 activation. Moreover, significant differences in experimental design, including differences in pharmacologic agent, timing of treatment, and murine strain, make comparisons with the current study difficult.

Although past research efforts have been directed at the use of exogenously administered antioxidants to prevent BPD, these strategies have not translated successfully to clinical care. Such approaches are likely plagued by inherent

difficulties in delivering exogenous antioxidants to appropriate cells and subcellular compartments, improper timing of administration, and/or insufficient efficacy of single antioxidants (2, 3). Novel approaches that enhance the expression of endogenous antioxidants could lead to protective effects in the highly susceptible preterm lung (2). We have observed consistently that TrxR1 inhibition is well tolerated in our experimental models. Cross-talk between the Trx and the GSH systems most likely explains the tolerance of TrxR1 inhibition *in vivo* and *in vitro* (20, 26, 28, 46–49). TrxR inhibition is detrimental only in settings of simultaneous GSH system disruption, and GSH system deficiencies, other than in dietary riboflavin deficiency, are virtually unknown in humans (50–52). Thus, the present data provide a compelling rationale for additional studies into the safety and efficacy of TrxR1 inhibitors to prevent neonatal lung injury. If pharmacologic TrxR1 inhibition can be used safely to attenuate oxygen-mediated lung injury in preterm neonates, this novel strategy could prevent BPD. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgments: The authors thank Dr. Elias S.J. Arnér, Dr. Edward Schmidt, Dr. Justin Prigge, and Dr. Tamas Jilling for provision of reagents and advice on experimental design.

References

1. Asikainen TM, White CW. Antioxidant defenses in the preterm lung: role for hypoxia-inducible factors in BPD? *Toxicol Appl Pharmacol* 2005; 203:177–188.
2. Asikainen TM, White CW. Pulmonary antioxidant defenses in the preterm newborn with respiratory distress and bronchopulmonary dysplasia in evolution: implications for antioxidant therapy. *Antioxid Redox Signal* 2004;6:155–167.
3. Welty SE. Is there a role for antioxidant therapy in bronchopulmonary dysplasia? *J Nutr* 2001;131:947S–950S.
4. Stenmark KR, Abman SH. Lung vascular development: implications for the pathogenesis of bronchopulmonary dysplasia. *Annu Rev Physiol* 2005;67:623–661.
5. Baydas G, Karatas F, Gursu MF, Bozkurt HA, Ilhan N, Yasar A, Canatan H. Antioxidant vitamin levels in term and preterm infants and their relation to maternal vitamin status. *Arch Med Res* 2002;33:276–280.
6. Lee JW, Davis JM. Future applications of antioxidants in premature infants. *Curr Opin Pediatr* 2011;23:161–166.
7. Smith CV, Hansen TN, Martin NE, McMicken HW, Elliott SJ. Oxidant stress responses in premature infants during exposure to hyperoxia. *Pediatr Res* 1993;34:360–365.
8. Jain A, Mehta T, Auld PA, Rodrigues J, Ward RF, Schwartz MK, Mårtensson J. Glutathione metabolism in newborns: evidence for glutathione deficiency in plasma, bronchoalveolar lavage fluid, and lymphocytes in prematures. *Pediatr Pulmonol* 1995;20:160–166.
9. Nguyen T, Nioi P, Pickett CB. The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. *J Biol Chem* 2009;284:13291–13295.
10. Kensler TW, Wakabayashi N, Biswal S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu Rev Pharmacol Toxicol* 2007;47:89–116.
11. Cho HY, Kleeberger SR. Nrf2 protects against airway disorders. *Toxicol Appl Pharmacol* 2010;244:43–56.
12. Boutten A, Goven D, Artaud-Macari E, Boczkowski J, Bonay M. NRF2 targeting: a promising therapeutic strategy in chronic obstructive pulmonary disease. *Trends Mol Med* 2011;17:363–371.
13. Walters DM, Cho HY, Kleeberger SR. Oxidative stress and antioxidants in the pathogenesis of pulmonary fibrosis: a potential role for Nrf2. *Antioxid Redox Signal* 2008;10:321–332.
14. Reddy NM, Suryanaraya V, Yates MS, Kleeberger SR, Hassoun PM, Yamamoto M, Liby KT, Sporn MB, Kensler TW, Reddy SP. The triterpenoid CDDO-imidazolide confers potent protection against hyperoxic acute lung injury in mice. *Am J Respir Crit Care Med* 2009; 180:867–874.
15. Cho HY, van Houten B, Wang X, Miller-DeGraff L, Fostel J, Gladwell W, Perrow L, Panduri V, Kobzik L, Yamamoto M, *et al*. Targeted deletion of *Nrf2* impairs lung development and oxidant injury in neonatal mice. *Antioxid Redox Signal* 2012;17:1066–1082.
16. McGrath-Morrow S, Lauer T, Yee M, Neptune E, Podowski M, Thimmulappa RK, O'Reilly M, Biswal S. Nrf2 increases survival and attenuates alveolar growth inhibition in neonatal mice exposed to hyperoxia. *Am J Physiol Lung Cell Mol Physiol* 2009;296:L565–L573.
17. Suvorova ES, Lucas O, Weisend CM, Rollins MF, Merrill GF, Capecchi MR, Schmidt EE. Cytoprotective Nrf2 pathway is induced in chronically txnrd 1-deficient hepatocytes. *PLoS One* 2009;4:e6158.
18. Mandal PK, Schneider M, Kölle P, Kuhlencordt P, Förster H, Beck H, Bornkamm GW, Conrad M. Loss of thioredoxin reductase 1 renders tumors highly susceptible to pharmacologic glutathione deprivation. *Cancer Res* 2010;70:9505–9514.
19. Fourquet S, Guerois R, Biard D, Toledano MB. Activation of NRF2 by nitrosative agents and H_2O_2 involves KEAP1 disulfide formation. *J Biol Chem* 2010;285:8463–8471.
20. Arner ES. Focus on mammalian thioredoxin reductases–important selenoproteins with versatile functions. *Biochim Biophys Acta* 2009; 1790: 495–526.
21. Iverson SV, Eriksson S, Xu J, Prigge JR, Talago EA, Meade TA, Meade ES, Capecchi MR, Arnér ES, Schmidt EEA. A Txnrd1-dependent metabolic switch alters hepatic lipogenesis, glycogen storage, and detoxification. *Free Radic Biol Med* 2013;63:369–380.
22. Chew EH, Nagle AA, Zhang Y, Scarmagnani S, Palaniappan P, Bradshaw TD, Holmgren A, Westwell AD. Cinnamaldehydes inhibit thioredoxin reductase and induce Nrf2: potential candidates for cancer therapy and chemoprevention. *Free Radic Biol Med* 2010;48: 98–111.
23. Cebula M, Schmidt EE, Arnér ES. TrxR1 as a potent regulator of the Nrf2-Keap1 response system. *Antioxid Redox Signal* 2015;23:823–853.
24. Smith AD, Guidry CA, Morris VC, Levander OA. Aurothioglucose inhibits murine thioredoxin reductase activity *in vivo*. *J Nutr* 1999; 129:194–198.
25. Gromer S, Arscott LD, Williams CH Jr, Schirmer RH, Becker K. Human placenta thioredoxin reductase. Isolation of the selenoenzyme, steady state kinetics, and inhibition by therapeutic gold compounds. *J Biol Chem* 1998;273:20096–20101.
26. Tipple TE, Welty SE, Rogers LK, Hansen TN, Choi YE, Kehrer JP, Smith CV. Thioredoxin-related mechanisms in hyperoxic lung injury in mice. *Am J Respir Cell Mol Biol* 2007;37:405–413.
27. Britt RD Jr, Velten M, Locy ML, Rogers LK, Tipple TE. The thioredoxin reductase-1 inhibitor aurothioglucose attenuates lung injury and improves survival in a murine model of acute respiratory distress syndrome. *Antioxid Redox Signal* 2014;20:2681–2691.
28. Locy ML, Rogers LK, Prigge JR, Schmidt EE, Arnér ES, Tipple TE. Thioredoxin reductase inhibition elicits Nrf2-mediated responses in Clara cells: implications for oxidant-induced lung injury. *Antioxid Redox Signal* 2012;17:1407–1416.
29. Velten M, Heyob KM, Rogers LK, Welty SE. Deficits in lung alveolarization and function after systemic maternal inflammation and neonatal hyperoxia exposure. *J Appl Physiol (1985)* 2010;108: 1347–1356.
30. Park MS, Rieger-Fackeldey E, Schanbacher BL, Cook AC, Bauer JA, Rogers LK, Hansen TN, Welty SE, Smith CV. Altered expressions of fibroblast growth factor receptors and alveolarization in neonatal mice exposed to 85% oxygen. *Pediatr Res* 2007;62: 652–657.
31. Farrell MR, Rogers LK, Liu Y, Welty SE, Tipple TE. Thioredoxin-interacting protein inhibits hypoxia-inducible factor transcriptional activity. *Free Radic Biol Med* 2010;49:1361–1367.

32. Rengby O, Cheng Q, Vahter M, Jörnvall H, Arnér ES. Highly active dimeric and low-activity tetrameric forms of selenium-containing rat thioredoxin reductase 1. *Free Radic Biol Med* 2009;46:893–904.
33. Cenas N, Prast S, Nivinskas H, Sarlauskas J, Arnér ES. Interactions of nitroaromatic compounds with the mammalian selenoprotein thioredoxin reductase and the relation to induction of apoptosis in human cancer cells. *J Biol Chem* 2006;281:5593–5603.
34. Arnér ES, Zhong L, Holmgren A. Preparation and assay of mammalian thioredoxin and thioredoxin reductase. *Methods Enzymol* 1999;300: 226–239.
35. Warner BB, Stuart LA, Papes RA, Wispé JR. Functional and pathological effects of prolonged hyperoxia in neonatal mice. *Am J Physiol* 1998;275:L110–L117.
36. Gromer S, Merkle H, Schirmer RH, Becker K. Human placenta thioredoxin reductase: preparation and inhibitor studies. *Methods Enzymol* 2002;347:382–394.
37. Chapple SJ, Keeley TP, Mastronicola D, Arno M, Vizcay-Barrena G, Fleck R, Siow RC, Mann GE. Bach1 differentially regulates distinct Nrf2-dependent genes in human venous and coronary artery endothelial cells adapted to physiological oxygen levels. *Free Radic Biol Med* 2016;92:152–162.
38. Cho HY, Gladwell W, Wang X, Chorley B, Bell D, Reddy SP, Kleeberger SR. Nrf2-regulated PPARγ expression is critical to protection against acute lung injury in mice. *Am J Respir Crit Care Med* 2010;182:170–182.
39. Sakurai R, Villarreal P, Husain S, Liu J, Sakurai T, Tou E, Torday JS, Rehan VK. Curcumin protects the developing lung against long-term hyperoxic injury. *Am J Physiol Lung Cell Mol Physiol* 2013;305:L301–L311.
40. Sakurai R, Li Y, Torday JS, Rehan VK. Curcumin augments lung maturation, preventing neonatal lung injury by inhibiting TGF-β signaling. *Am J Physiol Lung Cell Mol Physiol* 2011;301:L721–L730.
41. Reddy NM, Potteti HR, Vegiraju S, Chen HJ, Tamatam CM, Reddy SP. PI3K-AKT signaling via Nrf2 protects against hyperoxia-induced acute lung injury, but promotes inflammation post-injury independent of Nrf2 in mice. *PLoS One* 2015;10:e0129676.
42. Chen B, Nelin VE, Locy ML, Jin Y, Tipple TE. Thioredoxin-1 mediates hypoxia-induced pulmonary artery smooth muscle cell proliferation. *Am J Physiol Lung Cell Mol Physiol* 2013;305:L389–L395.
43. Tipple TE, Welty SE, Nelin LD, Hansen JM, Rogers LK. Alterations of the thioredoxin system by hyperoxia: implications for alveolar development. *Am J Respir Cell Mol Biol* 2009;41: 612–619.
44. Floen MJ, Forred BJ, Bloom EJ, Vitiello PF. Thioredoxin-1 redox signaling regulates cell survival in response to hyperoxia. *Free Radic Biol Med* 2014;75:167–177.
45. McGrath-Morrow SA, Lauer T, Collaco JM, Lopez A, Malhotra D, Alekseyev YO, Neptune E, Wise R, Biswal S. Transcriptional responses of neonatal mouse lung to hyperoxia by Nrf2 status. *Cytokine* 2014;65:4–9.
46. Prigge JR, Eriksson S, Iverson SV, Meade TA, Capecchi MR, Arnér ES, Schmidt EE. Hepatocyte DNA replication in growing liver requires either glutathione or a single allele of *txnrd1*. *Free Radic Biol Med* 2012;52:803–810.
47. Scarbrough PM, Mapuskar KA, Mattson DM, Gius D, Watson WH, Spitz DR. Simultaneous inhibition of glutathione- and thioredoxin-dependent metabolism is necessary to potentiate 17AAG-induced cancer cell killing via oxidative stress. *Free Radic Biol Med* 2012;52: 436–443.
48. Rubinstein I, Langevitz P, Pras M. Aurothioglucose overdosage in five patients with rheumatoid arthritis. *Clin Rheumatol* 1987; 6:583–587.
49. Du Y, Zhang H, Lu J, Holmgren A. Glutathione and glutaredoxin act as a backup of human thioredoxin reductase 1 to reduce thioredoxin 1 preventing cell death by aurothioglucose. *J Biol Chem* 2012;287: 38210–38219.
50. el-Hazmi MA, Warsy AS. Glutathione reductase deficiency in association with sickle cell and thalassaemia genes in Saudi populations. *Hum Hered* 1985;35:326–332.
51. el-Hazmi MA, Warsy AS. Riboflavin status in Saudi Arabia–a comparative study in different regions. *Trop Geogr Med* 1989;41: 22–25.
52. el-Hazmi MA, Warsy AS. Glutathione reductase in the south-western province of Saudi Arabia–genetic variation vs. acquired deficiency. *Haematologia (Budap)* 1989;22:37–42.