Glutathione Reductase Targeted to Type II Cells Does Not Protect Mice from Hyperoxic Lung Injury

Kathryn M. Heyob¹, Lynette K. Rogers¹, and Stephen E. Welty¹

¹Center for Perinatal Research, The Research Institute at Nationwide Children's Hospital, Department of Pediatrics, The Ohio State University, Columbus, Ohio

Exposure of the lung epithelium to reactive oxygen species without adequate antioxidant defenses leads to airway inflammation, and may contribute to lung injury. Glutathione peroxidase catalyzes the reduction of peroxides by oxidation of glutathione (GSH) to glutathione disulfide (GSSG), which can in turn be reduced by glutathione reductase (GR). Increased levels of GSSG have been shown to correlate negatively with outcome after oxidant exposure, and increased GR activity has been protective against hyperoxia in lung epithelial cells *in vitro*. We tested the hypothesis that increased GR expression targeted to type II alveolar epithelial cells would improve outcome in hyperoxia-induced lung injury. Human GR with a mitochondrial targeting sequence was targeted to mouse type II cells using the SPC promoter. Two transgenic lines were identified, with Line 2 having higher lung GR activities than Line 1. Both transgenic lines had lower lung GSSG levels and higher GSH/GSSG ratios than wild-type. Six-week-old wild-type and transgenic mice were exposed to greater than 95% O₂ or room air (RA) for 84 hours. After exposure, Line 2 mice had higher right lung/body weight ratios and lavage protein concentrations than wild-type mice, and both lines 1 and 2 had lower GSSG levels than wild-type mice. These findings suggest that GSSG accumulation in the lung may not play a significant role in the development of hyperoxic lung injury, or that compensatory responses to unregulated GR expression render animals more susceptible to hyperoxic lung injury.

Keywords: glutathione reductase; lung epithelium; hyperoxia; type II cells; mitochondria

Patients with respiratory failure, including preterm infants and adult patients with acute respiratory distress syndrome, are routinely supported with positive pressure ventilation and supplemental oxygen. Unfortunately, while life sustaining, these supportive therapies can be injurious to the lung. Hyperoxia has been shown to injure both the respiratory epithelium and the microvascular endothelium, but the specific mechanisms are still not clearly understood. In general, studies have indicated that the formation of reactive oxygen species generated as a result of the high concentrations of inspired oxygen can overwhelm the lung's antioxidant defenses (1). These events can result in apoptosis of alveolar epithelial cells, recruitment of inflammatory cells, and deteriorating lung mechanics dominated by increased fluid filtration (2–5).

The lung epithelium acts as the first line of defense against hyperoxia-induced lung injury, secreting surfactants and antioxidant enzymes into the alveolar space. Type II cells compose

CLINICAL RELEVANCE

Mice with glutathione reductase targeted to the mitochondria of type II cells were more susceptible to hyperoxic lung injury, suggesting that glutathione disulfide accumulation in the lung may not play a significant role, or that compensatory responses render them more susceptible.

a relatively small fraction of the lung epithelium, but have been shown to proliferate and differentiate into type I cells in response to oxidant stress to effectively remodel the damaged epithelium (6, 7). Increased antioxidant enzyme activity has been observed in type II cells in response to hyperoxia (8).

Glutathione (γ -glutamylcysteinyl glycine, GSH) is a thiol-containing tripeptide essential to lung antioxidant defenses, with GSH levels in the lung alveolar space 140-fold higher than plasma levels (9). Hydrogen peroxide and other hydroperoxides are reduced by the concurrent oxidation of GSH to glutathione disulfide (GSSG) as a result of the enzyme activities of glutathione peroxidases (GPx). GSSG can then be reduced back to GSH by the activity of glutathione reductase (GR), be exported out of the cell in which it is formed, or react with intracellular protein thiols to form mixed protein disulfides. Formation of mixed protein disulfides and alterations in the relative ratio of GSH/GSSG have been correlated with oxidant stress and lung injury (10). Mitochondria, in particular, are sensitive to disruptions in glutathione status because they are unable to synthesize GSH and therefore require either active import of GSH from the cytoplasm or the reducing capacity of GR to maintain appropriate levels of reduced GSH (11).

In human populations, GR insufficiencies due to genetic polymorphisms, inhibition with the chemotherapy drug 1,3-bis(chloroethyl)-1-nitrosourea (BCNU), or riboflavin deficiency have been associated with hemolytic anemia and reduced total glutathione content in red blood cells (12–15). BCNU increases toxicity in mice in response to hyperoxia (16) and in lung epithelial cells in response to zinc toxicity (17). In GR knockout mice, increased lung injury in hyperoxia was observed, but only after thioredoxin reductase was also inhibited pharmacologically (18, 19). *Drosophila* expressing transgenic GR had reduced GSSG levels and improved longevity with exposure to 100% oxygen (20). Targeted overexpression of GR to the mitochondria in lung epithelial cells *in vitro* was also protective against t-butyl hydroperoxide and hyperoxia (21, 22).

Based on the evidence that GR expression targeted to mitochondria is protective against oxidant injury *in vitro*, we tested the hypothesis that targeting GR expression to mitochondria in alveolar epithelial type II cells *in vivo* would have a protective effect against hyperoxic lung injury in mice.

MATERIALS AND METHODS

Mice

FVB/N zygotes were microinjected with a construct consisting of the 3.9-kb mouse SPC promoter followed by an intronic sequence (KCR,

(Received in original form March 21, 2008 and in final form June 5, 2008)

This work was supported by a grant from the National Institutes of Health, HL068948 (S.E.W.).

Correspondence and requests for reprints should be addressed to Stephen E. Welty, M.D., Center for Perinatal Research, The Research Institute at Nationwide Children's Hospital, Section of Neonatology, Department of Pediatrics, The Ohio State University, 700 Children's Drive, Columbus, OH 43205. E-mail: Stephen.Welty@nationwidechildrens.org

Am J Respir Cell Mol Biol Vol 39, pp 683–688, 2008

Originally Published in Press as DOI: 10.1165/rcmb.2008-01120C on June 19, 2008

Internet address: www.atsjournals.org

0.64 kb), the human GR (hGR) cDNA containing the 5' endogenous human mitochondrial targeting sequence (MTS-hGR, 1.5 kb), and the human growth hormone poly-A tail (hGRpa, 0.3 kb) (Figure 1A). Founders were screened after isolation of DNA using PCR primers directed to the hGR gene, and founder lines were confirmed by RNA and protein expression in F1 offspring. All studies were conducted after approval of the protocol from The Research Institute at Nationwide Children's Hospital's Institutional Animal Care and Use Committee.

Hyperoxia Exposure

Cages of 6-week-old mice were placed in Plexiglas chambers with a flow of 100% oxygen at 10 L/minute for 84 hours. The length of hyperoxia exposure was established during an initial time-course experiment, and 84 hours was chosen as the time at which the animals exhibited symptoms of lung injury but at which no mortality was observed. Soda lime was placed in the chambers to absorb excess CO₂. Oxygen levels were monitored daily to document that the concentration remained above 95% throughout the exposure, and mice were observed at least twice daily for signs of undue distress (extreme lethargy and respiratory distress). Age-matched controls were exposed to room air (RA) for the same time period. Before starting the exposures and after 84 hours of exposure, body weights were recorded. Mice were killed by intraperitoneal injection of 200 mg/kg of sodium pentobarbital followed by exsanguination. Right lungs were ligated, removed, weighed, and immediately snap-frozen in liquid nitrogen-cooled aluminum tongs, followed by storage in liquid nitrogen and long-term storage at -80°C. To collect bronchoalveolar lavage fluid samples, tracheas were cannulated with PE-50 tubing (Becton Dickinson, Franklin Lakes, NJ), and 300 µl of sterile saline was infused in and out of the left lung three times before collection of lavage contents. Lavage was centrifuged at 2,000 × g for 10 minutes to remove cellular infiltrates. Left lungs were then removed and snap-frozen for subsequent protein analysis. In separate animals, left lungs were inflation-fixed by tracheal cannulation and infusion of Histochoice (Amresco, Solon, OH) at 20 cm H₂O pressure and paraffin-embedded for subsequent histologic analyses. For subcellular fractionation, both lungs were removed, weighed, and kept in ice-cold 0.25 M sucrose until homogenization.

RT-PCR

RNA was isolated from frozen right lungs using TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's published protocols. Two micrograms of RNA was reverse-transcribed using Superscript III Reverse Transcriptase kit and Oligo d(T) primers (Invitrogen) according

to manufacturer's published protocols. Primers specific for human GR (forward: 5' TCAGCCCTGGGTTCTAAGAC, reverse: 5' TGTGAC CAGCTCTTCTGAAG) and the house-keeping gene glyceraldehyde-3 phosphate dehydrogenase (GAPDH, forward: 5' ACCACAGTC CATGCCATCAC, reverse: 5' TCCACCACCCTGTTGCTGTA) were used in separate reactions. Components of the 25-µl reaction were 2 µl Taq DNA polymerase (5 Prime, Gaithersburg, MD), 1× Taq buffer with magnesium chloride, 0.4 µM dNTP, 0.8 µM forward primer, 0.8 µM reverse primer, and 1 µl cDNA from the reverse-transcriptase reaction. For both hGR and GAPDH, 20 cycles were run at 94°C 45 seconds, 61°C for 45 seconds, and 72°C for 1 minute, with a final extension step for 10 minutes at 72°C. RNA samples were checked for genomic contamination by running PCR on RNA samples without reverse transcriptase. PCR products were separated by electrophoresis on a 1.5% agarose gel and visualized after staining with ethidium bromide.

Protein Quantitation

Protein concentrations of tissue homogenates and lavage fluids were analyzed using the Bio-Rad protein reagent (Bio-Rad, Hercules, CA). Lavage samples were not assessed if less than 70% of the instillate was recovered. Tissues were homogenized with a Dounce homogenizer in a 10% solution in KPO₄-EDTA, incubated with 0.1% Triton-X to lyse cells, and centrifuged. Pellets were discarded and 25 µl diluted homogenate or lavage was mixed with 200 µl 1:5 diluted dye. Absorbance was read at 600 nm, and sample readings were compared with a standard curve of known quantities of bovine serum albumin.

Western Blotting

SDS-PAGE was performed by running 30 µg protein on 4–12% bis-tris gels in MOPS buffer at 200V for 45 minutes using Invitrogen's XCell Surelock mini-gel system. Proteins were transferred to PVDF membranes. Western analysis was performed by blocking membranes in 10% nonfat dry milk in TBS-T (20 mM Tris buffer, pH 7.4, 150 mM NaCl, and 0.1% Tween-20) for 1.5 hours at room temperature and incubating overnight at 4°C in 5% nonfat dry milk in TBS-T containing primary antibody, which was either rabbit antisera against hGR or mGR developed in our lab (19) diluted 1:300, or mouse monoclonal anti-β-actin (Abcam, Cambridge, UK) diluted 1:5,000. Membranes were washed in TBS-T and incubated in secondary antibody, either goat anti-rabbit IgG diluted 1:10,000 in 5% nonfat dry milk in TBS-T or goat anti-mouse IgG diluted 1:15,000 in TBS-T (both secondary antibodies from Bio-Rad), washed again in TBS-T, and visualized by exposing the membranes to film after ECL detection (GE Healthcare, Buckinghamshire, UK).

Subcellular Fractionation

Mitochondria, cytosol, and nuclei were separated from fresh lungs by differential centrifugation as previously described (23).

Biochemical Assays

GSH and GSSG concentrations were determined in tissue homogenates using previously published enzyme recycling methods (23).

GR activities were measured as described (24) with or without the addition of riboflavin (FADH; Sigma-Aldrich, St. Louis, MO) at a final concentration of 1.25 µM. Briefly, 200 µl reaction buffer (0.1M Tris, pH 8.0, 1.14 mM EDTA, 6.95 mM GSSG in 0.1M Tris, pH 7.0, and 0.26 mM NADPH, with or without 1.25 µM FADH) was added to 50 µl of sample, and the rates of absorbance change were measured for 5 minutes at 340 nM on a microtiter plate reader (Dynex Technologies, Chantilly, VA).

Statistics

All experiments were repeated at least twice, with $n \geq 6$ per treatment group for statistical analysis. Subcellular fractionation studies were performed twice with total $n = 6$ per treatment group. Right lung/body weight ratios, lavage protein concentrations, lung GSH and GSSG concentrations, and GSH/GSSG ratios were analyzed by two-way ANOVA followed by modified *t* tests *post hoc*. Whole-homogenate GR activities were analyzed by two separate one-way ANOVAs, fixing either by transgenic line or treatment, with Student-Neumann-Keuls *post hoc* testing. For the subcellular fraction GR activity data, separate one-way ANOVA tests were performed for each fraction, followed by Student-Neumann-Keuls. Data were log-transformed when variances were unequal. $P < 0.05$ was chosen to indicate statistical significance.

Figure 1. Surfactant protein-C (SPC)-mitochondrial targeting sequence (MTS)-human glutathione reductase (hGR) gene construct and tissue expressions. (A) A construct containing hGR (including the MTS) under the SPC promoter was injected into FVB embryos for creation of transgenic founder lines. (B) Two-step RT-PCR for hGR and GAPDH was performed on lungs from 6-week-old wild-type (WT) mice and Lines 1 and 2 SPC-MTS-hGR mice. PCR product was separated on a 1.5% agarose gel and visualized using ethidium bromide and ultraviolet detection. (C) Rabbit antisera against hGR and mouse GR (mGR) and mouse monoclonal antibody against β-actin were used to probe Western blots from lungs of 6-week-old wild-type, Line 1 and Line 2 mice. Endogenous mGR expression was not affected by hGR expression.

RESULTS

Two founders were identified by genomic PCR, and mRNA and protein expressions were confirmed in the F1 generation by RT-PCR and Western blotting (Figure 1B). Although Line 1 had higher hGR mRNA levels than did Line 2 (Line 1: 1.11 ± 0.23 , Line 2: 0.35 ± 0.07 , hGR/GAPDH densitometry ratio), the protein expression, as measured by densitometry ratios of hGR/ β -actin protein, indicated no differences between Line 1 and Line 2 (Figure 1C). Furthermore, endogenous GR protein levels in the lung were not affected by transgene expression in either of the two founder lines (Figure 1C). A small subgroup of mice had all organs assessed for human GR mRNA and protein expression, and human GR was not expressed in other tissues examined (data not shown). No gross morphologic differences were observed in the lungs of either line of transgenic or wild-type mice in room air (Figure 2B).

To test the hypothesis that increased expression of the antioxidant glutathione reductase in type II cells would protect mouse lungs against oxidant-induced lung injury, 6-week-old transgenic and wild-type littermates were exposed to RA or hyperoxia for 84 hours. No mortality was observed during 84 hours of hyperoxia exposure. An effect of hyperoxia and no effect of transgene or interaction were observed upon right lung/body weight ratios, and in *post hoc* testing no differences were observed in right lung/body weight ratios (WT: 4.37 ± 0.12 , Line 1: 4.32 ± 0.24 , Line 2: 4.85 ± 0.35 mg/g) in mice exposed to room air, but in hyperoxia, Line 2 mice had higher right lung/body weight ratios (WT: 7.78 ± 0.56 , Line 1: 8.96 ± 1.44 , Line 2: 9.79 ± 0.57 mg/g; Figure 2A). An effect of hyperoxia and an interaction between hyperoxia and transgene were observed in lavage protein concentrations. Line 2 mice

had lower lavage protein concentrations than wild-type mice in RA (WT: 0.1188 ± 0.038 , Line 1: 0.0653 ± 0.021 , Line 2: 0.0393 ± 0.025 mg/mL; Figure 2A) and higher lavage protein concentrations than wild-type mice in hyperoxia (WT: 2.46 ± 0.32 , Line 1: 2.28 ± 0.31 , Line 2: 4.84 ± 0.53 mg/mL; Figure 2A). As would be typical with the higher right lung/body weight ratios and lavage protein concentrations observed in Line 2 mice, morphology after 84 hours of hyperoxia exposure indicated greater pleural edema and neutrophil accumulation in Lines 1 and 2 compared with wild-type mice, with the greatest level of lung injury visually in Line 2 (Figure 2B).

GR activities were measured in right lung homogenates using enzyme recycling methods (Table 1). Wild-type and Line 1 mice had higher tissue GR activities than Line 2 mice in RA. To test whether the differences were because of riboflavin limitation in the tissue homogenates, riboflavin (FADH) was added to the assay medium as described in MATERIALS AND METHODS. After addition of FADH, GR activities were highest in Line 2 mice, with GR in Line 1 mice also being higher than in wild-type mice. No differences were observed in lungs from hyperoxia-exposed mice, but after FADH addition GR activities were again highest in Line 2 mice, and GR activities in Line 1 mice were also higher than in wild-type mice.

The specificity of transgenic GR mitochondrial targeting was characterized by measuring GR activities in subcellular fractions isolated from fresh lung tissues (Figure 3).

Mitochondrial lung GR activities were highest in Line 2 mice, although Line 1 mice also exhibited higher GR activities than wild-type mice (WT: 7.527 ± 0.918 , Line 1: 47.407 ± 2.025 , Line 2: 192.175 ± 25.289 mU/mg protein). Lines 1 and 2 mice both had higher lung cytosolic (WT: 9.1472 ± 0.18965 , Line 1: 15.815 ± 0.231 , Line 2: 15.100 ± 1.231 mU/mg protein) and lung

Figure 2. Hyperoxic lung injury in SPC-MTS-hGR and wild-type mice. Six-week-old mice were exposed to greater than 95% O_2 or room air (RA) for 84 hours and killed by pentobarbital administered intraperitoneally. Right lungs were ligated, removed, and weighed, and left lungs were lavaged three times with saline or inflation-fixed. (A) Right lung weight to body weight ratios (mg/g) are expressed as an indicator of lung edema, and lung permeability due to injury is indicated by lung lavage protein concentrations. Data were analyzed by two-way ANOVA with modified *t* tests *post hoc*. For right lung/body weight ratios, an effect of hyperoxia and no effect of transgene or interaction were observed. An effect of hyperoxia and an interaction between hyperoxia and transgene were observed in lavage protein concentrations. Data are displayed as means $\pm$ SEM, with differing letters indicating statistical significance from wild-type mice. (B) Inflation-fixed lungs were paraffin-embedded and stained with hematoxylin and eosin; representative $\times 40$ images are shown.

TABLE 1. LUNG GLUTATHIONE REDUCTASE ACTIVITIES (mU/mg TISSUE) WITH (FINAL CONCENTRATION 125 μ M) AND WITHOUT ADDITIONAL RIBOFLAVIN (FADH) IN ASSAY MEDIUM IN MICE EXPOSED TO ROOM AIR OR OXYGEN FOR 84 h

	WT	Line 1	Line 2
RA	0.5250 $\pm$ 0.0837	0.6063 $\pm$ 0.0886 ^a	0.0855 $\pm$ 0.0354 ^{a*}
+ FADH	0.6799 $\pm$ 0.1207	2.3217 $\pm$ 0.2193 ^{*c}	8.6523 $\pm$ 0.5061 ^{†c}
O ₂	0.6632 $\pm$ 0.0106	0.7598 $\pm$ 0.05732 ^{ab}	0.4807 $\pm$ 0.1632 ^b
+ FADH	0.5540 $\pm$ 0.0401	1.0350 $\pm$ 0.1169 ^{*b}	5.7233 $\pm$ 0.9869 ^{†c}

* indicates differences between Line 1 and WT.

† indicates differences between Line 2 and Line 1.

^a, ^b, and ^c indicate differences within lines when letters are different.

nuclear (WT: 2.356 $\pm$ 0.273, Line 1: 22.839 $\pm$ 5.16, Line 2: 17.1438 $\pm$ 2.531 mU/mg protein) GR activities than wild-type mice. Subcellular fractions isolated from lungs of mice exposed to hyperoxia for 84 hours exhibited a similar trend. Mitochondrial fraction GR activities were highest in Line 2 mice, and they were also higher in Line 1 mice than in wild-type mice (WT: 9.2511 $\pm$ 2.79, Line 1: 47.46 $\pm$ 28.48, Line 2: 266.07 $\pm$ 66.75 mU/mg protein). Additional riboflavin in the assay medium did not alter GR activities in lung subcellular fractions (data not shown). GR activities were again higher in the nuclei of the transgenic mice than wild-type mice. Activities in Line 1 mice were higher than in Line 2 or wild-type mice, and activities were higher in Line 2 mice than in wild-type mice (WT: 4.39 $\pm$ 1.21, Line 1: 28.89 $\pm$ 5.09, Line 2: 16.51 $\pm$ 3.32 mU/mg protein). No differences were observed in cytosolic GR activities be-

Figure 3. GR activities in lung subcellular fractions. Mitochondrial, cytosolic, and nuclear fractions were isolated from fresh lungs of transgenic mice from Lines 1, 2, and wild-type littermates exposed to RA or greater than 95% O₂ for 84 hours. GR activities were measured by enzymatic assay, and results are displayed as means $\pm$ SEM. Data were analyzed by one-way ANOVA within each fraction followed by Student-Newman-Keuls (SNK) *post hoc* testing, with different letters indicating different SNK subsets.

tween the transgenic and wild-type mice (WT: 8.64 $\pm$ 1.47, Line 1: 12.28 $\pm$ 1.27, Line 2: 12.46 $\pm$ 1.25 mU/mg protein).

GSH levels in whole lungs of mice exposed to RA were not different (WT: 1.040 $\pm$ 0.016, Line 1: 1.06 $\pm$ 0.0305, and Line 2: 0.94 $\pm$ 0.015 μ mol/g tissue; Figure 4A), but when exposed to hyperoxia Line 2 mice had lower GSH levels than wild-type mice (WT: 1.52 $\pm$ 0.12, Line 1: 1.14 $\pm$ 0.22, Line 2: 0.806 $\pm$ 0.11 μ mol/g tissue; Figure 4A). By two-way ANOVA there was an effect of transgene and hyperoxia exposure on lung GSSG levels. In RA-exposed mice, GSSG levels were lower in both transgenic lines than in wild-type mice (WT: 18.52 $\pm$ 3.38, Line 1: 4.86 $\pm$ 2.07, and Line 2: 3.83 $\pm$ 1.41 nmol/g tissue), and both transgenic lines exposed to hyperoxia also had lower lung GSSG concentrations than wild-type mice (WT: 19.56 $\pm$ 2.30, Line 1: 9.26 $\pm$ 2.35, Line 2: 8.37 $\pm$ 1.34 nmol/g tissue; Figure 4B). An effect of transgene, no effect of hyperoxia, and an interaction were noted when GSH/GSSG ratios from whole lung homogenates were analyzed by two-way ANOVA (Figure 4C). In both transgenic strains GSH/GSSG ratios were significantly higher than in wild-type mice in RA (WT: 85.75 $\pm$ 26.94, Line 1: 260.74 $\pm$ 96.79, Line 2: 489.11 $\pm$ 218.78); however, there were no differences in lungs exposed to hyperoxia (WT: 86.07 $\pm$ 7.16, Line 1: 138.46 $\pm$ 20.06, Line 2: 144 $\pm$ 37.54).

DISCUSSION

Oxygen toxicity is frequently a complication of supportive care for infants and patients with respiratory failure, and this complication is independent of the cause of respiratory failure. Oxygen toxicity in studies of adult animals is characterized by

Figure 4. Glutathione (GSH) and glutathione disulfide (GSSG) levels in lungs of hyperoxia and RA-exposed transgenic SPC-MTS-hGR and wild-type mice. (A) GSH and (B) GSSG levels in lungs from wild-type and transgenic mice exposed to RA or greater than 95% O₂ were measured by enzymatic assay. Two-way ANOVA analysis of GSH levels indicated no effects of hyperoxia or transgene and no interaction, and effects of transgene and hyperoxia but no interaction were observed on GSSG levels. (C) Data are also expressed as GSH/GSSG ratio, and an effect of transgene, no effect of hyperoxia, and an interaction between transgene and hyperoxia were observed. Data were analyzed by two-way ANOVA followed by modified *t* tests, and expressed as means $\pm$ SEM, with differing letters indicating statistical significance at *P* < 0.05.

a period in which overt lung injury is not apparent, followed by an inflammatory phase involving neutrophil recruitment, and finally by acute deterioration in lung function secondary to the development of pulmonary edema (25, 26). In hyperoxia-exposed mice, pulmonary edema is manifested by increases in lung weights and bronchoalveolar lavage (BAL) protein concentrations (27). In the present study, the increases in lung weights and BAL protein concentrations, and observed increases in pleural edema and neutrophil infiltration in the mice expressing the highest activities of GR in the lung, suggest that increasing GR to the extent observed in these animals hastens the development of hyperoxic lung injury.

While the biological role for lung inflammation in the development of lung injury in models of adult murine hyperoxia are somewhat controversial, neutrophil accumulation is routinely observed in the later phases of lung injury. The earlier neutrophil accumulation in the transgenic mice is entirely consistent with a greater susceptibility to hyperoxic lung injury, and the accumulation may be causally related to both increased lung weights and lavage protein concentrations. While we cannot exclude the possibility that increasing GR activity in the lung causes a more pronounced lung injury in hyperoxia via an entirely different mechanism than is observed in wild-type animals, the fact that the characteristics of lung injury and histology are consistent with classic hyperoxic lung injury makes this possibility unlikely.

The exact mechanism(s) for the development of hyperoxic lung injury have not been elucidated, but there is extensive evidence for a direct effect of oxygen (26, 28). Investigators have observed increased production of oxidants in the mitochondria of rodents exposed to hyperoxia (29–31) and a protective role for antioxidants targeted to the mitochondria of mice (32). We targeted transgenic GR expression to lung mitochondria using the endogenous GR mitochondrial leader sequence, and targeting success was indicated by increased GR activities in lung mitochondrial fractions (Figure 3). The increases in whole-homogenate GR activities seemed to be primarily due to the increases in the mitochondria, although there was a slight but statistically significant increase in the cytosolic fraction GR activities in our transgenic lines. Whether this is the case *in situ* cannot be ascertained, but the increase is most likely related to leakage of GR into the cytosolic fraction during the isolation procedure. The increases in the nuclear GR activities are less likely the result of the isolation procedure and suggest that the cDNA we used may have also contained a nuclear targeting signal.

The fact that the transgenic animals were not protected from hyperoxic lung injury was in contrast to overexpression of GR *in vitro*. In experiments using transformed Clara cell-like lung epithelial cells (H441) transfected with a similar mitochondrial-targeted cDNA, increased GR activities, and protection against hyperoxia and tert-butyl hydroperoxide were observed (21, 22).

GR reduces glutathione disulfide to glutathione using the reducing equivalents of NADPH, and requires riboflavin (FADH) as a cofactor. In our transgenic mice, higher levels of lung GR activities were not uniformly detected unless exogenous FADH was added to the lung homogenate preparations (Table 1). We doubt that FADH is rate limiting in the lungs *in vivo*, as low GSSG contents in the lungs of transgenic mice most likely represent increased GR activity, and exogenous FADH was not necessary for measurements of GR in subcellular fractions. The fact that exogenous FADH was necessary for GR activity measurements in whole homogenates but not in subcellular fractions is not easily explained. Although we cannot exclude the possibility that the transgenic mice may be FADH deficient and unable to express the full transgene potential, addressing this

possibility would require further studies into the effects of dietary riboflavin supplementation and are beyond the scope of the present studies. It is also possible that mice with increased GR have rate-limited quantities of NADPH, as this molecule is added to the assay to measure GR activity; however, low GSSG contents in the lung would be unlikely if NADPH contents were not ample as a substrate for GR.

The observation that lungs of Line 2 mice in hyperoxia had lower GSH levels than wild-type mice was unexpected, but is likely due to the increase in lung weights when they accumulate lung water (Figure 4A). When GSH was expressed as $\mu\text{mol}/\text{whole right lung}$, GSH concentrations in hyperoxia were not statistically different (data not shown). Alternatively, GSH levels could be lower because GSH was consumed in hyperoxia. This is unlikely as consumption should have been associated with an increased GSSG level, which was not observed.

The greater susceptibility of transgenic mice expressing mitochondrial-targeted, lung-specific GR was not anticipated, and a nonspecific effect of gene integration cannot be excluded. However, this possibility is extremely unlikely in that a GR transgene expression dose response was observed during the development of hyperoxic lung injury. Line 2 mice with the highest GR activities had higher right lung/body weight ratios than wild-type mice at 84 hours of hyperoxia exposure, and Line 1 mice with intermediate levels of GR activity had intermediate right lung/body weight ratios not different than either wild-type or Line 2 mice (Figure 2A).

In contrast to the increased susceptibility to hyperoxic lung injury observed in the mice expressing GR targeted to the alveolar epithelium, similarly targeted transgenic expressions of manganese superoxide-dismutase (MnSOD) and EC-SOD have been shown to be protective (32–34). These apparent discrepancies may provide important clues as to the mechanisms involved in hyperoxic lung injury. Hyperoxia exposure increases superoxide production, which can be reduced to hydrogen peroxide by the activity of SOD. Furthermore, mice overexpressing GPx are less susceptible to other forms of oxidant injury (35, 36). Taken together, these findings may indicate a pivotal role for peroxide-reducing mechanisms. However, GPx knockout mice are not more susceptible to hyperoxia (37), and likewise catalase knockout mice are also not more susceptible to hyperoxic lung injury (38). These findings and the findings from the present study suggest that the pathways distal to superoxide dismutase, including peroxides and products of the glutathione pathway (which include GSSG and protein mixed disulfides), may not be directly involved in the development of hyperoxic lung injury. These interpretations probably represent an oversimplification of the role of oxidation and antioxidants in lung injury and do not entirely take into account other possible reasons for our findings.

It is possible that our findings may represent a compensatory response to up-regulated GR that deleteriously affects the lungs exposed to hyperoxia. GR-deficient mice have actually been reported to be less susceptible to hyperoxia than wild-type mice, and findings from our laboratory suggest that the protection is related to a compensatory up-regulation of thioredoxin (18). When GR knockout mice were rendered deficient in thioredoxin reductase by autothioglucose (ATG) administration, they were far more susceptible to hyperoxia than were GR knockouts administered saline or wild-type mice administered ATG (18). Thus it is possible that in mice with targeted overexpression of GR, there is a modulation of the thioredoxin system or some other molecule involved in thiol disulfide chemistry or cell signaling in the lung, rendering these mice more susceptible to hyperoxia.

In summary, targeted expression of GR to the lung increased the activity of this enzyme *in vivo* with an associated functional

effect as indicated by a decrease in lung GSSG contents. These mice were not protected from hyperoxic lung injury; in fact, the mice with the highest expression were more susceptible to hyperoxia than wild-type mice. These findings suggest that GSSG accumulation in the lung may not play a significant role in the development of hyperoxic lung injury or that compensatory responses to unregulated GR expression render animals more susceptible to hyperoxic lung injury.

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.

Acknowledgments: The authors thank XiaoMei Meng, Ryan Farrell, and Katherine Backes for their technical assistance and expertise.

References

1. Saugstad OD. Bronchopulmonary dysplasia-oxidative stress and antioxidants. *Semin Neonatal* 2003;8:39–49.
2. Town IG, Phillips GJ, Murdoch E, Holgate ST, Kelly FJ. Temporal association between pulmonary inflammation and antioxidant induction following hyperoxic exposure of the preterm guinea pig. *Free Radic Res Commun* 1993;18:211–221.
3. McGrath-Morrow SA, Stahl J. Apoptosis in neonatal murine lung exposed to hyperoxia. *Am J Respir Cell Mol Biol* 2001;25:150–155.
4. Arkovitz MS, Garcia VF, Szabo C, McConnell K, Bove K, Wispe JR. Decreased pulmonary compliance is an early indicator of pulmonary oxygen injury. *J Surg Res* 1997;67:193–198.
5. Wolfe WG, Robinson LA, Moran JF, Lowe JE. Reversible pulmonary oxygen toxicity in the primate. *Ann Surg* 1978;188:530–543.
6. Evans MJ, Cabral LJ, Stephens RJ, Freeman G. Transformation of alveolar type 2 cells to type 1 cells following exposure to NO₂. *Exp Mol Pathol* 1975;22:142–150.
7. Evans MJ, Dekker NP, Cabral-Anderson LJ, Freeman G. Quantitation of damage to the alveolar epithelium by means of type 2 cell proliferation. *Am Rev Respir Dis* 1978;118:787–790.
8. Bhandari V, Maulik N, Kresch M. Hyperoxia causes an increase in antioxidant enzyme activity in adult and fetal rat type II pneumocytes. *Lung* 2000;178:53–60.
9. Cantin AM, North SL, Hubbard RC, Crystal RG. Normal alveolar epithelial lining fluid contains high levels of glutathione. *J Appl Physiol* 1987;63:152–157.
10. O'Donovan DJ, Fernandes CJ. Mitochondrial glutathione and oxidative stress: implications for pulmonary oxygen toxicity in premature infants. *Mol Genet Metab* 2000;71:352–358.
11. Griffith OW, Meister A. Origin and turnover of mitochondrial glutathione. *Proc. Natl. Acad. Sci. USA* 1985;82:4668–4672.
12. 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.
13. el-Hazmi MA, Warsy AS. Riboflavin status in Saudi Arabia—a comparative study in different regions. *Trop Geogr Med* 1989;41:22–25.
14. Frischer H, Ahmad T. Severe generalized glutathione reductase deficiency after antitumor chemotherapy with BCNU (1,3-bis(chloroethyl)-1-nitrosourea). *J Lab Clin Med* 1977;89:1080–1091.
15. Loos H, Roos D, Weening R, Houwerzijl J. Familial deficiency of glutathione reductase in human blood cells. *Blood* 1976;48:53–62.
16. Kehrer JP, Paraidathathu T. Enhanced oxygen toxicity following treatment with 1,3-bis(2-chloroethyl)-1-nitrosourea. *Fundam Appl Toxicol* 1984;4:760–767.
17. Walther UI, Czermak A, Muckter H, Walther SC, Fichtl B. Decreased GSSG reductase activity enhances cellular zinc toxicity in three human lung cell lines. *Arch Toxicol* 2003;77:131–137.
18. 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.
19. Rogers LK, Tamura T, Rogers BJ, Welty SE, Hansen TN, Smith CV. Analyses of glutathione reductase hypomorphic mice indicate a genetic knockout. *Toxicol Sci* 2004;82:367–373.
20. Mockett RJ, Sohal RS, Orr WC. Overexpression of glutathione reductase extends survival in transgenic *Drosophila melanogaster* under hyperoxia but not normoxia. *FASEB J* 1999;13:1733–1742.
21. O'Donovan DJ, Katkin JP, Tamura T, Husser R, Xu X, Smith CV, Welty SE. Gene transfer of mitochondrially targeted glutathione reductase protects H441 cells from t-butyl hydroperoxide-induced oxidant stresses. *Am J Respir Cell Mol Biol* 1999;20:256–263.
22. O'Donovan DJ, Katkin JP, Tamura T, Smith CV, Welty SE. Attenuation of hyperoxia-induced growth inhibition in H441 cells by gene transfer of mitochondrially targeted glutathione reductase. *Am J Respir Cell Mol Biol* 2000;22:732–738.
23. Jopper-Davis KS, Park MS, Rogers LK, Backes CH Jr, Lim IK, Smith CV. Compartmental inhibition of hepatic glutathione reductase activities by 1,3-bis(2-chloroethyl)-N-nitrosourea (BCNU) in Sprague-Dawley and Fischer-344 rats. *Toxicol Lett* 2004;147:219–228.
24. Rogers LK, Gupta S, Welty SE, Hansen TN, Smith CV. Nuclear and nucleolar glutathione reductase, peroxidase, and transferase activities in livers of male and female Fischer-344 rats. *Toxicol Sci* 2002;69:279–285.
25. Smith LJ. Hyperoxic lung injury: biochemical, cellular, and morphologic characterization in the mouse. *J Lab Clin Med* 1985;106:269–278.
26. Crapo JD. Morphologic changes in pulmonary oxygen toxicity. *Annu Rev Physiol* 1986;48:721–731.
27. Wong YL, Smith CV, McMicken HW, Rogers LK, Welty SE. Mitochondrial thiol status in the liver is altered by exposure to hyperoxia. *Toxicol Lett* 2001;123:179–193.
28. Wolfe WG, DeVries WC. Oxygen toxicity. *Annu Rev Med* 1975;26:203–217.
29. Turrens JF, Freeman BA, Crapo JD. Hyperoxia increases H₂O₂ release by lung mitochondria and microsomes. *Arch Biochem Biophys* 1982; 217:411–421.
30. Turrens JF, Freeman BA, Levitt JG, Crapo JD. The effect of hyperoxia on superoxide production by lung submitochondrial particles. *Arch Biochem Biophys* 1982;217:401–410.
31. Freeman BA, Crapo JD. Hyperoxia increases oxygen radical production in rat lungs and lung mitochondria. *J Biol Chem* 1981;256:10986–10992.
32. Wispe JR, Warner BB, Clark JC, Dey CR, Neuman J, Glasser SW, Crapo JD, Chang LY, Whittsett JA. Human Mn-superoxide dismutase in pulmonary epithelial cells of transgenic mice confers protection from oxygen injury. *J Biol Chem* 1992;267:23937–23941.
33. Ahmed MN, Suliman HB, Folz RJ, Nozik-Grayck E, Golson ML, Mason SN, Auten RL. Extracellular superoxide dismutase protects lung development in hyperoxia-exposed newborn mice. *Am J Respir Crit Care Med* 2003;167:400–405.
34. Folz RJ, Abushamaa AM, Suliman HB. Extracellular superoxide dismutase in the airways of transgenic mice reduces inflammation and attenuates lung toxicity following hyperoxia. *J Clin Invest* 1999; 103:1055–1066.
35. Ran Q, Liang H, Gu M, Qi W, Walter CA, Roberts LJ II, Herman B, Richardson A, Van Remmen H. Transgenic mice overexpressing glutathione peroxidase 4 are protected against oxidative stress-induced apoptosis. *J Biol Chem* 2004;279:55137–55146.
36. Thiruchelvam M, Prokopenko O, Cory-Slechta DA, Richfield EK, Buckley B, Mirochnitchenko O. Overexpression of superoxide dismutase or glutathione peroxidase protects against the paraquat + maneb-induced Parkinson disease phenotype. *J Biol Chem* 2005;280: 22530–22539.
37. Ho YS, Magnenat JL, Bronson RT, Cao J, Gargano M, Sugawara M, Funk CD. Mice deficient in cellular glutathione peroxidase develop normally and show no increased sensitivity to hyperoxia. *J Biol Chem* 1997;272:16644–16651.
38. Ho YS, Xiong Y, Ma W, Spector A, Ho DS. Mice lacking catalase develop normally but show differential sensitivity to oxidant tissue injury. *J Biol Chem* 2004;279:32804–32812.