Thioredoxin-Related Mechanisms in Hyperoxic Lung Injury in Mice

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Reduction of glutathione disulfide (GSSG) to glutathione (GSH) by glutathione reductase (GR) enhances the efficiency of GSH-dependent antioxidant activities. However, GR-deficient (a1Neu) mice are less susceptible to acute lung injury from continuous exposure to > 95% O₂ (96 h: 6.9 ± 0.1 g right lung/kg body versus room air 3.6 ± 0.3) than are C3H/HeN control mice (10.6 ± 1.3 versus 4.2 ± 0.3, *P* < 0.001). a1Neu mice have greater hepatic thioredoxin (Trx)1 and Trx2 levels than do C3H/HeN mice, suggesting compensation for the absence of GR. a1Neu mice exposed to hyperoxia for 96 hours showed lower levels of inflammatory infiltrates in lungs than did similarly exposed C3H/HeN mice. Pretreatment with aurothioglu-ucose (ATG), a thioredoxin reductase (TrxR) inhibitor, exacerbated the effects of hyperoxia on lung injury in a1Neu mice (11.6 ± 0.8, *P* < 0.001), but attenuated hyperoxic lung edema and inflammation in C3H/HeN mice (6.3 ± 0.4, *P* < 0.001). No consistent alterations were observed in lung GSH contents or liver GSH or GSSG levels after ATG pretreatment. The data suggest that modulation of Trx/TrxR systems might provide therapeutically useful alterations of cellular resistance to oxidant stresses. The protective effects of ATG against hyperoxic lung injury could prove to be particularly useful therapeutically.

Keywords: hyperoxia; glutathione; thioredoxin; auranofin; aurothio-glucose

The use of high concentrations of supplemental oxygen is necessary in many disease states. Pulmonary oxygen toxicity, a frequent consequence of prolonged treatment with supplemental oxygen administration, has been implicated in the pathogenesis of acute respiratory distress syndrome (ARDS) and may play a role in the pathogenesis of bronchopulmonary dysplasia (1, 2). Although oxygen supplementation often is life-saving, hyperoxic exposures can cause toxicities in human patients and in experimental animals. The mechanisms that mediate oxygen-induced injury have been studied extensively, and oxygen toxicity is generally thought to be mediated through the actions of reactive oxygen species (ROS), such as superoxide (O₂⁻), hydrogen peroxide (H₂O₂), peroxynitrite (ONOO⁻), and hydroxyl radical (HO[•]), which are produced at rates greater than normal during exposure to hyperoxia (3).

Glutathione (GSH) acts as an antioxidant in several ways. In reactions that produce glutathione disulfide (GSSG), glutathione peroxidases (GPx) catalyze the GSH-dependent reduction of

CLINICAL RELEVANCE

The initiation of hyperoxic lung injury is not closely correlated with alterations in global thiol/disulfide ratios, and modulation of Trx/TrxR systems might provide therapeutically useful alterations of cellular resistance to oxidant stresses.

H₂O₂ and other substrate oxidants (GSH + H₂O₂ → GSSG + H₂O). The flavoenzyme glutathione reductase (GR) [EC 1.8.1.7] is pivotal to the normal functioning of GSH-dependent antioxidant defense mechanisms, by catalyzing the reduction of GSSG back to GSH (NADPH + H⁺ + GSSG → NADP⁺ + 2 GSH). This reduction is essential for the maintenance of high intracellular GSH:GSSG ratios, typically > 99:1 (4, 5). If not reduced to GSH or excreted into plasma, lymph, or bile, cellular GSSG can undergo reversible thiol-disulfide exchange reactions with protein thiol groups resulting in the formation of protein-SS-glutathione (PSSG). Glutaredoxins (Grx) are GSH-dependent enzymes responsible for reducing glutathionylated proteins. (6)

Thioredoxin (Trx) is a dithiol-disulfide protein with a conserved -Cys-Gly-Pro-Cys- active site that is maintained in a reduced state by the enzyme thioredoxin reductase (TrxR). The two major isoforms of thioredoxin characterized to date are thioredoxin-1 (Trx1) and thioredoxin-2 (Trx2), which are localized in cell cytosolic and mitochondrial compartments, respectively (7–9). The dithiol form of Trx can undergo thiol-disulfide exchange reactions with protein mixed disulfides (PSSX), that, coupled with reduction by TrxR, result in net reduction of PSSX by NADPH:

Trx can act more directly as an antioxidant via reduction of H₂O₂ by enzymes known as thioredoxin peroxidases, also called periredoxins (10). Other physiologic functions performed by Trx include the donation of reducing equivalents to ribonucleotide reductase, control of protein folding, and redox regulation of enzymes and transcription factors (9–11). Whereas reduction of disulfide bonds in glutathionylated proteins can be catalyzed by Grxs, the reduction of disulfides in proteins by Trx and TrxR provides a system for regulating cellular protein thiol redox status that is complementary to GSH-dependent systems. The GSH and Trx systems likely have overlap in functions, but these relationships have not been studied extensively to date (6, 7, 10, 12–15).

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GSH and GSSG levels historically have been measured as parameters for quantitative characterization of oxidant stresses. Most investigators appear to assume the importance of a functional GSH redox cycle in the antioxidant capacities of cells or organisms, and reduction of GSSG to GSH by GR would seem to be essential for optimal functioning of GSH-dependent reductive functions. *Gr1^{a1Neu}* (a1Neu) mice, which were generated by Pretsch, exhibit rates of GSSG-dependent NADPH oxidation that are readily measurable, but are less than 10% of the rates observed in assays with corresponding tissue homogenates and fractions from wild-type mice (16). However, a1Neu mice do not exhibit measurable levels of immunoreactive GR protein and were found by genomic sequence analyses to exhibit genetic deletions that are incompatible with expression of a catalytically active GR protein (17). The fact that a1Neu mice are functional GR knockouts suggested to us that the GSSG-stimulated oxidation of NADPH we observed might be due to Trx/TrxR-mediated transfer of reducing equivalents from NADPH to GSSG, as demonstrated in other species lacking functional GR (15, 18). Similar processes *in vivo* also could account for the surprising viability of the a1Neu mice, but would imply that the a1Neu mice confronted with oxidant challenges would be more dependent on Trx/TrxR activities than would mice that express active GR at normal levels.

Aurothioglucose (ATG) and auranofin (AFN), anti-inflammatory gold compounds used in the treatment of rheumatoid arthritis, inhibit TrxR through irreversible binding to the active site selenocysteines (19). Both ATG and AFN also inhibit GR and GPx *in vitro*, by binding to the active site cysteine and selenocysteine residues, respectively, but with affinities that are three orders of magnitude lower than are the corresponding affinities for TrxR. The AFN inhibition assay that is used to determine TrxR activities in biological samples is based upon the differences in reactivities of AFN with TrxR, GR, and GPx (20, 21). Furthermore, Smith and coworkers demonstrated that a single intraperitoneal injection of 25 to 200 mg ATG/kg in C3H/HeJ mice inhibited TrxR activities in heart, liver, pancreas, and kidney for up to 126 hours, without affecting GR or GPx activities (22).

The present studies used a mouse model of acute hyperoxic lung injury that produces responses similar to those seen in human patients with ARDS (23). Our studies were designed to test the hypothesis that a1Neu mice are more dependent on Trx-dependent mechanisms for protection against hyperoxic lung injury than are wild-type C3H/HeN mice. Greater exacerbation of hyperoxic lung injury in a1Neu mice than in C3H/HeN mice by pretreatment with ATG would suggest a greater dependence on Trx/TrxR-dependent mechanisms in a1Neu than in the GR-sufficient control mice. Effects of ATG pretreatment on the hyperoxic sensitivities of C3H/HeN mice would reflect the relative roles of the GSH/GR and Trx/TrxR systems in antioxidant defense functions in these animals.

a1Neu and C3H/HeN mice were treated with saline or ATG and exposed to > 95% O₂ for up to 96 hours. ATG treatment inhibited TrxR activities, but did not alter measured GR or GPx activities. Right lung/body weight ratios were calculated as indices of lung injury, and lung and liver GSH and GSSG contents were determined as biomarkers of redox responses. The results suggest that the Trx/TrxR systems have important protective functions against hyperoxic lung injury in the GR-deficient a1Neu mice, whereas the net protective effects of ATG against hyperoxic lung injury in the GR-sufficient C3H/HeN mice are more logically attributed to anti-inflammatory effects of the ATG. Complete absence of expression of GR, as observed in the a1Neu mice, has not been observed in humans, which suggests that the net protective effects of ATG or similar

agents against hyperoxic lung injury may be adaptable for therapeutic uses in human patients.

MATERIALS AND METHODS

Animal Protocol

Gr1^{a1Neu} mice, backcrossed at least 15 generations into a C3H background, were received as a generous gift from Professor Walter Pretsch. After import, the a1Neu mice were rederived in our facility. C3H/HeN mice were obtained from Harlan Sprague-Dawley to be used as controls. Both a1Neu and C3H/HeN strains were housed and bred in identical conditions in the Columbus Children's Research Institute Animal Facility. All animals were kept in polycarbonate cages with wood chip bedding. The animals were permitted access to food and water *ad libitum*, and a 12:12 hour day:night cycle was maintained throughout the study. The Institutional Animal Care and Use Committee of the Columbus Children's Research Institute approved the experimental protocols used in this study.

Western Blot Analyses

Trx1 contents were assessed by western analyses, using goat anti-Trx1 IgG primary antibody (R&D Systems, Minneapolis, MN) and HRP-conjugated rabbit anti-goat IgG secondary antibody. Trx2 contents were assessed by Western analyses, using rabbit polyclonal anti-Trx2 IgG primary antibody (gift from Dr. J. Yodoi at Kyoto University). The membranes were developed with ECL+ reagent (GE Healthcare, Buckinghamshire, UK) and read with a phosphorimager (GE Healthcare, Buckinghamshire, UK), with band quantitation using Image-Quant software (Molecular Dynamics, Sunnyvale, CA).

Pharmacodynamic Studies

Six- to 9-week-old a1Neu and C3H/HeN mice ($n = 4$ for each treatment group) were given single intraperitoneal injections of 0, 25, 50, 100, or 200 mg/kg of ATG (Research Diagnostics, Flanders, NJ) in saline. A single mouse from each treatment group was then killed at 2, 24, 48, or 96 hours, after anesthesia with 200 mg/kg pentobarbital administered intraperitoneally. As 25 mg/kg ATG was determined to be the best dose for subsequent pharmacologic studies, additional 6- to 9-week-old a1Neu mice ($n = 1-3$ for each time point) were given 25 mg/kg ATG and were killed at 2, 24, 48, and 96 hours. For animals that received 25 mg/kg ATG, livers and right lungs were removed, weighed, freeze-clamped, and stored at -80°C . Portions of 0.1 g of frozen tissues were homogenized in 0.9 ml 0.1 M NaHPO₄ with 5 mM EDTA in a Dounce homogenizer. Ten microliters of 10% Triton X-100 in water were added and mixed, and the homogenization mixtures were chilled on ice for 15 minutes. The samples were then centrifuged at $13,000 \times g$ for 15 minutes at 4°C . Supernatants were collected, protein concentrations were determined by the Bradford assay, and TrxR, GPx, and GR activities were determined (24). Twenty-five mg/kg ATG data were combined for pharmacodynamic statistical analyses (total $n = 2-4$ per treatment group).

Hyperoxia Experiments

All treatments and exposures were performed in parallel. Six-week-old C3H/HeN and a1Neu mice ($n = 4-6$ for each treatment group) received single intraperitoneal injections at time 0 of either saline or 25 mg/kg of ATG in saline. Mice were allowed to remain in room air or were placed in sealed Plexiglas containers with a continuous flow of oxygen (10 L/min) to sustain > 95% O₂. Oxygen levels were measured twice daily (Hudson RCI, Temecula, CA). Soda lime (Fisher Scientific, Fair Lawn, NJ) was placed in the hyperoxia exposure chamber, to prevent accumulation of CO₂. Mice were killed at either 72 or 96 hours after ATG administration, and tissues were processed as previously described.

Tissues for Histopathologic Examination

For histopathologic examination of lung tissues, tracheas of mice were cannulated with 25-gauge silastic catheters, and 10% buffered formalin (Fisher Scientific, Fair Lawn, NJ) was instilled at 20 cm H₂O pressure. After 5 minutes, right lungs were removed and fixed overnight in 10% formalin. The next day, lung samples were washed in PBS and serially

dehydrated in increasing concentrations of ethanol, then embedded in paraffin. Five-micron tissue sections were stained with hematoxylin and eosin (H&E).

TrxR Assay

TrxR activities were determined by the AFN inhibition assay (21). Briefly, in a total assay volume of 1 ml, 30 µl of 100 mM 5,5'-dithiobis(2-nitrobenzoic acid) (Sigma-Aldrich, St. Louis, MO, in DMSO) were mixed with T-buffer (100 mM potassium phosphate, 2 mM EDTA; pH 7.4) and tissue homogenate (for liver: 100 µl of a 10% [~ 20 mg/ml] homogenate; for lung: 125 µl of a 20% [~ 10 mg/ml] homogenate). The enzymatic reaction was initiated by adding 50 µl NADPH (4 mM in T-buffer), and the increases in absorbance at 412 nm were measured for 3 minutes. A second assay was performed as described above, with the replacement of 1 µl of buffer with 1 µl of 1 mM AFN (Prometheus Laboratories, San Diego, CA) in DMSO. TrxR activities were calculated as the differences between the activities measured in the absence and presence of AFN.

GR Assay

The assay mixture contained 83 µmol tris(hydroxymethyl)aminomethane (Tris), pH 8.0, 0.8 µmol of EDTA, 5.70 µmol GSSG (Sigma-Aldrich) in 0.1 M Tris, pH 7.0, and 0.2 µmol NADPH. To this mixture, 0.05 ml of sample was added, mixed rapidly, and the rates of change in absorbance at 340 nm were measured (25). The proportion of measured GR activity attributable to TrxR was calculated from the ΔA340 in the absence and presence of 1 µM AFN (final concentration based upon the AFN inhibition assay as described above), added as 1 mM solutions in DMSO.

GSH and GSSG Concentrations

The concentrations of GSSG and GSH (tGSH = GSH + 2GSSG) in lung and liver homogenates were measured by enzyme recycling assays and experimentally derived standard curves, as described by Rogers and Smith (26). GSH concentrations were calculated by subtracting GSSG concentrations from the total GSH concentrations.

Statistics

Data were analyzed by Levene's test of equality for error variances and were log transformed when appropriate. For Western blot analyses, data were analyzed by unpaired Student's *t* tests. For hepatic GSSG reductase and a1Neu ATG pharmacodynamic studies, data were analyzed by one-way ANOVA, with Student-Newman-Keuls (SNK) tests *post hoc*. For liver homogenate GSSG-reductase activity measurements, data were analyzed by two-way ANOVA, with SNK tests *post hoc* (27). For subsequent hyperoxia experiments, data were analyzed by three-way ANOVA, with SNK tests *post hoc*. Statistical analyses were performed using SPSS Version 13.01 (Chicago, IL). Differences were noted at *P* < 0.05.

RESULTS

Rates of GSSG-dependent oxidation of NADPH by homogenates from livers of the a1Neu mice were readily measurable, but were only 5% of the activities observed in livers from the C3H/HeN mice (Table 1). However, GR protein was not detectable by

TABLE 1. HEPATIC GLUTATHIONE DISULFIDE REDUCTASE ACTIVITY (mU/mg PROTEIN)

Strain	Auranofin (µM)	
	0	1
a1Neu	1.2 ± 0.1 ^{ab}	0.7 ± 0.1 ^{ac}
C3H/HeN	23.5 ± 1.5 ^b	22.7 ± 1.0 ^c

Glutathione disulfide-dependent oxidation of NADPH by liver tissue homogenates in the absence and presence of auranofin as described in MATERIALS AND METHODS (*n* = 6). Data, expressed as means ± SEM, were analyzed by two-way ANOVA, with SNK tests *post hoc*. Differences were noted at *P* < 0.05. Groups sharing common superscripted letters are different from each other.

Western blotting in tissue homogenates from a1Neu mice, and the alterations of the GR gene in the a1Neu mice are not compatible with expression of an active enzyme (16, 17). Addition of AFN to the GR enzyme assay mixtures *in vitro* decreased apparent GR activities in liver homogenates from a1Neu mice by 42%, but did not measurably alter liver GR activities in homogenates from the C3H/HeN mice.

Trx1 and Trx2 levels in livers from a1Neu mice were twice and four times the corresponding levels in C3H/HeN mice, respectively (Figure 1). In initial studies of inhibition of TrxR activities *in vivo* by administration of ATG to a1Neu and C3H/HeN mice not exposed to hyperoxia, differences between strains were observed in animal lethality. C3H/HeN mice did not exhibit mortality at any ATG dose tested, up to 200 mg/kg, but intraperitoneal administration of 200 mg/kg ATG to a1Neu mice was fatal by 24 hours, and all a1Neu mice that received 50 or 100 mg/kg ATG died by 48 hours. No mortality was observed through 96 hours in a1Neu mice that received 25 mg/kg ATG, and this dose was selected for further study. TrxR activities in liver and lung homogenates from a1Neu mice treated with 25 mg/kg ATG were 7 and 8% of the corresponding activities in saline-treated control animals, respectively, at 2 hours, with recovery to 27 and 14%, respectively, through 96 hours (Table 2). Lung and liver GPx and GR activities in a1Neu and C3H/HeN mice treated with 25 mg/kg ATG were not different than in saline-treated controls at any time point (data not shown).

No effects on lung to body weight ratios, used as an index of lung injury and edema, were observed after 72 hours of hyperoxia (FiO₂ > 0.95) in either strain (a1Neu or C3H/HeN) or treatment group (0 or 25 mg/kg of ATG) (Figure 2A). In both a1Neu and C3H/HeN mice, exposure to hyperoxia for 96 hours increased right lung/body weight ratios. The data showed effects of FiO₂; a three-way interaction of strain, ATG, and FiO₂; and two-way interactions between strain and ATG and between

Figure 1. Hepatic Trx-1 and Trx-2 contents in a1Neu and C3H/HeN mice. Western blot analyses of whole liver homogenates, using goat anti-Trx1 (A) and anti-Trx2 (B) IgG primary antibodies, respectively. The membranes were developed, and band densities quantified, as described in MATERIALS AND METHODS. Density data are expressed as means ± SEM (*n* = 4) and were analyzed by unpaired Student's *t* test, with differences noted at *P* < 0.05.

TABLE 2. TISSUE THIOREDOXIN REDUCTASE ACTIVITY (mU/mg PROTEIN)

Tissue	Hours after ATG Injection				
	Control	2	24	48	96
Liver	3.03 ± 0.07 ^a	0.20 ± 0.07 ^a	0.36 ± 0.06 ^a	0.39 ± 0.06 ^a	0.81 ± 0.16 ^a
Lung	1.60 ± 0.16 ^{ab}	0.12 ± 0.06 ^b	0.12 ± 0.04 ^b	0.19 ± 0.08 ^b	0.23 ± 0.03 ^b

Definition of abbreviation: ATG, aurothioglucose.

Pharmacodynamics of 25 mg/kg ATG in a1Neu mice as described in MATERIALS AND METHODS ($n = 2-4$). Data, expressed as means ± SEM, were analyzed by one-way ANOVA, with SNK tests *post hoc*. Differences were noted at $P < 0.05$. Groups sharing common superscripted letters are different from each other.

strain and FiO_2 (Figure 2B). Saline-pretreated C3H/HeN mice showed greater right lung/body weight ratios after 96 hours of hyperoxia than did saline-treated a1Neu mice. a1Neu mice treated with ATG before exposure to hyperoxia for 96 hours exhibited greater lung/body weight ratios than did saline-pretreated a1Neu mice exposed to hyperoxia for 96 hours.

In contrast, C3H/HeN mice pretreated with ATG before exposure to hyperoxia for 96 hours showed lower lung/body

weight ratios than did mice of the same strain that had been pretreated with saline vehicle and exposed to hyperoxia. Thus, pretreatment with ATG exacerbated hyperoxic lung injury in a1Neu mice, but attenuated hyperoxic lung damage in C3H/HeN mice. a1Neu mice pretreated with ATG and exposed to hyperoxia for 96 hours exhibited decreased ambulation and signs of respiratory distress, including labored respirations, gasping, and chest wall retractions. These effects were not observed in any other treatment group in either strain.

The most striking effects of hyperoxia exposure on lung histology observed were in the ATG-pretreated a1Neu mice exposed to hyperoxia for 96 hours (Figure 3D) and in the saline-pretreated C3H/HeN mice exposed to hyperoxia for 96 hours (Figure 3F). Although the effects of strain, ATG, and hyperoxia on total contents of inflammatory cells are striking, even more intriguing is that the lungs of a1Neu mice pretreated with ATG and exposed to hyperoxia exhibited edema and inflammatory cells primarily in the interstitial spaces, whereas edema and inflammatory cells in lung sections of saline-pretreated C3H/HeN mice exposed to hyperoxia were observed principally in the alveoli. Lung sections from C3H/HeN mice pretreated with ATG before hyperoxic exposure (Figure 3H) exhibited a profound attenuation of the extensive inflammatory infiltrates that were observed in lung sections from saline-treated controls exposed to hyperoxia (Figure 3F).

Lung GSH concentrations ($\mu\text{mol/g}$ tissue) indicated effects of FiO_2 , no effects of strain or dose of ATG, and interactions between strain and ATG, as well as between ATG and FiO_2 (Figure 4A). Lung GSH concentrations were lowest in the groups of animals with the highest right lung/body weight ratios, which were the ATG-treated a1Neu and saline-treated C3H/HeN mice exposed to hyperoxia for 96 hours. In a1Neu mice kept in room air, lung GSH concentrations also were lower in mice treated with ATG than in the mice given the saline vehicle alone.

Lung GSSG concentrations indicated effects of strain and FiO_2 , a three-way interaction of strain, ATG, and FiO_2 , and two-way interactions between strain and FiO_2 and between ATG and FiO_2 (Figure 4B). In all treatment groups, a1Neu mice had higher concentrations of GSSG in their lungs than did C3H/HeN mice. The GSSG concentrations in the lungs of the a1Neu mice were not affected by any of the treatments studied, whereas lung GSSG levels were increased in lungs of C3H/HeN mice exposed to hyperoxia for 96 hours. Administration of ATG had no effect on lung GSSG concentrations in either strain kept in room air, but exacerbated the effects of hyperoxia in C3H/HeN mice.

Lung GSH/GSSG ratios indicated effects of strain and FiO_2 and an interaction between strain and FiO_2 (Figure 4C). In saline-pretreated mice kept in room air, lung GSH/GSSG ratios were much lower in a1Neu than in C3H/HeN mice, but the ratios were not altered in a1Neu mice by any treatment studied. ATG alone had no effect on lung GSH/GSSG ratios in C3H/HeN mice. In C3H/HeN mice, lung GSH/GSSG ratios were decreased by exposure to hyperoxia for 96 hours, but this effect of hyperoxia was not affected by pretreatment with ATG.

Lung GSH contents (μmol of GSH in right lungs/kg of animal body weight) were calculated as a means of correction for dilution by GSH-poor edema fluids in mice sustaining hyperoxic lung injury. The data indicated effects of ATG and FiO_2 , no effect of strain, and interactions between strain and ATG, as well as between ATG and FiO_2 (data not shown). a1Neu mice that received 25 mg/kg ATG and were kept in room air had lower lung GSH contents than did saline-treated mice kept in room air. Similar effects were not seen in C3H/HeN mice.

Figure 2. Right lung/body weight ratios in a1Neu and C3H/HeN mice after exposure to hyperoxia. Mice received single doses of 0 or 25 mg/kg ATG, intraperitoneally in saline, and were kept in room air or placed in > 95% O_2 , for 72 (A) or 96 hours (B), as described in MATERIALS AND METHODS. Data are expressed as means ± SEM ($n = 5-9$), and were log-transformed and analyzed by three-way ANOVA and SNK tests *post hoc*, with differences noted at $P < 0.05$. (A) No effects of strain, ATG, or FiO_2 . (B) Effects of strain, ATG, and FiO_2 ; a three-way interaction of strain, ATG, and FiO_2 ; two-way interaction between strain and FiO_2 . Groups sharing common superscripted letters are different from each other.

Figure 3. H&E-stained lung sections from a1Neu (A–D) and C3H/HeN (E–H) mice. Mice were treated as described in Figure 2B, and left lungs were inflation fixed at 20 cm H₂O. Sections were prepared and stained with H&E. A–D are from a1Neu mice, and E–H are from C3H/HeN mice, with doses of ATG (mg/kg) and FiO₂, respectively, of (A, E) 0, 0.21; (B, F) 0, 0.95; (C, G) 25, 0.21; (D, H) 25, 0.95. The most notable effects evident in these lung sections are the greater inflammation and edema in the C3H/HeN mice treated with saline and exposed to hyperoxia (F) than in similarly treated a1Neu mice (B) and the remarkable attenuation of the effects of hyperoxia on the C3H/HeN mice pretreated with ATG (H), versus the marked potentiation of lung injury by hyperoxia in a1Neu mice pretreated with ATG (D) (×40).

Liver GSH concentrations (μmol/g tissue) showed effects of FiO₂ and an interaction between strain and ATG (Figure 5A). Concentrations were not different between saline-treated a1Neu and C3H/HeN controls. After exposure to hyperoxia, hepatic GSH concentrations in both strains were lower than in animals kept in room air, and this effect of hyperoxia was not changed by ATG pretreatment. ATG treatment decreased liver GSH concentrations in a1Neu mice, but not in C3H/HeN mice.

Hepatic GSSG concentrations indicated effects of strain and FiO₂ and an interaction between strain and ATG (Figure 5B). GSSG concentrations were substantially greater in livers of a1Neu mice than in livers of C3H/HeN mice, regardless of treatment or exposure. ATG pretreatment did not alter liver GSSG concentrations in either strain. Saline-pretreated C3H/HeN mice exposed to hyperoxia exhibited lower liver GSSG

Figure 4. Lung GSH and GSSG concentrations in a1Neu and C3H/HeN mice after exposure to > 95% O₂ for 96 hours. Mice were treated as described in Figure 2B, GSH (A) and GSSG (B) concentrations were determined, and GSH/GSSG ratios (C) calculated. Levels were normalized to tissue weights, and data, expressed as means ± SEM (*n* = 3–6), were log-transformed and analyzed by three-way ANOVA and SNK tests *post hoc*, with significance noted at *P* < 0.05. (A) Effects on GSH were noted for FiO₂, as were interactions between strain and ATG, and between strain and FiO₂. (B) Effects on GSSG observed were of strain and FiO₂ and interactions between strain and FiO₂; ATG and FiO₂; and a three-way interaction of strain, ATG, and FiO₂. (C) GSH/GSSG ratios indicated effects of strain and FiO₂ and an interaction between strain and FiO₂. Groups sharing common superscripted letters are different from each other.

concentrations than did mice kept in room air, but similar effects of hyperoxia were not observed in saline-pretreated a1Neu mice. In the ATG-pretreated a1Neu mice exposed to hyperoxia, the mean hepatic GSSG concentrations were about half of the concentrations observed in the other three groups of

Figure 5. Liver GSH and GSSG concentrations in a1Neu and C3H/HeN mice after exposure to > 95% O₂ for 96 hours. Mice were treated as described in Figure 2B, and hepatic GSH and GSSG levels measured, as described in MATERIALS AND METHODS. Data are expressed as means $\pm$ SEM ($n = 5-6$) and were log-transformed and analyzed by three-way ANOVA and SNK tests *post hoc*, with differences noted at $P < 0.05$. Effects of FiO₂ and an interaction between strain and ATG were indicated. Groups sharing common superscripted letters are different from each other.

a1Neu mice studied; however, these apparent differences were not identified as statistically significant ($P = 0.053$) by the methods and criteria applied in the present studies.

DISCUSSION

The low rates of GSSG-dependent oxidations of NADPH effected by homogenates of livers and other tissues isolated from a1Neu mice were interpreted by us initially as expression of inefficient and/or unstable forms of GR. Loos and colleagues suggested such a mechanism in their description of a cohort of three siblings with similarly low (apparent) GR activities in erythrocytes and nucleated blood cells (28). However, subsequent studies by Rogers and coworkers indicated that a1Neu mice express no measurable GR, which indicates that the activities responsible for GSSG-dependent oxidation of NADPH observed in tissues of a1Neu mice (Table 1) are more reasonably attributed to other enzymes (17). The effects of AFN *in vitro* on these activities are consistent with Trx/TrxR catalysis of the reactions observed. Addition of AFN to the assay mixtures decreased the measured GSSG reductase activities in liver homogenates of a1Neu mice, but effects of AFN were not ob-

served in homogenates from C3H/HeN mice. The lack of effects of AFN on measured activities in the homogenates from the C3H/HeN mice is not necessarily due to an inability of AFN to inhibit TrxR in these tissues, but is more readily attributable to the inherent difficulties in detecting small differences in the much higher capacities of the GR-dependent GSSG-reductase activities in these mice. The GR-independent GSSG reductase activities measured in a1Neu mouse liver homogenates represent $\sim 5\%$ of the total GSSG reductase activities (GR-dependent plus GR-independent) measured in the wild-type mice. The AFN-inhibited activities in a1Neu homogenates account for $\sim 2\%$ of the wild-type levels, thereby providing estimates of the relative capacities of the GR and TrxR systems in the C3H/HeN animals. The greater levels of expression of Trx1 and Trx2 in the a1Neu than in the C3H/HeN mice (Figure 1) suggest that the contributions of Trx/TrxR to GSSG reduction in the C3H/HeN mice represent even lower fractions of the total capacities in the wild-type animals.

The substantial and sustained inhibition of TrxR activities through 96 hours in both liver and lung tissues that was observed after a single dose of 25 mg/kg of ATG provided a useful experimental model for investigation of the relative importance of Trx-dependent mechanisms in hyperoxia. As with any pharmacologic interdiction, the possible inhibition and/or induction of enzymes other than TrxR by ATG treatment *in vivo* are potential confounding effects that should be considered in interpretation of the data. However, with the data presently available, the effects of ATG treatment on responses to hyperoxia are most reasonably interpreted as resulting primarily from inhibition of TrxR. As such, the data are consistent with Trx-dependent mechanisms serving significant functions in resistance to hyperoxia in the a1Neu mice, but not serving similarly critical functions in antioxidant resistance in the C3H/HeN mice.

Enhanced mortality in normoxia in a1Neu but not C3H/HeN mice after the administration of greater than 25 mg/kg ATG suggests that Trx-dependent mechanisms are of greater functional importance in a1Neu than in GR-sufficient C3H/HeN mice. The GSH and Trx systems likely have overlaps in functions in mammalian species, but these relationships have not been studied extensively to date (10, 13–15, 29). In *Escherichia coli*, the Trx and GSH pathways can be disabled independently, without serious detriment to the organism. However, if both pathways are interrupted simultaneously through the deletion of *E. coli* TrxR (*trxB*) and GR (*gor*) genes, aerobic growth of the organism is almost completely eliminated (30).

The a1Neu mice are not exquisitely sensitive to hyperoxia (Figures 2 and 3), despite the complete absence of GR expression, and the limited capacities of Trx/TrxR-dependent reduction of GSSG. These findings suggest a limited importance of high capacities of reduction of GSSG in organismal defenses against hyperoxia-induced oxidant stresses (16, 17). The greater resistance of the GR-deficient a1Neu mice than of the wild-type C3H/HeN mice to hyperoxic lung injury correlates with the greater expressions of Trx1 and Trx2, but this correlation does not prove a causative relationship or mechanism. Nevertheless, the data clearly indicate that resistance to hyperoxic lung injury does not correspond to GSSG reduction capacities.

The greater inflammatory responses observed in lung sections from saline-treated C3H/HeN mice exposed to hyperoxia for 96 hours than from similarly treated a1Neu mice may indicate attenuated inflammatory responsiveness in the a1Neu mice (Figures 3B and 3F). However, the lower levels of inflammation observed in the a1Neu mice may be secondary to lower levels of primary injury, possibly resulting from the greater levels of Trx1 and Trx2 in the a1Neu mice. Exacerbation of hyperoxic lung injury in the a1Neu mice pretreated with ATG (Figures 2 and 3)

further suggests that Trx is critical for antioxidant defense functions in these mice. The histologic differences between ATG-treated a1Neu and saline-treated C3H/HeN are striking, and detailed quantitative comparisons of the histologic and inflammatory responses are needed.

The attenuation of hyperoxic lung injury in C3H/HeN mice pretreated with ATG (Figures 2 and 3) suggests that Trx-dependent antioxidant functions are not as important in the C3H/HeN mice as in the a1Neu mice and that the dominant effects of ATG treatment in the GR-sufficient mice may be mediated by anti-inflammatory functions. In addition to inhibition of TrxR, gold compounds such as ATG and AFN act upon several types of immune cells, modulate the secretion of cytokines, and inhibit the traffic of leukocytes into inflamed areas (31, 32). Development of therapeutic applications of the effects evidenced by the attenuation by ATG of hyperoxic lung injury in the C3H/HeN mice would be aided greatly by delineation of the mechanisms responsible. However, the results of the present studies do not define the contributions of inhibition of TrxR, anti-inflammatory effects, or other properties of ATG administration.

The GSH and GSSG levels in lungs and livers of the animals studied appear to contradict most simple hypotheses relating depletion of GSH or accumulation of GSSG as events participating in the initiation of hyperoxic lung damage. Although a number of statistical differences are noted in the data on GSH and GSSG levels, the only consistent observation is that GSSG levels in the lungs and livers of the a1Neu mice are uniformly higher than in the C3H/HeN mice, regardless of treatment or extent of lung damage. The apparent hyperoxia-induced decreases in concentrations of GSH in the lungs of saline-pretreated mice of both strains are more reasonably ascribed to dilution by GSH-poor edema fluid. The decreases in hepatic GSH levels (Figure 5) in saline-pretreated mice of both strains are not accompanied by marked changes in organ weights and are therefore likely to reflect real metabolic effects, but the bases and potential therapeutic usefulness of these observations are not evident at the present time.

The fact that the GR-deficient a1Neu mice are not markedly more susceptible to hyperoxic lung injury than are the wild-type C3H/HeN mice is quite different than what would be expected for mechanisms of injury that arise from simple shifts in relatively singular thiol/disulfide ratios. Alterations in thiol status are the most commonly offered parameters used to define oxidant stresses. The results of the present studies thus have two rather fundamental implications, the first being that global thiol/disulfide ratios are not closely coupled with initiation of injury by what is arguably the purest experimental model of oxidant stress *in vivo*. The second implication of the present results is that, at least in lungs and livers, hyperoxia does not overwhelm disulfide reduction capacities that sustain tissue levels of thiols and restrain accumulation of disulfides. These observations do not lead us to conclude that thiol/disulfide shifts are unimportant in the biological effects of oxidant stresses, but the data do indicate that pathophysiologically important thiol/disulfide shifts can be quite small quantitatively, and specific and sensitive methods of analyses will be needed in the study of such effects.

The simplest interpretation of the greater resistance of the a1Neu mice than of the C3H/HeN mice to hyperoxic lung injury would be more facile reduction by a1Neu mice of protein disulfides formed through actions of oxidants generated in greater amounts during exposure to hyperoxia. However, Trx1 has both intracellular and extracellular functions and Trx1 in plasma and other extracellular compartments has exhibited striking cytoprotective effects in a range of experimental models of tissue damage (11, 33–55). The protective effects observed in these studies have

been more closely related to the immunomodulatory effects of extracellular Trx1 using transgenic overexpression of hTrx1 or exogenous administration of rhTrx1, which markedly attenuates cell injury in mouse and rat models. To date, the lack of availability of specific methods and reagents necessary for measuring murine plasma Trx1 has limited direct studies of endogenous extracellular Trx1 responses and effects. Thus, the role of extracellular Trx1 in the modulation of inflammatory responses to hyperoxia in a1Neu mice is unclear. Nevertheless, the much greater effects on lung injury in the a1Neu mice than in the C3H/HeN mice by ATG treatment suggests that Trx/TrxR-dependent functions are the primary critical mechanisms.

Although the present data might be interpreted as suggesting that enhanced expression of Trx and/or TrxR could be useful in efforts to augment antioxidant defense functions, the associations between Trx and TrxR levels and neoplastic states indicate a reason for considerable caution in any such efforts (56–59). The potentiation of hyperoxic lung injury in the a1Neu mice by pretreatment with ATG suggests that these mice are able to manage normal rates of disulfide reduction through Trx/TrxR, but that this system is unable to meet the increased demands arising during hyperoxia. In contrast, the substantial attenuation of hyperoxic lung injury observed in the wild-type C3H/HeN mice by pretreatment with ATG indicates that comparable levels of inhibition of TrxR in GR-sufficient mice are tolerated. The net protective effects observed in the wild-type mice further suggest that the anti-inflammatory effects of ATG dominate in the GR-sufficient animals. GR deficiencies are virtually unknown in humans, other than dietary riboflavin deficiencies, which are readily detectable and correctable (60–62). The protective effects of ATG against hyperoxic lung injury could prove to be most useful therapeutically and relatively straightforward to investigate and implement.

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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