Extracellular glutathione inhibits oxygen-induced permeability changes in alveolar epithelial monolayers

J. H. ROUM,1 A. S. ALEDIA,1 L. A. CARUNGCONG,1 K.-J. KIM,2–6 AND Z. BOROK2,3

1Department of Medicine, University of California Irvine Medical Center, Orange 92868; and 2Will Rogers Institute Pulmonary Research Center and Departments of 3Medicine, 4Physiology and Biophysics, 5Biomedical Engineering, and 6Molecular Pharmacology and Toxicology, University of Southern California, Los Angeles, California 90033

Received 7 July 2000; accepted in final form 5 March 2001

Roum, J. H., A. S. Aledia, L. A. Carungcong, K.-J. Kim, and Z. Borok. Extracellular glutathione inhibits oxygen-induced permeability changes in alveolar epithelial monolayers. J Appl Physiol 91: 748–754, 2001.—Exposure to high fractional inspired oxygen for 24 h increases permeability of the alveolar epithelium, contributing to the clinical manifestations of oxygen toxicity. Utilizing a model of the alveolar epithelium in which isolated rat type II cells form polarized monolayers on polycarbonate filters [transepithelial resistance (Rt) > 1 kΩ·cm2 by day 4], we evaluated the ability of reduced glutathione (GSH) to ameliorate these changes. On day 4, apical fluid was replaced with culture medium containing 1) no additives, 2) GSH (500 μM), or 3) GSH (500 μM) + glutathione reductase (0.5 U/ml) + nicotinamide adenine dinucleotide phosphate (250 μM). Monolayers were exposed (for 24 h) to room air (control) or 95% O2, each containing 5% CO2. After 24 h of hyperoxia, Rt for condition 1 decreased by 45% compared with control (P < 0.001). In conditions 2 and 3, Rt did not decrease significantly (P = not significant). Hyperoxia-induced decreases in active ion transport were observed for conditions 1 and 2 (P < 0.05), but not for condition 3 (P = not significant). These findings indicate that extracellular GSH may protect the alveolar epithelium against hyperoxia-induced injury. Addition of glutathione reductase and nicotinamide adenine dinucleotide phosphate may further augment these protective effects of GSH.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: J. H. Roum, University of California Irvine Medical Center, Bldg. 53, Rm. 119, 101 City Dr. South, Orange, CA 92868 (E-mail: jroum @uci.edu).
BACKGROUND

GSH, which is a ubiquitous, nonprotein thiol, is synthesized in the liver and transported to the lungs via the bloodstream (54). Basolateral fluid was replaced with culture medium alone. AEC monolayers were then exposed in parallel incubator chambers (Lab-line dual CO2 Imperial II incubator 422, Melrose Park, IL) circulated with 21% O2-5% CO2 or 95% O2-5% CO2 for 24 h. Rt and PD were measured before and after the 24-h exposure as described above.

Statistical Analysis

Values are means ± SE; n is number of monolayers. Differences among group means (e.g., media conditions due to room air or oxygen exposure) were evaluated by one-way ANOVA. Post hoc analyses using Student-Newman-Keuls procedure were performed to contrast group means. P < 0.05 was considered significant.

RESULTS

Baseline Resistances, PD, and Ieq

On day 4 of culture (before exposure to room air or hypoxic conditions), the Rt of monolayers was 1.73 ± 0.05 kΩ·cm² (n = 46). With the apical side as reference, the PD of monolayers was 5.6 ± 0.3 mV before exposure, with a calculated Ieq of 3.22 ± 0.11 μA/cm². There were no differences in the Rt or Ieq among individual preexposure groups [P = not significant (NS)]. Because there were no differences in the Rt and Ieq among the preexposure groups, postexposure values on day 5 were compared.

Oxygen Exposure

After 24 h of room air exposure, slight elevations in Rt and Ieq relative to day 4 values were noted, although the changes were not statistically significant (P = NS; Figs. 1 and 2). Similar increases in Rt and Ieq from days 4 through 5 under baseline conditions have been previously reported for rat AEC monolayers over time in culture (16). In contrast, monolayers exposed to 95% O2 for 24 h showed significant decreases in Rt (45%, P < 0.001; Fig. 1) and Ieq (37%, P < 0.05; Fig. 2) compared with room air controls.

Downloaded from http://jap.physiology.org/ by guest on June 11, 2017
Effect of Apical GSH, GR, and NADPH

Room air exposure. After 24 h of exposure to room air, monolayers showed no differences in $R_t$ when media conditions 1, 2, and 3 were compared ($P = NS$; Fig. 3). Likewise, no statistical differences in $I_{eq}$ were found when conditions 1, 2, and 3 were compared after room air exposure for 24 h ($P = NS$; Fig. 4). These data demonstrate that individual media conditions did not differentially affect $R_t$ or $I_{eq}$ after 24 h of room air exposure.

Oxygen exposure. In contrast to the marked decrease in $R_t$ after 24 h of exposure to 95% $O_2$ seen in cells bathed in media alone compared with room air controls, $R_t$ did not significantly decrease in the presence of GSH in the apical fluid ($P = NS$ compared with media alone after 24 h of hyperoxia). Likewise, when GSH, GR, and NADPH were present in the apical fluid, there was no significant decrease in $R_t$ compared with room air controls ($P = NS$; Fig. 3).

The $I_{eq}$ observed in hyperoxia-treated monolayers in the absence or presence of GSH in the apical fluid was significantly less than that in monolayers exposed to room air for 24 h ($P < 0.01$; Fig. 4). In contrast, when GSH, GR, and NADPH were present in the apical fluid, no significant decrement in $I_{eq}$ was observed in response to hyperoxia compared with 24-h room air control ($P = NS$).

**DISCUSSION**

Exposure of AEC monolayers in primary culture on polycarbonate filters to 95% $O_2$ for 24 h resulted in decreases in $R_t$ and $I_{eq}$. These findings are consistent with previous animal studies, in which hyperoxia causes an increase in the permeability of the air-blood barrier as reflected by an increase in the movement of small solutes across the lung epithelial barrier (45, 57). Previous studies of epithelial cells in culture have demonstrated similar increases in epithelial permeability after oxidative stress (42, 53). The increase in epithelial permeability and subsequent decrease in $R_t$ caused by hyperoxia observed in this study are accompanied by a significant decrease in $I_{eq}$. These findings are consistent with a previous study, in which hyperoxia-induced decreases in $I_{eq}$ were accompanied by reduced Na$^+$ pump mRNA and protein levels (8).

Although there appears to be some variability in the effects of oxygen depending on species (29, 45), exposure duration (8, 22, 45, 48), and exposure methods or models used (47), in this study we demonstrate that 24 h of oxygen exposure leads to acute permeability changes reflected by decrements in $R_t$ in this in vitro model of the alveolar epithelium. The present data are inconsistent with the occurrence of major cell death or “desquamation” from the polycarbonate filters, since relatively high resistance (>1 kΩ·cm$^2$) was still maintained after oxygen exposure in the absence of exogenous glutathione. Sprague-Dawley rats are reportedly more resistant to oxygen toxicity than other strains (32, 60), and it is conceivable that the oxygen-induced effect on permeability presented here might have been greater if a different strain was used.

Consistent with the role of GSH as an antioxidant, we demonstrate that the presence of GSH in apical fluid protects against oxygen-induced changes in $R_t$ and $I_{eq}$, suggesting that glutathione ameliorates oxidant-induced permeability changes. GSH is a tripeptide antioxidant, normally present in high concentrations in ELF (15, 46). In ELF, glutathione is found predominantly in the reduced form (15), which is its active antioxidant form. Among the antioxidant properties of GSH is its ability to protect cells against free radicals and ROS because of its strongly reducing thiol group (46). Furthermore, glutathione in ELF has been reported to be deficient in a number of disease states that are characterized by alterations of the alveolar epithelium [e.g., adult respiratory distress syndrome (49)] or feature an increased oxidant burden on the alveolar epithelial surface [e.g., cystic fibrosis (CF) and idiopathic pulmonary fibrosis (IPF) (14, 56)].
diseases often place the patient at risk for oxygen toxicity because of the need for supplemental oxygen. The possibility of potentiating acute lung injury by oxygen toxicity is supported by results of studies in animal models (59).

In addition to changes in permeability and active ion transport with hyperoxia, other measurements of alveolar epithelial dysfunction due to hyperoxia have been reported previously for similar exposures (24 h, >90% O₂). These include a decrease in ATP content (1), decreased cell proliferation (21), decreased DNA, thymidine, GSH, and superoxide dismutase levels, decreased synthesis of dipalmitoylphosphatidylcholine, and increased lactate dehydrogenase levels (33). Although they are not evaluated here, it may be of interest to further study effects of GSH supplementation on these and other hyperoxia-induced processes.

Analysis of the role of glutathione in oxygen toxicity is complex; this complexity is related to the unique characteristics of GSH and its metabolism: 1) it likely functions as an intracellular and an extracellular antioxidant (5, 11, 14, 15, 49, 56); 2) it interacts with several other molecules in the extracellular milieu (other thiols including thiolated proteins, nitric oxide, and other nitrogen oxides, e.g., S-nitrosothiols); 3) it has a complete system of enzymes and substrates [GR, glutathione peroxidase (GPx), and NADPH], extracellularly (ELF) (15) and intracellularly, to enhance its activity and restore it to its reduced (active antioxidant) form (46); 4) it has complex means of cellular uptake, involving other substrates such as cysteine (25) and cellular enzymes such as γ-glutamyl transpeptidase (28); and 5) it has a multienzyme intracellular synthesis pathway (46). These multiple characteristics have led to the study of the role of GSH from multiple different aspects including oxidative-reductive state, activity of related enzymes, absolute concentrations intracellularly and extracellularly, synthesis, cellular uptake, and release. In this study, the effects of exogenous extracellular GSH were investigated on the basis of the knowledge that ELF normally contains large GSH concentrations (15), deficiencies in ELF GSH in disease have been demonstrated (14, 49, 56), and the feasibility of in vivo ELF supplementation with GSH has been demonstrated (5, 56). Likewise, study of extracellular GR and NADPH was pursued because of their known presence at high concentrations in normal ELF (15) and to evaluate the hypothesis that maintaining glutathione in its reduced form would improve its antioxidant protective role. It is, however, conceivable that extracellular glutathione or GR and NADPH may also in part exert their effect by affecting any of the characteristics listed above.

Several investigative approaches have supported an important role for intracellular and extracellular GSH in preventing lung oxygen toxicity. Studies based on whole animal and in vitro models suggest that depletion of intracellular GSH (24, 40, 58) or blocking of extracellular GSH uptake (28, 50, 58) potentiates oxygen toxicity, suggesting mechanisms by which sufficient extracellular GSH concentrations may be important in preventing oxygen toxicity. Furthermore, a greater susceptibility of Fischer 344 than of Sprague-Dawley rat lung to hyperoxic damage has been attributed to significantly lower levels of GSH in bronchoalveolar lavage fluids (60). With regard to direct effects of GSH, studies show that 95% O₂ for 24 h leads to a decrease in GSH content of AEC (33), accompanied by an increased sensitivity to oxygen toxicity compared with fibroblasts. Additional studies showed a correlation between an increase in markers of AEC damage due to hyperoxia and decreased GSH cell content (1).

Other studies have demonstrated that perfusion of isolated rat lungs with GSH or N-acetylcysteine enhanced intracellular synthesis of glutathione (3). In addition, several investigators have shown that extracellular application of GSH or its synthetic substrates can enhance intracellular levels of GSH (25, 61, 62). Inhibition of GSH synthesis enhances oxygen toxicity (19), while supplementation with N-acetylcysteine protects against oxygen toxicity in distal fetal rat lung epithelial cells (19) and preterm guinea pig lung (41), as does GSH supplementation in preterm rabbits (10).

Extracellular exogenous GSH also appears to preserve rat type II cell signal transduction pathways in the face of oxidant stress (9). Thus the literature supports the concept that depletion of cellular GSH increases oxygen toxicity, extracellular supplementation with exogenous GSH or its analogs can enhance extracellular GSH and thiol content as well as intracellular GSH, and these interventions may be protective against oxidant-induced damage.

Studies of nutritional supplementation with GSH also indicate protection against oxygen toxicity (10, 61). Supplementation with antioxidant analogs of GSH, such as N-acetylcysteine (19, 41) or other nutritional substrates that could enhance GSH synthesis (18), confers protection against hyperoxic lung injury. Also, direct administration of extracellular GSH appears to provide an antioxidant effect in response to other oxidants. For example, bathing type II cells in 1 mM GSH protected cells against t-butyl hydroperoxide (9). In addition, extracellular GSH protected alveolar type II cells against paraquat toxicity (31). GSH also has been shown to decrease the release of oxidants from bronchoalveolar lavage inflammatory cells from patients with CF (55).

Our findings that GR and NADPH added to the apical fluid appear to further enhance the protective effect of GSH against oxygen-induced epithelial dysfunction support the importance of GSH as an antioxidant defense against epithelial injury. Normal ELF contains substantial activity of GR and GPx, as well as NADPH (15). In the present study, we chose activities of GR to approximate physiological conditions (15) and concentrations of NADPH (a substrate for GR-dependent reduction of oxidized glutathione) to be in excess of physiological concentrations (~10× concentration) (15). Together, intracellular GR and NADPH help maintain glutathione in the reduced form, thus keeping the concentration of the oxidized form low (46). Extracellular glutathione in ELF collected from nor-
mal individuals is also found to be predominantly in its reduced state (15). Because GR activity and substantial NADPH are present in ELF from normal individuals (15), these are thought to be important in maintaining glutathione in the reduced form at steady state in ELF.

In hyperoxia, the presence of adequate extracellular GR and NADPH may become particularly important. Although it appears from the present study that the presence of GSH alone in the apical surface of the alveolar epithelium may be capable of partially abrogating the harmful effects of hyperoxia, GR and NADPH appear to further enhance the protective effect. In an in vitro model, inactivation of GR appears to exacerbate the effect of oxygen toxicity (51). Moreover, glutathione content and GR activity appear to be up-regulated in the lung in animal models of prolonged hyperoxia (37, 52), suggesting a compensatory antioxidant response to hyperoxia.

Another important enzyme in the glutathione redox cycle, GPx (46), has been studied extensively with regard to its role in prevention of oxygen toxicity. Several lines of evidence with regard to GPx provide additional support for the role of GSH as an important antioxidant in ELF and in the intracellular compartment. First, ELF has been shown to contain substantial amounts of GPx (15). Also, alveolar macrophages and lung cell lines (A549) express the extracellular-type GPx (2). Furthermore, whole lung homogenates demonstrate an increase in GPx after 100% O2 for 72 h (39), while GPx activity more than doubled in isolated type II pneumocytes from adult and fetal rats exposed to 95% O2 (4). Moreover, older rats had elevated GPx activity in whole lung homogenates compared with younger rats, suggesting a mechanism for their increased resistance to oxygen toxicity (12). Finally, 1,3-bis(2-chloroethyl)-1-nitrosourea, an inhibitor of GPx activity, increases susceptibility of rat type II pneumocytes to oxidant stress (35). Thus, although the present study specifically addressed the role of GR and NADPH in maintenance of GSH to protect against oxygen toxicity, it is conceivable that addition of exogenous GPx activity to ELF may further potentiate the function of GSH in preventing alveolar epithelial oxygen-induced damage.

The site of action of exogenously administered GSH/GR/NADPH is suggested by the properties of the alveolar epithelial model employed in this study. The transepithelial permeability to GSH in this model appears to be negligible (regardless of whether GSH is added to the apical or basolateral surface). Therefore, because GSH was added to the apical bathing fluid in the present study, it is unlikely that its primary protective effect was on the extracellular basolateral cell surface. An intracellular effect is a possibility, however, since increased extracellular GSH may increase intracellular GSH concentrations (25). With regard to GR activity, it is unlikely that this relatively large protein enzyme substantially crosses the epithelial monolayer or gains access to the intracellular space. Thus it is likely that GR added to the apical bathing fluid maintained the glutathione contained in this fluid in its reduced form (GSH), and the GR-provided protection for \( I_{eq} \) was dependent on these changes in the apical bathing fluid compartment (ELF).

The finding that protection of \( I_{eq} \) required the presence of GR and NADPH in addition to GSH suggests that at least two separate oxidant or oxygen-related “injuries” occur. For example, \( R_t \), which is considered to be primarily dependent on the integrity of the paracellular tight junctions (17), may have a high threshold for oxygen toxicity, and therefore the presence of GSH alone may be sufficient for protection. \( I_{eq} \), on the other hand, which is dependent on \( R_t \), as well as ion transport mechanisms (apical surface channels and basolateral Na\(^+\)-K\(^+\)-ATPase active transport) (17), may have a lower threshold for oxygen toxicity and, thus, may require additional GR and NADPH activity to provide adequate antioxidant protection.

A deficiency of GSH in ELF is found in a number of diseases characterized by an increased oxidant burden on the alveolar epithelial surface, including the adult respiratory distress syndrome (49), CF (56), and IPF (14); this deficiency may play an important role in the pathophysiology of these disorders. The increased oxidant burden that is characteristic of these pulmonary diseases, together with the hyperoxia encountered during oxygen therapy, may make the GSH deficiency even more consequential to the respiratory epithelial injury associated with these disease states. Aerosol delivery of GSH to the lower respiratory tract has been used to correct some of the oxidant-antioxidant imbalance in CF and IPF (5, 55). In this regard, supplementation of GSH levels in ELF may be a viable therapeutic approach in augmenting the antioxidant defense of the respiratory epithelial surface to protect against oxidant-induced damage to the alveolar epithelial barrier. Codelivery of GR and NADPH may further enhance therapeutic effects of GSH against oxygen toxicity in the respiratory epithelial tract.

This work was supported in part by the Baxter Foundation, Hastings Foundation, American Lung Association, American Heart Association-National Center Grants-in-Aid 9950172N and 9950442N, and National Heart, Lung, and Blood Institute Research Grants HL-38658, HL-62569, and HL-64365.

REFERENCES


