Normal alveolar epithelial lining fluid contains high levels of glutathione

ANDRÉ M. CANTIN, SUSAN L. NORTH, RICHARD C. HUBBARD, AND RONALD G. CRYSTAL
Pulmonary Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892

CANTIN, ANDRÉ M., SUSAN L. NORTH, RICHARD C. HUBBARD, AND RONALD G. CRYSTAL. Normal alveolar epithelial lining fluid contains high levels of glutathione. J. Appl. Physiol. 63(1): 152-157, 1987.—The epithelial cells on the alveolar surface of the human lower respiratory tract are vulnerable to toxic oxidants derived from inhaled pollutants or inflammatory cells. Although these lung cells have intracellular antioxidants, these defenses may be insufficient to protect the epithelial surface against oxidants present at the alveolar surface. This study demonstrates that the epithelial lining fluid (ELF) of the lower respiratory tract contains large amounts of the sulfhydryl-containing antioxidant glutathione (GSH). The total glutathione (the reduced form GSH and the disulfide GSSG) concentration of normal ELF was 140-fold higher than that in plasma of the same individuals, and 96% of the glutathione in ELF was in the reduced form. Compared with nonsmokers, cigarette smokers had 80% higher levels of ELF total glutathione, 98% of which was in the reduced form. Studies of cultured lung epithelial cells and fibroblasts demonstrated that these concentrations of reduced glutathione were sufficient to protect these cells against the burden of H₂O₂ in the range released by alveolar macrophages removed from the lower respiratory tract of nonsmokers and smokers, respectively, suggesting that the glutathione present in the alveolar ELF of normal individuals likely contributes to the protective screen against oxidants in the extracellular milieu of the lower respiratory tract.

Like other cells, the epithelial cells of the lower respiratory tract likely utilize intracellular antioxidants such as superoxide dismutases, catalase, and the glutathione system to protect themselves against the toxic effects of oxidants generated within the cells (8, 19). However, because these are intracellular antioxidants, they may only play a limited role in protecting the epithelial surface that is exposed to inhaled oxidants and inflammatory cells in the alveolar lumen. In this context, the present study was designed to determine whether the epithelial surface of the lower respiratory tract might be additionally protected by extracellular antioxidants that could function to suppress the toxic effects of oxidants present at the epithelial surface.

As an initial approach to this concept, we hypothesized that the epithelial lining fluid (ELF) of the lower respiratory tract might contain sufficient concentrations of glutathione (L-γ-glutamyl-L-cysteinyl-glycine) to contribute to the antioxidant protection of the epithelial surface of the lung. The theoretical basis for this hypothesis rests in the knowledge that, although glutathione is a major component of intracellular antioxidant defenses, it is exported extracellularly (20, 21). Specifically, 1) among the cell types known to export glutathione are mononuclear phagocytes, lymphocytes, and fibroblasts, cells present in the lower respiratory tract (5, 11, 26); 2) although peripheral blood plasma levels of glutathione are very low, the glutathione concentration in hepatic vein plasma is much higher, consistent with the concept that the concentrations of glutathione may vary at different sites in the body (3); 3) γ-glutamyl transpeptidase, a cell surface enzyme that functions to remove glutathione from the extracellular space, is present in the lung, but in far lower concentrations in the lung parenchyma than in the kidney, the major site of glutathione removal from plasma (2, 14); and 4) although the epithelial lining fluid of the lower respiratory tract is replenished constantly, this process is relatively sluggish so that materials released into this compartment likely remain in the local milieu for some time (7, 29).

To evaluate this concept, the concentration of glutathione was quantified in the epithelial lining fluid of the lower respiratory tract of nonsmoking and smoking healthy individuals. The results indicate that the ELF of the lower respiratory tract of normal individuals contains markedly increased concentrations of glutathione compared with those of plasma and that cigarette smokers...
have significantly higher levels of ELF glutathione than nonsmokers. Furthermore, the glutathione in ELF is almost entirely in the reduced form and glutathione at concentrations equal to those found in ELF protects lung parenchymal cells against the burden of H₂O₂ estimated to be present in the epithelial lining fluid of cigarette smokers.

METHODS

Study population. The study population consisted of 19 healthy nonsmoking individuals (9 men, 10 women; age: 32 ± 2 yr) and 12 healthy smoking individuals (6 men, 6 women; age: 31 ± 2 yr) with an average smoking history of 22 ± 4 pack-years. All individuals were free of lung disease as determined by the combined criteria of normal histories, physical examinations, chest X-rays, and lung function tests including volumes, flow rates and diffusing capacity. Data are presented as means ± SE, and all comparisons were made using the Student’s t test.

Bronchoalveolar lavage. All individuals participating in this study underwent a bronchoscopy with bronchoalveolar lavage according to methods previously described (17). The total number of cells recovered from nonsmokers was 16 ± 2 × 10⁶/ml fluid recovered, and the differential cell count showed 82 ± 2% macrophages, 16 ± 4% lymphocytes, and 1.0 ± 0.2% neutrophils, whereas the total number of cells recovered from the lungs of smokers was 29 ± 6 × 10⁶/ml fluid recovered and included 94 ± 1% macrophages, 4 ± 1% lymphocytes, and 2.0 ± 0.4% neutrophils.

The volume of ELF recovered by bronchoalveolar lavage was quantified by the urca method (28). Urca concentrations in bronchoalveolar lavage fluid and in plasma were determined with the Urea Nitrogen 65-UV Kit (Sigma Chemical, St. Louis, MO).

Total glutathione in ELF and plasma. Total glutathione (the reduced form GSH + the disulfide form GSSG) was determined in the bronchoalveolar lavage fluid supernatant (2,000 g for 5 min) immediately after bronchoscopy, according to the assay described by Sies and Akerboom (28). Bronchoalveolar lavage fluid, 100 μl, was added to 1.1 ml of 0.1 M sodium phosphate buffer, pH 7.0, containing 1 mM EDTA, 200 μM NADPH, 64 μM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and 100 μM thioglutathione reductase (all reagents from Sigma Chemical, St. Louis, MO), and the rate of reduction of DTNB was recorded spectrophotometrically at a wavelength of 412 nm. Sample glutathione concentrations were determined according to curves generated from standards of known concentrations of GSSG (0.25–4 μM) in 0.15 M NaCl. Because inhibitors of the assay of glutathione have been reported in some biological fluids (28), internal standards consisting of known concentrations of GSSG were added to the lavage fluids and assayed. However, the standard curves were identical to those in buffer, i.e., no inhibitor that interfered with the assay was detected in the lavage fluids.

Glutathione concentrations in plasma were determined using the assay described above, by replacing 100 μl of lavage fluid with 100 μl of plasma. Because plasma
the alveolar structures, an in vitro model of oxidant injury was developed utilizing a hydrogen peroxide-generating system as a source of oxidants, cultured lung cells as the targets, and increasing concentrations of reduced glutathione to compare with actual concentrations of reduced glutathione measured in ELF. The H$_2$O$_2$-generating system consisted of glucose and glucose oxidase in concentrations known to generate 90–150 nmol H$_2$O$_2$/ml media. This range of H$_2$O$_2$ burden for the target cells was chosen because alveolar macrophages recovered from the lungs of cigarette smokers release 4.4–9.0 nmol H$_2$O$_2$/h $^{-1}$ (see RESULTS), and the ELF of cigarette smokers contains 20 ± $6 \times 10^6$ alveolar macrophages/µl ELF (25); i.e., a reasonable estimate of the H$_2$O$_2$ burden of the lower respiratory tract of these individuals from the inflammatory cells on the epithelial surface is between 100 and 300 nmol.h$^{-1}$.ml ELF$^{-1}$. The target cells utilized included AKD, a diploid feline alveolar epithelial cell with characteristics that include both type I and type II lung epithelial cells (18), and HFL-1, a diploid human fetal lung fibroblast (6). The lung parenchymal cells were cultured in DMEM with 10% calf serum (Biofluids, Rockville, MD) in 24-well tissue culture plates (Falcon Labware, Div. of Becton Dickinson, Cockeysville, MD) in 10% CO$_2$ at 37°C. At confluence, the cells were labeled with $^{51}$Cr (5 µCi/well, Amersham, Arlington Heights, IL) for 12 h, rinsed three times with phosphate-buffered saline solution followed by the addition of 500 µl of Earle’s balanced salt solution (EBSS) supplemented with $55$ mM glucose/well. EBSS, 100 µl, containing varying concentrations of reduced glutathione (Boehringer Mannheim Biochemicals, Indianapolis, IN) was added to each well. To generate H$_2$O$_2$, this was followed by 50 µl of a glucose oxidase solution (Boehringer Mannheim) at a concentration of 5 mU/ml for experiments with the HFL-1 cells and 20 µU/µl for experiments with the AKD cells. The cells were incubated in 5% CO$_2$, at 37°C, for 8 h. At the end of the incubation period, the amount of radioactivity released into the supernatant was measured and the cytotoxicity index was calculated as

$$\text{cytotoxicity index} = \frac{([\text{dpm sample} - \text{dpm background}])}{([\text{dpm maximum} - \text{dpm background}])] \times 100$$

where background represents wells with EBSS without glucose oxidase and maximum represents wells with a 2% Triton-X solution. Glutathione, glucose, and glucose oxidase alone did not augment $^{51}$Cr release above the background.

**Glutathione reductase, peroxidase, and NADPH in ELF.** Glutathione reductase and peroxidase were quantitated in bronchoalveolar lavage fluid, concentrated 50-fold after ultrafiltration on a YM-10 membrane (Amicon, Lexington, MA) by use of standard methods (16, 32, 43). NADPH was quantitated in bronchoalveolar lavage fluid by incubating 500 µl sample with 50 µl 6.5 mM GSSG, 50 µl 10 mM glucose 6-phosphate, 20 µl 4 mM DTNB, 10 µl 3 U/ml glutathione reductase, 10 µl 100 U/ml glucose-6-phosphate dehydrogenase in 0.1 M potassium phosphate buffer pH 7.0, containing 1 mM EDTA. The rate of reduction of DTNB was monitored spectrophotometrically at 412 mM, and a standard curve was derived from known concentrations of NADPH within each assay. To express the amounts of glutathione reductase, glutathione peroxidase and NADPH per microliter ELF, the albumin content of the concentrated fluid was measured and the amount of albumin referenced to that in ELF determined by the urea method (25).

**RESULTS**

**Total glutathione in ELF and in plasma.** Total glutathione (GSH + GSSG) concentrations in the plasma were low (3.0 ± 0.6 µM). In marked contrast, the concentration of total glutathione in the epithelial lining fluid of the lower respiratory tract was 429 ± 34 µM, a 140-fold higher concentration than in the plasma of the same individuals (Fig. 1, $P < 0.001$). Furthermore the vast majority (96 ± 2%) of the ELF glutathione was in the reduced (GSH) state. Thus the concentration of glutathione in the ELF of the lower respiratory tract is markedly higher than in plasma, and the glutathione in the ELF is in the appropriate state to function as an antioxidant.

Although the mechanisms by which GSH in ELF is maintained in the reduced form are unknown, it is apparent that other components of the glutathione system are present in ELF, including NADP (22 ± 3 PM) and glutathione reductase (0.05 ± 0.0 mU/µl), which together can regenerate GSH from GSSG, as well as glutathione peroxidase (0.05 ± 0.0 mU/µl), which catalyzes the removal of H$_2$O$_2$ by converting GSH to GSSG.

Several lines of evidence demonstrated that the high concentrations of GSH found in ELF did not result from artifactual elevation resulting from cell lysis during the
lavage procedure, including the following. 1) Of the average of 16 × 10^6 cells recovered by bronchoalveolar lavage with 300 ml of saline, >99% were inflammatory cells; of this total cell population, on average, 95% were viable. 2) Erythrocytes were rarely seen in the lavage analyses, and measurement of the hemoglobin concentration of the lavage fluid revealed that it was <20 nM. Since the ratio of intracellular GSH to hemoglobin in erythrocytes is 0.35 ± 0.10 nmol GSH/nmol hemoglobin, even lysis of all of the erythrocytes recovered by lavage would result in ELF GSH concentrations of <1 μM, far less than the observed 429 ± 34 μM. 3) The inflammatory cells recovered by lavage contained 3.76 ± 0.65 nmol GSH/10^6 cells; even total lysis of all of these cells would result in apparent ELF GSH concentrations of only 60 ± 10 μM, also far less than that observed.

Evaluation of a group of cigarette smokers without demonstrable lung disease revealed ELF total glutathione levels that were even greater than observed in the nonsmokers (Fig. 1). The plasma level of total glutathione of those smokers was low (2.8 ± 0.8 μM) and similar to those of the nonsmokers (P > 0.5). In contrast, although the ELF concentration of total glutathione of the smokers was far greater than that in their plasma (ELF 775 ± 119 μM, P < 0.001 compared with plasma), it was also higher than the total glutathione concentration in the ELF of the nonsmokers (P < 0.005). No correlation was found between the number of pack-years and ELF GSH. Furthermore, like the nonsmokers, the ELF glutathione in the smokers was dominated by glutathione in the reduced form (98 ± 1% of total glutathione; P > 0.5 smokers compared with nonsmokers).

*Extracellular GSH at concentrations found in normal ELF can protect lung parenchymal cells against oxidants.* When glucose oxidase was incubated with the HFL-1 and AKD cells in amounts that generated amounts of H₂O₂ equivalent to those estimated to be released by inflammatory cells of cigarette smokers, cytotoxicity indexes of 18 ± 2 and 14 ± 1% were generated, respectively. However, incubation of the cells in the presence of the oxidant-generating system and increasing concentrations of reduced glutathione resulted in a marked reduction in the cytotoxicity index (Fig. 2). At concentrations of reduced GSH equal to those found in normal non-smoking individuals (i.e., 400 μM), the cytotoxicity indexes were reduced to 4 ± 2% for the HFL-1 cells (P < 0.01) and 6 ± 1% for the AKD cells (P < 0.01). Furthermore, for both cell types the concentrations of reduced GSH equal to those of ELF of cigarette smokers (i.e., 800 μM) reduced the cytotoxicity indexes to 0 ± 1% for both cell types (P < 0.01) compared with the cells exposed to oxidants without added GSH.

![Figure 2](http://jap.physiology.org/DownloadedFrom leaned from http://jap.physiology.org/)  
**FIG. 2.** Ability of glutathione, at concentrations in range of those observed in epithelial lining fluid of nonsmoking and smoking individuals to provide lung parenchymal cells with antioxidant protection. Human lung fibroblasts (HFL-1, A) and cat lung epithelial cells (AKD, B) were cultured with oxidant-generating system (glucose, glucose oxidase) in absence and presence of increasing concentrations of reduced glutathione. At end of incubation period, degree of parenchymal cell injury was determined by quantitating release of ⁵¹Cr into supernatant, expressed as a cytotoxicity index. Arrows indicate amounts of reduced glutathione found in epithelial lining fluid (ELF) of nonsmokers and smokers.

Interestingly, cigarette smokers, individuals who clearly have an enhanced oxidant burden at their alveolar surface (15), have significantly higher levels of reduced GSH in ELF than do nonsmokers. Furthermore, the concentrations of ELF GSH in nonsmokers and smokers are sufficient to protect lung parenchymal cells against the burden of oxidants estimated to be derived from inflammatory cells in the lower respiratory tract of cigarette smokers.

*Mechanisms contributing to the presence of high levels of GSH in ELF.* Although the mechanisms responsible for the relatively high concentration of GSH in ELF are unknown, several lines of evidence suggest that the balance of GSH export into and removal from ELF is normally set to favor the presence of high concentrations of this tripeptide on the epithelial surface of the lower respiratory tract.

First, a variety of cell types are known to export GSH, including lymphocytes, macrophages, and fibroblasts, cells found in large numbers in the lower respiratory tract (5, 11, 26).

Second, within organs, GSH is thought to be normally

**DISCUSSION**

Glutathione is a sulfhydryl-containing tripeptide which plays a critical role in defending cells against toxic oxidants (4, 20, 21). The evaluation of the epithelial lining fluid of the human lower respiratory tract indicates that high levels of GSH are present in vivo in the extracellular milieu of the lower respiratory tract and that most of this glutathione is in the reduced form.
removed from the extracellular space by uptake and subsequent catabolism by cells (19, 20). For an organ such as the lung, this likely occurs by two processes: uptake by lung parenchymal cells in the local milieu and diffusion into the blood, where it is eventually taken up and catabolized mostly by kidney cells. In the lung, however, it is probable that both processes are relatively suppressed compared to some other organs. Within the lung, as in other organs, glutathione is likely taken up by cells by virtue of the plasma membrane-bound γ-glutamyl transpeptidase, an enzyme that cleaves the γ-glutamyl bond of GSH during the uptake process (20, 21). However, although this enzyme is found in very high concentrations in the kidney, the lung contains 300-fold less, likely resulting in a relatively lower rate of glutathione metabolism in the lower respiratory tract (2).

Consistent with the concept that the relative amount of γ-glutamyl transpeptidase is a rate-limiting step in the uptake of GSH is the knowledge that when there is a hereditary deficiency of this enzyme, the plasma concentrations of GSH are markedly elevated (27). Thus, in the lung, the relative deficiency of γ-glutamyl transpeptidase likely contributes to a decreased local catabolism of GSH and hence higher concentrations of GSH on the epithelial surface. Furthermore, although some GSH undoubtedly passes from the epithelial surface to the pulmonary circulation, to do so it must pass between the epithelial cells, the connective tissue extracellular matrix of the alveolar wall, and then between the endothelial cells lining the pulmonary capillaries. Although permeable, the alveolar-capillary barrier is relatively tight, so as to prevent the accumulation of fluid in the air spaces; i.e., the lung is relatively impermeable compared with many other organs (7, 29).

**ELF GSH is increased in cigarette smokers.** The observation that the glutathione concentration in the ELF of the lower respiratory tract of cigarette smokers is ~60% higher than that of nonsmokers is of interest in the context of the knowledge that the epithelial surface of the lower respiratory tract of cigarette smokers is likely exposed to a higher oxidant burden than that of nonsmokers. This oxidant burden is from two sources. First, cigarette smoke itself contains oxidants; it is estimated that there are 10^{14} free radicals per puff, many of which have sufficient half-lives to theoretically reach the lower respiratory tract (23). Second, the alveolar surface of individuals who smoke cigarettes is exposed to a chronic inflammatory process that is dominated by alveolar macrophages that spontaneously are releasing oxidants capable of injuring normal lung parenchymal cells (13, 15).

Since at least some antioxidant defenses can be induced by an oxidant burden (8), it is possible that the increased ELF glutathione concentration represents an adaptive response of the lung to an oxidative stress. Consistent with this hypothesis is the recent observation that erythrocytes of cigarette smokers have higher GSH concentrations and better protect endothelial cells from H_2O_2 than erythrocytes from nonsmokers (31). Though parenchymal cells may contribute to this increase in ELF glutathione, it is probable that the alveolar macrophage also contributes to the process. In this context, Rouzer et al. (26) have demonstrated that, when macrophages phagocytose zymosan, they export glutathione at a rate approximately threefold greater than resting macrophages. Thus the increased ELF glutathione concentrations in smokers may be derived in part from the increased number of alveolar macrophages on the epithelial surface as well as the increased oxidative metabolism of these cells in response to cigarette smoke at the alveolar surface.

**Most of the glutathione in ELF is in reduced state.** Strikingly, >95% of the ELF “total glutathione” in both smoking and nonsmoking individuals is in the reduced form (GSH). Since the alveolar surface is directly exposed to the environment, and to any oxidants derived from this exposure, it is reasonable to hypothesize that the lung must have mechanisms to maintain such a high level of glutathione in the reduced state. One possible mechanism is that glutathione may be actively maintained in the reduced state by an enzymatic system in the extracellular space. Consistent with this hypothesis, active glutathione reductase and NADPH were detectable in ELF. However, since the concentrations of ELF NADPH were only 5% of the ELF GSH concentrations, and since NADPH is not known to be exported from cells, it is unlikely that ELF NADPH could provide the necessary reducing equivalents to maintain ELF GSH in the reduced state in the presence of an acute oxidant stress. A more likely explanation is that glutathione may be exported from cells in a reduced state at a rate exceeding the rate of formation of extracellular GSSG. Although cigarette smokers have an accentuated oxidant burden in their lower respiratory tract, GSSG was not found to accumulate in their ELF. Since cigarette smoking represents a chronic form of lower respiratory tract oxidant burden, it is possible that the rate of oxidation of ELF GSH is not sufficient to alter the ELF GSSG/GSH. However, in experimental models of acute oxidative stress, the extracellular GSSG/GSH in plasma is markedly increased (1), suggesting that an acute oxidant stress in the lung could increase ELF GSSG levels.

Importantly, the concentrations of glutathione measured in the ELF of both nonsmokers and smokers were in the range sufficient to protect lung fibroblasts and alveolar epithelial cells against H_2O_2-mediated damage. Thus, bearing in mind the difficulty in extrapolating from these in vitro studies to the in vivo situation, it is reasonable to conclude that the concentrations of GSH present on the epithelial surface of the lower respiratory tract are in the range to provide at least some antioxidant protection to the lung parenchyma. Consistent with this concept is the observation by Green (12) that GSH, in the same concentration range as found in normal ELF (200–400 μM), protected alveolar macrophages against the inhibitory effect of cigarette smoke on phagocytosis and bactericidal activity.

In addition to its role as an antioxidant, glutathione also plays a role in the metabolism of carcinogens and other toxins. Since the lung can be directly exposed to potentially toxic substances from the environment, the presence of high concentrations of glutathione in the ELF may contribute to detoxification of inhaled toxins.
In this context, it is important to note that the lung, as well as isolated lung cells, has been shown to be able to utilize extracellular glutathione to metabolize xenobiotics (10).

In view of the high concentrations of reduced GSH in normal ELF and the ability of GSH to inactivate a variety of toxic oxidants potentially present at the alveolar surface, it is likely that GSH is an important component of the antioxidant defenses in the alveolar epithelial lining fluid. Studies of ELF GSH in disorders of the lower respiratory tract in which oxidant-mediated injury is thought to play a role may provide further insight into the pathogenesis of oxidant-mediated lung parenchymal cell damage.

Present address of A. M. Cautin: Hospitalier Universitaire de Sherbrooke, Unite de Recherche Pulmonaire, Piece 3601, Sherbrooke, Quebec J1H 5N4, Canada.

Address for reprint requests: R. G. Crystal, Pulmonary Branch, Building 8, Room 6F03, National Institutes of Health, Bethesda, MD 20892.

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