Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 4063–4067, June 1990
Medical Sciences

Augmentation of glutathione in the fluid lining the epithelium of the lower respiratory tract by directly administering glutathione aerosol

(lung/antioxidant/therapy/bronchoalveolar lavage)

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Communicated by Martha Vaughn, February 20, 1990 (received for review November 9, 1989)

ABSTRACT **Glutathione (GSH), a cysteine-containing tripeptide, functions as an antioxidant, provides cells with cysteine, and is required for optimal function of the immune system. Because the epithelial-lining fluid (ELF) of the lower respiratory tract normally contains high GSH levels and lung ELF GSH deficiency states can exist, we evaluated the feasibility of augmenting lung ELF GSH levels by (*i*) administering GSH to sheep i.v. and by direct aerosolization and then (*ii*) measuring the GSH levels in lung ELF, lung lymph, venous plasma, and urine. When GSH (600 mg) was administered i.v. ($n = 11$), GSH levels in venous plasma, lung lymph, and ELF rose, but only transiently, suggesting the i.v. route would not deliver adequate GSH to the alveolar epithelial surface. For directly administering GSH to the lung by the aerosol route, *in vitro* studies were first conducted to show that >50% of a GSH solution could be converted to droplets <3 μm in aerodynamic diameter without oxidizing the GSH. To target functional GSH to the lower respiratory tract, an aerosolized solution of GSH (600 mg) was administered to sheep ($n = 12$). Significantly, the GSH level in ELF increased 7-fold at 30 min (preaerosol, 45.7 ± 10 μM; 30-min postaerosol, 337 ± 64 μM; $P < 0.001$). The ELF GSH levels remained above baseline at 1 hr ($P < 0.01$), returning toward baseline over a 2-hr period. In contrast, GSH levels in lung lymph, venous plasma, and urine were not significantly increased during the period—i.e., aerosol therapy selectively augmented the GSH levels only at the lung epithelial surface. Thus, functional GSH can be delivered by aerosol to directly augment the ELF GSH levels of the lower respiratory tract. Such an approach may prove useful in treating a variety of lung disorders.**

Glutathione (γ-glutamylcysteinylglycine, GSH), a ubiquitous sulfhydryl-containing tripeptide produced by most mammalian cells, functions as an efficient intra- and extracellular antioxidant by scavenging toxic oxygen radicals (1–3). Thus cells are thought to be protected by extracellular GSH from oxidants produced by inflammatory cells and by intracellular GSH from oxidants generated in a variety of normal biochemical processes, as well as from xenobiotics (1–9). GSH is also the most abundant extracellular form of cysteine and is a major intracellular source of this amino acid through the action of γ-glutamyltranspeptidase, an enzyme in the plasma membrane of most cells (1–3). Therefore, GSH can modulate the behavior of many cell types, including critical cellular components of the host defense system (1–3, 10–14); for example, adequate extracellular GSH levels are required for the function of T and B lymphocytes (11–14).

In the lung, GSH is found in high concentrations in the epithelial-lining fluid (ELF) of the lower respiratory tract; normal levels in humans are >40-fold those in plasma (8, 15). Thus, ELF GSH is a key component of the antioxidant screen that protects the pulmonary epithelium from oxidants released by inflammatory cells as well as inhaled oxidants (16–21). In addition, ELF GSH probably helps maintain the normal function of the immune components of the pulmonary epithelial host defense system (15). The importance of these functions in humans is emphasized by two acquired lung ELF "deficiency states," idiopathic pulmonary fibrosis (IPF) and infection with the human immunodeficiency virus (HIV). In IPF, a fatal disease characterized by an enhanced inflammatory cell-produced oxidant burden at the alveolar epithelial surface and concomitant chronic epithelial cell damage (22), ELF GSH levels are decreased 4-fold compared with normal (23). In individuals seropositive for HIV infection but without clinical evidence of AIDS, there is a 1.6-fold ELF GSH deficiency (15).

These lung ELF GSH deficiency states are only part of the biologic processes culminating in lung epithelial cell damage in IPF and the high risk of HIV-seropositive individuals for pulmonary opportunistic infections. Nevertheless, the importance of GSH as an antioxidant and a modulator of normal immune function leads to the hypothesis that raising ELF GSH levels by administering GSH should help reestablish the lower respiratory tract antioxidant screen in IPF and augment lung-epithelial-surface immune function in individuals with HIV infection. However, this rational strategy of augmenting lung ELF GSH levels is difficult to achieve in practice. The problem is not how to obtain the GSH, which is readily synthesized by chemical methods, but how to deliver it to the epithelial surface of the lower respiratory tract. The purpose of this study is to evaluate the feasibility of directly augmenting lower-respiratory-tract ELF levels of GSH by the aerosol route as compared with i.v. administration. To directly administer aerosol, we used an aerosol generator that produced droplets capable of reaching the lower respiratory tract and chose sheep as our experimental animal, an animal large enough for easy aerosol administration and one in which lung ELF, lung interstitial fluid (lymph), plasma, and urine could be repeatedly sampled.

METHODS

GSH Preparation. GSH was obtained as a free acid (tissue culture grade; Sigma) and stored at 4°C. The percentage of

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Abbreviations: GSH, glutathione (a reduced substance); GSSG, glutathione, oxidized; HIV, human immunodeficiency virus; ELF, epithelial-lining fluid; IPF, idiopathic pulmonary fibrosis; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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GSH in this preparation determined (see below) before each experiment was always >96%.

Experimental Model. Female mixed-breed sheep (Ovine Biotechnology) (n = 23; 33 ± 1 kg; all data are presented as mean ± SEM; all statistical comparisons were made using the two-tailed Student's t test) were anesthetized with i.v. sodium thiopental. Catheters were inserted into a jugular vein and a vein of the hind limb, and a catheter was also inserted into the bladder. The trachea was intubated with a cuffed endotracheal tube attached to a positive-pressure ventilator. Animals were ventilated at a tidal volume of 12 ml/kg of body weight with 5 cm of H_2O (1 cmH_2O = 98 Pa) positive end-expiratory pressure at a rate of 16/min. Electrocardiogram and inspiratory pressure were monitored throughout the study. Anesthesia was maintained with 1.0–1.5% Fluothane/50% oxygen. Lung lymph was collected from the caudal efferent lymphatic duct cannulated with a heparinized Silastic catheter by the technique of Staub *et al.* (24), a method that permits selective sampling of lung lymph draining the pulmonary interstitium. Lower respiratory tract ELF was obtained by bronchoalveolar lavage through a fiberoptic bronchoscope [length, 1 m; o.d., 5.3 mm; Machida (Orangeburg, NY)] by using a single 50-ml wash of 0.9% NaCl. Blood samples were obtained from the jugular catheter, and urine samples were obtained from the bladder catheter. Venous plasma, lung lymph, ELF, and urine were obtained before GSH administration and at intervals up to 180 min after i.v. administration or aerosolization of 600 mg of GSH. A total of 23 sheep were evaluated; the number of sheep used for each set of experiments is indicated with the data. All i.v. and aerosol studies were done with the chest closed and the animal prone. After baseline bronchoalveolar lavage fluid, blood, lung lymph, and urine samples were obtained, 600 mg of glutathione in 4 ml of saline was administered either into a vein of the hind leg or by aerosol into the inspiratory limb of the ventilation circuit over a 25-min period. Bronchoalveolar lavage fluid, blood, lymph, and urine samples were then obtained at intervals over a 3-hr period. Lavage samples at different times were obtained from different sites. All measurements were done in duplicate. Concentrations in lymph, plasma, blood, and urine are reported in μM; concentrations (μM) in ELF were calculated from concentrations in bronchoalveolar lavage fluid and the volume of ELF recovered in it, as assessed by the urea method (25). To further test the safety and efficacy of very large doses of GSH, 4.2 g of GSH was aerosolized over a 3-hr period to sheep (n = 3) that had not undergone thoracotomy and lymph-catheter placement. Bronchoalveolar lavage was done 3 hr after completion of the aerosol, and GSH, oxidized glutathione (GSSH), and GSH/GSSH were determined in ELF.

Aerosol Generating System. GSH was put into a form capable of reaching the lower respiratory tract with a nebulizer (Ultravent; Mallinckrodt) that generated aerosol droplets appropriate for alveolar deposition (26). To generate the aerosol containing GSH, 4 ml of a GSH solution of 150 mg/ml in 0.9% NaCl was placed in the reservoir of the nebulizer, and the nebulizer was driven at 40 psi (1 psi = 6.9 kPa) with compressed air. The sizes of aerosol droplets determined by laser particle-size analysis indicated a mass median aerodynamic diameter of 2.8 μm with a geometric SD of 1.3 μm. The relative proportion of the GSH preparation that remained in the reduced form was evaluated by collecting the aerosolized droplets in phosphate-buffered saline (pH 7.4) as described (26).

GSH Levels and Form. GSH levels in venous plasma, lung lymph, bronchoalveolar lavage fluid, and urine were quantified with minor modifications of standard methods (8, 27–29). In brief, to determine the GSH/GSSG levels, the sample was mixed with an equal amount of 10 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB)/0.1 M potassium phosphate, pH 7.5/17.5 mM EDTA and centrifuged at 2000 × g for 10 min. A 50-μl sample of the supernatant was added to a cuvette containing 0.5 unit of glutathione reductase in 0.1 M potassium phosphate, pH 7.5/5 mM EDTA. After 1-min incubation at 25°C, the assay reaction was started by adding 220 nM NADPH/0.1 M potassium phosphate, pH 7.5/5 mM EDTA in a final volume of 1 ml. The rate of reduction of DTNB was recorded spectrophotometrically at a wavelength of 412 nm (Beckman DU-70 spectrophotometer). Determination of the GSH/GSSG concentration was based on standard curves generated from known concentrations of GSSG (0.125–4 μM) in phosphate-buffered saline (pH 7.4).

To quantify GSSG, a sample of each fluid was mixed immediately after recovery with an equal volume of 10 mM N-ethylmaleimide/0.1 M potassium phosphate, pH 6.5/17.5 mM EDTA and centrifuged at 2000 × g for 10 min; 250 μl of the supernatant was passed through a Sep-pak C_{18} cartridge (Waters) that had been washed with 3 ml of methanol followed by 3 ml of distilled water, and the effluent was collected. GSSG was eluted from the column with 1 ml of 0.1 M potassium phosphate, pH 7.5/5 mM EDTA. A 750-μl sample of the combined effluent and eluate was mixed with 250 μl of 0.1 M potassium phosphate, pH 7.5/5 mM EDTA/800 μM DTNB/glutathione reductase at 2 units/ml/1 mM NADPH, and the rate of reduction of DTNB was recorded spectrophotometrically at 412 nm. Standard curves were derived from dilutions of known concentrations of GSSG (0.125–4 μM) mixed with 10 mM N-ethylmaleimide and chromatographed with Sep-pak C_{18} cartridges, as described above. When values were above the range of the standard curve, samples diluted with 0.9% NaCl were run in parallel. The amount of GSH was obtained by subtracting the amount of GSSG from the GSH/GSSG levels. For both the GSH/GSSG and GSSG assays, standard curves generated in the various fluids were parallel—i.e., values from all fluids were comparable.

RESULTS

Baseline GSH Levels in Sheep. The GSH levels in venous plasma and in lung lymph were in a similar range, although the plasma levels were slightly higher ($P > 0.1$; Fig. 1). Urine levels were much lower. Strikingly, as in humans (8, 15, 23), lung ELF GSH concentrations were more than twice those in plasma or lung lymph. In both plasma and lung lymph, GSH accounted for >95% of the GSH/GSSG (plasma, 97 ± 2% GSH; lymph, 95 ± 3% GSH). In ELF 75 ± 4% of the baseline GSH/GSSG was in the reduced form, whereas in urine 59 ± 6% was in the reduced form.

i.v. Administration of GSH. To evaluate the i.v. route as a way of augmenting GSH levels of lung ELF, single doses of 600 mg of GSH were given i.v. to sheep. Levels of GSH in plasma increased after the injection but returned to baseline by 45 min ($P < 0.05$; 15 min compared with baseline; all other values $P > 0.1$; Fig. 2). Some GSH diffused into the lung, transiently raising lung lymph levels ($P < 0.05$; 15 min compared with baseline), but like the plasma GSH, by 30 min the values returned to baseline ($P > 0.1$; all times other than 15 min). Although the average lung ELF values were variable, they did not change significantly during the observation period ($P > 0.1$; all comparisons with baseline values). As expected, urine excretion was rapid with high levels at 15 min ($P < 0.02$; compared with baseline). This change was also transient, returning to baseline thereafter ($P > 0.1$). Evaluation of the GSH/GSSG levels in plasma, lymph, ELF, and urine demonstrated a pattern similar to that of GSH (data not shown). Together, these observations suggest that the i.v. route is not an effective means to deliver GSH to the lung epithelial surface.

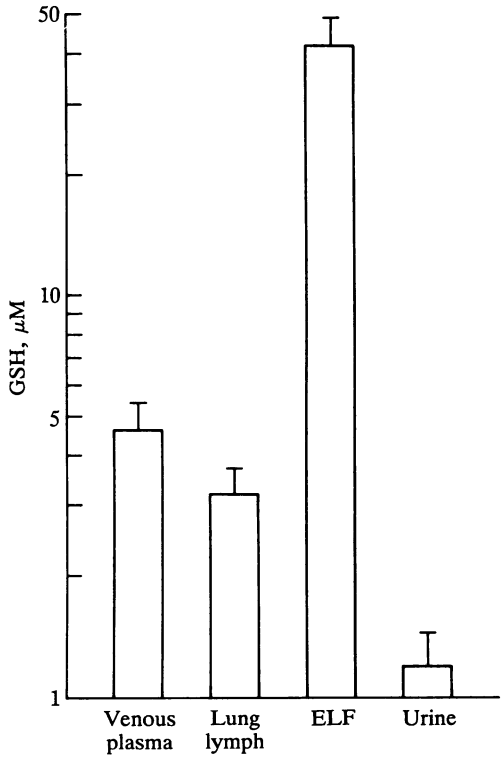

FIG. 1. Baseline GSH levels in sheep. Data represents the means (± SEM) of values from 23 sheep.

Effect of Aerosolization on GSH. To use aerosolization to deliver GSH to the lower respiratory tract, two main criteria must be fulfilled. (*i*) The GSH solution must be placed into an aerosol composed of droplets of an optimal size for deposition on the alveolar surface. This requirement can be fulfilled with the Ultravent nebulizer used in these studies. (*ii*) The aerosol should not alter the GSH—i.e., GSH must remain reduced and therefore functional. *In vitro* evaluation of the aerosolized GSH revealed that the aerosolization process itself did not alter the structure of the GSH molecule. In this regard, the GSH/GSSG in the preaerosol preparations contained 98.2 ± 0.1% GSH, whereas GSH in the collected aerosolized droplets was 97.0 ± 0.6% ($P > 0.1$). Together, these observations show that the aerosol was composed of fully functioning GSH within droplets of an optimal size for reaching the lung alveolar regions.

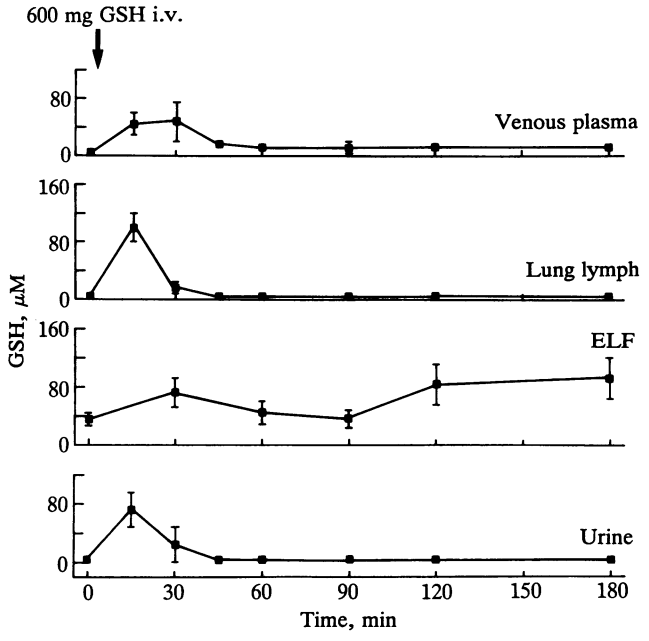

FIG. 2. GSH levels in sheep after i.v. administration of 600 mg of GSH. GSH concentrations were evaluated before and at intervals after i.v. GSH administration. Arrow denotes GSH administration over a 1-min interval. Each data point represents the mean (± SEM) of values from 11 sheep.

Aerosol Administration of GSH. Single doses of 600 mg of GSH in 4 ml of 0.9% NaCl given by aerosol over 25 min to sheep ($n = 12$) increased GSH levels in the ELF of the lower respiratory tract over 7-fold within 30 min ($P < 0.001$, preaerosol compared with 30 min postaerosol; Fig. 3). ELF GSH levels remained above baseline for up to 2 hr ($P < 0.01$, 60 min and 90 min postaerosol; both comparisons with baseline). Levels in lung lymph appeared to increase at 30 min but were not significantly different from baseline ($P > 0.1$), and levels in venous plasma and urine did not change significantly after aerosolization ($P > 0.1$; all comparisons to baseline). As a control, only saline was aerosolized; GSH levels in ELF remained unchanged from baseline over a 3-hr period ($P > 0.1$; all comparisons).

At each time point where ELF GSH levels were significantly above baseline, GSH/GSSG in ELF was significantly higher than GSH. For example, at 30 min the GSH in ELF was 65 ± 7% of GSH/GSSG ($P < 0.001$). Overall, at 60, 90, and 120 min, GSH was 62–65% of GSH/GSSG ($P < 0.005$; all comparisons). Because the aerosolization process itself did not alter the relative proportion of GSH from that in the preaerosol preparation and yet a proportion of the recovered GSH/GSSG was not reduced, the GSH probably encountered an oxidant burden when in the ELF. However, independent of this observation, there is no question that GSH aerosolization markedly augmented GSH levels in ELF (Fig. 3). Aerosolization of very large doses of GSH showed that the delivery of "superphysiologic" doses of GSH to the lower

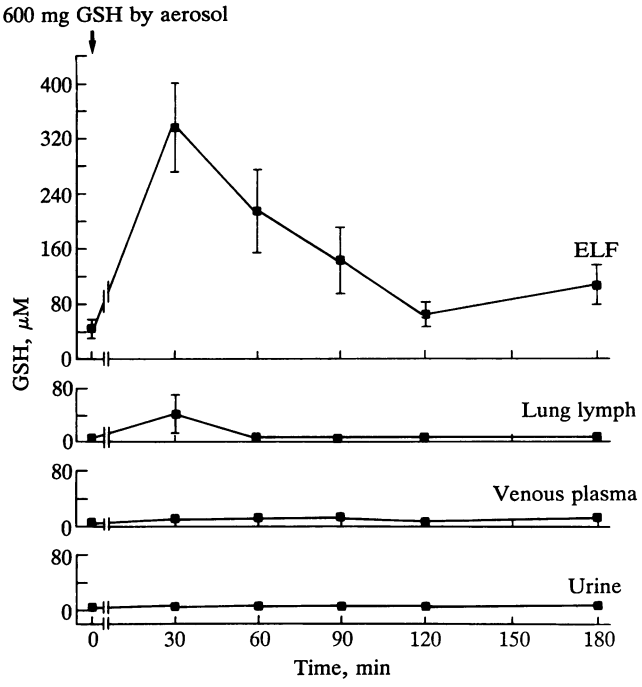

FIG. 3. GSH levels in sheep after aerosol administration of 600 mg of GSH. Concentrations of GSH were evaluated before and at intervals after the end of aerosol GSH administration. Time 0 is just before aerosolization of GSH, which continued for 25 min; subsequent times refer to time after completion of aerosol administration. Each data point represents the mean (± SEM) of values from 12 sheep.

respiratory tract to obtain higher, sustained levels of ELF GSH is possible. Three hours after administering 4.2 g of GSH by aerosol, the ELF level was increased to 4259 ± 2160 μM, a value 25-fold higher than preaerosol value, and over 10-fold higher than the 30-min peak level achieved with aerosolization of 600 mg of GSH.

Together, these data show that aerosolization of GSH is an effective method for targeting GSH to the ELF of the lower respiratory tract. The therapy appeared safe in that the mean and peak inspiratory pressures remained stable throughout the procedure. Aerosolization of GSH did not cause inflammation of the lower respiratory tract, as judged by visual inspection of the mucous membranes of the respiratory tract. Further, there was no apparent "leak" of the epithelial barrier, as determined by measurements of ELF volumes of pre- and postaerosol bronchial lavage fluids ($P > 0.1$; all comparisons with baseline values).

DISCUSSION

The present study shows that GSH levels can be augmented in the pulmonary ELF by directly administering aerosolized GSH. Several reasons suggest that augmentation of GSH levels in the ELF of the lower respiratory tract would be a useful therapeutic approach for a variety of lung disorders.

(*i*) GSH is a major component of the antioxidant defenses of ELF, where it serves to detoxify H_2O_2, a central component of the array of reactive oxygen species participating in biologic processes (8, 15–21).

(*ii*) Because GSH is the major source of cysteine for most cells (1–3), ELF GSH probably plays a major role in modulating the intracellular GSH levels of the pulmonary epithelium. Consistent with this concept, γ-glutamyltranspeptidase, the surface enzyme that metabolizes glutathione, is found at lung cell surface; this enzyme facilitates the entry of cysteine into cells, permitting intracellular resynthesis of GSH (29, 30). Further, transport of GSH against a concentration gradient has been demonstrated in a number of lung cell types, including pulmonary alveolar type II epithelial cells (31).

(*iii*) GSH is important in modulating immune function and, thus, probably participates in the pulmonary epithelial host defense system (10–15). Depletion of intracellular GSH suppresses lymphocyte activation by mitogens, and GSH is important in lymphocyte-mediated cytotoxicity (11, 13, 14).

(*iv*) A number of lung disorders are associated with an increased oxidant burden on the pulmonary epithelial surface and pulmonary epithelial cell damage, including IPF, asbestosis, cigarette smoking, adult respiratory distress syndrome, cystic fibrosis, and acute and chronic bronchitis (21, 22, 32–36). Consistent with the fact that GSH administration is helpful in experimental disorders of other organs associated with an increased oxidant burden [e.g., free radical damage in ischemic acute renal failure in rats (37), doxorubicin-induced myocardial toxicity in mice (38), myocardial ischemia-reperfusion injury in swine (39)], enhancement of antioxidant protection in ELF may be beneficial in disorders in which pulmonary epithelial cell damage is associated with an increased oxidant burden at the pulmonary epithelial surface (21, 40, 41).

(*v*) Two clinical disorders, IPF (23) and the HIV-seropositive state (15), are associated with "GSH deficiency" in lung ELF. Although the therapeutic efficiency of GSH in IPF would probably be associated with the GSH antioxidant function, augmentation of ELF GSH in HIV infection could help reestablish host defense function in the lung.

Although the concept of augmenting ELF GSH levels seems clinically useful on rational grounds, the major problem is a practical one. In the present study, a single dose of 600 mg of GSH given i.v. to sheep raised the GSH levels in plasma, lung lymph, and urine but not in lung ELF. Further, these increases were short-lived, in accordance with the short plasma half-life of GSH of ≈1.6 min in humans (42). Although these observations rest on only one dose of GSH, it is improbable that repeated i.v. injections could significantly augment GSH levels on the alveolar surface because most GSH may be metabolized by γ-glutamyltranspeptidase on the surface of endothelial cells; this is particularly true in the kidney (1–3, 29).

Knowing that i.v. therapy was unlikely to augment ELF GSH levels, we explored the direct targeting of GSH to the alveolar epithelial surface of the lower respiratory tract by aerosol administration, an approach used successfully to administer proteins, such as plasma and recombinant α_1-antitrypsin to α_1-antitrypsin-deficient individuals (43, 44). The data show that GSH can be included in droplets of optimal size for alveolar deposition while retaining its functional integrity—i.e., without being oxidized. Thus it seems feasible to administer GSH by the aerosol route. Consistent with the *in vitro* data, one aerosol dose of 600 mg of GSH markedly augmented lower respiratory tract ELF levels of GSH: the aerosol-delivered GSH augmented functional GSH levels in lung ELF. Furthermore, the aerosol administration not only permits targeting of GSH delivery to the alveolar epithelial surface, but the increased levels remain for a reasonable time; the half-life of GSH in ELF is much longer than in plasma. The levels remain elevated for up to 2 hr after aerosol delivery, suggesting independence of the ELF and plasma in GSH metabolism. It is conceivable that this approach can be adapted to humans. Human ELF is known to contain all elements of the redox cycle of the GSH system, including glutathione peroxidase, glutathione reductase, and NADPH. Thus, human ELF contains all the necessary components to maintain, in a reduced state, the GSH delivered to the ELF (8).

Other modifications may be necessary for GSH aerosol therapy to be clinically applicable. One goal would be to prolong the lifetime of aerosolized GSH in ELF. Approaches include (*i*) increasing the GSH dose deliverable within a reasonable time by altering the pharmacologic preparation to increase solubility or (*ii*) aerosolization of a crystalline form of GSH, which would then be released more slowly after deposition on the alveolar epithelial surface.

Together, these observations suggest that aerosol administration of GSH is a practical way to significantly augment GSH levels on the epithelial surface of the human lower respiratory tract. Importantly, the aerosol administration of GSH not only augmented ELF GSH levels but it did so with no adverse effects. No evidence of local inflammation was seen, and no changes occurred in the clinical parameters monitored during the study period. Such therapy may prove useful in a variety of lung disorders associated with an increased oxidant burden and/or decreased antioxidant protection on the pulmonary epithelial surface, as well as in augmenting pulmonary host defense in HIV infection.

R.B. is a Fellow of the International Molecular Medicine Foundation.

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