Determination of low-molecular-mass antioxidant concentrations in human respiratory tract lining fluids

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1Division of Pulmonary/Critical Care Medicine, University of California, Davis Medical Center, Sacramento 95817; 2Center for Comparative Respiratory Biology and Medicine, Department of Internal Medicine, University of California, Davis 95616; 3Toxicology Department, Genentech, Inc., So. San Francisco, California 94080; and 4Pharmacology Group, International Antioxidant Research Centre, King's College, London SW3 6LX, United Kingdom

Van der Vliet, Albert, Charles A. O'Neill, Carroll E. Cross, John M. Koostra, William G. Volz, Barry Halliwell, and Samuel Louie. Determination of low-molecular-mass antioxidant concentrations in human respiratory tract lining fluids. Lung Cell. Mol. Physiol. 276: L289–L296. 1999—Antioxidants present within lung epithelial lining fluids (ELFs) constitute an initial line of defense against inhaled environmental oxidants such as ozone, nitrogen oxides, and tobacco smoke, but the antioxidant composition of human ELFs is still incompletely characterized. We analyzed ELF concentrations of the low-molecular-mass antioxidants ascorbate, urate, glutathione (GSH), and α-tocopherol by obtaining bronchoalveolar lavage (BAL) and nasal lavage fluids from healthy nonsmoking volunteers and compared two different BAL procedures. ELF dilution by the lavage procedures was estimated by measurement of urea in recovered BAL fluids in comparison with those in blood plasma from the same subjects. The results indicated that a recently developed single-cycle BAL procedure minimizes influx of non-ELF urea into the instilled fluid and thus allows for a more accurate determination of ELF antioxidant concentrations. Using this procedure, we determined that bronchoalveolar ELF contains 40 ± 18 (SD) μM ascorbate, 207 ± 167 μM urate, 109 ± 64 μM GSH, and 0.7 ± 0.3 μM α-tocopherol (n = 12 subjects). Similar analysis of nasal lavage fluid yielded nasal ELF levels of 28 ± 19 μM ascorbate and 225 ± 105 μM urate (n = 12 subjects), whereas GSH was undetectable (<0.5 μM). Our results demonstrate that ascorbate and urate are major low-molecular-mass ELF antioxidants in both the upper and lower respiratory tract, whereas GSH is present at significant concentrations only in bronchoalveolar ELF.

THE EPITHELIAL LINING FLUIDS (ELFs) within the respiratory tract form an interface between the respiratory tract epithelial cells and the external environment and represent the first biological matrix to interact with inhaled environmental toxic gases such as tobacco smoke, ozone, and nitrogen oxides. ELFs contain multiple antioxidant enzymes, including superoxide dismutase, catalase, glutathione peroxidase, the iron-binding proteins lactoferrin and transferrin, and the copper-binding protein ceruloplasmin (10, 18), as local defense mechanisms against oxidative reactions initiated by inhaled pollutants or against endogenous oxidants generated during inflammatory-immune processes at respiratory tract surfaces. Additionally, ELFs also contain substantial amounts of the low-molecular-mass antioxidants ascorbate, urate, and GSH that presumably serve to protect important biomolecules within the ELF (such as α1-antiproteinase inhibitor and surfactant) as well as the underlying airway and parenchymal cells from oxidative injury. Respiratory tract mucous and reduced protein sulfhydryl groups may also contribute antioxidant activity to the ELFs (7, 17). Although a number of studies have attempted to determine antioxidant levels in ELFs, information about ELF antioxidant concentrations within distinct regions of the respiratory tract is still incomplete (4, 5, 8, 27, 30, 36, 40). Several research groups (5, 27, 30, 36) have determined bronchoalveolar ELF levels of GSH, with results ranging from 90 to 500 μM, although much less is known about GSH levels in more proximal ELFs. Moreover, there is as yet little information about ELF concentrations of other important low-molecular-mass antioxidants such as ascorbate and urate in any region of the respiratory tract.

Procedures to obtain ELF commonly utilize nasal lavage (NL) or bronchoalveolar lavage (BAL) techniques that involve instillation of varying amounts of saline that are then recovered by aspiration and gentle suction. This dramatically dilutes the ELF in the lavaged region and thus compromises accurate determination of ELF constituents (16, 25, 35). Various efforts have been made to estimate the extent of ELF dilution during lavage procedures; however, approaches to quantitate ELF constituents with dilution markers such as 99mTc-diethylenetriaminepentaacetic acid, 51Cr-ETDA, methylene blue, or urea are recognized to have major technical restrictions (12, 13, 20, 25, 35, 42). First, there is considerable exchange of these dilution markers between the instilled saline and non-ELF compartments (respiratory tract cells, interstitium, and plasma), the extent of which increases with longer dwell time of the instilled fluid. Second, increases in alveolar epithelial and/or endothelial permeability, secretion of products from cells, and perhaps some cell lysis during BAL are all events that may affect estimations of normal ELF antioxidant concentrations (12, 13, 42).
A convenient and commonly used procedure to estimate ELF dilution during lavage procedures has been proposed by Rennard et al. (35) and is based on the assumption that the ELF concentration of urea is similar to that in blood plasma and that minimal diffusion of urea into the instilled saline occurs during the lavage procedure. To satisfy the latter assumption, lavage procedures need to be performed rapidly because the high concentration gradient caused by instillation of urea-free lavage fluids promotes rapid diffusion of urea into the instilled fluid (12, 42). Commonly used BAL procedures involve repeated instillations of saline into the lavaged lung segment to completely recover distal ELFs and cells, but such procedures are known to result in substantial diffusion of urea and other constituents from non-ELF compartments into the instilled saline (12, 25, 35), which compromises accurate determination of ELF solute concentrations. In an attempt to minimize this problem, Peterson et al. (34) employed a single-cycle BAL procedure, which involves a single instillation of saline into the lung that is then retrieved in four to five separate fractions. The final fractions obtained by this procedure, although relatively small in volume, are more concentrated and permit more accurate determination of ELF constituents. Furthermore, because the single-cycle BAL procedure can be performed rapidly (within 40 s), errors in the estimation of ELF dilution due to diffusion of urea into the instilled fluid are likely to be small. Similar urea measurements were also found to be applicable in the determination of ELF dilution during NL because the levels of urea in undiluted nasal secretions were found to be similar to those in plasma (21). Moreover, NL procedures are commonly performed within 30 s, thus minimizing the influx of urea from non-ELF sources into the instilled fluid (21).

It was the aim of the present study to determine ELF concentrations of the major low-molecular-mass antioxidants in two different regions of the human respiratory tract and to establish potential regional differences in extracellular antioxidant composition within the respiratory tract. Such information may give insights into the potential presence of local antioxidant secreting or recycling mechanisms within the respiratory tract. Moreover, determination of actual ELF antioxidant concentrations rather than uncorrected lavage fluid levels will correct for changes in ELF volume due to exudative processes in response to respiratory tract injury and thus better reflect local extracellular antioxidant status.

EXPERIMENTAL PROCEDURES

Lavage procedures. Twelve healthy nonsmoking volunteers (9 men and 3 women; age range 18–62 yr) entered into the study after giving informed consent. Subjects were not taking any medication or antioxidant supplements. All volunteers were studied under a protocol approved by the Human Subjects Review Committee at the University of California, Davis. Fiber-optic bronchoscopies were performed in the Bronchoscopy Laboratory at University of California, Davis Medical Center in accordance with guidelines recommended by a National Institutes of Health workshop (2). All bronchosopies and BAL procedures were done transnasally after local anesthesia of the mouth, pharynx, nose, and throat with 4% lidocaine. Except for supplemental oxygen (2 l/min), supplied via a nasal cannula inserted into one of the nares, and 15–20 ml of 1% lidocaine without epinephrine applied directly to the larynx and tracheobronchial tree, no other medications were used. A flexible fiber-optic bronchoscope (Olympus model BF-P20) was passed into a subsegmental bronchoscope of either the right middle lobe or lingula. With the tip of the bronchoscope wedged in the bronchus, aliquots of sterile 0.9% saline at 25°C were infused and immediately aspirated back into a syringe by gentle suction. All subjects were monitored by continuous electrocardiogram, pulse oximetry, and blood pressure.

Two separate BAL procedures were performed on each subject. In the first procedure, five 20-ml aliquots of sterile saline were instilled sequentially and collected separately, thus obtaining five separate fractions. This sequential BAL procedure was usually completed within 2–3 min. The second BAL procedure, single-cycle BAL, was performed after the bronchoscope was wedged into the contralateral lung where a single 60-ml aliquot of saline was infused and immediately withdrawn into a series of four 15-ml syringes with a manifold (Fig. 1). Single-cycle BAL was completed in ~1 min. The lung subsegment lavaged first was alternated between study subjects to avoid potential bias. All subjects were monitored for 30 min after the procedures for any complications before being released.

Twelve healthy human subjects (9 men and 3 women; age range 18–62 yr) were also selected for NL, which was per-
formed essentially as previously described (21, 23). Briefly, 5 ml of warm sterile saline were instilled into each nostril, after which the subjects tilted their heads back at a 60o angle with the glottis closed. After 10 s, the lavage fluid was aspirated, forcibly expelled into a collection vessel, and kept on ice until further processing.

Processing of lavage fluids and biochemical assays. Recovered BAL fractions were stored on ice in the dark until further processing, which was performed within 1–2 h (time of transportation of BAL fluids from the Bronchoscopy Laboratory in Sacramento to the Research Laboratory in Davis). Spiking experiments with antioxidants demonstrated >80% recovery for ascorbate and >90% recovery for the other antioxidants after storage of unprocessed BAL fluids on ice for up to 2 h (the maximal time between BAL collection and removal of lavaged cells by centrifugation). NL fluids were processed immediately after collection. Lavage fluids were centrifuged at 200 g for 10 min at 4°C to remove cells, and the supernatants were used for analysis of antioxidants, urea, and protein content. For comparison, similar analyses were also performed on blood plasma obtained by centrifugation (1,000 g for 10 min at 4°C) of venous blood, which was collected from each subject before the lavage procedures.

Ascorbate and urate were determined by HPLC with amperometric electrochemical detection, with a detector potential of 500 mV. Plasma or lavage samples were mixed with four volumes of cold methanol to precipitate proteins, and the supernatant was injected on a 250 × 4.6-mm SUPELCOSIL LC-NH2 column (Supelco, Bellefonte, PA) and eluted with methanol–40 mM NaH2PO4 (95:5 vol/vol) as the mobile phase (15). Alternatively, samples were mixed with an equal volume of 5% metaphosphoric acid to precipitate proteins, and 50 µl of the supernatant were mixed with 15 µl of 2.6 M K2HPO4 and 185 µl of the mobile phase and injected onto a 250 × 4.6-mm Spherisorb ODS-2 column (Alltech, Deerfield, IL) with 40 mM sodium acetate (pH 4.75) containing 7.5% methanol and 1.5 mM dodecyltriethylammonium phosphate as the mobile phase (14). Both procedures gave similar results, with an on-column detection limit of 1 pmol. Control experiments with solutions of ascorbate in saline (1 or 10 µM) demonstrated that only minimal autoxidation of ascorbate occurred during storage on ice in the dark for up to 4 h (~20%), the maximal time between BAL or NL collection and ascorbate and urate analyses.

Low-molecular mass thiols (cysteine and GSH) were analyzed by HPLC with fluorescence detection after thiol derivatization (29) by reacting plasma or lavage fluids with an equal volume of 8 mM monobromobimane (Calbiochem, San Diego, CA) in 50 mM N-ethylmorpholine (pH 8.0). After protein precipitation with trichloroacetic acid (5% final concentration), the supernatants were injected onto a 10-µm Bondapak RP-18 RadialPak column (Waters, Milford, MA), eluted with 8% acetonitrile in 0.25% acetic acid at 1 ml/min, and analyzed by fluorescence detection (excitation at 394 nm and emission at 480 nm). The on-column detection limit for derivatized GSH or cysteine was ~1 pmol.

For analysis of α-tocopherol, 1 ml of BAL fluid or 100 µl of plasma were extracted with four volumes of cold methanol and five volumes of n-hexane. The collected hexane layer was dried under N2 and reconstituted in 100 µl of methanol–tert-butanol (50:50 vol/vol) for HPLC analysis, with ultraviolet detection at 290 nm (15).

The protein content of recovered BAL fluids and plasma was determined according to Lowry et al. (24), with bovine serum albumin as a standard.

Calculation of ELF dilution by urea analysis. The dilution of ELF fluids with the lavage procedures was estimated by the determination of urea levels in BAL and NL fluids and plasma from the same subject with a diagnostic kit (Sigma, St. Louis, MO) based on the complexation of urea with diacetyl monoxime (6). EL291 Dilution was determined by dividing the plasma urea concentration by that in the various lavage fractions (35), and ELF concentration of each measured solute was calculated as [solute]ELF = [solute]BAL × (urea)PLASMA/[urea]BAL, where [solute] and [urea] are the concentrations of solute and urea, respectively, in ELF, BAL, or plasma as indicated.

Because prolonged dwell time of instilled lavage fluids causes a substantial influx of urea from sources outside the bronchoalveolar luminal compartment (cells, interstitial spaces, and plasma), this will lead to inappropriately high BAL urea concentrations and result in an underestimate of ELF dilution (12, 25, 35).

RESULTS

Comparison of BAL procedures. Recovery of instilled saline in the standard sequential BAL procedure (5 × 20 ml) was 60 ± 7% (SD), consistent with previous studies (25, 35) in which similar BAL procedures were used. In agreement with earlier findings (9, 25, 26), the recovered volume per instillation increased with each successive fraction (Table 1), most likely resulting from the filling of residual volumes of the lavaged area with each instillation, with improved recovery after each filling. The instilled 60 ml of saline in the single-cycle BAL procedure were retrieved in four roughly equal fractions, with a total recovery of 41 ± 10% (Table 1), which was similar to the total recovery after three 20-ml instillations in the sequential BAL procedure.

Table 1 also lists the measured total protein content of each recovered BAL fraction and indicates a progressive increase in protein concentration in each successive fraction.
sive fraction obtained by sequential BAL, in close agreement with a previous report by Marcy et al. (25) using an identical BAL procedure (5 × 20 ml). Similarly, consistent with a previous study in sheep (34), the concentration of total protein was also found to increase in each successive fraction obtained by single-cycle BAL (Table 1). Although differences in protein concentration between BAL fractions were not significant, this increasing trend was very consistent for each subject studied and may be related to variations in the residence time of the instilled fluid in the wedged lung segment, which ranges from ~5 (fraction 1) to 15–20 s (fraction 4), the longer residence time allowing more efficient mixing of ELF constituents with the instilled saline. Alternatively, the final fractions may have sampled more distal segments of the bronchoalveolar region, which are expected to be more concentrated because of the lower air space volume-to-airway surface area ratio in the more distal regions of the respiratory tract (34). Because of the relatively short dwell time of the instilled saline in this single-cycle BAL protocol, diffusion or leakage of acellular components and/or constituents from non-ELF compartments is thought to be minimal.

Similar to the observed total protein levels, there was a progressive increase in urea concentration in each successive fraction obtained by the single-cycle BAL procedure (Table 1), consistent with the results of Peterson et al. (34). This phenomenon may again be related to more efficient mixing of the instilled saline with ELF constituents, although it is difficult to completely rule out influx of urea from other non-ELF sources into the instilled saline during this lavage procedure. A progressive increase in urea concentration in each successive BAL fraction was also observed with sequential BAL (5 × 20 ml; Table 1), and detected urea concentrations compared well with those reported by Marcy et al. (25), who used an identical BAL procedure. These authors attributed this progressive increase in BAL urea concentration to an influx of urea from non-ELF sources into the instilled saline. Because <50% of the initial instillations are recovered, the remainder dwells in the air spaces during the entire lavage procedure (which lasts 2–3 min), allowing substantial diffusion of urea into these residual lavage fluids, causing an inappropriate increase in urea levels in following lavage aliquots (25). As demonstrated in Table 1, average ELF dilution factors, based on urea measurements, ranged from ~100-fold in the initial recovered fractions to 30–40-fold in the final fractions for either BAL procedure.

In summary, general parameters of BAL fluid recovery and total protein and urea contents of recovered lavage fluids compare well with a number of previous reports and indicate that influx of non-ELF urea into the instilled fluids during sequential BAL may undermine calculation of ELF dilution.

Analysis of ELF antioxidants. Each lavage fraction obtained by either BAL procedure was analyzed for the low-molecular-mass antioxidants ascorbate, urate, GSH, and α-tocopherol, and the results are presented in Figs. 2 and 3. As shown, average antioxidant levels were found to be similar in each fraction obtained by the sequential BAL procedure (Fig. 2), although there was considerable variation among subjects. In contrast, a progressive increase in antioxidant concentrations was apparent in each successive fraction obtained after single-cycle BAL (Fig. 3). Although differences between successive fractions were not significant, this increasing pattern was very consistent for each individual subject. We also detected α-tocopherol in most of the BAL fractions obtained after both sequential and single-cycle BAL (<10–25 nM), although no consistent differences between lavage fractions were found. Compared with the determination of total thiols in BAL fractions with 5,5'-dithiobis(2-nitrobenzoic acid) (data not shown), we found that GSH accounted for >90% of the total thiols detected in BAL fluid, although low amounts of cysteine (0.1 ± 0.1 µM) could be detected in the final two single-cycle BAL fractions.

For each individual subject, ELF antioxidant concentrations were calculated with ELF dilution factors as
determined for each BAL fraction obtained by either lavage procedure. As illustrated in Fig. 4A, thus calculated ELF levels of ascorbate and urate were found to decrease markedly with each successive fraction obtained by the sequential BAL procedure. This apparent washout is consistent with diffusion of non-ELF urea into the instilled saline, resulting in an underestimation of ELF solute concentrations, and this "error" is more pronounced in the later BAL fractions obtained (25). These results also suggest that the influx of urea from non-ELF sources into the instilled saline, resulting in an underestimation of ELF solute concentrations, and this "error" is more pronounced in the later BAL fractions obtained (25). These results also suggest that the influx of urea from non-ELF sources into the instilled saline is more rapid than that of these low-molecular-mass antioxidants. This "washout" phenomenon was less pronounced for GSH (Fig. 4d), thus calculated ELF levels of ascorbate and urate were found to decrease markedly with each successive fraction obtained by the sequential BAL procedure. This apparent washout is consistent with diffusion of non-ELF urea into the instilled saline, resulting in an underestimation of ELF solute concentrations, and this "error" is more pronounced in the later BAL fractions obtained (25). These results also suggest that the influx of urea from non-ELF sources into the instilled saline is more rapid than that of these low-molecular-mass antioxidants. This "washout" phenomenon was less pronounced for GSH (Fig. 4d) and total ELF protein (levels ranging from 4.9 to 7.2 mg/ml), perhaps because of an influx of albumin and GSH from non-ELF sources during sequential or rewash lavage procedures (34). Because plasma protein and albumin concentrations are over 10 times higher than those in ELFs (e.g., Refs. 34, 35), protein leakage from plasma into the alveolar air space will dramatically affect measured protein concentrations in BAL fluids. In contrast to the results with sequential BAL, similar calculations of ELF antioxidant concentrations from each successive single-cycle BAL fraction gave very consistent results (Fig. 4B). Moreover, calculated antioxidant levels from the final three BAL fractions were markedly higher when the single-cycle BAL procedure was used instead of the conventionally used sequential BAL procedure, the latter resulting in a more extensive influx of non-ELF urea into the instilled saline, which yields lower calculated ELF solute concentrations. Hence the observed progressive increases in BAL solute concentrations in each successive single-cycle BAL fraction (Table 1, Fig. 3) are most likely not due to an influx of these solutes from non-ELF sources but rather are caused by a more effective mixing of ELF constituents with the instilled saline or by sampling of more distal areas within the respiratory tract with a greater surface area-to-volume ratio in the later lavage fractions. Interestingly, ELF antioxidant levels calculated from the first sequential BAL fraction (Fig. 4A) compare well with those calculated from single-cycle BAL fluids (Fig. 4B), again indicating that the increased antioxidant concentrations in the final single-cycle BAL fractions are unlikely to be due to antioxidant influx from non-ELF sources. Calculation of ELF protein concentration from the various fractions obtained by single-cycle BAL also gave consistent results (7.7-8.6 mg/ml), in accordance with a previous study in sheep (34). Despite the fact that we did not take steps to remove mucus (e.g., no filtering of BAL fractions), calculated ELF protein concentrations with either BAL technique are comparable to values reported in the literature (20, 25).

Collectively, our results support the conclusion of Peterson et al. (34) that the single-cycle BAL procedure is a more accurate and reproducible procedure for the quantitation of ELF constituents compared with more conventional sequential BAL procedures. Calculated bronchoalveolar ELF antioxidant and protein concentrations for each individual subject from single-cycle BAL fluids are summarized in Table 2 together with comparative measurements in blood plasma. 

Table 2. Comparison of antioxidant concentrations in human ELF and plasma

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>Bronchoalveolar</th>
<th>Nasal</th>
<th>Plasma</th>
</tr>
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<tbody>
<tr>
<td>Ascorbate, µM</td>
<td>40 ± 18</td>
<td>28 ± 19</td>
<td>67 ± 25</td>
</tr>
<tr>
<td>Urate, µM</td>
<td>207 ± 167</td>
<td>225 ± 105</td>
<td>387 ± 132</td>
</tr>
<tr>
<td>GSH, µM</td>
<td>109 ± 64</td>
<td>&lt;0.5</td>
<td>1.0 ± 0.7</td>
</tr>
<tr>
<td>α-Tocopherol, µM</td>
<td>0.7 ± 0.3</td>
<td>ND</td>
<td>16 ± 5</td>
</tr>
<tr>
<td>Total protein, mg/ml</td>
<td>9.5 ± 6.1</td>
<td>ND</td>
<td>74 ± 19</td>
</tr>
</tbody>
</table>

Values are means ± SD from 12 subjects. Single-cycle BAL (1 x 60 ml) or nasal lavage was performed on healthy volunteers as described in EXPERIMENTAL PROCEDURES, and ELF antioxidant and protein levels were calculated from urea analysis and compared with corresponding plasma levels. Bronchoalveolar ELF concentrations were calculated from the final 2 fractions obtained by single-cycle BAL. ND, not determined.
30, 36). No significant correlation was observed between plasma and ELF concentrations of either ascorbate or urate (r < 0.30).

Antioxidant levels in nasal ELF. We also determined antioxidant concentrations in nasal ELF obtained by NL, and NL fluid was found to contain 1.8 ± 1.1 (SD) μM ascorbate and 15.6 ± 6.9 μM urate (n = 12 subjects). In contrast to earlier reports by others (3, 41), we did not detect GSH in any NL fluid obtained (<0.03 μM). For calculation of nasal ELF dilution by the NL procedure, urea was measured in plasma and NL fluid from each subject. The urea level in NL fluid was 13.6 ± 8.3 μg/ml, in close agreement with previously published data (21), and comparison with the plasma urea level in the same subject (173 ± 61 μg/ml) indicated that dilution of nasal ELF by this NL procedure was ~10- to 20-fold. Calculated ELF concentrations of low-molecular-mass antioxidants, based on these urea measurements, are summarized in Table 2. Although variations are large, nasal ELF concentrations of ascorbate were invariably lower than those in plasma, and nasal ELF urate levels were lower than plasma levels in 10 out of 12 subjects. Again, there was no significant correlation between plasma or nasal ELF levels of either ascorbate or urate (r < 0.20). Nasal ELF ascorbate and urate levels are roughly similar to those in bronchoalveolar ELFs, although nasal and bronchoalveolar ELF levels were not always determined within the same subject at the same time, so no direct correlations can be made. In contrast, GSH was found to be at least 100 times more abundant in bronchoalveolar ELF compared with nasal ELF (Table 2).

DISCUSSION

A recent study by Peterson et al. (34) in sheep has indicated that a recently designed single-cycle BAL procedure is more suitable for the determination of ELF solute concentrations than more conventionally used sequential BAL procedures. Our studies, which provide the first comparison of these BAL procedures in human subjects, are in general agreement with this notion. Based on results obtained from single-cycle BAL fluid, we determined ELF concentrations of the low-molecular-mass antioxidants ascorbate, urate, and \( \alpha \)-tocopherol, which were generally found to be lower than those in plasma, whereas ELF GSH concentrations were considerably higher. Such comparisons are more difficult to make for lipid-soluble antioxidants such as \( \alpha \)-tocopherol because the distribution of these antioxidants depends on the presence of extracellular lipids such as in surfactant or lipoproteins. Indeed, \( \alpha \)-tocopherol is known to be secreted by alveolar type II cells together with surfactant lipids (38). Comparison of water-soluble antioxidant levels in various extracellular fluids is more meaningful and may perhaps indicate the presence of local secretory and/or recycling pathways. These issues as well as a comparison with other literature reports are discussed below.

Ascorbate. Uncorrected measured levels of ascorbate in BAL fluid from our study are generally consistent with those reported by others (22), although Slade et al. (40) and Bui et al. (4) reported BAL fluid ascorbate levels that are considerably higher than ours (1.75 and 1.7 ± 0.4 μM, respectively). Other studies (e.g., see Ref. 22) yielded BAL fluid ascorbate levels (0.47–0.61 μM) that are more similar to those obtained in the present study. Similarly, our measurement of ascorbate level in NL fluid agrees well with several previous reports (3, 18, 19). However, simultaneous plasma ascorbate determinations were often not performed, and no estimations of actual ELF concentrations of ascorbate have been made previously. Our results indicate that nasal and bronchoalveolar ELF ascorbate levels in humans are similar to or somewhat lower than those in plasma. Nasal ELF ascorbate levels were consistently lower than those in simultaneously obtained plasma but were not significantly different from ascorbate levels in bronchoalveolar ELFs. Of note, reduction of NL fluid with 2,3-dimercapto-2-propanol to reduce dehydroascorbate (11) did not affect measured NL ascorbate levels, indicating that ascorbate was almost exclusively present in its reduced form, consistent with a previous study in rats (39). The redox state of extracellular ascorbate is most likely maintained by reducing mechanisms at respiratory tract epithelial surfaces or by active uptake of oxidized ascorbate, intracellular reduction, and subsequent transfer to extracellular fluids (28, 37). The generally lower ELF ascorbate levels compared with those in plasma would suggest the absence of active ascorbate-secreting mechanisms within the human respiratory tract because respiratory tract lining fluid ascorbate could theoretically be maintained by leakage from plasma. The absence of a significant correlation between plasma and ELF ascorbate levels, however, is not consistent with the maintenance of ELF ascorbate by passive diffusion from plasma and would suggest the presence of local ascorbate secreting and/or recycling mechanisms within the respiratory tract. In this regard, it would be useful to attempt correlations utilizing a larger number of subjects with more divergent plasma ascorbate concentrations.

Urate. It has been well recognized that urate is a predominant ELF antioxidant, especially in the upper respiratory tract (3, 8, 19, 32, 33) where it is thought to be cosecreted with mucus by submucosal nasal glands after uptake from plasma (32, 33). Analysis of NL fluids for low-molecular-mass antioxidants has indeed shown that uric acid is the major low-molecular-mass antioxidant present (18, 29, 32). This is confirmed in the present study, which furthermore demonstrates that urate is also the most abundant low-molecular-mass antioxidant in bronchoalveolar ELF. The present study also provides the first determination of ELF urate concentrations, which are similar in both nasal and bronchoalveolar ELFs and somewhat lower than those in plasma. Thus the proposed uptake of urate from plasma by submucosal nasal glands, which are thought to concentrate and secrete urate in upper airway ELFs (33), is not directly supported by our findings because nasal ELF urate levels are generally lower than those in plasma and bronchoalveolar ELF urate levels are
similarity to those found in nasal ELF. Respiratory tract epithelial cells could also contribute to the maintenance of urate in ELF by purine metabolism via xanthine oxidoreductase (31). No significant correlation between plasma and ELF urate concentrations was observed in our study, which further supports the presence of local mechanisms that maintain ELF urate levels.

GSH. Measurement of total reduced thiols and HPLC analysis of low-molecular-mass thiols indicate that GSH is the predominant extracellular thiol in bronchoalveolar ELF. Our calculation of alveolar ELF GSH level (109 ± 64 µM) is generally lower than that in several reports in the literature (5, 27, 30, 36), which ranges from 90 to >500 µM. These previous studies have employed a relatively lengthy sequential BAL procedure, which may have stimulated GSH efflux from cellular sources or some cell lysis, thereby artifically increasing BAL fluid GSH levels. In fact, calculation of total antioxidant recovery by either BAL procedure used in the present study resulted in very similar results for either ascorbate or urate, but GSH recovery was much higher when sequential BAL was used, consistent with potential GSH efflux from cellular sources during the more lengthy sequential BAL procedure. Our findings are nevertheless consistent with those of others (5, 27, 30, 36) in that ELF concentrations of GSH are much higher than those in plasma. In contrast, no GSH could be detected in any NL fluid obtained (<0.03 µM), which indicates that nasal ELF contains <0.5 µM GSH. These findings contrast with earlier reports by Blomberg et al. (3) and Testa et al. (41), who reported NL fluid GSH levels of 0.6–2.5 and 1.3 ± 1.7 µM, respectively. Both research groups measured GSH by spectrophotometric assay procedures, which may account for differences with the present results obtained by HPLC analysis. Similar to the situation in plasma, GSH in upper ELFs is perhaps present largely as GSSG or in the form of mixed disulfides, with the abundant mucin thiols present in upper ELFs. Because alveolar ELFs contain much less mucin and much lower levels of albumin compared with plasma (e.g., Ref. 34), formation of mixed protein disulfides with GSH will be less prominent in the more distal ELFs. Furthermore, efflux of GSH from respiratory tract cellular sources, such as alveolar type II cells (1), and/or reducing mechanisms at respiratory tract cell surfaces are expected to contribute to the maintenance of bronchoalveolar ELF GSH levels.

Concluding remarks. Collection of ELF by BAL procedures is associated with a substantial dilution that compromises detection of various ELF constituents, and an influx of solutes from non-ELF sources into the instilled saline may represent a significant source of error in attempts to calculate ELF solute concentrations. Our results indicate that these problems are lessened by using a single-cycle BAL procedure, which is performed rapidly and results in the collection of relatively concentrated BAL fluids. Using this single-cycle BAL procedure and NL, we have established concentrations of various ELF antioxidants in various regions of the respiratory tract in comparison to those in blood plasma. Determination of actual ELF antioxidant concentrations rather than uncorrected BAL fluid levels is critical in the assessment of the antioxidant status within ELFs because they are corrected for changes in ELF volume caused by exudative processes during respiratory tract injury, which would artifically elevate BAL fluid antioxidant concentrations.

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REFERENCES


