Thioredoxin and dihydrolipoic acid inhibit elastase activity in cystic fibrosis sputum

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Excessive neutrophil elastase activity within airways of cystic fibrosis (CF) patients results in progressive lung damage. Disruption of disulfide bonds on elastase by reducing agents may modify its enzymatic activity. Three naturally occurring dithiol reducing systems were examined for their effects on elastase activity: 1) Escherichia coli thioredoxin (Trx) system, 2) recombinant human thioredoxin (rTrx) system, and 3) dihydrolipoic acid (DHLA). The Trx systems consisted of Trx, Trx reductase, and NADPH. As shown by spectrophotometric assay of elastase activity, the two Trx systems and DHLA inhibited purified human neutrophil elastase as well as the elastolytic activity present in the soluble phase (sol) of CF sputum. Removal of any of the three Trx system constituents prevented inhibition. Compared with the monothiols N-acetylcysteine and reduced glutathione, the dithiols displayed greater elastase inhibition. To streamline Trx as an investigational tool, a stable reduced form of rhTrx was synthesized and used as a single component. Reduced rTrx inhibited purified elastase and CF sputum sol elastase without NADPH or Trx reductase. Because Trx and DHLA have mucolytic effects, we investigated changes in elastase activity after mucolytic treatment. Unprocessed CF sputum was directly treated with reduced rTrx, the Trx system, DHLA, or DNase. The Trx system and DHLA did not increase elastase activity, whereas reduced rTrx treatment increased sol elastase activity by 60%. By contrast, the elastase activity after DNase treatment increased by 190%. The ability of Trx and DHLA to limit elastase activity combined with their mucolytic effects makes these compounds potential therapies for CF.

Thioredoxin (Trx) and dihydrolipoic acid (DHLA) are dithiol reducing systems. In this three-component system, NADPH is the reducing agent in vivo and is present in both the cytosolic and mitochondrial compartments. Reduced Trx is a ubiquitous 12-kDa dithiol-containing protein that acts as a potent reducing agent in vivo and is present in both the cytosolic and mitochondrial compartments. Reduced Trx catalyzes the proteolysis of a variety of extracellular matrix proteins, including collagen and elastin. Normally this proteolytic activity is modulated by a number of antiproteases, including α1-antiprotease and secretory leukoprotease inhibitor (SLPI) (40). However, in certain pathological states such as cystic fibrosis (CF), neutrophil elastase escapes the action of these endogenous protease inhibitors because of a protease/antiprotease imbalance (7).

CF is a genetic lung disease characterized by the accumulation of thick inspissated mucus and chronic neutrophilic inflammation (2, 22, 28). Neutrophils entering the airways release elastase and other proteases that exceed the capacity of the antiprotease defenses (1). This protease/antiprotease imbalance is believed to play an important role in the development of structural lung damage such as bronchiectasis, progressive pulmonary function decline, and early death (16). Reducing this protease/antiprotease imbalance may be beneficial in altering this inexorable decline.

Enhancement of mucus clearance remains a key part of CF management, with DNase (Pulmozyme) being the most commonly used mucolytic compound in the United States. Despite aggressive use of DNase, CF disease progression to bronchiectasis, respiratory failure, and death or transplant continues to occur routinely. DNase acutely increases the elastolytic activity of CF sputum in vitro (4) as well as in patients (33). Minimizing the increase in elastase activity while effecting mucus clearance is a desirable goal in the care of the CF patient. The dithiol compounds thioredoxin (Trx) and dihydrolipoic acid (DHLA) have been shown to decrease the viscoelasticity of CF mucus, and further investigation of these compounds as potential mucolytic therapies has been proposed (19, 26). Their direct effects on elastase have not been studied.

Human neutrophil elastase has four disulfide bonds that are important for maintaining tertiary structure and enzymatic activity (34). The dithiol dithiothreitol (DTT) is able to inhibit the activity of elastase, presumably by disrupting these disulfide linkages (21). We hypothesized that Trx and DHLA would also inhibit elastase activity.

Trx is a ubiquitous 12-kDa dithiol-containing protein that acts as a potent reducing agent in vivo and is present in both the intra- and extracellular spaces (12, 14, 39). DHLA is another dithiol with significant reducing potential. Like Trx, DHLA is found within all mammalian cells, although it is primarily restricted to the mitochondria (23, 24). We investigated the ability of these naturally occurring dithiol reducing compounds to directly inhibit human neutrophil elastase activity within the sputum of CF patients.

DHLA is commercially available in reduced form. By contrast, Trx requires NADPH and Trx reductase to act as a reducing system. In this three-component system, NADPH is

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the source of reducing equivalents. We hypothesized that the reduced form of Trx would be the active component of the Trx system and that the need for NADPH and Trx reductase could be eliminated if Trx was used in a reduced form. Such a single-component form of Trx has the potential for therapeutic use as a mucolytic, particularly in disease processes with excessive elastase activity.

MATERIALS AND METHODS

Reagents

Lyophilized recombinant Escherichia coli thioredoxin-1 (E. coli Trx), E. coli thioredoxin reductase (E. coli TR), DHLA, reduced β-nicotinamide adenine dinucleotide phosphate (NADPH), DTT, reduced glutathione (GSH), N-acetylcysteine (NAC), human neutrophil elastase, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and N-methoxy-succinyl-Ala-Ala-Pro-Val 4-nitroanilide were obtained from Sigma (St. Louis, MO). DHLA (25 mg) was dissolved in aqueous solution by adding 1 ml of Chelex resin-treated 5% NaHCO3. After thorough mixing by repeated pipetting, 200 μl of Chelex resin-treated 10× phosphate-buffered saline (PBS) plus 800 μl of Chelex resin-treated water were added. pH was corrected to 8.0 with concentrated hydrochloric acid. Recombinant human thioredoxin-1 (rhTrx) and a plant recombinant NAPD-thioredoxin reductase (raTR) from Arabidopsis were prepared as detailed below. All other chemicals were of the highest possible grade. Throughout this article, the term “E. coli Trx system” refers to E. coli Trx + E. coli TR + NADPH, and the term “rhTrx system” refers to rhTrx + raTR + NADPH.

Cloning of rhTrx and raTR Genes

The rhTrx gene was amplified by PCR out of human single-strand cDNA from breast tissue and HeLa cells (Stratagene, La Jolla, CA). The raTR gene was obtained in the same manner from leaf Arabidopsis single-strand cDNA (Torry Mesa Research Institute, San Diego, CA). In both cases, two primers were designed containing a NdeI restriction site at the 5′ end and an Xhol restriction site at the 3′ end. The PCR fragments were then cloned into the TOPO-TA vector (Invitrogen, Carlsbad, CA) and sequenced for gene accuracy. The genes were subcloned NdeI/Xhol in the pET-22b vector for overexpression in bacteria or site-directed mutagenesis.

Microbial Protein Overexpression

B21 DE3 (rhTrx) or B21 DE3 pLys (raTR) bacteria were transformed and plated in Luria-Bertani (LB) agar plates containing carbenicillin. Five colonies of freshly transformed bacteria were used to inoculate 100 ml of LB-carbenicillin and were grown in a shaker overnight at 37°C. The 100-ml preculture was added to 1 liter of LB-carbenicillin and grown to an optical density of 0.6–0.8. The bacteria were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and grown until the end of their exponential phase. rhTrx was released by osmotic shock without cell disruption (31). raTR was extracted by disrupting the cells with either 0.1 mg/ml lysozyme or five freeze/thaw cycles. This cell lysate was incubated in the presence of 10 μg/ml Dnase I and 10 μg/ml Rnase I in 50 mM Tris·HCl (pH 8.0) supplemented by 10 mM MgCl2 and 100 μM PMSF.

rhTrx and raTR Purification

A two-step ammonium sulfate (AS) fractionation was used to precipitate the rhTrx or raTR. AS was added to the protein mixture to yield a 40% AS solution. Precipitated material was removed by centrifugation (17,000 g for 15 min). Additional AS was added to the supernatant to yield a 90% solution. After centrifugation, the pellet was resuspended in <10 ml of 20 mM K-phosphate-200 mM NaCl buffer (pH 7.0). The 40% AS pellet contained contaminating proteins, and the 90% AS pellet contained either rhTrx or raTR. The resulting protein extracts were clarified by centrifugation (20,000 rpm, 4°C), and the supernatant was retained for further purification steps. Both protein solutions were separated by size-exclusion chromatography in a 450-ml Superdex 75 HP column equilibrated in 20 mM K-phosphate-200 mM NaCl buffer (pH 7.0). The raTR was retrieved close to the void volume and was identified either by a bright yellow color absorbing at 460 nm or with the DTNB assay as described by Florencio et al. (10). rhTrx was retrieved in the 10-kDa size range and was identified with the corn NAPD-malate dehydrogenase assay as described by Jacquot et al. (15). The active fractions were combined and purified to homogeneity as described below.

rhTrx. The protein sample was supplemented with 2 M AS and separated close to homogeneity on a 6-ml Resource phenyl custom column by using a 120-ml gradient ranging from 2.2 to 0 M AS in 100 mM K-phosphate buffer (pH 7.0). rhTrx was retrieved at ~1 M AS, and the active fractions were combined and dialyzed against 20 mM Na-acetate (pH 4.7) overnight at 4°C in 8,000 molecular weight cutoff (MWCO) dialysis bags. Proteins were then separated on a 6-ml Resource Q column in a 120-ml gradient of 0–300 mM NaCl-20 mM Na-acetate (pH 4.7), and rhTrx was recovered at ~100 mM NaCl. The protein was dialyzed in 8,000 MWCO dialysis bags against 20 mM K-phosphate buffer (pH 7.0) and stored at −80°C.

raTR. The protein sample was supplemented with 1.4 M AS and then separated on a 6-ml Resource phenyl custom column in a 120-ml gradient of 1.4−0 M AS in 200 mM K-phosphate buffer (pH 7.0). The raTR was eluted at ~0.5 M AS. The raTR positive fractions were retrieved by UV monitoring at 460 nm. Active fractions were dialyzed against 20 mM Tris·HCl (pH 8.0) overnight at 4°C in 50,000 MWCO dialysis bags. The proteins were then purified to homogeneity on a 6-ml Resource Q column in a 140-ml gradient of 0−600 mM NaCl in 20 mM Tris·HCl (pH 8.0). The raTR was recovered at ~180 mM NaCl and stored at −80°C.

Purified rhTrx was incubated in excess DTT (millimolar concentration) for 1 h at 37°C. DTT was removed from the protein solution by using a PD-10 desalting column (Amersham Bioscience, Uppsala, Sweden). Reduced protein was stored at −80°C.

Measurement of Reducing Activity

The reducing activity of different Trx formulations was tested using the DTNB reduction assay. For the NADPH-dependent Trx system, 50 μl of assay buffer (100 mM potassium phosphate, pH 7.0, 10 mM EDTA, and 0.05 mg/ml bovine serum albumin) containing 5 or 50 mM rhTrx and 0.5 mM raTR were added to a 96-well flat-bottom plate (Costar, Corning, NY). Next, 175 μl of sample buffer containing 720 mM NAPD were added, followed by 25 μl of 6 mM DTNB in sample buffer. To measure the reducing ability of purified rhTrx (p-rhTrx), we added 50 μl of 2.5 mM p-rhTrx to a 96-well plate, followed by 175 μl of sample buffer and 25 μl of 6 mM DTNB. After all reactions were initiated by the simultaneous addition of the DTNB, the change in kinetic absorbance at 412 nm due to DTNB reduction was monitored using a Spectra Max 340 microplate reader ( Molecular Devices, Sunnyvale, CA). Temperature was maintained at 30°C, and the duration of the assay was set to 12 min.

Purified Neutrophil Elastase Studies

To assess direct effects on elastase activity, we conducted studies by exposing purified human neutrophil elastase to the various reductant systems. The reductant to be tested (Trx, DHLA, GSH, or NAC) was diluted to the desired concentration in PBS (pH 7.2). The mixture was added to purified human neutrophil elastase (100 μg/ml in PBS, pH 7.2, 0.01% Triton X-100) and incubated at 37°C for 1 h. The final volume during incubation was 210 μl, and the concentration of
neutrophil elastase was 1.6 μg/ml. After incubation, 60-μl aliquots were tested in triplicate for elastolytic activity.

**Sputum Collection**

Sputum was obtained from adult and pediatric patients with CF at the Children’s Hospital (Denver, CO). Patients were diagnosed with CF if they had sweat chloride values in excess of 60 mM in two separate pilocarpine iontophoresis sweat tests and exhibited two allelic CF-producing mutations in subsequent genetic analysis. All samples were donated by either spontaneous expectoration or hypertonic saline induction. Sputum samples containing visibly detectable saliva were discarded. Sputum collection protocol, data collection, and consent/assent forms were approved by the Institutional Review Board of the University of Colorado Health Sciences Center. Sputum was kept on ice until delivered to the laboratory and was then kept at −80°C.

**Soluble-Phase Sputum Experiments**

For experiments with sputum in the soluble phase (sol), sputum samples were centrifuged at 50,000 g for 90 min at 4°C. The supernatant (sol phase) was removed and stored at −80°C. Incubation mixture was vortexed briefly (<1 s) and incubated for 20 min. A 20-min time frame was selected because this was a sufficient period to allow measurable mucolysis. The concentrations of DNase and p-rhTrx were chosen to give a similar mucolytic effect as assessed using an AR-1000 rheometer (TA Instruments, New Castle, DE) in oscillation mode with an angular velocity of 1 rad/s at 37°C. After incubation, the mixture was centrifuged at 22,000 g for 15 min. The supernatant was removed, and 5-μl aliquots were tested for elastase activity.

**Elastase Activity Assay**

Elastase activity was determined by adding 120 μl of 0.8 mM N-methoxysuccinyl-Ala-Ala-Pro-Val 4-nitroanilide in PBS (pH 7.2) to the experimental sample and monitoring kinetic absorbance at 405 nm at 37°C over 4 min (6). The concentration of elastase used in these experiments was determined to be within the linear range. The percent elastase inhibition was determined as follows: %inhibition = [1 − (ΔOD/ΔOD0)], where ΔOD and ΔOD0 are the change in absorbance for the experimental group and PBS control group, respectively.

**Statistics**

Data involving multiple group comparisons were analyzed using a one-way analysis of variance (ANOVA) with a two-tailed Student’s t-test for comparisons between individual groups. The percent elastase inhibition equal to 0 was used as the null hypothesis. For data with a nonnormal distribution, a logarithmic transformation was performed before statistical analysis. We set α = 0.05 for all analyses. Data variability is expressed as either SD or 95% confidence interval (CI).
dependent pattern (Fig. 2C). Sputum sol elastase inhibition reached a maximum at 4 mM NADPH (percent elastase inhibition: 94%, SD 4.4, \( P < 0.0001 \)), but statistically significant inhibitory effects on elastase activity were noted with NADPH concentrations as low as 0.0625 mM (percent elastase inhibition: 24%, SD 7.3, \( P = 0.0005 \)).

Each of the three components of the Trx system was essential for inhibition of elastase. The absence of any of the three Trx system components resulted in the elimination of the inhibitory effect (percent inhibition with no Trx: −5.5%, SD 4.3; no Trx reductase: −15.6%, SD 12.1; no NADPH: −11.6%, SD 12.2).

Comparison of Elastase Inhibition After Treatment With Dithiol and Monothiol Reductants

Trx’s conserved active site has two cysteine groups in a C-G-P-C conformation. DHLA is a small molecule with two closely positioned free thiol groups in a similar orientation. A concentration-response curve was developed by incubating increasing concentrations of DHLA overnight with CF sputum sol. DHLA inhibits sol elastase activity in a concentration-dependent manner (Fig. 3). At the highest concentration tested (2 mM), DHLA inhibited 80% (SD 18, \( P = 0.0001 \)) of sol elastase activity. With a similar concentration of reducing equivalents on a molar basis in the rhTrx system (2 mM NADPH), the extent of elastolytic activity inhibition by rhTrx was almost identical (Fig. 2C; percent elastase inhibition: 83%, SD 19). Oxidized lipoic acid (LA) demonstrated substantially less ability to inhibit elastase activity than its reduced counterpart (percent inhibition: 2%, SD 9 at 250 \( \mu \)M LA; 8%, SD 10 at 500 \( \mu \)M LA).
In contrast to the dithiols *E. coli* Trx, rhTrx, and DHLA, the monothiols NAC and GSH did not show the same ability to inhibit elastase, particularly at low concentrations. For this comparison, the concentration of NADPH within the *E. coli* Trx system was varied, because NADPH is the primary source of reducing equivalents. At concentrations between 0.125 and 2 mM, neither NAC nor GSH significantly inhibited purified human neutrophil elastase (Fig. 4). By contrast, the *E. coli* Trx system caused potent inhibition throughout this concentration range. DTT showed significant elastase inhibition at low millimolar concentrations (2 mM) but was less effective at lower micromolar concentrations (Fig. 4).

**Prereduced Trx**

In the previous experiments, NADPH provided the reducing equivalents to Trx via TR as part of a three-component Trx system. To confirm that the reduced form of Trx alone was sufficient to inhibit elastase activity, we prepared a prereduced form of rhTrx (p-rhTrx). The reducing activity of p-rhTrx was verified by spectrophotometer with DTNB (Fig. 5). With the use of similar amounts of reductant activity (500 μM p-rhTrx or 500 μM NADPH as part of the rhTrx system), p-rhTrx demonstrated very rapid reduction of DTNB with over 50% of the DTNB being reduced in the time taken for the spectrophotometer to take the first measurement. By contrast, the reduction kinetics of the rhTrx system were not nearly as rapid and were dependent on rhTrx concentration.

p-rhTrx was a potent inhibitor of purified neutrophil elastase (Fig. 6A). Progressive inhibition of elastase activity was noted up to a maximum of 79% (SD 6.6, *P* < 0.0002) at 0.5 mM p-rhTrx after a 1-h incubation at 37°C. Treatment of CF sputum sol for the same time period with p-rhTrx also caused a concentration-dependent inhibition (Fig. 6B), but the absolute inhibition was less (elastase percent inhibition: 27.8%, SD 22.6, *P* = 0.03).

**Fig. 4.** Comparison of dithiol and monothiol compounds on purified human neutrophil elastase activity. Elastase was incubated with *E. coli* Trx system, DTT, N-acetylcysteine (NAC), or GSH at 37°C for 1 h. Elastase activity is expressed as the percentage of inhibition relative to PBS control. In the case of the Trx system, *E. coli* Trx (10 μM) + *E. coli* Trx reductase (0.1 μM) was held constant and the concentration of NADPH was varied. The Trx system showed significant inhibition of elastase at all concentrations tested, whereas inhibition by DTT was concentration dependent. Neither monothiol (NAC and GSH) at concentrations up to 2 mM demonstrated any sizeable elastase inhibition. Data are presented as means ± SD; n = 3 determinations per condition. *

**Fig. 5.** Reduction of DTNB by rhTrx system and prereduced rhTrx (p-rhTrx). A representative curve is shown. Either 10 or 1 μM rhTrx + 0.1 μM Trx reductase + 500 μM NADPH or 500 μM p-rhTrx was tested for the ability to reduce DTNB. DTNB was added to each experimental sample to achieve a final concentration of 600 μM. The reaction was performed at 30°C over a time (T) of 12 min.

**Fig. 6.** Elastase activity after treatment with p-rhTrx. A: purified human neutrophil elastase incubated at 37°C with p-rhTrx for 1 h. No Trx reductase or NADPH was present. p-rhTrx inhibited elastase in a concentration-dependent manner (*P* < 0.0001). Data are presented as means ± SD; n ≥ 3 determinations per data point. *P* < 0.05 vs. PBS-treated control group. B: sputum sol from CF patients (1:14 dilution) incubated for 1 h at 37°C with p-rhTrx. Increasing concentrations of p-rhTrx resulted in progressive inhibition of elastase activity (*P* = 0.0005). Elastase activity is expressed as the percentage of inhibition relative to PBS control. Data are presented as means ± SD; n = 6 determinations per condition. *P* < 0.05 vs. PBS-treated control group.
Treatment of Whole Sputum From Patients With CF

Trx has been shown to have potent mucolytic effects in vitro (26). In vitro treatment of whole sputum with the mucolytic DNase has been shown to increase elastolytic activity (4). To investigate the possibility that Trx or other dithiol agents may show a similar effect, we incubated whole mucus from CF patients for 20 min with 2.7 μM DNase, 100 μM p-rhTrx, rhTrx system (10 μM rhTrx + 0.1 μM raTrx) with 100 μM or 2 mM NADPH, 2 mM DHLA, or 2 mM DTT. The sol phase was then collected by centrifugation. The concentration of DNase selected was equivalent to that in the medication Pulmozyme. Based on oscillation rheometry, where G is the mechanical impedance or vector sum of viscosity and elasticity, the concentrations of DNase and p-rhTrx tested yielded similar mucolysis (100 μM p-rhTrx: log ΔG* = -0.70, SD 0.15; 2.7 μM DNase: log ΔG* = -0.61, SD 0.37; n = 4). DNase and p-rhTrx showed significant increases in sol elastase activity (relative multiples of increase in elastase activity vs. control: DNase: 2.9, CI: 1.8–4.6, P = 0.0001; p-rhTrx: 1.6, CI: 1.3–1.9, P = 0.0003). The increase after DNase treatment was much more pronounced than after any di-thiol, including p-rhTrx (Fig. 7). Sol collected from sputum treated with DNase had 2.6 times the elastase activity relative to p-rhTrx treated sputum (Wilcoxon rank sum, P = 0.006, n = 10).

DISCUSSION

This study provides evidence that Trx and DHLA can directly inhibit human neutrophil elastase activity. Elastase has four disulfide bonds that are important in maintaining its tertiary structure and enzymatic activity (34). Although the mechanism by which Trx and DHLA inhibit elastase activity was not definitively determined in this study, these potent reducing agents likely disrupt the disulfide linkages within elastase. The presence of reduced sulphydryl groups would appear to be an important part of this mechanism, because the oxidized forms of Trx and lipoic acid do not effectively inhibit elastase, whereas the reduced forms do. Other groups have also documented decreases in elastase activity with the use of reduced thiols. Significant reductions in neutrophil elastase activity with the use of micromolar amounts of DTT have been demonstrated in vitro (21, 35). Factors influencing the inhibition of elastase included DTT concentration, time, and reaction temperature (35). Del Val et al. (9) showed that the three-component Trx system in the presence of α1-antitrypsin was able to act as a potent porcine pancreatic elastase inhibitor. The data presented in this article extend this finding by demonstrating a direct inhibitory effect of Trx on elastase even in the absence of an antiprotease such as α1-antitrypsin.

Both rhTrx and E. coli Trx systems caused significant inhibition of elastase. In the three-component Trx system (Trx, TR, and NADPH), TR serves to transfer reducing equivalents (electrons) from NADPH to Trx. Trx required both TR and NADPH to inhibit elastase. Decreasing the NADPH caused a concentration-dependent impairment of elastase inhibition, and complete removal of either NADPH or TR eliminated inhibition altogether. However, if prereduced rhTrx (p-rhTrx) was used as a single component, effective elastase inhibition could be demonstrated in the absence of TR and NADPH. This finding supports the reduced form of Trx as the active constituent of the Trx system.

Trx and DHLA have two sulphydryl moieties at their active sites and are strong dithiol reductants with redox potentials of −280 and −320 mV, respectively. By contrast, monothiols such as GSH and NAC have one sulphydryl group with redox potentials of −240 and −150 mV, respectively. In our experiments, we noted that in the concentration range at which dithiols inhibited elastase (up to 2 mM), the monothiols had little inhibitory effect. This is consistent with previous work showing that neither GSH nor the monothiol β-mercaptethanol had a significant effect on elastase activity at low millimolar amounts (21). However, higher concentrations of monothiols can inhibit elastase. To achieve a 50% reduction of purified neutrophil elastase activity, ~60 mM NAC (35) is required, whereas even 120 mM of the monothiol mercaptethanesulfonate (Mistabron) does not achieve this level of elastase inhibition (36). For comparison, p-rhTrx showed a 50% reduction in purified neutrophil elastase activity at 0.03 mM.

To explore the possible clinical application of our findings, we examined sputum from CF patients. A major component of airway inflammation in CF is an influx of neutrophils, which...
release large quantities of elastase. Excess elastase overwhelms the antielastase protective mechanisms (α1-antiprotease, secretary leukoprotease inhibitor, α2-macroglobulin, and eglin), resulting in dramatically elevated free elastase levels in the sputum of CF patients (30). Even patients with mild CF disease have large amounts of free neutrophil elastase present in bronchoalveolar lavage fluid (17). This free elastase has numerous effects, including direct structural damage to airways, increased mucus secretion, impaired opsonophagocytosis as a result of complement and immunoglobulin cleavage, and increased cytokine expression, especially IL-8 (16). Elastase impairs clearance of apoptotic neutrophils through cleavage of phosphatidyl serine receptors on macrophages (37, 38) and inactivates the pulmonary surfactant proteins A (29) and D (13). Regulation of the airway surface liquid layer may be influenced by elastase through the epithelial sodium channel (ENaC). Human neutrophil elastase activates ENaC and may contribute to the sodium hyperabsorption in the CF airway (3). Inhibition of elastase activity could help mitigate these various adverse consequences. Our experiments demonstrated significant decreases in the elastolytic activity of CF sputum sol treated with the three-component Trx system, the single-component p-rhTrx, or DHLA. This inhibitory effect was concentration dependent with micromolar concentrations of the reductants showing efficacy.

The three-component Trx system (26) and DHLA (19) have potent mucolytic effects and are being investigated as potential mucolytic therapies in CF. DNase (Pulmozyme) is the most commonly prescribed mucolytic in the United States for CF patients. Acute increases in elastase activity after DNase treatment have been documented in vitro (4) as well as in patients with CF (33). Our study confirmed these previous findings. After DNase treatment, the elastase activity within the sol phase increased almost threefold that of control sputum. As mucolytics, the dithiols may also have showed undesirable increases in elastase activity. However, in contrast to DNase, most of the dithiols did not affect elastase activity. A 60% increase in elastase activity was noted after p-rhTrx (100 μM) treatment, but this was less than one-third the increase in elastase activity in the DNase-treated samples.

Attempting to predict in vivo outcomes based on in vitro results involving CF mucus is problematic. Despite the acute increase in elastase activity noted in the above paragraph, long-term use of DNase by CF patients results in decreased sputum elastase load and activity relative to untreated patients (8, 25), presumably through enhanced airway clearance. The fact that dithiols are able to minimize the acute elastase increase raises the possibility that even greater elastase reductions may be realized with chronic use of these potential mucolytics. Currently there is no effective antielastase therapy available to physicians treating CF. Attempts to return the protease/antiprotease balance to normal have met with only marginal success. Konstan and Davis (18) noted that “since the neutrophils reach the airway by migrating through the epithelium, much of the release of proteases will occur beneath the mucus blanket and the inhibitors must penetrate this protective layer.” Because dithiols act as both potent mucolytics and elastase inhibitors, these agents have the potential to penetrate beneath this mucus layer.

Relatively high concentrations of dithiols are required for both their mucolytic (19, 26) and elastase-inhibitory effects. This may present a significant and potentially insurmountable barrier to their effective use. However, recent advances in nebulizer technologies have substantially enhanced intrathoracic deposition efficiencies to such an extent that delivering compounds requiring greater airway concentrations is now possible. Using a Pari LC Star nebulizer connected to an AKITA inhalation device, one investigative group (11) has demonstrated an intrathoracic deposition efficiency of 86.3%, well above the 10–20% pulmonary deposition noted for traditional nebulizers.

Trx is an endogenous dithiol that participates in a variety of cell activities, including modulation of transcription factors such as NF-κB, AP-1, and hypoxia-inducible factors-1 and -2 as well as nuclear glucocorticoid receptor activation and regulation of apoptosis (14). DHLA is also a naturally occurring dithiol, first identified by Lester Reed in 1951 (27). This small molecule (molecular weight 208.3) has been shown to have both antioxidant and prooxidant activities (20, 32). In its lipoamide form, it participates as a cofactor of the multienzyme complexes catalyzing the oxidative decarboxylation of α-keto acids (23). Both DHLA and its oxidized form (α-lipoic acid) have been proposed as treatments for a variety of conditions (24), including CF (5), and recent safety data regarding treatment of diabetic polynephropathy with α-lipoic acid are reassuring (41). As native mammalian reductants, Trx and DHLA hold promise for future medicinal development as treatments in CF.

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DISCLOSURES

The views expressed in this article are those of the authors and do not reflect the official policy or position of the Department of the Navy, Department of Defense nor the U.S. Government. Syngenta International AG (Basel, Switzerland) provided funding to C. W. White. U.S. patent 5,985,261 describing the induction of manganese superoxide dismutase by thioredoxin has been awarded to C. W. White, K. C. Das, and National Jewish Medical and Research Center. C. W. White, R. L. Lee, and G. del Val have a patent pending regarding the use of thioredoxin and DHLA as mucolytics and elastase inhibitors.

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