Antioxidant properties of cystic fibrosis sputum

Nurlan Dauletbaev, Jens Rickmann, Klaus Viel, Holger Diegel, Christian von Mallinckrodt, Jürgen Stein, Thomas O. F. Wagner, and Joachim Bargon. Antioxidant properties of cystic fibrosis sputum. Am J Physiol Lung Cell Mol Physiol 288: L903–L909, 2005. First published January 7, 2005; doi:10.1152/ajplung.00349.2004.—Oxidative stress is a likely contributor to the pathogenesis of cystic fibrosis (CF) lung disease. However, hydrogen peroxide (H2O2), a physiological oxidant, is not elevated in CF exhalates. H2O2 may be neutralized by antioxidants in CF airway secretions. The H2O2-detoxifying capacity of CF airway secretions, obtained via sputum induction, was studied in an in vitro H2O2 cytotoxicity model. 16HBE14o- cells were exposed to H2O2 in culture medium containing either 0 or 10% fetal bovine serum (FBS) or 10% CF sputum supernatant (extracted without use of dithiothreitol). The efficiency of H2O2 neutralization was estimated by measuring intracellular oxidant levels (dihydorhodamine 123) after 2 h and cell viability (propidium iodide) after 24 h of H2O2 exposure. Furthermore, the presence of reduced thiols (DTNB assay) and reduced glutathione (recycling assay) in CF sputum samples was evaluated. CF sputum extracts completely prevented intracellular oxidant accumulation seen in cells incubated with H2O2 in both control media (i.e., 0 or 10% FBS). Furthermore, CF sputum abolished cell death in 16HBE14o- cells exposed to up to 1 mM H2O2. In contrast, there was 100% cytotoxicity in cells exposed to 600 μM H2O2 in both control media. The H2O2-detoxifying potential of CF sputum was sustained after catalase, GSH, and glutathione peroxidase were inactivated by sodium azide, which does not affect glutathione peroxidase. In addition, reduced protein thiols were found in abundance in CF sputum. In conclusion, CF sputum is capable to neutralize H2O2 and abundant reduced thiols and/or glutathione peroxidase are fully sufficient to detoxify H2O2.

Cystic fibrosis (CF) is a lethal hereditary multiorgan disease caused by mutations affecting expression of the cystic fibrosis transmembrane regulator (CFTR) protein. In adult patients, CF lung disease is the primary cause of morbidity. Oxidative stress is a likely contributor to the advance of the CF lung disease (2, 3, 15, 16). Oxidative stress is a condition where oxidants exceed the neutralizing capacity of the antioxidant defense system. In CF, oxidative stress may be due to both elevated oxidant levels and impaired antioxidant defense. It is assumed that neutrophils, which are abundant in CF airways, are capable of producing high levels of oxidants such as hydrogen peroxide (H2O2). There is indeed evidence of irreversible oxidative modifications of, e.g., proteins in CF airway secretions (e.g., Ref. 15). In addition, proinflammatory cytokines (e.g., TNF-α) and bacterial products (pyocyanin and pyochelin), both present in copious amounts in CF airway secretions (19, 23), are known to promote intracellular oxidative stress (13, 18). On the other hand, antioxidant defense in CF airways may be disturbed as a result of impaired absorbance of fat-soluble vitamins, such as vitamin E (2, 16). Furthermore, excretion of reduced glutathione (γ-glutamyl-cysteinyl-glycine, GSH), which is the major low-molecular-weight thiol and crucial antioxidant (8), appears to be linked to the transport of chloride ion (11, 12), and CFTR is the major chloride channel. Indeed, GSH excretion was reported to be impaired in CF respiratory epithelium (11).

Given this as a background, it is surprising that, in sharp contrast to chronic obstructive pulmonary disease patients, H2O2 is not elevated in breath condensate samples of CF patients (14, 24). This lack of exhaled H2O2 may be explained by an efficient scavenging in CF airways. Previously (6), we reported high levels of GSH and glutathione peroxidase in CF sputum samples. These data are well in line with the findings by Worlitzsch et al. (24), who reported high levels of catalase in CF sputum samples. Catalase, GSH, and glutathione peroxidase are the principal antioxidants detoxifying H2O2. We hypothesized that antioxidants in CF airway secretions effectively detoxify H2O2 and tested the H2O2-scavenging properties of CF airway secretions, obtained via sputum induction, in an in vitro H2O2 cytotoxicity model.

MATERIALS AND METHODS

Sputum induction and obtaining of supernatants. Study protocol was approved by the Ethics Committee of the Hospital of Johann Wolfgang Goethe University (Frankfurt/Main, Germany). CF sputum supernatants were obtained from 12 stable adult CF patients (median age: 29 yr, median forced expiratory volume in 1 s: 1.7 l/49% predicted all ΔF508 homozygous and chronically infected with Pseudomonas aeruginosa) who received ambroxol (Mucofalkan, 30 mg, twice a day) as a mucolytic agent. Patients receiving recombinant human DNase I (Pulmozyme) or N-acetylcysteine as mucolytics were not included in this study. The samples were obtained via sputum induction as described (5, 6). Samples were incubated on melting ice and processed with chilled solutions and low temperatures to preserve antioxidants. Contaminating saliva was removed, and mucus plugs were pooled and resuspended in serum- and antibiotic-free minimal essential medium (MEM; Invitrogen, Karlsruhe, Germany). CF sputum patients (12 stable adult CF patients (median age: 29 yr, median forced expiratory volume in 1 s: 1.7 l/49% predicted all ΔF508 homozygous and chronically infected with Pseudomonas aeruginosa) who received ambroxol (Mucofalkan, 30 mg, twice a day) as a mucolytic agent. Patients receiving recombinant human DNase I (Pulmozyme) or N-acetylcysteine as mucolytics were not included in this study. The samples were obtained via sputum induction as described (5, 6). Samples were incubated on melting ice and processed with chilled solutions and low temperatures to preserve antioxidants. Contaminating saliva was removed, and mucus plugs were pooled and resuspended in serum- and antibiotic-free minimal essential medium (MEM; Invitrogen, Karlsruhe, Germany) to obtain a final dilution of 10% (10 ml of MEM per 1-g sample). Samples were vortexed and agitated to allow MEM to wash out substances trapped in mucus. Plugs were pelletted by centrifugation (300 g, 15 min, 4°C), and supernatants were aspirated, passed through four layers of sterile cotton gauze to remove remaining mucus plugs, and filter sterilized (Millipore Sterillip filter unit, 0.22 μm pore size; Millipore, Schwalbach, Germany). MEM containing 10% CF sputum extract, referred throughout the text as MEM (10% CF sputum), was immediately used.

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in the study. In some experiments, catalase and peroxidases in CF sputum extracts were inactivated by sodium azide (NaN₃) before incubation with cells.

**H₂O₂ cytotoxicity**. 16HBE140- cells (a gift from Dieter Gruenert, Univ. of California at San Francisco) were used to study H₂O₂ cytotoxicity in the presence of CF sputum. 16HBE140- cells were cultured in MEM supplemented with 10% fetal bovine serum (FBS) and antibiotics. The cells were collected by trypsinization, counted (Trypan blue exclusion), seeded on multiwell plates at concentration of 1.0×10⁶ viable cells/ml, and allowed to grow for 24 h in MEM (10% FBS). After 24 h of incubation, cells reached 100% confluence, cell culture medium was renewed, and cells were cultured for additional 24 h. Then, the cells were exposed to H₂O₂ diluted from the cell culture medium was renewed, and cells were cultured for additional (10% FBS). After 24 h of incubation, cells reached 100% confluence, cell culture medium was renewed, and cells were cultured for additional 24 h. Then, the cells were exposed to H₂O₂ diluted from the 30% stock solution (Sigma-Aldrich Chemie, Munich, Germany). Dilutions were made in serum-/antibiotic-free MEM or MEM (10% FBS, antibiotic-free) (both as controls) or MEM (10% CF sputum, antibiotic-free). The effects of H₂O₂ were examined as described below.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide test. Confluent cells grown on 96-well plates were incubated for 24 h with 0–1,000 μM H₂O₂ in either serum-/antibiotic-free MEM or MEM (10% FBS). After exposure, cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test (17). The viability was expressed as a percentage of the viability of control cells exposed to 0 μM H₂O₂. The H₂O₂ concentration that caused more than 50% decrease in cell viability was considered cytotoxic.

Detection of intracellular oxidants. Dihydorhodamine 123 (DHR123: Mo Bi Tec, Göttingen, Germany) was used to detect intracellular oxidants. DHR123 is a nonfluorescent, noncharged dye that easily penetrates cell membrane. Once inside the cell, DHR123 reacts with intracellular oxidants to yield rhodamine, a highly fluorescent compound. The rhodamine fluorescence is directly proportional to the amount of DHR123 oxidized by intracellular oxidants and can be detected by, e.g., flow cytometry.

For the current studies, 16HBE140- cells grown on 12-well plates were loaded for 30 min with 5 μM DHR123 in serum-/antibiotic-free MEM. Subsequently, the cells were exposed for 2 h to H₂O₂ in serum-/antibiotic-free MEM, MEM (10% FBS), or MEM (10% CF sputum). After incubation, cell culture supernatants were aspirated, cells were trypsinized, and cell fluorescence was analyzed using flow cytometer (FACS Calibur, BD Biosciences; excitation with a 488-nm argon laser, emission acquired in red channel). The instrument settings were adjusted to set the autofluorescence of unstained, unstimulated cells below 10⁶ and 10³ log on the x-axis. In each experiment, the fluorescence intensity of 10,000 cells was analyzed. Data are demonstrated as a histogram of fluorescence intensity (x-axis) vs. relative cell number (y-axis) using WinMDI version 2.8 software (http://facs.scripps.edu/software.html, developed by J. Trotter).

**Cell images.** 16HBE140- cells grown on LabTec cell chambers (Nunc, Wiesbaden, Germany) were exposed to H₂O₂ for 12 h. Cell images were captured using a phase-contrast inverted microscope equipped with a videocamera (Zeiss, Jena, Germany).

**Viability assay with propidium iodide.** 16HBE140- cells grown on 12-well plates were exposed to H₂O₂ for 24 h. After incubation, cell supernatants were transferred into sterile 12×75-mm polystyrene test tubes to collect detached cells. Attached cells were trypsinized and combined with respective supernatants. Cells were stained for 5 min in the dark with a solution of propidium iodide (PI, 1 μg/ml) in phosphate-buffered saline (PBS, Invitrogen). After incubation, cells were briefly washed with PBS to remove unbound PI, and fluorescence intensity was analyzed on flow cytometer (red channel).

Viable and dead cells can be easily distinguished from their fluorescence intensity (viable cells exhibiting low vs. dead cells with high fluorescence intensity) (20). In each experiment, 10,000 cells were analyzed, and distribution of PI fluorescence was demonstrated as a histogram of fluorescence intensity (x-axis) vs. relative cell number (y-axis).

**Reduced thiols and GSH in CF sputum extracts.** For this study, CF mucus plugs were diluted in PBS to obtain 10% solution. CF sputum extracts were obtained as described above. Reduced thiols in CF sputum samples were probed using 5,5′-dithio(bis)2-nitrobenzoic acid (DTNB) (10). In reaction 1:

\[ 2\text{RSH} + \text{DTNB} \rightarrow \text{RSSR} + \text{TNB} \quad (1) \]

RSH is a reduced low- or high-molecular-weight thiol, such as a GSH or SH group of protein, respectively; RSSR is a thiol disulfide, such as GSSG or protein disulfide. DTNB is reduced to yield thio(bis)2-nitrobenzoate (TNB), which at neutral pH has absorbance maximum at 412 nm (OD₄₁₂ nm) (10).

This reaction can be made specific for GSH by adding glutathione reductase (GR) and NADPH to reactions 2 and 3 (Ref. 1).

\[ \text{GSH} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+ \quad (2) \]

\[ 2\text{GSH} + \text{DTNB} \rightarrow \text{GSSG} + \text{TNB} \quad (3) \]

In the present studies, the OD₄₁₂ nm of 10% CF sputum extract, PBS as a blank, or 1–8 μM GSH in PBS (positive control) were measured by Beckman DU spectrophotometer (Beckman Coulter, Krefeld, Germany). Afterward, DTNB in 100 mM potassium phosphate buffer (pH 7.4) was added to samples (0.25 mM final concentration), the reaction mixture was inverted for mixing and incubated for 20 s to allow for the reaction to proceed, and OD₄₁₂ nm was registered again. At this step, all reduced thiols are evaluated. Then, a mixture of GSH and NADPH was added to the cuvette (final concentrations 1 U/ml and 0.25 mM, respectively). In the presence of GR and NADPH, GSH is recycled from GSSG and steadily reduces DTNB to TNB (reactions 2 and 3). The increase in OD₄₁₂ nm over time (i.e., ΔOD₄₁₂ nm) is directly proportional to the GSH content in the sample.

Inactivation of catalase and heme peroxidases by NaN₃ and exposure of 16HBE140- cells to H₂O₂. NaN₃ is a broad inhibitor of catalase and heme peroxidases. To inactivate these enzymes, CF sputum extracts were incubated with 1 mM NaN₃ (1 h, 4°C). In preliminary experiments, NaN₃ completely inhibited 1,000 mU/ml of catalase, the activity comparable to the one reported by Worlitzsch et al. (24).

16HBE140- cells were incubated with H₂O₂ in different tested media ± NaN₃. Exposed cells were analyzed for cell viability (PI staining).

**Data analysis.** Each experiment was repeated with at least four different CF sputum samples. Where appropriate, results are expressed as means ± SE. Data were compared by Student’s t-test for paired comparisons using the SPSS statistical package.

**RESULTS**

**H₂O₂ cytotoxicity.** The viability of 16HBE140- cells gradually decreased with increasing concentrations of H₂O₂ (0–500 μM). However, only at 500 μM was the desired cytotoxicity (i.e., 50% viable cells vs. 0 μM H₂O₂) reached. The concentration of 600 μM in both serum-free MEM and MEM (10% FBS) was cytotoxic to cells to nearly 100%. The subsequent experiments were performed with 0 or 600 μM H₂O₂.

**Intracellular oxidant levels.** It was assumed that exposure of cells to H₂O₂ will result in a significant accumulation of intracellular oxidants unless H₂O₂ is detoxified before reaching the cell layer. When the cells were exposed to 600 μM H₂O₂ in either serum-free MEM or MEM (10% FBS), there was a marked increase in fluorescence intensity due to oxidation of DHR123 by intracellular oxidants (Fig. 1, A and B). In contrast, this was not the case in cells overlaid with CF sputum and...
H$_2$O$_2$. DHR123 oxidation was completely abolished, and cells exposed to the oxidant exhibited the same fluorescent intensity as control cells (Fig. 1C).

Cell microscopy. Whereas cells exposed for 2 h to 600 μM H$_2$O$_2$ were still morphologically indistinguishable from control cells (data not shown), cells incubated for 12 h with 600 μM H$_2$O$_2$ in either serum-free MEM or MEM (10% FBS) exhibited visible signs of cell damage (Fig. 2, A and B). Cells shrank, the confluence of cell monolayer was lost, and many cells started detaching (Fig. 2, A and B). CF sputum extracts were not toxic for cells (Fig. 2C). Furthermore, the cell monolayers exposed to 600 μM H$_2$O$_2$ in CF sputum extracts remained morphologically intact (Fig. 2C), suggesting a complete detoxification of H$_2$O$_2$.

Cell viability. On the basis of the MTT test results, we assumed that a 24-h exposure to 600 μM H$_2$O$_2$ would result in 100% cell death and that the H$_2$O$_2$ cytotoxicity would be detected by PI staining. As expected, cells exposed to H$_2$O$_2$ in both serum-free MEM and MEM (10% FBS) exhibited nearly 100% cell death (Fig. 3, A and B).

Similar to the cell microscopy experiments, a prolonged exposure of 16HBE14o- cells to CF sputum extracts did not reveal any cytotoxicity of CF sputum (Fig. 3C). Furthermore, in a marked contrast to both control culture media, the viability of cells exposed to 600 μM H$_2$O$_2$ in CF sputum extracts did not change compared with control cells (Fig. 3C). In addition, it was found that CF sputum extracts effectively protected 16HBE14o- cells from even higher (up to 1 mM) H$_2$O$_2$ concentrations (data not shown).

Reduced thiols and GSH in CF sputum extracts. CF sputum extracts (10% in PBS) were probed for the presence of GSH and other reduced thiols. After DTNB was added, CF sputum extracts exhibited strong absorbance at 412 nm, indicating a high content of reduced thiols (Fig. 4). In the case of CF sputum extracts, the increase in OD$_{412}$ nm after incubation with DTNB in CF far exceeded the changes in OD$_{412}$ nm in positive controls (1 or 8 μM GSH) (Fig. 4).

After GR and NADPH were added to the cuvette, OD$_{412}$ nm increased over time in a fashion similar to that of positive controls (Fig. 4). This increase in OD$_{412}$ nm over time is specific for GSH. Therefore, the experimental findings suggest that GSH is present in CF sputum extracts and that there are other reduced thiols besides GSH in CF sputum.

Both low- (e.g., GSH, cysteine, etc.) and high-molecular-weight (i.e., SH-protein) reduced thiols can increase OD$_{412}$ nm in a reaction with DTNB. The contribution of protein thiols to the reduction of DTNB was estimated in the following experiment. Sputum proteins were acid precipitated and removed by high-speed centrifugation. After the pH of the supernatant, now containing only acid-soluble, low-molecular-weight thiols, was restored to 7.4 and DTNB was added, the increase in OD$_{412}$ nm was profoundly lower than in the presence of proteins (data not shown). This indicates that a significant portion of reduced thiols in CF sputum can be attributed to sputum proteins.

Fig. 1. Intracellular oxidant accumulation in 16HBE14o- cells exposed to exogenous H$_2$O$_2$ and tested culture media [flow cytometry, dihydorhodamine 123 (DHR123) as a probe]. The experiment is representative of 4 independent experiments. Fluorescence intensity of 10,000 cells was analyzed. In contrast to cells exposed to H$_2$O$_2$ in both control media, serum-free MEM, or MEM (10% FBS), intracellular oxidants are undetectable in cells incubated with MEM (10% CF sputum) + 600 μM H$_2$O$_2$. 

L905 CF SPUTUM NEUTRALIZES H$_2$O$_2$ CYTOTOXICITY

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Inactivation of catalase and heme peroxidases by NaN₃ and exposure of 16HBE14o- cells to H₂O₂. CF sputum extracts retained their H₂O₂-scavenging activity after catalase and heme peroxidases were inactivated (Table 1). 16HBE14o- cells exposed to CF sputum extracts and H₂O₂ demonstrated viability similar to control cells (Table 1), again, in a marked contrast to both control culture media (Table 1).

Fig. 2. Phase-contrast microscopy of 16HBE14o- cells exposed for 12 h to H₂O₂ and tested culture media. The cells incubated with MEM (10% CF sputum) and 600 μM H₂O₂ do not exhibit any signs of cell damage seen in both control media (0 or 10% FBS) + 600 μM H₂O₂. The photogaphic pictures are representative of another 4 independent observations.
DISCUSSION

The present study assessed the antioxidant properties of CF upper airway secretions (obtained via sputum induction) toward H$_2$O$_2$ cytotoxicity. It was found that sputum extracts from adult CF patients efficiently neutralize H$_2$O$_2$. Specifically, CF sputum abolished intracellular oxidant accumulation and protected cells from the H$_2$O$_2$-driven cytotoxicity.

Previously, high levels of antioxidants, such as GSH, glutathione peroxidase, and catalase, were found in CF sputum samples (6, 24). In addition, there are published data (21, 24) with respect to the in vitro oxidant-scavenging properties of CF sputum extracts. In the present study, we were able to confirm and extend the previous findings. The CF sputum extracts were obtained by a specific protocol to minimize cell damage and to maximally preserve antioxidants (5, 6). Techniques such as ultrafiltration and ultracentrifugation were not applied as they were found to damage sputum cells (22). The damage of sputum cells would result in a leakage of intracellular antioxidants and affect the evaluation of H$_2$O$_2$-neutralizing capacity of CF airway secretions. Furthermore, dithiothreitol (DTT), a synthetic thiol donor commonly used to liquefy sputum, for various reasons was not used during sputum processing in the

Fig. 3. Viability assessment by propidium iodide (PI) staining (flow cytometry) in 16HBE14o- cells exposed to H$_2$O$_2$ and tested culture media. Fluorescence intensity of 10,000 cells was analyzed. The cells incubated with 600 μM H$_2$O$_2$ in MEM (10% CF sputum) exhibit similar viability pattern (M1: viable cells vs. M2: dead cells) as cells exposed to MEM (10% CF sputum + 0 μM H$_2$O$_2$). The experiment is representative of 4 independent experiments.
oxidants and not a simple reflection of protein abundance. Therefore, it can be postulated that the antioxidant efficiency of min is, in fact, lower than that of reduced glutathione (4, 7).

3 other independent experiments. By the recycling assay. Similar absorbances and kinetics were obtained in the extract. Furthermore, GSH is present in the CF sputum extract as demonstrated by the recycling assay. Similar absorbances and kinetics were obtained in the other independent experiments.

The pronounced increase in $OD_{232 \text{ nm}}$ in 10% CF sputum extract after DTNB has been added is indicative of a high content of reduced thiols in the CF sputum extract. Furthermore, GSH is present in the CF sputum extract as demonstrated by the recycling assay. Similar absorbances and kinetics were obtained in the other independent experiments.

The present study. First, DTT was found to affect glutathione recycling assay (5). Next, DTT is a dithiol able to reduce DTNB. Therefore, sputum’s content of reduced thiols would be hugely overestimated by DTNB assay in the presence of molar excess of DTT (0.1% DTT, a commonly used concentration for sputum homogenization, corresponds to 6.5 mM DTT).

Furthermore, the urge to study CF upper airway secretions as close as possible to their natural environment also forced the authors not to include patients receiving N-acetylcysteine, which is a thiol, antioxidant, and a glutathione prodrug. The obtained data indicate, however, that CF upper airway secretions, even in the absence of a thiol donor, demonstrate a substantially reduced thiol content and possess a significant $H_2O_2$-detoxifying capacity. $H_2O_2$ concentrations as high as 1 mM were efficiently neutralized by CF sputum extract.

In a marked contrast, both control media (serum-free MEM and MEM containing 10% FBS) exhibited only a negligible antioxidant capacity in the present study. Interestingly, the FBS-containing culture medium was as inefficient in $H_2O_2$ detoxification as the serum-free MEM. Indeed, FBS is rich in albumin, and albumin contains one SH group that theoretically can interact with $H_2O_2$. However, there are reports in the literature demonstrating that the antioxidant capacity of albumin is, in fact, lower than that of reduced glutathione (4, 7). Therefore, it can be postulated that the antioxidant efficiency of CF airway secretions is due to the presence of specific antioxidants and not a simple reflection of protein abundance.

In normal human airway secretions, $H_2O_2$ can be consumed in several ways. First, there is an antioxidant enzymatic system (glutathione peroxidase and catalase) that neutralizes $H_2O_2$ to water. The second enzymatic system, based on heme peroxidases [myeloperoxidase (MPO), lactoperoxidase (LPO), and eosinophil peroxidase], uses $H_2O_2$ as a substrate to produce highly active antimicrobial compounds (hypochloride, thiocyanate, and hydrobromide, respectively). Furthermore, there are also nonenzymatic $H_2O_2$ scavengers, such as reduced glutathione and other reduced thiols, etc. All these substances have been identified in CF upper airways secretions. The presence of abundant H2O2-scavenging sputum factors is likely to be the reason why CF breath condensate does not exhibit elevated $H_2O_2$ levels.

In the present study, we did not attempt to compare the antioxidative potential of CF airway secretions with that of healthy individuals. Because healthy individuals do not produce induced sputum in sufficient quantities, it would have been difficult to perform experiments such as outlined above. Moreover, it is the authors’ experience that samples of healthy individuals exhibit a gel-like consistency that is in marked contrast to mucopurulent CF samples. Due to the mucopurulent character of CF sputum, it is easier to obtain sputum supernatant by dilution with PBS and centrifugation. In contrast, a complete liquefaction of a healthy sputum requires use of DTT, which was avoided in the present study due to the aforementioned reasons.

It is, however, interesting to compare the data obtained in the present study with the data from literature. El Chemaly et al. (9) analyzed tracheal secretions from healthy individuals and estimated that $H_2O_2$ detoxification predominantly occurs via the enzymatic route (i.e., via catalase, glutathione peroxidase, and heme peroxidases). According to their estimations, in upper airway secretions these enzymes neutralize up to 80% of exogenous $H_2O_2$. The nonenzymatic mechanisms account for the remaining 20%. Interestingly, the present and previous (6, 24) studies document both enzymatic (glutathione peroxidase and catalase) and nonenzymatic $H_2O_2$ scavengers (reduced glutathione and protein thiols) in CF sputum. The data from the present study suggest, however, that the reduced thiol and/or glutathione peroxidase system is fully capable to detoxify $H_2O_2$, as the $H_2O_2$-scavenging potential was sustained after both catalase and heme peroxidases were inactivated by NaN3. It is not clear yet what is the relative contribution of reduced thiol/glutathione peroxidase/catalase scavengers vs. MPO/LPO to $H_2O_2$ consumption in CF airways. It is interesting, though, that in the present study CF sputum extracts, with or without added $H_2O_2$, were not toxic for cells. In the authors’ opinion, the latter observation may be suggestive of $H_2O_2$’s being detoxified to water in the presence of sputum factors, i.e., via reduced thiol and/or glutathione peroxidase/catalase system, rather than to hypochlorous acid or thiocyanate. Therefore, it can be speculated that the abundant and efficient antioxidant system in CF upper airway secretions may be helpful to combat oxidant stress, but this may happen at the expense of producing antimicrobial substances deriving from $H_2O_2$.

Data from the present study provide no exact answer as to which high-molecular reduced thiols may be involved in $H_2O_2$ detoxification.

**Table 1. Percentage of dead cells in 16HBE14o- cells exposed to $H_2O_2$ and tested culture media**

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<td>600 mM $H_2O_2$, then 600 mM $H_2O_2$</td>
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Data are shown as means ± SE (4 independent experiments). *Catalase and heme peroxidases were inactivated by preincubation (1 h, 4°C) with 1 mM NaN3. †P < 0.001 vs. 0 mM $H_2O_2$. 

**Fig. 4. Detection of reduced thiols by DTNB and GSH** (by DTNB recycling assay) in a blank (PBS), in positive controls (1 and 8 μM GSH), and in 10% CF sputum extract. The data from the present study suggest, however, that the reduced thiol and/or glutathione peroxidase system is fully capable to detoxify $H_2O_2$, as the $H_2O_2$-scavenging potential was sustained after both catalase and heme peroxidases were inactivated by NaN3. It is not clear yet what is the relative contribution of reduced thiol/glutathione peroxidase/catalase scavengers vs. MPO/LPO to $H_2O_2$ consumption in CF airways. It is interesting, though, that in the present study CF sputum extracts, with or without added $H_2O_2$, were not toxic for cells. In the authors’ opinion, the latter observation may be suggestive of $H_2O_2$’s being detoxified to water in the presence of sputum factors, i.e., via reduced thiol and/or glutathione peroxidase/catalase system, rather than to hypochlorous acid or thiocyanate. Therefore, it can be speculated that the abundant and efficient antioxidant system in CF upper airway secretions may be helpful to combat oxidant stress, but this may happen at the expense of producing antimicrobial substances deriving from $H_2O_2$.

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</table>

Data are shown as means ± SE (4 independent experiments). *Catalase and heme peroxidases were inactivated by preincubation (1 h, 4°C) with 1 mM NaN3. †P < 0.001 vs. 0 mM $H_2O_2$. 

**Fig. 4. Detection of reduced thiols by DTNB and GSH** (by DTNB recycling assay) in a blank (PBS), in positive controls (1 and 8 μM GSH), and in 10% CF sputum extract. The data from the present study suggest, however, that the reduced thiol and/or glutathione peroxidase system is fully capable to detoxify $H_2O_2$, as the $H_2O_2$-scavenging potential was sustained after both catalase and heme peroxidases were inactivated by NaN3. It is not clear yet what is the relative contribution of reduced thiol/glutathione peroxidase/catalase scavengers vs. MPO/LPO to $H_2O_2$ consumption in CF airways. It is interesting, though, that in the present study CF sputum extracts, with or without added $H_2O_2$, were not toxic for cells. In the authors’ opinion, the latter observation may be suggestive of $H_2O_2$’s being detoxified to water in the presence of sputum factors, i.e., via reduced thiol and/or glutathione peroxidase/catalase system, rather than to hypochlorous acid or thiocyanate. Therefore, it can be speculated that the abundant and efficient antioxidant system in CF upper airway secretions may be helpful to combat oxidant stress, but this may happen at the expense of producing antimicrobial substances deriving from $H_2O_2$.

Data from the present study provide no exact answer as to which high-molecular reduced thiols may be involved in $H_2O_2$ detoxification.
detoxification. However, it can be speculated that sputum mucins may, at least partially, be responsible for this detoxification, since they are very abundant in sputum.

If the antioxidant system is so efficient in CF upper airway secretions, is oxidative stress a relevant feature of the CF lung disease? Recent studies appear to confirm this suggestion. There is abundant evidence of airway oxidative stress in CF (for review, see e.g., Ref. 3). It can be speculated that the mucus layer possesses sufficient antioxidant properties in CF whereas the sol layer may not. Due to the impaired absorption of fat soluble antioxidants, such as vitamin E, and affected secretion of GSH by the CF respiratory epithelium, the complex antioxidant network in the sol phase may be severely disturbed in CF. Therefore, H₂O₂ released in the vicinity of epithelial surface, i.e., in the sol phase of the airway surface liquid, may still reach the cells. In addition, proinflammatory cytokines, such as TNF-α, and bacterial products, such as pyocyanin and pyochelin, are known to trigger intracellular oxidative stress by disturbing mitochondrial respiration. These factors, in contrast to H₂O₂, are unlikely to be scavenged by extracellular CF mucus and antioxidants present in it and may, therefore, elicit oxidative insults in CF airways.

In conclusion, in the present study the in vitro cytotoxicity of up to 1 mM H₂O₂ was effectively prevented by CF sputum extracts. The reduced thiol and/or glutathione peroxidase system appears to be very efficient in H₂O₂ neutralization, even when catalase and heme peroxidases are inactivated. Further studies are needed to understand the complex nature of oxidative stress and inflammation in CF to better design therapeutic interventions.

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