Hypochlorous acid alters bronchial epithelial cell membrane properties and prevention by extracellular glutathione

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Hypochlorous acid alters bronchial epithelial cell membrane properties and prevention by extracellular glutathione. J Appl Physiol 95: 2444–2452, 2003. First published September 26, 2003; 10.1152/japplphysiol.00002.2003.—In chronic inflammatory diseases of the airways, such as cystic fibrosis, hypochlorous acid (HOCl) generated by neutrophils is involved in airway injury. We examined the effects of HOCl on 16HBE14o— bronchial epithelial cells by bolus addition or by generation with glucose oxidase plus myeloperoxidase. HOCl produced both carbonyl formation of a discreet number of proteins and modification of surface targets that were recognized by an antibody raised against HOCl-modified protein. Bolus or enzymatically generated HOCl decreased transepithelial resistance, but surprisingly bolus HOCl also increased short-circuit current. Glutathione in lung epithelial lining fluid is an excellent scavenger of HOCl; however, glutathione content is lower in cystic fibrosis epithelial lining fluid due to deficient glutathione transport to the apical side of bronchial-tracheal epithelial cells (Gao L, Kim KJ, Yankaskas JR, and Forman HJ. Am J Physiol Lung Cell Mol Physiol 277: L113–L118, 1999). We found that alteration of the GSH content of apical fluid above 16HBE14o— cells was protective because all HOCl-induced changes were delayed or eliminated by exogenous glutathione within the physiological range. Extrapolating this to cystic fibrosis suggests that HOCl can alter cell function without destruction but that elevating glutathione could be protective.

16HBE14o— cells; epithelial lining fluid

INVASION OF NEUTROPHILS INTO TISSUES with concomitant stimulation of these cells are common events that produce inflammation. When neutrophils are stimulated, several rapid processes occur. An NADPH oxidase is assembled in the plasma membrane that produces superoxide on the outside of the cell. Superoxide is then rapidly converted to H₂O₂ and O₂ by nonenzymatic dismutation. The neutrophil simultaneously releases myeloperoxidase (MPO) and very rapidly consumes H₂O₂ to form hypochlorous acid (HOCl), such that H₂O₂ is expected to have little biological effect (41). HOCl, with an acidic dissociation constant of 7.4, is found in two forms in physiological solution (HOCl and OCl⁻), the active components of laundry bleach. HOCl is far more potent than OCl⁻ in terms of bactericidal activity (37). HOCl has been shown to 1) chlorinate amines, unsaturated lipids, and protein-bound tyrosine (14, 50, 51); 2) oxidize (lipo)proteins (19, 27, 28); and 3) cause oxidative bleaching of heme and iron-sulfur proteins (1). The amine adducts chloramines are also reactive but, as longer-lived intermediates, can travel and cause damage at a distance from their site of generation (14). Gross oxidative insults or selective modification of critical molecules (DNA, proteins, and lipids) can lead to cellular necrosis.

Nonetheless, HOCl and chloramines can also profoundly influence the function of living cells without directly causing cell death. Indeed, this can include influencing or even stimulating signal transduction. Specific reported effects include 1) disruption of tight junctions (52), 2) increased transepithelial Cl⁻ secretion (32), 3) basolateral K⁺ channel activation (12), and 4) activation of mitogen-activated signal transduction pathways (30). This strongly suggests that HOCl and chloramines, which share some of the chemical attributes of H₂O₂, likely mimic the now well-established ability of H₂O₂ to act as a second messenger (15).

The epithelial lining fluid (ELF) bathing the epithelial surface of the lung normally contains a high concentration of reduced glutathione (GSH; 200–400 μM) compared with most other extracellular compartments (6, 40). Several reactions of GSH with HOCl lead to products that are either innocuous or far less reactive than HOCl. Reaction of GSH occurs with HOCl through both radical and nonradical pathways, resulting in the formation of GSH disulfide (GSGG), GSH thiosulfonate, and GSH sulfonamide (which is presumably formed from GSH sulfonate by an internal condensation) (9, 33, 55). Surprisingly, GSSG, which is not a potent scavenger of HOCl, does protect some enzymes against inactivation by HOCl, although only those with critical sites that react more slowly with...
HOCI than does GSSG (10). Because GSH is the major substance present in the extracellular fluid that can eliminate HOCI, a constitutive decrease in GSH ELF content would be predisposed to inflammation, whereas a decrease in GSH during inflammation could exacerbate injury.

Cystic fibrosis (CF) is a common autosomal recessive disease affecting ~1 in 2,000 Caucasians (29). The genetic basis of CF has been known for over 10 yr (34). The defective gene codes for an epithelial anion channel named CF transmembrane conductance regulator (CFTR) (34). Intestinal, pancreatic, sweat duct, vas deferens, and lung function are all compromised in CF. However, pulmonary disease accounts for most (~90%) of the mortality (29). Specifically, the airways of CF patients undergo chronic recurrent bacterial infection, and many are eventually colonized with Pseudomonas aeruginosa. These bacterial incursions evoke a neutrophil-mediated inflammatory response (23). MPO and HOCI are found in significant quantities in the sputum of CF patients showing symptoms (44). Although this HOCI would be expected to consume GSH, total GSH equivalents (GSH + 2 × GSSG) are reduced approximately twofold in bronchoalveolar lavage fluid from CF patients (36), which suggests that the lower GSH is caused by other factors in addition to consumption by HOCI. Supporting this, transgenic CF mice with defective CFTR also have decreased GSH in the absence of lung inflammation (47). During the past few years, several articles have appeared indicating that a defect in CFTR causes a decrease in GSH secretion to the apical surface of CF cells. This appears to be due to both a loss of conductance of GSH through normal CFTR (22, 26) and a decrease in chloride transport-linked GSH secretion independent of CFTR (16, 17). Thus, in CF, damage due to the production of HOCI is likely exacerbated by the defective GSH secretion.

In the present study, we examined the effects of HOCI on 16HBE14o− bronchial epithelial cells, a model for airway epithelium, by bolus addition or by generation with glucose oxidase plus MPO. The aims were to 1) determine what effect acute apical HOCI addition or generation has on the properties of cultured human bronchial epithelial cells and 2) quantify the efficacy of extracellular GSH within the physiological range in protecting human bronchial epithelial cells from HOCI exposure.

MATERIALS AND METHODS

Cell culture. 16HBE14o− cells were kindly provided by Dr. D. C. Gruenert (University of Vermont, Burlington, VT). This line was derived from a primary culture of human bronchial epithelial cells by using an origin-defective simian virus 40 (8). We obtained 16HBE14o− cells at indeterminate passage and selected them for study on the basis of their ability to form resistive monolayers and transport ions similar to second-generation bronchi as described previously (8). Cells were maintained in modified Eagle’s medium ( Gibco- In-Vitrogen, Grand Island, NY) containing Hank’s salts and supplemented with nonessential amino acids plus 5% FBS (Atlanta Biologicals, Atlanta, GA). We replenished the culture media every 1–2 days. Monolayers were obtained by seeding 4–6 × 10^6 cells/cm^2 on commercial porous filter inserts (Transwell, Corning Costar, Cambridge, MA) or Falcon (Becton-Dickinson, Franklin Lakes, NJ). Initially, cells were grown to confluence under submerged culture conditions. After 1 wk, monolayers were then cultured in the presence of an air-liquid interface to increase ion transport and differentiation (53). The electrophysiological properties of 16HBE14o− monolayers were similar regardless of filter source and remained stable during at least 10 cell passages.

Epithelial voltage clamp. These methods are described in detail in our laboratory’s previous reports (5, 16, 18). Briefly, small Transwell filter inserts (area = 0.4 cm^2) containing 16HBE14o− cells were mounted in modified water-jacketed Ussing chambers (Jim’s Instrument Manufacturing, Iowa City, IA). Monolayers were bathed on both sides with a Krebs PBS (K-PBS) solution containing (in mM) 144 NaCl, 8.5 Na2HPO4, 5 KCl, 5 p-glucose, 1.4 NaH2PO4, 1.3 CaCl2, and 1 MgCl2 (pH = 7.4). The volume of both solutions was 6 ml. Unless otherwise specified, all chemicals and reagents were purchased from Sigma Chemical. HEPES was not used as a pH buffer because it is expected to react with HOCI and form HOCl. Identical chambers and basolateral bath solutions were oxygenated and stirred with room air via gas lifts, and temperature was maintained at 37°C throughout an experiment. The potential difference across a monolayer was voltage clamped by using a Physiologic Instruments VCC-600 (San Diego, CA), which provided measures of the transepithelial resistance (Rt) and short-circuit current (Isc). These data were recorded continuously by using a strip chart (BD 112 Kipp & Zonen, Delft, Holland). We tested for effects of continual exposure to HOCI by adding MPO (1 U/ml, Calbiochem) plus glucose oxidase (GO; 10 mM/ml) to the apical bath. This enzymatic system is expected to generate H2O2 (and thereafter HOCI) at an initial rate of 1 μM/min. As MPO becomes inactivated over time, the rate of HOCI production will decline as it would for stimulated neutrophils. In other experiments, we added HOCI directly to apical bath solutions. Paired nontreated monolayers served as controls in both paradigms. The ionic basis for Isc was determined by adding apical amiloride to block Na+ absorption or basolateral bumetanide to block Cl− secretion, as described in previous studies (5, 18). All compounds were as a small volume of concentrated stock solutions in water or phosphate buffer.

GSH efflux assay. The experimental design used to measure apical GSH accumulation was generally similar to previous reports (16, 17). Transwell or Falcon filter inserts (24-mm diameter) containing confluent 16HBE14o− monolayers (300–600 fcm^2) were placed in six-well clusters and bathed on both sides with warmed K-PBS (37°C). The volume of fluid above the cells for these measurements was 0.5 ml. The solutions contained acivicin (2 mg/ml) to prevent GSH hydrolysis by γ-glutamyl transpeptidase. Monolayers were maintained in an incubator at 37°C in the presence of room air during the 3-h experimental period. Previously, our laboratory showed that GSH efflux from CFT1 CF airway cells depended on the apical Cl− conductance and that increasing the basolateral K+ conductance by adding chlorozazone further potentiated GSH secretion (16). Because 16HBE14o− cells express CFTR (8), we added forskolin (10 μM, Calbiochem, San Diego, CA) or forskolin plus chlorozazone (10 μM) to determine the effects on GSH efflux. Un- treated monolayers served as controls. Small volumes (50 μl) of the apical and basolateral solutions were sampled and replaced with identical solutions at defined intervals. At the end of the experiment, the cells were lysed by using ice-cold 0.1% Triton X-100 in 100 mM sodium phosphate buffer (pH =
Computations were performed in Excel, and data were corrected to account for the sample and to replace paradigm. Results were compared by using Student’s t-test or ANOVA depending on the number of comparisons. Differences were considered statistically significant if \( P < 0.05 \).

We measured GSH equivalents (GSH + 2 GSSG) by using an enzymatic-recycling assay (43) adapted for a microtiter plate reader (2). Samples were mixed with an equal volume of 10% (wt/vol) sulfosalicylic acid and centrifuged at 10,000 \( g \) for 10 min at \( 0 \)°C to remove precipitated materials. The resulting supernatants were neutralized with 33% (vol/vol) triethanolamine (100:22.5). Standards containing known amounts of GSH were prepared by using identical solutions and protocols. The assay was performed by pipetting 20 \( \mu l \) of samples, standards, or blanks into wells containing 30 \( \mu l \) of 100 mM sodium phosphate buffer. We then added 50 \( \mu l \) of a solution containing 4 mM diethylenetriamine-pentaacetic acid, 2 U/ml GSH reductase, and 0.8 mM NADPH in 100 mM sodium phosphate buffer (pH = 7.4) to each well. The recycling reaction was initiated by adding 100 \( \mu l \) of 1.2 mM DTNB in sodium phosphate buffer; the plate was immediately at room temperature. The rate of DTNB reduction was monitored at 412 nm.

Staining for HOCl-modified protein. Surface targets modified by HOCl were immunostained by methods similar to those used to localized CFTR in a previous study (5) by using an antibody raised against HOCl-modified protein. Briefly, 16HBE14o- cells were grown on 24-mm transparent filter inserts (Falcon or Costar). Filters containing confluent monolayers (300–600 \( \Omega \) cm\(^2\)) were placed into 6-well plates and washed on both sides with warmed K-PBS (37°C). MPO (20 U/ml) plus GO (200 mU/ml) were then added to the 1 mM apical K-PBS solutions. Because HOCl reacts irreversibly, it is important to keep the ratio of oxidant to cell mass relatively constant. Thus we added 20-fold more MPO and GO compared with Ussing chamber experiments to partly compensate for the 6-fold lower bath volume plus the 12-fold increase in filter area. The treated and control monolayers were incubated in a warmed incubator (37°C) containing room air for the duration of the experimental treatment (8 h). We periodically monitored the \( R_t \) by using chopstick electrodes (World Precision Instruments) to ensure that the outcomes of the \( I_{sc} \) and staining procedures were comparable. At the end of an experiment, monolayers were fixed in ice-cold methanol and postfixed in 3% formaldehyde in Dulbecco’s PBS. We used a primary monoclonal antibody raised against HOCl-modified low-density lipoprotein (28). This antibody (clone 6E10E11) is specific for HOCl-modified lipoprotein, as demonstrated by Western and immunoblotting analyses (28). The monolayers were also stained for CFTR by using a polyclonal antibody that was kindly provided by Dr. David Bedwell (University of Alabama at Birmingham, Birmingham, AL). The specificity of this antibody was previously confirmed by immunoprecipitation (5). Secondary antibodies labeled with Texas red X or Oregon green were purchased from Molecular Probes. Nuclei were counterstained with bisbenzimide H33258. Confocal fluorescence microscopy and image analysis was performed as described previously (5).

Western blot analysis. Protein carbonyls were detected by reaction with dinitrophenyl hydrazine and subsequent Western blot analysis for dinitrophenyl incorporation by using standard methods (25). Briefly, 16HBE14o- monolayers cultured on 24-mm-diameter permeable inserts were treated with apical HOCl (1 mM) in 1 ml of apical K-PBS for 1 h in the presence or absence of GSH (0, 25, 250 \( \mu M \)). Accounting for 70-fold difference in the oxidant-to-cell ratios, 1 mM HOCl corresponds to addition of 14 \( \mu M \) in an Ussing chamber experiment. Cells were then washed and collected by scraping. Cell extracts were obtained by sonication in a lysis buffer containing (in mM) 20 Tris-HCl, 150 NaCl, 1 MgCl\(_2\), 1 CaCl\(_2\), 2.5 EDTA, 2.5 EGTA, and 1 PMSF, plus 10 \( \mu M \) aprotinin. The protein carbonyls in the extracts were derivatized by reaction with dinitrophenyl hydrazine (25). Proteins were then separated by SDS-PAGE. The extract was mixed with 1 volume of 2 \( \times \) DTT loading buffer (0.125 M Tris-HCl, pH 6.5, 20% glycerol, 4% SDS, 0.0025% pyronin Y, and 15.4 mg/ml of ml-DTT) and applied to 10% SDS-PAGE. After electrophoresis, the proteins were transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA). The membranes were blocked with 5% nonfat milk at room temperature for 30 min and incubated with an anti-dinitrophenol IgE (Southern Biotechnology Associates, Birmingham, AL) as the primary antibody at 4°C overnight. After membranes were washed, they were treated with goat antimouse antibody tagged with horseradish peroxidase at room temperature for 1 h and then with an enhanced chemiluminescence (ECL, ECL Plus) reagent mixture for 5 min. Film exposure was carried out at room temperature by using Hyperfilm ECL film (Amersham).

## Results

### Regulation of transepithelial ion transport and GSH accumulation

This initial series of experiments was designed to demonstrate and compare the ion and GSH transport properties of 16HBE14o- monolayers. Previous studies using these cells showed that cAMP-mediated agonists like forskolin enhanced CFTR channel activity and transepithelial conductance without affecting active transport as measured by \( I_{sc} \) (8, 54). In contrast, addition of calcium-mediated agonists transiently stimulated \( I_{sc} \), suggesting an important role for basolateral \( K^+ \) channels in regulating \( Cl^- \) secretion across these cells (24). Nothing has been published regarding possible basal or agonist-regulated GSH secretion from 16HBE14o- monolayers. Thus we tested for effects of forskolin alone or plus the basolateral \( K^+ \) channel activator, chlorzoxazone (18, 38) on \( I_{sc}, R_t, \) and \( I_{sc} \), suggesting an important role for basolateral \( K^+ \) channels in regulating \( Cl^- \) secretion across these cells (24). Nothing has been published regarding possible basal or agonist-regulated GSH secretion from 16HBE14o- monolayers. Thus we tested for effects of forskolin alone or plus the basolateral \( K^+ \) channel activator, chlorzoxazone (18, 38) on \( I_{sc} \), suggesting an important role for basolateral \( K^+ \) channels in regulating \( Cl^- \) secretion across these cells (24). Nothing has been published regarding possible basal or agonist-regulated GSH secretion from 16HBE14o- monolayers. Thus we tested for effects of forskolin alone or plus the basolateral \( K^+ \) channel activator, chlorzoxazone (18, 38) on \( I_{sc} \), suggesting an important role for basolateral \( K^+ \) channels in regulating \( Cl^- \) secretion across these cells (24). Nothing has been published regarding possible basal or agonist-regulated GSH secretion from 16HBE14o- monolayers. Thus we tested for effects of forskolin alone or plus the basolateral \( K^+ \) channel activator, chlorzoxazone (18, 38) on \( I_{sc} \), suggesting an important role for basolateral \( K^+ \) channels in regulating \( Cl^- \) secretion across these cells (24). Nothing has been published regarding possible basal or agonist-regulated GSH secretion from 16HBE14o- monolayers. Thus we tested for effects of forskolin alone or plus the basolateral \( K^+ \) channel activator, chlorzoxazone (18, 38) on \( I_{sc} \), suggesting an important role for basolateral \( K^+ \) channels in regulating \( Cl^- \) secretion across these cells (24). Nothing has been published regarding possible basal or agonist-regulated GSH secretion from 16HBE14o- monolayers. Thus we tested for effects of forskolin alone or plus the basolateral \( K^+ \) channel activator, chlorzoxazone (18, 38) on \( I_{sc} \), suggesting an important role for basolateral \( K^+ \) channels in regulating \( Cl^- \) secretion across these cells (24). Nothing has been published regarding possible basal or agonist-regulated GSH secretion from 16HBE14o- monolayers. Thus we tested for effects of forskolin alone or plus the basolateral \( K^+ \) channel activator, chlorzoxazone (18, 38) on \( I_{sc} \), suggesting an important role for basolateral \( K^+ \) channels in regulating \( Cl^- \) secretion across these cells (24).
not entirely unexpected, because chlorzoxazone has previously been shown to activate both apical CFTR and basolateral K\textsuperscript{+} channels in non-CF epithelial cells (38). Thus the precise effect of chlorzoxazone on membrane voltage is difficult to predict in non-CF cells and requires additional study. More importantly, these data demonstrate that increased CFTR channel activity in human bronchial epithelial cells by forskolin addition enhanced GSH accumulation in the apical media. These data are in agreement with our laboratory’s previous studies showing that either CFTR expression or increased anion permeability using a Cl\textsuperscript{–} channel-forming peptide enhanced net GSH transport from CF airway (CFT1) cells (16, 17) and provides new evidence that alterations in intracellular cAMP can regulate GSH secretion acutely from normal airway epithelial cells.

Apical HOCl generation markedly decreased \( R_t \) and GSH was protective. HOCl is highly reactive and unstable at pH 7.4. Therefore, we used an enzyme system consisting of MPO (1 U/ml) plus GO (10 mU/ml) to determine the effects of a more prolonged exposure to low levels of HOCl. MPO catalyzes the formation of HOCl by using the GO-generated \( \text{H}_2\text{O}_2 \) plus chloride. The rate of hydrogen peroxide production by 10 mU/ml GO is expected to be rate limiting for HOCl production and is \( \sim 1 \mu\text{M/min} \) (45), although this will decline with time due to inactivation of the MPO. We observed that MPO plus GO caused a marked decline in \( R_t \) over 3 h, as summarized in Fig. 2. Figure 2A shows the \( R_t \) before treatment (\( n = 3 \)). Figure 2B depicts the \( R_t \) 3 h after treatment. The resistances of MPO, GO, and untreated monolayers all declined less than twofold during the

![Figure 1](image1.png)  
**Fig. 1.** Effects of forskolin and chlorzoxazone on short-circuit current \( (I_{sc}) \), transepithelial resistance \( (R_t) \), and glutathione (GSH) efflux from 16HBE14o– monolayers. **A:** representative tracings of \( I_{sc} \) (top) and \( R_t \) (bottom) measurements after addition of forskolin (10 \( \mu\text{M}, \) both sides) and chlorzoxazone (500 \( \mu\text{M}, \) both sides) to the monolayers. The K\textsuperscript{+} channel blocker clotrimazole (100 \( \mu\text{M}, \) both sides) was added to block \( I_{sc} \). Forskolin, chlorzoxazone, and clotrimazole were present continuously after addition. These records are representative of \( \geq 20 \) experiments. **B:** comparison of the time-dependent apical GSH accumulation from untreated monolayers with forskolin (10 \( \mu\text{M} \) or forskolin plus chlorzoxazone (500 \( \mu\text{M} \)). These experiments were performed on monolayers in 6-well plates under open-circuit conditions. Data report the GSH concentration measured in 0.5 ml of Krebs PBS (K-PBS) overlaying the 4.2-cm\textsuperscript{2} monolayer and are means \( \pm \) SE for \( n = 3 \). *Significantly different from untreated control (\( P < 0.05 \)).

![Figure 2](image2.png)  
**Fig. 2.** HOCl generation decreases \( R_t \), and GSH pretreatment slows this effect. We added glucose oxidase (GO; 10 mU/ml) and/or myeloperoxidase (MPO; 1 U/ml) to the apical bathing solutions of 16HBE14o– monolayers mounted in Ussing chambers. \( I_{sc} \) and \( R_t \) were monitored continuously in Ussing chambers before treatment and for 3 h thereafter. There was no remarkable change in \( I_{sc} \) during the experimental period (not shown). **A:** summary of resistance measurements before treatment. **B:** summary of the resistance measurements 3 h after treatment. Bars show means \( \pm \) SE; \( n = 3 \). *Significantly different from controls (\( P < 0.05 \)). *Significantly different from MPO + GO treatment without GSH (\( P < 0.05 \)).

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experimental period. Interestingly, H$_2$O$_2$ generation alone (i.e., GO) had no effect. Addition of MPO plus GO caused the $R_t$ to decrease to a very low value (28 Ωcm$^2$), which is similar to the resistance of the filter support (10 ± 5 Ωcm$^2$). GSH (25 μM) slowed the effect of MPO + GO, and $R_t$ was 165 Ωcm$^2$ after 3 h. Figure 3 shows that GO + MPO produced significant surface protein modification in 16HBE14o– cells, as demonstrated by immunostaining with an antibody raised against HOCl-modified protein. This result is an expected effect of HOCl, and these data serve to validate the MPO + GO enzymatic system.

Bolus HOCl addition decreased $R_t$ and caused a rapid increase in $I_{sc}$. Figure 4, shows representative records comparing the $I_{sc}$ and $R_t$ responses to addition of 10 or 50 μM HOCl. The mixed Na-H form of HOCl was added as the acidic dissociation constant of HOCl is in the neutral range (~7.0). Sodium hypochlorite is stable, but when added to a buffer at pH 7.4, the HOCl formed will begin to rapidly decompose (37). Addition of 50 μM HOCl decreased $R_t$, which is consistent with the data shown previously by using MPO + GO (Fig. 2). Somewhat unexpectedly, however, 50 μM HOCl also evoked a rapid increase in $I_{sc}$. The stimulated current was abolished by the K$^+$ channel blocker clemastimazole (100 μM) as shown. Basolateral bumetanide (100 μM) blocked a portion of the HOCl-stimulated current, whereas apical amiloride (100 μM) was without effect (data not shown). Thus the $I_{sc}$ response was due at least in part to Cl$^-$ secretion. The ability of 50 μM but not 10 μM HOCl to enhance $I_{sc}$ and decrease $R_t$ is suggestive of steep dose-response relations. This was indeed the case, as shown in the bottom panels. These data were poorly described by either Michaelis-Menten functions or Hill plots, and the curves were drawn from spline fits (SigmaPlot 5.0).

The fact that HOCl acts irreversibly also raises an important caveat regarding the interpretation of these data. Specifically, the response is predicted to depend on the HOCl mass (i.e., concentration × bath volume) and the cell mass (i.e., monolayer area). In this regard, preliminary experiments ($n = 4$) showed that 50 μM HOCl was completely ineffective when added to monolayers grown on slightly larger format filters (0.6 cm$^2$), which is the expected result.

GSH protected monolayers from apical HOCl addition. Figure 5A presents representative current records, which show that 50 μM GSH was effective in preventing the $I_{sc}$ response to a bolus HOCl initially at 100 μM. One monolayer was treated with 50 μM apical GSH. The second paired monolayer was not treated and served as a control. Addition of 100 μM HOCl to the apical bath of both monolayers evoked a $I_{sc}$ response from only the untreated control. These data are representative of four paired experiments. Figure 5B is a Western blot showing that protein carbonyls were formed after addition of 1 mM HOCl and that that carbonyl formation was also decreased by addition of GSH (25 or 250 μM). There were a number of proteins that were susceptible to carbonyl formation, which is consistent with known effects of HOCl (45). It is also important to note that 250 μM GSH is within the physiological concentration range in the ELF (6, 40).

DISCUSSION

Implications of HOCl exposure on airway function. Previously, it was reported that exposure to H$_2$O$_2$ or HOCl at relatively high concentrations (i.e., 1–5 mM) markedly increased the paracellular permeability across epithelial monolayers (52). The data presented in Figs. 2 and 4 show that HOCl markedly decreased $R_t$.
across cultured airway epithelial cells at lower concentrations (50–100 μM). Hence, HOCl is not only more relevant than H₂O₂ in CF airway disease (45) but it also appears to be more potent. Furthermore, it is likely that the reduction in Rᵣ is also mediated by increased paracellular permeability as HOCl-treated 16HBE14o− cells showed no gross morphological abnormalities (Fig. 3) and remained capable of vectoral transport (Fig. 4). Importantly, compromising the barrier function of airway epithelial cells (Rᵣ) has important pathological consequences, regardless of the underlying mechanism, because fluid is expected to flow from the pulmonary interstitium into the lung.

Previous studies also demonstrated that H₂O₂ increased anion secretion across cultured intestinal and airway epithelial cells at slightly lower concentrations (i.e., 0.5–1 mM) (7, 12). This effect was mediated partly by increases in the basolateral K⁺ conductance and in CFTR-mediated apical membrane anion permeability (7). Data presented herein also implicate activation of the basolateral K⁺ conductance (Fig. 4). Specifically, addition of HOCl increased Iₛ across 16HBE14o− monolayers (Fig. 4). This Iₛ response was similar to the effect of the K⁺ channel opener chlorzoxazone (18, 38) and was unlike the response to the cAMP-mediated agonist forskolin (see Fig. 1). The basolateral K⁺ conductance regulates mass and charge balance and plays a key role in vectorial transport (48). It is also possible that HOCl increased apical CFTR channel activity, but testing this possibility is beyond the scope of the present study.

It is unlikely that HOCl affects the basolateral K⁺ channels by direct modification because it has been shown to dissipate entirely at or near the apical cell surface due to its highly reactive nature (45). Thus there are two possible explanations for the chloroxzone-like effects of HOCl on Iₛ. First, HOCl may react with amines to form reactive chloramines that are longer lived and are able to act at a distance from their site of generation. There is precedence for this explanation because HOCl-generated chloramines have recently been shown to increase Cl⁻ secretion across intestinal epithelial cells via an enhanced basolateral K⁺ conductance (39). A second possibility is that either HOCl and/or chloramines, which share some of the chemical attributes of H₂O₂, alter intracellular signal transduction pathways (15). There is also precedence for this explanation since previous studies showed that oxidant-mediated increases in paracellular permeability in other cell lines could be blocked by addition of cytokines or protein kinase C inhibitors (3, 4, 49). Low concentrations of HOCl (10–20 μM) have also been shown to activate mitogen-activated signal transduction pathways (30). In summary, it is likely that the effects of HOCl are mediated at least in part by a secondary mechanism that is related to chloramine
HOCl (top) and reduced protein carbonyl formation. 

Previously, we showed that GSH secretion into the airway ELF depends on the apical membrane Cl⁻ permeability as other epithelial cells and will provide a convenient model to address these possibilities in future studies.

**GSH secretion from airway epithelial monolayers.**

First, we demonstrated the following: GSH secretion into the ELF of 16HBE14o– cells appears to respond similarly to oxidants as other epithelial cells and will provide a convenient model to address these possibilities in future studies.

**Fig. 5.** Low concentrations of GSH abolished the I_{sc} response to HOCl (top) and reduced protein carbonyl formation (bottom). Paired monolayers of 16HBE14o– cells were mounted in Ussing chambers. GSH (50 μM) was added to the apical bath of 1 monolayer (dotted line) and not the other (solid line). HOCl (100 μM) was added to both apical bath solutions. A: representative I_{sc} and R_{t} responses. GSH pretreatment blocked these effects. B: Western blot using an antibody against dinitrophenol (DNP) that shows significant carbonyl formation by 1 mM HOCl that was reduced by pretreatment with 25 or 250 μM of GSH.

that normal human bronchial epithelial cells are also capable of GSH secretion in vitro and that the cAMP-mediated agonist forskolin enhanced apical GSH accumulation. Because forskolin was previously shown to increase apical CFTR channel activity in 16HBE14o– cells, (54) the ability of forskolin to enhance GSH secretion acutely extends our previous observations that apical anion permeability indirectly regulates GSH secretion.

Furthermore, the GSH accumulated in the apical bathing solutions over 3 h compares favorably with the 100–400 μM of GSH measured in the ELF (6, 40). The 0.5-cm³ volume of fluid used to bathe the 4.2-cm² cell monolayer (i.e., 1.2-mm fluid depth) is ~60- to 80-fold larger than the 15- to 21-μm layer of fluid observed in cultured primary airway epithelial cells (21). Correcting for the increased volume in our experiments, the GSH concentrations after 3 h are calculated to be 51–72 μM under basal conditions, 84–118 μM in the presence of forskolin, and 97–136 μM with forskolin plus chlorzoxazone. These secretion rates indicate that the GSH in the airway ELF is capable of turning over every 3–12 h. The rates will be lower if GSH transport is substrate limited, but they may also be higher because GSH synthesis was limited by the absence of cysteine, glutamate, and glycine in the K-PBS solutions.

**Exogenous GSH is efficient in blocking the effects of HOCl on airway cells.**

Consistent with the role of GSH as an antioxidant, we demonstrate that addition of GSH to apical fluid protects against HOCl-induced changes in R_t, I_{sc}, and protein carbonyl formation. The reactions of GSH and HOCl are well described (see above). GSH is a relatively small molecule and reacts efficiently with HOCl to form innocuous products. Importantly, this study demonstrates that relatively small decreases in extracellular GSH concentration can markedly reduce the protection against HOCl generation as demonstrated by the extremely steep dose response relations shown in Fig. 4. We have also demonstrated that the apical fluid present in air-liquid cultures alone was able to block the effect of bolus HOCl addition (data not shown). Although there is evidence to suggest that this fluid may contain additional antioxidants including uric acid and proteins (46), these demonstrate the effectiveness of the endogenous extracellular defense against oxidant-mediated injury.

**Relevance to CF airway disease.** Finally, these data demonstrate the potential for GSH antioxidant therapy for CF (20). Recently, the use of GSH aerosol has been suggested to suppress inflammatory cell-derived oxidants in the lungs of CF patients (35). Presently, two clinical trials of aerosolized GSH are underway in Utah and Germany. Unfortunately, GSH administered by this route has been shown to rapidly oxidize to GSSG (35), which is far less efficient in converting HOCl to innocuous products compared with GSH. In addition, delivery of GSH to the interface between the neutrophils and the epithelium will be achieved most effectively by direct secretion from the epithelial cells.
Therefore, our goal is to increase apical GSH content where it is most needed. There are two potential strategies. First, we can use the peptide N-K2-M2GlyR to bypass the Cl⁻ permeability defect in CF and enhance GSH secretion (16). Second, we can identify the GSH transport protein(s) and pharmacologically target the mechanism that regulates GSH secretion. Restoration of GSH secretion by either of these approaches can lead to a potential clinical application for treatment against damage due to oxidative stress during chronic inflammation in CF patients.

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DISCLOSURES

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