Identification of Nrf2-dependent airway epithelial adaptive response to proinflammatory oxidant-hypochlorous acid challenge by transcription profiling

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Zhu L, Pi J, Wachi S, Andersen ME, Wu R, Chen Y. Identification of Nrf2-dependent airway epithelial adaptive response to proinflammatory oxidant-hypochlorous acid challenge by transcription profiling. Am J Physiol Lung Cell Mol Physiol 294: L469–L477, 2008. First published December 21, 2007; doi:10.1152/ajplung.00310.2007.—In inflammatory diseases of the airway, a high level (estimated to be as high as 8 mM) of HOCl can be generated through a reaction catalyzed by the leukocyte granule enzyme myeloperoxidase (MPO). HOCl, a potent oxidant, causes extensive tissue injury through its reaction with various cellular substances, including thiols, nucleotides, and amines. In addition to its physiological source, HOCl can also be generated by chlorine gas inhalation from an accident or a potential terrorist attack. Despite the important role of HOCl-induced airway epithelial injury, the underlying molecular mechanism is largely unknown. In the present study, we found that HOCl induced dose-dependent toxicity in airway epithelial cells. By transcription profiling using GeneChip, we identified a battery of HOCl-inducible antioxidant genes, all of which have been reported previously to be regulated by nuclear factor erythroid-related factor 2 (Nrf2), a transcription factor that is critical to the lung antioxidant response. Consistent with this finding, Nrf2 was found to be activated time and dose dependently by HOCl. Although the epidermal growth factor receptor-MAPK pathway was also highly activated by HOCl, it was not involved in Nrf2 activation. To further understand the functional significance of Nrf2 activation, small interference RNA was used to knock down Nrf2 level by targeting Nrf2 or enhance nuclear accumulation of Nrf2 by targeting its endogenous inhibitor Keap1. By both methods, we conclude that Nrf2 directly protects airway epithelial cells from HOCl-induced toxicity.

INFLAMMATORY DISEASES of the airway, such as bacterial infection, cystic fibrosis (CF), and chronic obstructive pulmonary disease, the neutrophil is the predominant inflammatory cell type. Activated neutrophils generate reactive oxygen species, such as superoxide anion radicals (O2•−) and H2O2, and secrete the enzyme myeloperoxidase (MPO) (24). In the presence of a physiological concentration of chloride, MPO can utilize H2O2 to generate the powerful oxidant hypochlorous acid (HOCl) (24). Because of the high reactivity of HOCl, accurate measurement of HOCl concentration in the human airway is difficult. In CF airway, on the basis of published estimates of the number of neutrophils in the sputum and the amount of HOCl produced by a stimulated neutrophil, airway HOCl concentration was estimated to be ~3 mM on average, with a maximum of ~8 mM (15). This estimate was close to that reported by Weiss (43), who showed that active neutrophils could produce up to 5 mM HOCl. Because the pKs of HOCl is 7.53, under physiological pH, HOCl exists in neutral (HOCl) and ionized (OCl−) forms (24). Throughout the present study, we use HOCl to represent this mixture. HOCl is a very potent oxidant that can react with numerous molecules, such as proteins, lipids, nucleotides, thiols, and amines (24). Thus, in addition to its primary function as a pathogen killer, HOCl also causes tissue injuries, which result in various human pathologies (24).

In addition to its endogenous source, a high level of HOCl can also be generated through hydrolysis of inhaled chlorine gas (12). Chlorine gas is a widely used industrial chemical and has been used as a chemical weapon dating back to World War I (12). Humans can be exposed to chlorine by virtue of occupational exposure (12), transportation accidents (12), misuse of cleaners (12), or deliberate terrorist attack (http://en.wikipedia.org/wiki/2007_chlorine_bombings_in_Iraq).

Despite the importance of HOCl, there have been very few studies of the molecular nature of HOCl-induced tissue injury. In the airway of patients with acute or chronic diseases, an enormous number of neutrophils are present in the bronchoalveolar lavage (24). Similarly, MPO levels are also highly elevated in the airway lumen (24). With its massive surface area, the airway epithelium is likely to be a major target of HOCl generated by the MPO-catalyzed reaction. The airway epithelium is the first barrier to inhaled chlorine and, thus, the first target of the large amount of HOCl generated from the chlorine hydrolytic reaction with airway surface fluids. Because epithelial injury and its corresponding adaptive response have a significant impact on airway inflammation and remodeling, we seek to understand the molecular basis of the airway epithelial response to HOCl challenge.

To vividly mimic in vivo airway epithelium, we used a state-of-the-art well-differentiated epithelial cell culture system that grows and differentiates cells under an air-liquid interface (5). The resulting culture maintains the morphological and physiological characteristics of in vivo epithelia (45, 46). Combining GeneChip profiling and genetic manipulation using...
an epithelial cell line model, we have demonstrated for the first time that a key nuclear factor erythroid-related factor 2 (Nrf2)-dependent antioxidant pathway is activated by HOCl and protects airway epithelial cells from HOCl-induced toxicity.

**MATERIALS AND METHODS**

**Chemicals, Inhibitors, and Antibodies**

HOCl was purchased from Sigma-Aldrich (St. Louis, MO). The concentration of HOCl was determined by measurement of its absorbance at 290 nm (13). Chemical inhibitors (AG-1478 and U-0126) were purchased from Calbiochem (EMD Biosciences, San Diego, CA); Nrf2 and actin antibodies from Santa Cruz Biotechnology (Santa Cruz, CA); and antibodies against phosphorylated proteins (phosphorylated epithelial growth factor receptor (pEGFR), ERK (pERK), p38 (pP38), and JNK (pJNK)) from Cell Signaling Technology (Danvers, MA).

**Cell Culture and HOCl Treatment**

**Differentiated primary cell culture.** Human tracheobronchial tissues were obtained from the National Disease Research Interchange. The Hamner Institute Health and Safety Committee approved all procedures involved in tissue procurement. We previously successfully established primary epithelial cultures from these tissues (5, 46). Normally, primary cells were plated on a 25-mm Transwell procedures involved in tissue procurement. We previously successfully established primary epithelial cultures from these tissues (5, 46). Normally, primary cells were plated on a 25-mm Transwell (Costar, Corning, NY) chamber at 1–2 × 10⁴ cells/cm² in Ham’s F-12-DMEM (1:1) supplemented with insulin (5 µg/ml), transferrin (5 µg/ml), epidermal growth factor (EGF, 10 ng/ml), dexamethasone (0.1 µM), cholera toxin (10 ng/ml), bovine hypothalamus extract (15 µg/ml), BSA (0.5 mg/ml), and all-trans-retinoic acid (30 nM). The cells were subjected to an immersion culture condition for 1 wk and then shifted to an air-liquid interface culture condition. Under this biphasic culture condition, high transepithelial resistance (>500 Ω·cm²), multiple cell layers, beating cilia, and the formation of mucus-secreting granules were observed (5, 46). Normally, experiments were performed 21 days or 2 wk after the culture condition was switched from immersion to air-liquid interface. The medium was routinely changed once every other day.

**Monolayer primary culture.** Primary epithelial cells were cultivated on regular tissue culture dishes in the medium described above. The cells were not differentiated and were morphologically similar to each other.

**NCI-H292 cells.** NCI-H292 cells were obtained from American Type Culture Collection and cultivated on regular tissue culture dishes in RPMI medium + 10% FBS.

**HOCl treatment.** For treatment of monolayer primary cells and NCI-H292 cells, HOCl was diluted into culture medium at the desired concentration immediately before treatment. For differentiated primary cell culture, 100 µl of medium containing the desired concentration of HOCl was added to the top of the cells, which mimicked the apical side of airway epithelia.

**Assay for Cell Viability**

Methanethiosulfonate (MTS) assay (Promega, Madison, WI) was carried out according to the manufacturer’s instructions to determine HOCl-induced toxicity in a monolayer culture. Briefly, 2 × 10⁴ primary epithelial cells or NCI-H292 cells were seeded onto a 96-well plate and grown to 90–95% confluence. After the cells were washed three times with fresh medium, medium containing different concentrations of HOCl were added, and the cells were incubated for 6 or 24 h. Then the cells were washed three times with medium without HOCl and incubated with medium containing freshly made MTS mixture according to the manufacturer’s instruction. MTS is bioreduced by cells to a formazan product that is soluble in tissue culture medium, and the absorbance was measured at 490 nm. The quantity of formazan product as measured by the amount of 490-nm absorbance is directly proportional to the number of living cells in culture. To obtain cell viability, readings from nontreated cells were designated 100%, and the readings of treated cells were then compared with those from nontreated cells to obtain their percentage. The final percent reading represents the number of metabolically viable (“living”) cells in the culture.

**Transcription Profiling by GeneChip Analysis**

The HG-U133A chip, which contains a 22,283-probe set, was used, and all protocols were based on the manufacturer’s instruction (Affymetrix). The double-extracted total RNA was submitted to the Gene Expression Core Facility of the Hamner Institute, where RNA samples were prepared and hybridized to these array chips and the hybridization signals were scanned using the standard protocols suggested by Affymetrix. For quality control, the scanned images of each array were visually inspected and found to be free of artifacts. Scatterplots of individual arrays were also used to assess the overall quality of the array data. All array data sets (total 32) were deposited into the National Center for Biotechnology Information Gene Expression Omnibus database (ID no. GSM245427-58).

**Biocorendor (14), a biological data analysis package based on R statistical programming language (Vienna University of Technology: http://www.r-project.org/), was used for array data analysis and integration with other gene annotations. Signal intensity and noise correction were performed using the robust microarray analysis algorithm (18). Differential expressions were determined using the LIMMA (R) package; the genes with statistically significant difference were determined using false detection rate-based adjusted P < 0.05, as calculated by the method described by Benjamin and Hochberg (3). The differential expression genes were then ordered using the hierarchical clustering method (using R package hclust) based on the Euclidean distance measures of the average difference of normalized values between the experimental and control conditions.

**Real-Time PCR**

Real-time PCR was performed as described previously (21). cDNA was prepared from 3 µg of total RNA with Moloney murine leukemia virus reverse transcriptase (Promega) by exposure to oligo(dT) primers for 90 min at 42°C in a 20-µl reaction solution and further diluted to 100 µl with water for the following procedures. Two microliters of diluted cDNA were analyzed using 2 × SYBR Green PCR Master Mix by an ABI 7500 or ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol. Primers (Table 1) were used at 0.2 µM. The PCR was performed in 96-well optical reaction plates, and each well contained 50 µl of reaction mixture. The SYBR green dye was measured at 530 nm during the extension phase. The relative amount of mRNA in each sample was calculated on the basis of the cycle threshold (ΔΔCt) method using the housekeeping gene GAPDH. Purity of the amplified product was determined from a single peak of a dissociation curve. Efficiency curves were performed for each gene of interest relative to the housekeeping gene according to the manufacturer’s instructions. Results were calculated as fold induction over control, as described previously (21).

**Measurement of Intracellular Reactive Oxygen Species by Live Cell Imaging**

Reactive oxygen species (ROS) production was determined using chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA; Molecular Probes, Eugene, OR) according to the manufacturer’s instruction. Briefly, cells were rinsed twice with warm phenol red-free medium and then loaded for 30 min with CM-H₂DCFDA (3 µM) in medium without phenol red. The cells were rinsed three times for removal of the extracellular dye, and the medium was replaced with phenol red-free medium. Then the cells were loaded into a live cell
keeping gene.

family; GCLM, modulator unit of glutamate-cysteine ligase; GAPDH, house-catabolism; NQO1, a member of the NAD(P)H dehydrogenase (quinone)

Measurement of Cellular Reduced and Oxidized Glutathione Levels

imaging incubator (Carl Zeiss, Thornwood, NY) at 37°C in a 5% CO2 atmosphere. Live cell images were continuously recorded before and after HOCI treatment by confocal microscopy (LSM 510 meta, Carl Zeiss).

Measurement of Cellular Reduced and Oxidized Glutathione Levels

Reduced and oxidized thiols (GSH and GSSG, respectively) were measured by a BioXYTECH GSH/GSSG-412 kit (OxisResearch, Foster City, CA) according to the manufacturer’s instruction. This measurement is essentially based on the enzymatic method for glutathione quantification developed by Tietze (39). The kit uses a patented thiol-scavenging reagent [1-methyl-2-vinylpyridinium trifluoromethanesulfonate (M2VP)], instead of N-ethylmaleimide, which also inhibits glutathione reductase, to rapidly scavenge GSH but does not interfere with the glutathione reductase reaction. Briefly, 10 µl of M2VP were added to 100 µl of cell extract and used for GSSG sample preparation. For GSH sample preparation, 50 µl of cell extract without M2VP were used. The mixture of sample (or blank or standard), chromagen, enzyme, and NADPH (200 µl of each) in a cuvette was examined for the change of absorbance at 412 nm for 3 min with a spectrophotometer. The reaction rate and calibration curves were used to calculate concentrations of GSH or GSSG sample, which will be standardized with total protein concentration. The concentration of GSH or GSSG was expressed as micromoles of GSH or GSSG per gram of protein. AGSSG or AGSH was calculated by subtraction of the GSSG or GSH value of nontreated cells from that of HOCI-treated cells.

Western Blot

Total cellular protein was collected as described previously (9). Nuclear proteins were collected using a nuclear protein extraction kit (Panomics, Fremont, CA). Equal protein load for total and nuclear proteins was confirmed by anti-actin antibody staining.

Small Interference RNA and Transient Transfection

Control small interference RNA (siRNA) was purchased from Ambion (Austin, TX). siRNA against Nrf2 (GTAAGAGGCCAGATGTAA) (35) or Keap1 (GGCGTGGCTGTCCCTCAAT) (35) was synthesized by Ambion. siRNA was transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instruction. Successful knockdown of the target was confirmed by real-time RT-PCR and Western blot.

Table 1. Real-time primers

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers</th>
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<tbody>
<tr>
<td>TXNRD1</td>
<td>AAAGCAGCTGGAACAGCAAT</td>
</tr>
<tr>
<td></td>
<td>AGCAACTGCGCCCTTAACCTTT</td>
</tr>
<tr>
<td>HMOX1</td>
<td>GCCCTGGAGACACGCTCTATGG</td>
</tr>
<tr>
<td></td>
<td>GGCGGTCTCAAAGAGATACCTT</td>
</tr>
<tr>
<td>NQO1</td>
<td>AGATCTCGGAGAGCTGTTTGG</td>
</tr>
<tr>
<td></td>
<td>TACTCGGAAAGGCTGTTTGG</td>
</tr>
<tr>
<td>GCLM</td>
<td>TGATGCGCAAGATTTGACTG</td>
</tr>
<tr>
<td></td>
<td>GTGCGCTTGAATGCGAGAT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CAATGAGCCCTGATTGACC</td>
</tr>
<tr>
<td></td>
<td>CACGAGTCTCGCGCTGCA</td>
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TXNRD1, thioredoxin reductase 1; HMOX1, HO-1, 1st enzyme in heme catabolism; NQO1, a member of the NAD(P)H dehydrogenase (quinone) family; GCLM, modulator unit of glutamate-cysteine ligase; GAPDH, housekeeping gene.

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Statistical Analysis

Experimental groups were compared using a two-sided Student’s t-test, with significance set at P < 0.05. When data were not distributed normally, significance was assessed with Wilcoxon’s matched-pairs signed-ranks test, and P < 0.05 was considered to be significant. Matlab 6.0 with statistics toolbox (MathWorks, Natick, MA) was used for analysis of the data.

RESULTS

HOCI-Dependent Antioxidant Cell Toxicity

We first examined whether HOCI could elicit a cytotoxic effect on airway epithelial cells. We used an MTS assay kit (Promega), which is similar to, but more convenient than, the traditional 4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide assay. This assay, by measuring the number of metabolically active (viable) cells in the culture, evaluates direct damage, which kills the cells, and indirect toxicity, which represses metabolic activity of the cells and eventually leads to cell growth inhibition and death. For convenience, we use the term “viability” to represent viable cells in the culture. HOCI induced a dose-dependent toxicity on primary airway epithelial cells grown as a monolayer (Fig. 1). At 6 h, ≤2 mM HOCI had no effect, whereas 4 mM HOCI elicited a 61% reduction of cell viability. Interestingly, low-dose, particularly 0.8 mM, HOCI had a slight promoting effect on cell viability. At 24 h, >0.8 mM HOCI had a significant toxic effect, which is also consistent with the light-microscopic observation of an increase in the number of floating cells. At 4 mM HOCI, most of the cells observed under light microscopy were dead (data not shown). Thus HOCI alone, which has been estimated to be as high as 8 mM in severely inflamed airways (15), should be sufficient to damage airway epithelial cells.

Nrf2-Dependent Antioxidant Gene Expression Was Identified by Transcription Gene Profiling in Differentiated Airway Epithelial Cell Culture Treated With HOCI

To further understand the epithelial response to HOCI-induced injury on the molecular level, we used state-of-the-art
GeneChip technology to characterize transcription profiling of HOCl-treated cells compared with nontreated controls. Our goal was to identify key genes or pathways that represented the cellular stress response, which led to epithelial injury and death, or the adaptive response, which protected cells from damage. We utilized a differentiated cell culture model grown on an air-liquid interface. As we demonstrated previously (5, 46), this model consists of major airway cell types and is morphologically and physiologically similar to the in vivo epithelial layer. We took into account human-to-human variability by using epithelial cells isolated from four different healthy individuals as replicates. Accurate measurement of viability (MTS assay) is difficult in the differentiated cell culture with multilayers, because the number of cells in the culture exceeds the maximum for linearity and because different layers are exposed to different concentrations. Since the initial epithelial injury tends to occur at the top layer, a viability test of the monolayered epithelial cell (Fig. 1) should provide the best approximation. Thus, on the basis of the cell viability measurements of monolayered epithelial cells (Fig. 1), we chose 0.4, 1, and 4 mM for our study: 0.4 mM represented a dose with no obvious toxicity; 1 mM caused some toxicity at 24 h but not at 6 h, suggesting that an underlying molecular event might have been triggered at an earlier time point; and 4 mM caused significant toxicity even at 6 h. Any higher dose (e.g., 6, 8, and 10 mM) was toxic (Fig. 1). Because we were particularly interested in the early response, we chose 2 and 6 h for this GeneChip study. A total of 83 differentially regulated genes were identified (Fig. 2A). Hierarchical analysis revealed several very interesting gene expression pattern changes depending on dose and time (Fig. 2A). In early induced genes (those that were elevated in 2 h but not in 6 h), we found two transcription factors, MAFF and MAFG, which were highly elevated by HOCl (Fig. 2B). In the late (those that were elevated in 6 h but not in 2 h) or persistent (those that were elevated in 2 and 6 h) induced genes, a battery of antioxidant genes, including TXNRD1, HMOX1, ALDH1A3, NQO1, and GCLM, were also significantly elevated by HOCl (Fig. 2C). Interestingly, all these genes are related to the Nrf2-dependent cellular antioxidant/adaptive pathway. MAFF and MAFG, DNA-binding

Fig. 2. Transcriptional profiling of HOCl-treated airway epithelial (differentiated primary) cells. A: heat map showing relative levels of expression of genes differentially regulated by 0.4, 1, and 4 mM HOCl for 2 and 6 h. Genes with adjusted $P < 0.05$ for comparison between untreated cell and cells with any treatment were clustered according to Euclidean distance between their expression levels and average agglomeration method using the R package hclust. Expression values are represented (in rows) for each gene at each HOCl concentration by color on a scale from green (underexpressed) through black (unchanged) to red (overexpressed) by using the heatmap.2 function of the R package ggplot2 (42). B: dose-dependent elevation of MAFF (B1) and MAFG (B2) at 2 h. $*P < 0.05$ vs. control ($n = 4$). C: dose-dependent expression of TXNRD1 (C1), HMOX1 (C2), ALDH1A3 (C3), NQO1 (C4), and GCLM (C5) at 6 h. $*P < 0.05$ vs. control ($n = 4$). Area enclosed in rectangle designates commonly significant dose.
partners of Nrf2 on the antioxidant response element site (19), can modulate Nrf2 binding preference and activity. TXNRD1 (37), HMOX1 (31), ALDH1A3 (11), NQO1 (31), and GCLM (31), phase II detoxification enzymes, are regulated by Nrf2. Thus activation of Nrf2 appeared to be a major epithelial adaptive response to HOCl. Because of the critical role of Nrf2 in airway diseases (8), we focused our effort on the role of this pathway in HOCl-induced epithelial injury.

**HOCl-Induced Nrf2 Activation is Not Dependent on the EGFR-MAPK Pathway**

We first decided to confirm whether Nrf2 was indeed activated by HOCl. Nrf2 induced a time- and dose-dependent elevation of nuclear Nrf2 (Fig. 3A), a hallmark of Nrf2 activation (23). In nontreated epithelial cells, Nrf2 was very low in the nucleus (Fig. 3A) and the cytosol (data not shown). With treatment, nuclear Nrf2 was significantly elevated within 2 h. At 6 h, nuclear Nrf2 was decreased by 1 mM HOCl but was further increased by 4 mM HOCl; this response might reflect high oxidative burden at this dose.

Next, we sought to understand the upstream event that activated Nrf2. Besides the classic Keap1 dissociation mechanism (40) (see Fig. 5), phosphorylation events caused by upstream kinases have been reported to activate Nrf2 in lung cells challenged by other oxidants (29). In our model, we indeed found that HOCl dose dependently activated EGFR and MAP kinases (e.g., ERK1/2, p38, and JNK; Fig. 3B). Only EGFR and ERK1/2 phosphorylations were correlated with Nrf2 activation, because HOCl activated p38 and JNK at 4 mM but not at 1 mM. To test whether activation of EGFR or ERK1/2 was responsible for Nrf2 activation, we used specific inhibitors: AG-1478 to inhibit EGFR activation and U-0126 to inhibit MEK1/2, the upstream kinase of ERK1/2. Neither AG-1478 nor U-0126 affected the level of nuclear Nrf2 (Fig. 3C) or Nrf2-dependent expression of HMOX1, NQO1, and GCLM, which showed similar responses (for simplicity, only HMOX1 is shown in Fig. 3D). Thus it appeared that EGFR and MAPK pathways were not responsible for HOCl-activated Nrf2 in airway epithelial cells.

**HOCl-Induced Nrf2 Activation is Dependent on Cellular Oxidative Stress**

Because HOCl is an oxidant, we sought to determine whether HOCl could induce generation of ROS and whether ROS could then activate Nrf2. HOCl induced cellular oxidative stress, as indicated by a significant decrease of GSH and increase of GSSG (Fig. 4A). A substantial amount of intracellular ROS was also detected by an oxidation-sensitive fluorescence dye in HOCl-treated cells (Fig. 4B). Treatment of the cells with the antioxidant N-acetylcysteine significantly decreased nuclear Nrf2 level (Fig. 4C) and Nrf2-dependent HMOX1 expression (Fig. 4D). Thus HOCl-induced Nrf2 activation was dependent on ROS-mediated signaling.

**Nrf2 Protects Epithelial Cells From HOCl-Induced Toxicity**

Nrf2 has been shown to play a protective role in animal studies (1, 6, 32, 33, 38). Since HOCl induced epithelial cell toxicity, we sought to determine whether the activation of Nrf2 provided any protection. Because primary cells are notoriously resistant to regular transfection procedures, we used an epithelial cell line, NCI-H292, to test the protective role of Nrf2 in...
HOCl-treated cells. Similar to its effect on primary cells, HOCl induced Nrf2 activation (Fig. 5A), Nrf2-dependent gene expression (Fig. 5B), and dose-dependent cellular toxicity (Fig. 5C). We specifically knocked down Nrf2 level by using siRNA against Nrf2 or enhanced nuclear Nrf2 level by using siRNA against Keap1 (Fig. 5A), a cellular inhibitor of Nrf2, the primary function of which is believed to be trapping Nrf2 in cytosol and targeting Nrf2 for proteasomal degradation (23). Western blot analysis confirmed that the change of Nrf2 was indeed consistent with our manipulation (Fig. 5A). Modulation of nuclear Nrf2 level also affected Nrf2-dependent HMOX1 expression, which was correlated with the change of nuclear Nrf2 level (Fig. 5B). Interestingly, knockdown of Nrf2 also reduced basal (untreated) HMOX1 expression when Nrf2 level was very low. This finding was consistent with the Nrf2-knockout mouse study, in which the expression of many Nrf2-dependent genes was significantly lower than in the wide-type mouse, even without challenge (6).

We then tested whether Nrf2 could protect HOCl-induced epithelial toxicity. Using MTS assay, we found that elevation of nuclear Nrf2 level by Keap1 knockdown could protect cells treated with 0.6–1.5 mM HOCl. Consistently, loss of Nrf2 elicited significantly exacerbated toxicity with similar treatment. At high doses (e.g., 2 and 4 mM), there was no protection or exacerbation under either manipulation. Under such conditions, HOCl might already overwhelm all defense systems. Notably, NCI-H292 cells were more susceptible to HOCl treatment than primary epithelial cells (Fig. 1). Nevertheless, these findings demonstrate the protective role of Nrf2 activation in HOCl-induced epithelial toxicity.

DISCUSSION

HOCl is a powerful oxidant that can be generated by environmental exposure to chlorine (12) or catalytic reaction by a major neutrophilic granular enzyme, MPO (24). One of the major clinical manifestations of high-dose chlorine inhalation is acute lung injury (ALI), which may lead to acute respiratory distress syndrome (25). An animal study of chlorine inhalation confirmed that the progress from ALI to acute respiratory distress syndrome was correlated with inhaled chlorine dose (2). In addition, intratracheal infusion of glucose peroxidase (as
an H$_2$O$_2$ source) and MPO into rats generated ALI, which was not affected by peroxidase or MPO alone (20). Under either condition, HOCl was the major product of chemical/biochemical reactions. Also, HOCl-induced epithelial damage might represent the first event that eventually leads to inflammation and progressive lung injury. Consistently, the levels of 3-chlorotyrosine, a specific marker for the HOCl reaction with protein, were high in infants who developed chronic lung diseases (4). Although there are no reports of 3-chlorotyrosine levels in adult patients with lung injury, numerous studies have demonstrated the positive correlation between HOCl-generating activated neutrophils, as well as its catalyzing enzyme MPO, and lung injury in various lung diseases (24). Thus HOCl is very likely to play an essential role in the epithelial injury in those disease contexts.

The most important finding in the present study is the involvement of a key antioxidant factor, Nrf2, in the adaptive response to HOCl. In addition to epithelial cells reported in the present study, we previously observed HOCl-activated Nrf2 in macrophages (30). Thus HOCl appears to be an Nrf2 activator in various cell types. Interestingly, Nrf2 was previously identified as a susceptible locus for hyperoxia-induced lung injury (7). Later, with use of a gene-knockout approach, Nrf2 was found to be a master transcription factor that regulates most of the phase II detoxification enzyme and appears to be critical for the antioxidant response (8). Consistently, in HOCl-treated cells, a battery of phase II genes, including TXNRD1, HMOX1, ALDH1A3, NQO1 and GCLM, were highly regulated. All these genes have been reported to be Nrf2 dependent (11, 31, 37) and have the specific role of detoxification of different deleterious oxygen intermediates. TXNDR1 (thioredoxin reductase 1), is a selenoprotein that catalyzes the NADPH-dependent reduction of thioredoxin (TRX) (27), a ubiquitous small peptide with a redox-active thiol group. The TRX-TXND system is a critical antioxidant system that is essential for intracellular signaling pathways by catalyzing protein disulfide-dithiol exchange (28). Because HOCl is known to oxidize protein thiols groups (24), activation of the TRX-TXND system may facilitate the reduction of those oxidized thiols and restore the normal protein function. HMOX1, also called HO-1, is the first enzyme in heme catalabolism, producing CO, Fe$^{2+}$, and biliverdin, which can further be reduced to bilirubin by biliverdin reductase (47). Biliverdin and bilirubin are antioxidants that can prevent H$_2$O$_2$/O$_2^-$-induced lipid peroxidation and cell death (22). CO was identified as a signaling molecule that prevented nitric oxide toxicity in HeLa cells (34). In HOCl-treated cells, these HMOX1-related antioxidant products may help eliminate intracellular ROS. ALDH1A3, also known as ALDH6, plays a major role in the detoxification of aldehydes generated by, for example, lipid peroxidation and alcohol metabolism (16). In our case, lipid peroxidation by HOCl is likely to be the source of aldehydes. NQO1 is a member of the NAD(P)H dehydrogenase (quinone) family and encodes a cytoplasmic two-electron reductase, which detoxifies toxic quinone by reducing it to hydroquinones (36). Because HOCl has no quinine-like structure, the substrate of NQO1 in our study is unknown. However, it is very likely that some toxic quinone or quinine-like molecules were generated from the HOCl-induced oxidative reaction on various cell components. GCLM is the modulator unit
of GCL, also known as γ-glutamylcysteine synthetase, which is the first rate-limiting enzyme of glutathione synthesis (10). The glutathione system provides the most important antioxidant capacity in the intracellular and extracellular environment. Because HOCl reduced GSH level and increased GSSG level, upregulation of GCLM may contribute to rebalancing the whole system to restore the cellular antioxidant capacity. Interestingly, GSH is a very efficient scavenger of HOCl (44). Therefore, an increased level of GSH may be directly used to neutralize HOCl.

Despite the importance of Nrf2 signaling in the HOCl effect, the upstream pathway that triggered Nrf2 activation is not entirely clear. In lung cells, other studies demonstrated that EGFR-MAPK-mediated signaling was essential for hyperoxia-induced Nrf2 activation (29). Although HOCl appeared to activate the EGFR-MAPK pathway and Nrf2, we failed to find any connection between these events. Other studies (17, 29) have also indicated the involvement of phosphatidylinositol 3-kinase-Akt and PKC pathways in Nrf2 activation. However, the specific phosphatidylinositol 3-kinase inhibitor LY-203580 (Calbiochem) and Akt inhibitors I and II (Calbiochem), as well as a pan-PKC (calphostin C, Calbiochem), failed to inhibit Nrf2 activation (unpublished observation). Thus HOCl-induced Nrf2 activation appeared not to be associated with these pathways. Our data only indicated that the oxidant-dependent signaling upstream of Nrf2, whatever it is, could be inhibited by N-acetylcysteine. This unknown signaling was likely to activate Nrf2 by disrupting the Keap1-Nrf2 interaction and facilitate the nuclear accumulation of Nrf2. This mechanism was corroborated by our siRNA study on an epithelial cell line. Because Keap1 can be an oxidant sensor, it was very likely that HOCl activated Nrf2 by direct oxidation of key cysteine residues in Keap1 (41). However, we cannot exclude the possibility that Nrf2 activation by HOCl was mediated through some unidentified signaling pathway other than EGFR-MAPK.

Consistent with the proposed protective role of Nrf2, increased sensitivity to lung oxidative stress in Nrf2-deficient mice has been reported in several disease models (1, 6, 32, 33, 38). However, because of the nature of systematic protection, the level at which this protection occurs is unclear. No study has been done to determine whether Nrf2 can directly protect airway epithelium from oxidative injury. The present study has filled in this knowledge gap by demonstrating that modulation of Nrf2 activation by siRNA could directly affect the sensitivity of epithelial cells to HOCl-induced toxicity. To our knowledge, this is the first study that conclusively demonstrates the causal link between Nrf2-Keap1 signaling and the protection of epithelial cells from HOCl-induced toxicity. Most importantly, our model and approach can be readily applied to the study of epithelial injury induced by other oxidants, which is a very interesting topic to pursue in the future. One weakness of the present study is the use of the epithelial cell line NCI-H292 as a substitute for the primary cell because of to the difficulty of transfecting these cells. Nonetheless, NCI-H292 cells could recapitulate all HOCl responses of primary cells, including dose-dependent toxicity, Nrf2 activation, and Nrf2-dependent gene activation (see RESULTS). Thus, NCI-H292 cells provide the most convenient and close surrogate of primary epithelial cells for study of the detailed molecular mechanism of HOCl-induced epithelial response. Application of this in vitro finding to an animal model is needed to further investigate its physiological role in vivo.

Identification of epithelial Nrf2 activation in HOCl challenge has significant implications in the areas of biomedicine and risk assessment. In the area of biomedical research, the present study suggests that Nrf2 activation provides critical protection against HOCl-induced airway epithelial injury. HOCl is abundantly present in the airways of neutrophilic inflammation associated with many chronic diseases, including chronic obstructive pulmonary disease and CF. Since it has been shown that Nrf2 polymorphism is directly associated with susceptibility to lung injury (26), it is tempting to develop therapeutic reagents that can modulate Nrf2 level to prevent lung injury in these chronic diseases. In the area of risk assessment, since Nrf2 activation is dose dependent and occurred much earlier than the actual injury, it provides an early biomarker to detect lung injury induced by chlorine inhalation. This is particularly important, because the chlorine-induced ALI is delayed and has few symptoms immediately after exposure (25). It is also attractive to examine whether other major toxicants, most of which generate ROS, induce Nrf2 activation, which will expand the scope of using Nrf2 as a valuable biomarker for risk assessment of various oxidant-generating toxicants.

In summary, by using transcription profiling, we have identified the Nrf2-dependent adaptive response that protects epithelial cells from HOCl-induced injury. Further study of this pathway in an animal model, as well as extension of this pathway to other toxicant challenges, will affect biomedicine and risk assessment.

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