Mechanisms and Modification of Chlorine-induced Lung Injury in Animals

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Chlorine (Cl₂) is a reactive oxidant gas used extensively in industrial processes. Exposure of both humans and animals to high concentrations of Cl₂ results in acute lung injury, which may resolve spontaneously or progress to acute respiratory failure. Injury to airway and alveolar epithelium may result from chemical reactions of Cl₂ from HOCl (the hydrolysis product of Cl₂), and/or from the various reaction products, such as chloramines, that are formed from the reactions of these chlorinating species with biological molecules. Subsequent reactions may initiate self-propagating reactions and induce the production of inflammatory mediators compounding injury to pulmonary surfactant, ion channels, and components of lung epithelial and airway cells. Low-molecular-weight antioxidants, such as ascorbate, glutathione, and urate, present in the lung epithelial lining fluid and tissue, remove Cl₂ and HOCl and thus decrease injury to critical target biological targets. However, levels of lung antioxidants of animals exposed to Cl₂ in concentrations likely to be encountered in the vicinity of industrial accidents decrease rapidly and irreversibly. Our measurements show that prophylactic administration of a mixture containing ascorbate and desferal N-acetyl-cysteine, a precursor of reduced glutathione, prevents Cl₂-induced injury to the alveolar epithelium of rats exposed to Cl₂. The clinical challenge is to deliver sufficient quantities of antioxidants noninvasively, after Cl₂ exposure, to decrease morbidity and mortality.

Keywords: ascorbate; N-acetyl-cysteine; chlorine; alveolar epithelium; hypochlorous acid

CL₂ AND ITS REACTIVE INTERMEDIATES: CHEMISTRY

Chlorine (Cl₂) is a dense, acrid, pungent, greenish-yellow gas that is easily recognized by both color and odor at very low concentrations. It is noncombustible and moderately soluble at room temperature and atmospheric pressure. It is the ninth largest produced chemical by volume in the United States, transported mainly by rail to manufacturing plants for pulp bleaching, waste sanitation, and pharmaceutical manufacturing. It is also added in swimming pools to limit the number of pathogens (1). However, accidental or deliberate release of Cl₂ into the atmosphere during industrial accidents has been associated with significant morbidity and mortality (1, 2). During the last few years, Cl₂ cylinders have been bundled with

traditional explosives, raising significant concerns regarding the possible reemergence of this agent as a chemical weapon against both combatants and civilians (3). The density of Cl₂ is greater than that of air; therefore, it has a tendency to settle in low-lying areas, which may affect exposures on a microenvironmental scale.

Cl₂ is a highly reactive oxidant gas and has higher solubility in water than nitrogen dioxide and ozone, two well-known environmental pollutants (4). When inhaled, Cl₂ dissolves in the epithelial lung lining fluid (ELF), a thin layer of fluid covering the apical surfaces of airway and distal lung epithelial cells, and then reacts with biological molecules, such as low-molecular-weight antioxidants (5). As the concentrations of antioxidants are decreased, Cl₂ undergoes hydrolysis to generate hypochlorous (HOCl) and hydrochloric (HCl) acids according to the following reactions:

Bicarbonate in the ELF buffers and neutralizes HCl, limiting its toxicity (6). Consequently, Cl₂ toxicity has been attributed to HOCl and its conjugate base (7). We have recently shown that Cl₂ reacts with low-molecular-weight antioxidants in the ELF with rates exceeding its hydrolysis (5). Both Cl₂ and HOCl/OCl⁻ may contribute to the initiation and propagation of lung injury.

REACTIONS OF CL₂ AND ITS REACTIVE INTERMEDIATES WITH BIOLOGICAL TARGETS

The main targets of Cl₂, HOCl, and OCl⁻ are functional groups of proteins and amino acids such as cysteine (8, 9), methionine, side-chain amino groups in amino acids, terminal amino groups, yielding the corresponding chloramines (10), and aromatic amino acids (such as tyrosine), yielding 3-chlorotyrosine (11–13). Chloramines are toxic and both contribute to and compound the injurious effects of Cl₂/HOCl/OCl⁻ to biological tissues (14–17). Accidental inhalation of chloramines formed by mixing cleaning products containing sodium hypochlorite with ammonia may cause respiratory injury that may require emergency treatment (18). HOCl also damages DNA and poly-ADP-ribose, a DNA repair enzyme (19, 20).

There is also evidence to suggest that chloramines and HOCl may activate inflammatory cascades. For example, HOCl activates the mitogen-activated protein kinase pathway (21), while the cell-permeable glycine and taurine chloramines regulate NF-κB activity via oxidation of its inhibitor IκBα (22). Activated inflammatory cells, attracted into the alveolar space by chemokines and cytokines, release myeloperoxidase and large amounts of HOCl (0.1–1 mM) in the vicinity of airway and alveolar epithelial cells (23). Also, exposure of mice to Cl₂ gas up-regulates the inducible form of nitric oxide synthase (iNOS) in alveolar epithelial cells and macrophages (24). Thus, exposure to Cl₂ gas initiates a series of biochemical reactions that could be detrimental to the host, even after the end of the exposure period.

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Figure 1. Schematic representation of lung epithelial targets and reactive intermediates formed in the lung epithelial fluids during inhalation of Cl₂. Diagram on top shows sites of penetration of Cl₂ (upper airways for inhaled Cl₂ < 50 ppm; distal lung regions for Cl₂ > 50 ppm). The depths of the airway (in blue color) and alveolar (yellow color) epithelial lining fluids are not in scale (thickness of ALF and epithelial lining fluid [ELF] = 5–10 and 0.1–0.24 μm, respectively; reviewed in Reference 61). Some potential cellular targets such as cilia, surfactant monolayer, tubular myelin, surfactant protein A and D, ion channels, Na⁺/K⁺-ATPase, airway and alveolar cells, alveolar macrophages, and NF-κB are shown in the diagram. GSH = reduced glutathione; ASC = ascorbate; X = secondary reactive intermediates (such as chloramines) formed by the interaction of HOCl and Cl₂ with proteins and lipids. A sensory nerve that contains TRPA1 channels (red dots) is also shown. Cl₂ molecules (prior to being hydrolyzed to HOCl) may attack components of the surfactant monolayer at the surface of the alveolar epithelial lining fluid, tubular myelin, surfactant proteins, and proteins on the surface of alveolar type II cells because of the closer proximity of these targets to the air-liquid interface (5).

Inhalation of Cl₂ at concentrations less than 50 ppm causes reversible bronchospasm, increased mucous production and airway resistance, and decreased respiratory rate in humans and animals (25–30). There is little alveolar injury, since most Cl₂ penetrates no deeper than the upper airways (31). Bessac and coworkers (32, 33) showed that low concentrations of aerosolized HOCl, as well as other oxidants, activate transient receptor potential ankyrin 1 (TRPA1) ion channels, located in a subset of airway sensory neurons, through covalent modification of the channel protein. *Trpa1*^{−/−} mice exhibited reduced respiratory depression in response to low concentrations of aerosolized HOCl compared with wild-type mice (32, 34). These findings suggest that activation of TRPA1 channels by low

concentrations of Cl₂, HOCl, and other oxidants contributes to the neurological and airway responses mentioned above. In the short term, decreased respiratory rate can limit the amount of inhaled Cl₂ and thus lower the extent of lung injury. The role of TRPA1 channels in the initiation and progression of lung injury in mice exposed to higher concentrations of Cl₂ (> 100 ppm) is currently under investigation.

At higher concentrations (those encountered during industrial accidents or terrorist attacks), inhaled Cl₂ molecules penetrate into distal lung regions (31) and may damage critical components of alveolar epithelial cells and the pulmonary surfactant system, resulting in severe hypoxemia, pulmonary edema, and even death from respiratory failure (28, 35–40). In additional studies, we

exposed surfactant protein (SP)-A to various concentrations of HOCl. This resulted in oxidized and chlorinated amino acids in its carbohydrate recognition domain and decreased its ability to bind mannose residues, an important step in the binding and killing of pathogens (41). SP-D, like SP-A, is a lectin and plays diverse and important roles in innate immunity and pulmonary homeostasis. Exposure of SP-D to HOCl decreased its ability to aggregate pathogens, and resulted in the generation of abnormal disulfide cross-linked oligomers (42). These findings indicate that animals exposed to Cl₂ may be at risk of bacterial infections due to compromised innate immunity.

Lung epithelial cells actively transport Na⁺ ions from the alveolar to the interstitial spaces: Na⁺ ions enter the apical membranes of epithelial cells mainly through amiloride-sensitive Na⁺ (ENaC) channels and are transported across the basolateral membranes by the Na⁺,K⁺-ATPase (43). Active Na⁺ transport limits the degree of alveolar edema following damage to the alveolar epithelium; patients with acute lung injury that maintain the ability to concentrate alveolar proteins (as a result of active Na⁺ reabsorption) have better prognosis than those who cannot (44, 45). Exposure of C57BL/6 and BALB/c mice to Cl₂ (50–500 ppm for 30 min) decreased Na⁺-dependent alveolar fluid clearance (AFC) from 28 ± 0.7% to 15 ± 4% (% of instilled volume/30 min, n = 3–23, P < 0.05), at 1 hour after exposure. This decrease was due to specific injury to ENaC, the rate-limiting pathways in the vectorial transport of Na⁺ ions across epithelial cells (17). Na⁺,K⁺-ATPase was most likely damaged as well. Major lung airway and alveolar epithelial targets of Cl₂ are shown in Figure 1.

Recent findings suggest that inhaled Cl₂ inhibits eNOS-dependent vessel dilation via decreased eNOS expression (Rakesh P. Patel, University of Alabama; personal communication). It has been reported that exposure of mice to ozone causes vascular dysfunction, oxidative stress, mitochondrial damage, and atherogenesis (46). The deleterious effects of inhaled oxidant gases may extend to extrapulmonary sites, resulting in long-term systemic abnormalities.

LUNG ANTIOXIDANT DEFENSES

During normal metabolism, steady-state levels of reactive oxygen and nitrogen species are kept at very low levels by the existence of various antioxidant enzymes (such as superoxide dismutase, glutathione peroxidase, glutathione-S-transferase, catalase, and thioredoxin) as well as low-molecular-weight antioxidants (such as glutathione, urate, ascorbate, Vitamin E, etc.) (47–50). Significant levels of enzymatic antioxidants (such as the extracellular form of superoxide dismutase), as well as low-molecular-weight scavengers (reduced glutathione, ascorbate, and urate) also exist in the ELF and help decrease steady-state concentrations of extracellular and intracellular oxidants to low levels (49, 51). A variety of transition metal ion binding molecules, such as ceruloplasmin, transferrin, ferritin, and

bilirubin, prevent the formation of hydroxyl radicals and secondary self-propagating reactions by maintaining very low levels of free iron. Normal ELF contains plasma proteins (mainly albumin), the concentration of which increase significantly after inhalation of Cl₂ (35). Albumin has significant antioxidant capacity (52), and administration of albumin to patients with acute lung injury decreased carbonyl content in their plasma, an index of oxidative stress (53). Concentrations of major antioxidants in the human and rodent bronchoalveolar lavage fluid (BAL) are shown in Table 1.

There is significant interest in identifying FDA-approved agents that decrease acute lung injury. Vitamin C or L-ascorbic acid is the natural L-enantiomer of ascorbic acid. Ascorbic acid is a facile reductant and an important hydrophilic antioxidant. At physiological pH most of the ascorbic acid is present as ascorbate, acting as an antioxidant by donating one or two electrons to oxidants or free radicals. Ascorbate reacts directly with hydroxyl radicals and reduces Vitamin E radicals (generated during reduction of lipid hydroperoxides or peroxy radicals [54]). It also reduces HOCl, yielding Cl[–] and H₂O. Reduced glutathione (GSH) is the most abundant of the intracellular free thiols, is present in high concentrations in the normal ELF (51), and reduces lipid peroxides and hydrogen peroxide via a reaction catalyzed by glutathione peroxidase. In the process GSH is oxidized to its disulfide (GSSG) (55). A decrease of GSH/GSSG ratio is considered an index of generation of oxidative stress and has been shown to be a risk factor for the development of acute respiratory distress syndrome in alcoholics (56). N-acetyl-L-cysteine (NAC) is a precursor for GSH synthesis, interacts with electrophiles via its cysteine content, and reduces cystine to cysteine, an important mechanism for intracellular lung GSH elevation *in vivo* (57). Administration of NAC mitigates oxidative stress in a number of organs (reviewed in References 57 and 58). Ascorbate is the predominant low-molecular-weight antioxidant in rats and rabbits (which synthesize ascorbate), while GSH plays an important role in the human lung (see Table 1). A detailed account of the various enzymatic and nonenzymatic antioxidant systems in the lung can be found in a number of recent reviews (57).

EXPOSURE TO CL₂ DEPLETES LUNG ASCORBATE LEVELS AND DAMAGES THE ALVEOLAR EPITHELIUM

To test the hypothesis that lung antioxidants play an important role in the responses of animals to Cl₂, we pretreated rats with a mixture of antioxidants and exposed them to 184 or 400 ppm Cl₂ for 30 minutes in an environmental chamber in a whole body mode (59). They were then returned to room air and the extent of lung injury was assessed by measuring the partial pressure of arterial oxygen and the concentration of protein in BAL fluid. One hour after Cl₂ exposure, ascorbate levels and GSH/GSSG ratios in BAL and tissue compartments were

TABLE 1. CONCENTRATIONS OF LOW-MOLECULAR-WEIGHT ANTIOXIDANTS IN THE BRONCHOALVEOLAR LAVAGE OF RATS, RABBITS, AND HUMANS

	Rats (35*)	Rabbits (62)	Humans (63)	Humans (62)	Humans (64)
Asc (μM)	17 ± 1.5	2.5 ± 0.1	0.5 ± 0.05	0.5 ± 0.05	0.64 (0.4–0.9)
Urate (μM)	0.7 ± 0.2	0.4 ± 0.04	0.7 ± 0.04	0.7 ± 0.04	0.74 (0.6–0.8)
GSH (mM)	0.5 ± 0.05	1.1 ± 0.2	1.6 ± 0.2	1.5 ± 0.2	0.7 (0.45–0.9)
(GSH/GSSG)	10.5 ± 2	2.2	49	50	68
Protein (mg/100 ml)	16 ± 1.5	–	7	7 ± 1	8

Definition of abbreviations: Asc = ascorbate, GSH = reduced glutathione; GSH/GSSG = ratio of reduced to oxidized glutathione.

Values are means ± 1 SEM or means with ranges shown in parentheses.

* Numbers in parentheses in column headings indicate reference numbers.

Figure 2. Systemic administration of antioxidants decreases lung injury. Rats were injected with a mixture of antioxidants (ascorbate, NAC, and deferoxamine) or an equivalent amount of vehicle (saline), 18 hours and 1 hour before being exposed to 184 ppm Cl₂ for 30 minutes, returned to room air, and killed 1 hour later. Values are means $\pm$ 1 SEM. In each case, *open bars* represent values from rats exposed to air, *solid bars* from rats exposed to Cl₂ and pretreated with saline, and *hatched bars* from rats pretreated with antioxidants and then exposed to Cl₂. PaO₂ was measured in arterial samples from the carotid artery drawn during anesthesia; protein and ascorbate concentrations were measured in BAL samples. **P* < 0.05 compared with the corresponding saline values. Adapted by permission from Reference 35.

decreased. In rats exposed to 400 ppm Cl₂, ascorbate levels in BAL and lung tissues, as well as GSH/GSSG ratios in lung tissues, remained low at 24 hours after exposure, which may compromise the ability of these animals to survive a subsequent exposure to Cl₂ (35).

Based on these observations and extensive literature regarding antioxidant treatment of animals with various forms of acute lung injury (48), we tested the hypothesis that enhancing lung antioxidant defenses in rats may decrease the extent of Cl₂-induced lung injury. Rats were injected twice with 0.3 ml saline containing ascorbate (80 mg/kg body weight), NAC (Acetadote; 150 mg/kg), and deferoxamine (deferoxamine mesylate; 15 mg/kg). The first injection was administered intramuscularly 18 hours before Cl₂ exposure, the second intravenously 1 hour before Cl₂ exposure. Deferoxamine limits the production of hydroxyl radicals by scavenging iron. Both deferoxamine and NAC help maintain ascorbate in its reduced state. As shown in Figure 2 and reported previously (35), rats developed significant levels of hypoxemia and hypercapnia and increased protein levels in their BAL fluid (albumin, IgG, and IgM) 1 hour after exposure to 184 ppm Cl₂ for 30 minutes. Levels of ascorbate in BAL fluid also decreased significantly. Prophylactic administration of antioxidants as described above restored PaO₂ and ascorbate to normal levels and decreased BAL fluid protein concentration

by about 35% (Figure 2). These studies provide proof of concept that prophylactic administration of antioxidants (commonly administered in patients for a variety of conditions) blunts Cl₂-induced injury to the alveolar epithelium.

ANTIOXIDANTS, ADMINISTERED AFTER CL₂ EXPOSURE, REPLENISH DEPLETED STORES OF RAT LUNGS

Prophylactic administration of antioxidants for the prevention of Cl₂-induced lung injury may benefit first responders and other personnel likely to be exposed to Cl₂ in the immediate future. There is also a need to develop effective treatments for people that have been exposed to Cl₂. We are assessing the efficacy of low-molecular-weight scavengers in decreasing lung injury when administered to animals at various intervals after Cl₂ exposure. We exposed rats to 300 ppm Cl₂ for 30 minutes, and then returned them to room air. At the end of exposure respiration was labored, with flaring of the nostrils during inspiration. Within 5 to 10 minutes upon return to room air, rats received an intravenous (tail vein) injection of 200 μl of sterile saline containing 20 mg ascorbic acid. One hour later, the rats were killed, a blood sample was drawn from the left ventricle, and their lungs were lavaged with 8 ml of normal saline as previously described (35). Concentrations of ascorbate

Figure 3. Augmentation of lung ascorbate levels by post-exposure, intravenous administration of ascorbate. Rats were exposed to 300 ppm Cl₂ for 30 minutes and then returned to room air. Within 10 minutes after exposure, they received a single injection of 20 mg ascorbic acid in 0.2 ml of normal saline. They were killed 1 hour later, their lungs were lavaged, and a blood sample was drawn from the left ventricle. Urea concentrations were measured in both the bronchoalveolar lavage (BAL) fluid and the plasma and ELF volume was calculated from the dilution of urea as previously described (60). The amounts of ascorbate in the BAL fluid and lung tissues were then measured by HPLC as previously described (35). Values are means $\pm$ 1 SEM for *n* = 6 rats in each group. *Significantly different from the corresponding saline group under the same conditions; #significantly different from the corresponding variable in the air group.

in the BAL fluid and lung tissues were measured by high-pressure liquid chromatography as previously described (35), and total amounts of ascorbate in the BAL were calculated. In addition, we calculated the volume of the ELF by measuring the concentrations of urea in both the plasma and recovered BAL fluid as previously described (60). From these data we calculated the ascorbate concentration in the ELF by the following formula:

$$[\text{asc}]_{\text{ELF}} = \frac{(\text{BAL} + \text{ELF}) \times [\text{asc}]_{\text{BAL}}}{(\text{ELF})},$$

where BAL is the volume of saline used for lavage, $[\text{asc}]_{\text{BAL}}$ is the concentration of ascorbate in the BAL, and ELF is the volume of the epithelial lining fluid calculated from the following formula as previously described (60):

$$\text{ELF} = \frac{(\text{BAL}) \times [\text{urea}]_{\text{BAL}}}{[\text{urea}]_{\text{plasma}} - [\text{urea}]_{\text{BAL}}},$$

where $[\text{urea}]_{\text{BAL}}$ and $[\text{urea}]_{\text{plasma}}$ are the concentrations of urea in the BAL and plasma, respectively.

As shown in Figure 3, intravenous injection of ascorbate in rats after Cl_2 exposure returned ELF ascorbate to control levels. Currently, we are evaluating whether replenishing decreased ascorbate levels in the alveolar spaces and lung tissues of Cl_2 -exposed rats and mice, by post-exposure administration of ascorbate, decreases the extent of alveolar and airway injury up to 7 days after exposure.

CONCLUSIONS

Inhalation of Cl_2 concentrations likely to be encountered in the vicinity of major industrial accidents results in severe lung injury, which may cause death. Ascorbate, an important lung antioxidant in rodents, forms the first line of defense and limits injury to vital components of the alveolar epithelium such as surfactant, epithelial tight junctions, ion channels, cilia, and so on. However, lung ascorbate levels are depleted, which decreases the ability of the pulmonary system to defend from subsequent oxidant insult. Prophylactic administration of ascorbate prevents the decrease of lung ascorbate and blunts lung injury by exposure to Cl_2 at concentrations likely to be encountered in industrial accidents. It remains to be seen whether post-exposure administration of antioxidants will prevent the progression of lung injury in animal models. The clinical challenge is to find noninvasive routes of antioxidant administration, and optimization of delivery methods to mass casualties within a reasonable time frame after an exposure, provided that preliminary work in animal models suggest benefit for the validation of human studies.

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