Free Radical Biology and Medicine 85 (2015) 83–94

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Upregulation of autophagy decreases chlorine-induced mitochondrial injury and lung inflammation

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ARTICLE INFO

Article history:
Received 20 January 2015
Received in revised form
6 March 2015
Accepted 24 March 2015
Available online 13 April 2015

Keywords:
Chlorine
Mitochondrial dysfunction
Lung injury
MitoQ
Trehalose
Autophagy
Bioenergetics
3-Methyladenine
Free radicals

ABSTRACT

The mechanisms of toxicity during exposure of the airways to chlorinated biomolecules generated during the course of inflammation and to chlorine (Cl_2) gas are poorly understood. We hypothesized that lung epithelial cell mitochondria are damaged by Cl_2 exposure and activation of autophagy mitigates this injury. To address this, NCI-H441 (human lung adenocarcinoma epithelial) cells were exposed to Cl_2 (100 ppm/15 min) and bioenergetics were assessed. One hour after Cl_2, cellular bioenergetic function and mitochondrial membrane potential were decreased. These changes were associated with increased MitoSOX signal, and treatment with the mitochondrial redox modulator MitoQ attenuated these bioenergetic defects. At 6 h postexposure, there was significant increase in autophagy, which was associated with an improvement of mitochondrial function. Pretreatment of H441 cells with trehalose (an autophagy activator) improved bioenergetic function, whereas 3-methyladenine (an autophagy inhibitor) resulted in increased bioenergetic dysfunction 1 h after Cl_2 exposure. These data indicate that Cl_2 induces bioenergetic dysfunction, and autophagy plays a protective role in vitro. Addition of trehalose (2 vol%) to the drinking water of C57BL/6 mice for 6 weeks, but not 1 week, before Cl_2 (400 ppm/30 min) decreased white blood cells in the bronchoalveolar lavage fluid at 6 h after Cl_2 by 70%. Acute administration of trehalose delivered through inhalation 24 and 1 h before the exposure decreased alveolar permeability but not cell infiltration. These data indicate that Cl_2 induces bioenergetic dysfunction associated with lung inflammation and suggests that autophagy plays a protective role.

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Introduction

Mitochondria are now emerging as key regulators of cell survival in response to stress [1], but little is known of their role in lung injury. This is particularly important for exposure to reactive toxicants, which can be released in industrial accidents or acts of terrorism. An important example is chlorine (Cl_2), which is a highly irritative and reactive gas produced in large quantities throughout the world and used extensively for pulp bleaching, for waste sanitation, and in the manufacturing of various pharmaceuticals. It also poses a significant threat to public health when inhaled. Between 1940 and 2007, the accidental release of large amounts of Cl_2 in 30 large cities worldwide (such as the train derailment in Graniteville, SC [2]; the industrial accident in a chemical plant near Apex, NC; and the malfunction of Cl_2 delivery systems to a water park near Sacramento, CA, USA (described in the local press)) caused significant lung injury, which in some cases progressed to adult respiratory distress syndrome and death from respiratory failure [2,3]. For example, 60 t of Cl_2 were released in Graniteville, South Carolina, after a train derailment. Average Cl_2 levels during a 30-min exposure period were 6, 868, 837, and 89 ppm at 0.2, 0.5, 1, and 2 km downwind of the epicenter of the accident [4]. Eight persons died before reaching medical care; of the 71 persons hospitalized for acute health effects because of chlorine exposure, 1 died in the hospital. Twenty-five (35%) persons were admitted to the intensive care unit; the median length of stay was 3 days [2]. In addition to these public disasters, from 2000 to 2004, there were about 6000 calls to U.S. poison control centers for Cl_2-related injuries each year.

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http://dx.doi.org/10.1016/j.freeradbiomed.2015.03.039
0891-5849/Published by Elsevier Inc.

Our previous studies suggested increased production of superoxide in the mitochondria of alveolar type II cells exposed to Cl_2 [5]. However, the contribution of mitochondria-originated reactive species to cellular injury after exposure of lung epithelial cells to sublethal concentrations of Cl_2 gas has not been elucidated. Exposure of cells to oxidants causes bioenergetic dysfunction, and increased production of mitochondrial superoxide and hydrogen peroxide can further compromise the healthy mitochondrial population, leading to increased inflammation and, in extreme cases, cell death [6–8].

Animals that survive Cl_2 exposure develop severe reactive airway disease syndrome and mucous hyperplasia [9]. There is no safe exposure to Cl_2: even domestic exposure to low levels of Cl_2 may result in wheezing and exacerbate the clinical outcome of asthma and chronic obstructive pulmonary disease [10]. When inhaled, Cl_2 reacts with water in the epithelial lining fluid and generates hypochlorous (HOCl) and hydrochloric acid (HCl) according to the following equation [11]:

$$Cl_2 + H_2O \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} HOCl + H^+ + Cl^- \quad K_{H2O} = 1.8 \times 10^{-3} M^2 \qquad (1)$$

At the pH of epithelial lining fluid (6.9), more than 99% of inhaled Cl_2 will be converted to HOCl, which exists in equilibrium with its conjugated base hypochlorite (OCl^-). As reviewed previously [12], millimolar concentrations of HOCl may be generated by activated neutrophils and eosinophils by the catalytic actions of neutrophil- and eosinophil-derived peroxidases on chloride (Cl^-) and hydrogen peroxide (H_2O_2) in close proximity to the apical and basolateral membranes of epithelial cells [13,14]. The main targets of HOCl and OCl^- are sulfhydryl groups [15,16], free amino groups of proteins, plasma amino acids [17], and aromatic amino acids (yielding chlorotyrosine [18–20]). In addition, the interaction of HOCl with surface plasmalogens, which exist both in pulmonary surfactant and on the surfaces of epithelial cells, generates a variety of chlorinated lipids, including chlorinated sterols and fatty acids, chlorohydrins, and α-chloro fatty aldehydes [21]. These compounds may propagate injury after the cessation of Cl_2 exposure through damage to amiloride-sensitive epithelial channels, contributing to the formation of pulmonary edema [12]. Our previous studies show that the effects of these reactive intermediates can be partially ameliorated by the administration of low-molecular-weight antioxidants after the cessation of Cl_2 exposure [5,9,22,23]. However, the low bioavailability and lack of targeting to specific regions of oxidative damage limit the efficacy of these interventions.

Proteins or organelles modified by reactive species are targeted for removal by the lysosomal–autophagy systems, and the selective removal of damaged mitochondria by mitophagy is a critical step in maintaining mitochondrial quality control [1,24,25]. However, mitophagy can fail or become overwhelmed by increased mitochondrial superoxide/hydrogen peroxide formation, suggesting that therapeutic intervention to enhance mitochondrial quality control by controlling oxidative stress may be beneficial. In support of this concept, delivery of MnSOD, an antioxidant enzyme targeting the mitochondria, protected cells and animals from hyperoxic lung injury [26,27].

In the present study, we tested the hypothesis that acute exposure of human Clara-cell-like epithelial cells (H441) to Cl_2, in concentrations likely to be encountered near industrial accidents, resulted in the formation of mitochondrial superoxide and affected mitochondrial oxygen consumption, membrane potential, and glycolysis and inhibited the activity of complexes in the mitochondrial transport chain. We then tested the ability of a mitochondria-targeted redox modulator (MitoQ) to mitigate injury to the mitochondria of intact epithelial cells and evaluated the contribution of autophagy in mitigating Cl_2-induced injury to the mitochondria by treating cells with either trehalose (an autophagy activator) or 3-methyladenine (3-MA; an autophagy inhibitor). Finally, we treated animals by administration of oral trehalose over 6 weeks or acute intratracheal trehalose before Cl_2 exposure. We found that chronic treatment with trehalose decreased inflammatory cell infiltration, whereas acute treatment attenuated leakage of protein into bronchoalveolar lavage fluid (BALF). Our data offer new insights into the mechanisms by which Cl_2 damages epithelial cells and suggest that autophagy may play an important role in limiting the extent of injury in vitro and in vivo.

Material and methods

Reagents

Oligomycin, antimycin A, FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone), pyruvate, malic acid, rotenone, sodium azide, succinate, ADP, ascorbate, saponin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 3-MA, *N,N*-diethyl-*p*-phenylenediamine (DPD), and trehalose were purchased from Sigma–Aldrich (St. Louis, MO, USA). MitoSOX red, MitoTracker green, and tetramethyl rhodamine methyl ester (TMRM) were purchased from Invitrogen (Carlsbad, CA, USA). Protease inhibitor cocktail (complete, Mini) was purchased from Roche (Indianapolis, IN, USA). Polyclonal antibodies against microtubule-associated protein 1B light chain 3 (LC3B) was purchased from Sigma–Aldrich. Full-range rainbow molecular weight markers, 4–20% SDS–PAGE gels, and secondary antibodies were obtained from Bio-Rad Laboratories (Hercules, CA, USA).

Cell lines and cell culture

Human airway Clara-cell-like H441 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and were maintained in RPMI 1640 (Roswell Park Memorial Institute, Buffalo, NY, USA) medium (Invitrogen) with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, and 1% penicillin–streptomycin. Cells were kept at 37 °C in a humidified incubator vented with 95% air/5% CO_2 and were used between passages 10 and 20. Cell cycle analysis by FACS revealed that 51% of the cells were in G1, 36% in G2, and 13% in S phase. Removing the FBS for 29 h did not alter the cell cycle. Thus, we opted to culture cells with complete medium for all experimental measurements. For all extracellular flux measurements and Western blot measurements, H441 cells were plated at a seeding density of 40–80,000 cells/well on specialized XF24 tissue culture plates and maintained at 37 °C in a humidified incubator vented with 95% air/5% CO_2 for up to 24 h. For immunofluorescence and FACS analysis studies, H441 cells were cultured on a μ-Dish 35-mm Grid-500 (ibidi, Madison, WI, USA) for 24 h.

Exposure of cells to Cl_2

Immediately before exposure to Cl_2, the normal growth medium was replaced with 50 μl of normal Ringers (120 NaCl, 25 $NaHCO_3$, 3.3 KH_2PO_4, 0.83 K_2HPO_4, and 1.2 mM $CaCl_2$ and $MgCl_2$) containing ascorbic acid (1 mM), reduced glutathione (GSH; 0.12 mM), and urate (0.03 mM) (referred to as the exposure medium). The concentrations of the antioxidants were similar to those in the rat epithelial lining fluid [3,28]. Cells were then placed in a glass chamber inside a water-jacketed incubator maintained at 37 °C and exposed to the desired Cl_2 concentration (75–200 ppm in 5% CO_2) for 15 min as previously described [5]. The presence of 25 mM HCO_3 in the medium and 5% CO_2 in the gas mixture maintained the pH of the exposure medium at 7.4 despite the presence of a significant acid load generated by the hydrolysis of Cl_2. The concentration of Cl_2 in the chamber was measured continuously with an Interscan Corp. (Model RM34-1000 m) Cl_2 detector as described previously [23]. In addition, the consistency of the Cl_2 exposure to 100 ppm for 15 min was assessed