Published in final edited form as:

Toxicol Appl Pharmacol. 2014 July 1; 278(1): 53–64. doi:10.1016/j.taap.2014.02.006.

Acute Chlorine Gas Exposure Produces Transient Inflammation and a Progressive Alteration in Surfactant Composition with Accompanying Mechanical Dysfunction

Christopher B Massa, Pamela Scott, Elena Abramova, Carol Gardner, Debra L Laskin, and Andrew J Gow

Departments: Pharmacology & Toxicology. Ernest Mario School of Pharmacy, Rutgers University

Abstract

Acute Cl₂ exposure following industrial accidents or military/terrorist activity causes pulmonary injury and severe acute respiratory distress. Prior studies suggest that antioxidant depletion is important in producing dysfunction, however a pathophysiologic mechanism has not been elucidated. We propose that acute Cl₂ inhalation leads to oxidative modification of lung lining fluid, producing surfactant inactivation, inflammation and mechanical respiratory dysfunction at the organ level. C57BL/6J mice underwent whole-body exposure to an effective 60 ppm-hour Cl₂ dose, and were sacrificed 3, 24 and 48 hours later. Whereas pulmonary architecture and endothelial barrier function were preserved, transient neutrophilia, peaking at 24 hours, was noted. Increased expression of ARG1, CCL2, RETLNA, IL-1b, and PTGS2 genes was observed in bronchoalveolar lavage (BAL) cells with peak change in all genes at 24 hours. Cl₂ exposure had no effect on NOS2 mRNA or iNOS protein expression, nor on BAL NO₃⁻ or NO₂⁻. Expression of the alternative macrophage activation markers, Relm-α and mannose receptor was increased in alveolar macrophages and pulmonary epithelium. Capillary surfactometry demonstrated impaired surfactant function, and altered BAL phospholipid and surfactant protein content following exposure. Organ level respiratory function was assessed by forced oscillation technique at 5 end expiratory pressures. Cl₂ exposure had no significant effect on either airway or tissue resistance. Pulmonary elastance was elevated with time following exposure and demonstrated PEEP refractory derecruitment at 48 hours, despite waning inflammation. These data support a role for surfactant inactivation as a physiologic mechanism underlying respiratory dysfunction following Cl₂ inhalation.

Keywords

Respiratory impedance; Pulmonary mechanics; Nitric Oxide; Alternative activation; Bronchoalveolar lavage

© 2014 Elsevier Inc. All rights reserved.

Corresponding Author name, e-mail and address: Andrew J Gow, Gow@rci.rutgers.edu, Telephone: (848) 445-4612, Fax: (732) 445-1191, Department of Pharmacology & Toxicology, Ernest Mario School of Pharmacy, Rutgers University, Piscataway, NJ 08854.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Acute inhalation of chlorine gas (Cl_2) is known to produce respiratory dysfunction, although the underlying pathophysiological mechanism remains undefined (Malo *et al.*, 2009). Such exposures - though presently rare - have occurred both in the setting of industrial and transportation accidents as well as in chemical warfare/terrorism scenarios (Jones *et al.*, 2010). Clinically, exposures are characterized by hyperacute respiratory irritation, followed by respiratory distress and increased work of breathing (Leroyer *et al.*, 1998). In the setting of human exposure incidents individuals are subjected to a wide range of doses. Severe exposures may produce acute lung injury, necessitating mechanical ventilation and increasing the likelihood of significant morbidity or death (Hedges and Morrissey, 1979). Among those exposed are also persons receiving lower-doses that produce minimal tissue injury, yet cause respiratory distress that may require mechanical ventilator support (White and Martin, 2010). In these patients the mechanism by which Cl_2 inhalation results in increased work of breathing and organ level dysfunction are unclear (Malo *et al.*, 2009).

In humans, chlorinated oxidant inhalation has principally been examined in the context of recurring, low-dose exposure in either occupational settings or as experienced through common use as swimming pool disinfectants. Such exposures produce asthma-like obstructive lung disease, characterized by chronic cough and airway hyperresponsiveness following methacholine challenge (Bherer *et al.*, 1994). Pathology is thought to involve recurrent disruption of redox homeostasis, with antioxidant depletion and consequent inflammation (Squadrito *et al.*, 2010; Yadav *et al.*, 2010). Recent studies (Martin *et al.*, 2003; Leustik *et al.*, 2008; Tian *et al.*, 2008; Hoyle, 2010; Hoyle *et al.*, 2010; Song *et al.*, 2011; Zarogiannis *et al.*, 2011) have examined the effect of acute high-dose Cl_2 inhalation on pulmonary inflammation and injury in rodents. These studies have employed exposure regimens that produce profound tissue injury, epithelial barrier disruption and alveolar flooding, carrying a high risk of mortality. Treatment with antioxidants (Zarogiannis *et al.*, 2011; Fanucchi *et al.*, 2012), sodium nitrite (Yadav *et al.*, 2011; Samal *et al.*, 2012) and restoration of transepithelial fluid balance through pharmacologic manipulation of cAMP dependent signaling (Song *et al.*, 2011; Chang *et al.*, 2012) have been demonstrated effective at reducing lung pathology and mortality in these exposure conditions. Little is known regarding acute effects of Cl_2 exposure on inflammation and mechanobiology below the threshold for overt injury with epithelial barrier damage.

Due to its high water solubility Cl_2 readily partitions into the lung lining fluid and may exert prolonged or delayed toxicologic effects (Nodelman and Ultman, 1999; Squadrito *et al.*, 2010). The chemistry of Cl_2 in aqueous environments favors the production of both hydrochloric acid and hypochlorous acid/hypochlorite anion (Wang and Margerum, 1994). The pH change that this reaction produces can likely be mitigated by the buffering capacity of the lung lining fluid. Oxidative reactions between hypochlorite and nucleophilic lung lining constituents may also occur, with thiols being particularly sensitive to these oxidations (Squadrito *et al.*, 2010). Disruption of local redox homeostasis may promote the inflammatory response through induction of redox sensitive stress signaling, or as a secondary response to protein oxidation with consequent alteration in function. Through its proposed oxidative reactions with lung lining fluid (Yadav *et al.*, 2010) Cl_2 gas exposure

may result in impaired surfactant activity with consequent mechanical organ dysfunction either from direct oxidative modification of lung lining components or potentially through signaling which alters type II pneumocyte function as a secondary effect.

To examine the proposal that pulmonary dysfunction results from altered lung lining fluid we have employed a murine whole-body Cl₂ gas exposure model that produces inflammation and mechanical dysfunction in the absence of overt injury. As outcomes we have measured the inflammatory response within the tissue and the BAL cells, surfactant composition and function, as well as organ-level lung mechanical function via forced oscillation technique and inverse modeling. Using broadband respiratory impedance measurements with increasing positive end expiratory pressure to recruit collapsed lung units allowed measurement of pathophysiologic changes arising from heterogeneous responses to inhalation. Empiric modeling of respiratory impedance allowed direct comparison of resistance and elastance spectra between exposure conditions, with curve parameterization separating the contributions of proximal airways from distal/tissue components. Comparing measured surfactant function with computational analysis of respiratory mechanics supports the notion that small airway collapse contributes to the physiological mechanism underlying the mechanical dysfunction arising from low-dose Cl₂ inhalation.

Methods

Exposure and ventilation protocol

For a target chlorine exposure of 300 ppm for 1 hour, 1% Cl₂ gas in N₂ (Scott Specialty gasses) was blended with compressed room air (Praxair) at 0.16 L.min⁻¹ and 5 L.min⁻¹ respectively using bubble flow meter. The two gas flow streams were mixed in a round-bottom flask with stir agitation prior to entry into the whole-body exposure chamber and allowed to equilibrate for 10 minutes before mice entered the chamber. All experiments using Cl₂ gas were conducted in a designated fume hood for toxic gas exposures with safety protocols developed in consultation with Rutgers Environmental Health Services. Protocol was developed for proper tank storage, transport, maintenance, regulator use and gas stream purging. As Cl₂ gas is a potent oxidizing agent, traffic near exposures was reduced; personnel in proximity to exposures were informed of risks of exposure as per the MSDS, proper emergency procedure in event of gas leak, required PPE (laboratory coat, gloves and protective eye wear) and monitoring of ambient gas concentration within the hood. All exposures were performed with chamber top closed and 4 outlet ports open to atmospheric air. In-chamber concentrations were monitored via one outlet port both with and without mice for 1 hour, by drawing 0.6 L.min⁻¹ gas directly from the chamber into an ITX-4 multiple gas monitoring system (Industrial Scientific Corporation, Pittsburgh, PA). The Cl₂ concentration-time profile was fit to a logarithmic regression line and integrated in order to determine the effective exposure within the chamber. Pathogen free, male 10-week-old C57BL/6J mice (Jackson Labs, Bar Harbor, ME) received whole body exposure to Cl₂ gas in room air or room air alone for one hour. Five mice were exposed in the chamber at a given time, with animals in each exposure randomized to measurement time points. All

animal procedures were performed in accordance with institutional IACUC approved protocols as outlined in the *NIH Guide for the Care and Use of Laboratory Animals*.

At 3, 24 and 48 hours following exposure mice were anesthetized with 300 µg ketamine/15 µg xylazine per g of body weight via i.p. injection. Mice were tracheostomized and maintained on mechanical ventilation using the Flexivent-small animal ventilator (SCIREQ, Montreal, QC). Mechanical ventilation was performed using a quasi-sinusoidal inspiratory flow waveform to deliver tidal volume of 10 mL/kg of body weight at a rate of 120 breaths/minute and 2:3 ratio of inspiratory to expiratory time. The protocol for measurement of pulmonary mechanical function was created to assess the effect of Cl₂ inhalation on two principal endpoints: A) basal airway constriction as determined by increases in high-frequency resistance in the absence of methacholine challenge, and, B) small airway collapse assessed by an increase in effective pulmonary elastance that may be overcome with airway pressure support. To examine the pressure-responsiveness of these endpoints, measurements of respiratory mechanical function were made in triplicate at 5 levels of Positive End Expiratory Pressure (PEEP = 0, 1, 3, 6, 9 cm H₂O). Prior experiments demonstrated that 1 minute of ventilation is sufficient to allow for mechanical equilibration following step increases in PEEP. At each level of PEEP respiratory mechanical function was assessed using two approaches: broad-band respiratory impedance measurement for the partitioning of mechanical function into tissue and airway components and quasi-static pressure-volume loops for the evaluation of PEEP-dependent changes in effective elastance. Measurement of respiratory impedance and pressure-volume curves are detailed below.

Following ventilation, mice were euthanized by exsanguination and bronchoalveolar lavage (BAL) was performed with 1 ml of isotonic HEPES buffered saline × 4 washes. BAL cells were isolated by centrifugation (300g × 10 minutes) and resuspended in 1 mL of sterile, phosphate buffered saline at pH 7.4 and counted using a Multisizer particle counter (Beckman Coulter, Brea CA) with particles between 4 and 20 µm diameter considered as cells. The BAL cells were spun onto glass slides (30,000 cells/slide, 800 × g for 3 minutes), air dried for 24 hours, methanol fixed then stained with Diff-Quik buffered modified Wright-Giemsa stain for manual analysis of cell differentials. Remaining cells were lysed in Trizol for RNA collection. BAL supernatant was stored at -80 C for protein, nitric oxide (NO) metabolite, phospholipid and surface tension analysis. Right lung lobes were isolated and tied off at the mainstem bronchus, then frozen at -80 C for Western Blot and RT-PCR, while the left lung was inflated fixed with 3% paraformaldehyde in 2% sucrose and paraffin embedded for hematoxylin and eosin (H+E) stain and immunohistochemistry. All reagents purchased from Sigma-Aldrich unless stated otherwise.

Histology and tissue inflammation scoring

Tissue sections were obtained from paraffin embedded lung tissue following inflation/fixation. Sections were H+E stained, examined by light microscopy and scored on a five point scale [0–4] for airway epithelial thickening/membrane blebbing, peribronchial/perivascular infiltration, septal thickening and interstitial destruction/airspace enlargement (Rudmann *et al.*, 1998). The extent of inflammation and injury were independently scored by two blinded observers. Comparison of injury severity scores from Cl₂ exposed lungs to

control was performed using the Wilcoxon Rank sum test and reported as median with 25th and 75th percentile range.

BAL nitrite/nitrate analysis

Cell-free BAL was examined for total protein content and for products of NO oxidation: nitrate (NO₃⁻) and nitrite (NO₂⁻). Diethylenetriamine-pentaacetic acid was added to 300 µL of BAL to reduce the oxidation of nitrite to nitrate. BAL protein concentrations were determined by the Bradford method. NO metabolites were detected using ozone chemiluminescence (Seivers Instruments, Boulder CO) following a chemical reduction by either KI in acetic acid for nitrite detection or VCl₃ in 1M HCl for nitrate. Levels are reported as mean concentrations in µM ± standard deviation and compared ANOVA, followed by Dunnet's post-hoc test vs control where appropriate.

RT-PCR

RNA was prepared from both frozen lung tissue and BAL cells. Frozen lung was pulverized, homogenized by sonication and suspended in Trizol, while BAL cells were isolated by centrifugation and lysed in Trizol. Isolation of RNA was performed by sequential chloroform/2-propanol/70% ethanol extraction. RNA was converted to cDNA by reverse transcription using Superscript III polymerase RT-kit (Invitrogen). cDNA was mixed with random nucleotides and TAQman primers and amplified by thermocycling (AB Biosciences, Allston, MA). All cycle numbers were normalized to β-actin and fold change and reported as mean of treatment condition relative to control by computing the CT.

Immunohistochemistry

Lung sections were deparaffinized in xylene and ethanol and antigen retrieval was performed with 0.1M citrate. Sections were probed for two markers of alternative activation – the Mannose Receptor and RELM-α. Detection of Mannose receptor used a 60-minute incubation of 1:10 dilution of mouse monoclonal antibody (Abcam, Cambridge, MA. Cat# ab64693) with the Vectastain mouse-on-mouse kit (Vector Laboratories, Burlingame, CA). RELM-α detection was achieved using a 1:50 dilution of rat-monoclonal antibody (Alexis Biochemical, Farmingdale, NY. Cat# ALX-211-052). Antigen was visualized using hydrogen peroxide and DAB. Slides were counterstained with hematoxylin and coverslipped. Images were acquired at 200× magnification.

Capillary Surfactometry

To examine the surface active function of the lung lining fluid, BAL was separated into large aggregate (LA) and small aggregate (SA) fractions by centrifugation at 20,000×g for 1 hour. LA phospholipid content was quantified by measuring inorganic phosphate (Rouser *et al.*, 1966) acquired from the lipid phase (Bligh and Dyer, 1959). Total protein content in BAL, as well as LA and SA fractions, was measured by the Bradford method using bovine albumin as a standard. Relative content of specific proteins in BAL was assessed by western blot as described below.

Pulmonary surfactant function was assessed by measuring critical opening pressure in a capillary surfactometer (Liu *et al.*, 1991; Enhorning and Holm, 1993). The capacity of the

surfactant to reduce surface tension was determined by the addition of a volume of LA fraction to produce a phospholipid concentration within the capillary tube equal to 0.5 µg/µl.

Surfactant protein composition

Relative surfactant protein content was determined using western blotting. SP-D content was measured in the whole BAL while SP-B content was measured in large aggregate fraction reconstituted in phosphate free HEPES buffer. BAL samples were denatured in LDS, reduced with DTT, then loaded into 10–20% tricine SDS gradient gels (Invitrogen); whole BAL was loaded with 2 µg total protein per well, while large aggregate fraction was loaded for constant phospholipid level (4 µg/well). Protein was transferred to PVDF membrane and blocked in 10% milk for 45 minutes. Antibodies against SP-B (Guttentag *et al.*, 1998) and SP-D (Cao *et al.*, 2004) were provided courtesy of Michael Beers, University of Pennsylvania. Primary antibodies were diluted in 1% non-fat milk and membranes incubated at 4°C overnight. HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz CA) applied for 2 hours at room temperature in 1% NFM. Membranes were developed following 5 minute incubation with ECL Plus reagent (Amersham) and intensity quantified using Kodak 440 imaging platform. Background corrected SP-B and SP-D signal intensity was determined for each sample and reported as mean ± standard error, normalized to mean intensity of control within each blot. Experimental time points were compared to control using Dunnett's test ($\alpha < 0.05$) following one-way ANOVA.

Respiratory Impedance

Determination of respiratory impedance was performed using the forced oscillation technique, whereby normal tidal ventilation was interrupted and the respiratory system was stimulated using an 8 second, low-amplitude flow signal with pressure measured at airway opening. As single frequency forced oscillation is insufficient in partitioning lung mechanics into the contributions of airway and tissue components, mechanical measurements were made using a broadband input flow signal containing 17 sinusoidal frequencies between 0.5 Hz and 20 Hz. Frequencies were chosen to satisfy non-sum non-difference criteria for minimization of harmonic cross talk. Respiratory impedance (Z_{rs}) was calculated at each frequency, f , by taking the ratio of the Fourier transforms of the Pressure and Flow signal. Z_{rs} spectra are thus represented as complex numbers as a function of frequency and can be decomposed into Resistance (R_L), and Elastance (E_L) spectra as follows:

$$R_L = Re\{Z_{rs}\} \quad \text{Eqn. 1}$$

$$E_L = -(2\pi f)Im\{Z_{rs}\} \quad \text{Eqn. 2}$$

where Re and Im represent the real and imaginary components of Z_{rs} respectively.

To quantify the impact of Cl₂ exposure on airway and tissue mechanical properties, a model fitting approach was employed to characterize the frequency dependence of the impedance spectra. Z_{rs} data have been shown to deviate from the constant phase model (Hantos *et al.*, 1992) in the presence of pathology (Ito *et al.*, 2004; Kaczka *et al.*, 2005), particularly when regional heterogeneity is present (Kaczka *et al.*, 2007). In this setting, application of the

constant phase model often produces significant model error and unphysiologic parameter estimates. At extremes of lung volume, Z_{rs} demonstrated increased frequency dependence – a cardinal sign of heterogeneity (Lutchen *et al.*, 1996; Ito *et al.*, 2004; Kaczka *et al.*, 2007).. As PEEP-dependent changes in Z_{rs} are critical to the evaluation of small airway recruitment spectral comparison and parameterization was performed using an empiric curve-fitting approach (Golden *et al.*, 2012; Groves *et al.*, 2012; Groves *et al.*, 2013).

Based on asymptotic and frequency-dependent behavior of Z_{rs} data in the <20 Hz frequency range, empiric functions have been used for independent characterization of R_L and E_L (Groves *et al.*, 2012). A rational equation was chosen to model R_L due to its ability to reliably represent the high frequency asymptote with a single parameter while characterizing the magnitude and curvature of the frequency-dependent change:

$$\hat{R}_L = \frac{a+bf}{c+f}. \quad \text{Eqn. 3}$$

Using this functional form, estimated values of b predict the contribution of airway caliber to R_L while the ratio of a/c estimates the total effective lung resistance at the static limit. Isolated changes in the value of b result from changes in the contractile state of conducting airways. Independent examination of parameters a and c show differential effects on the frequency dependence of R_L , with changes in a increasing the magnitude of low-frequency dependence and changes in c producing translation of the curve along the frequency axis.

In order to determine the extent of airway collapse in our exposure model the PEEP responsiveness of E_L spectra was examined. E_L spectra were fit to a three parameter exponential equation over the 0.5 to 20 Hz frequency range. Examination of the shape of the E_L curve necessitates a model with defined offset, and a frequency dependent increase to a high-frequency plateau:

$$\hat{E}_L = E_0 + \Delta E(1 - e^{-\beta f}). \quad \text{Eqn. 4}$$

In this model, the intrinsic tissue stiffness in the static limit is given by E_0 , somewhat analogous to the constant-phase model H . Values of ΔE represent the magnitude of change of elastance over the 0.5 to 20Hz frequency range, while values of β reflect the rapidity with which this plateau is reached. Isolated increases in any of these three parameters represent an increase in the effective lung elastance within frequencies which largely contribute to normal breathing (2–3 Hz).

Optimal parameter values to obtain best fit curves were estimated using the Nelder-Mead Simplex method implemented in Matlab's (Mathworks, Natick, MA) `fminsearch` command to minimize the sum of squared residuals, ϕ , between data and model.

$$\phi_R = \sum_{k=1}^{17} \left[\text{Re}\{Z_{RS}(f_k)\} - \frac{a+bf_k}{c+f_k} \right]^2 \quad \text{Eqn. 5}$$

$$\phi_E = \sum_{k=1}^{17} \left[\text{Im}\{Z_{RS}(f_k)\} - (E_0 + \Delta E (1 - e^{-\beta f})) \right]^2 \quad \text{Eqn. 6}$$

Modeled E_L and R_L spectra were directly compared between treatment groups and air-control at each PEEP using the Pearson's χ^2 test. For spectra determined significantly different by χ^2 test, parameter values were compared using Welch's t-test with $\alpha < 0.05$ set as limit of significance.

Quasi-static Pressure-Volume Loops

Following the 3 forced oscillation measurements pressure-volume (PV) curves were produced under quasi-static conditions. As repeated excursions to TLC at each PEEP may alter histology and effect future measurements of pulmonary mechanics, each loop produced submaximal lung inflation, with each PV loop terminating at a pressure 10 cm H₂O above PEEP. Seven pressure-controlled step changes were used to produce both the inspiratory and expiratory limbs of the PV curve. Pressure was maintained for 1.25 seconds per step in order allow sufficient time for mechanical equilibration but minimize the time that mice are not receiving effective ventilation. As PV curves were made above the effective lung volume at each PEEP, the volume axis represents relative volume change within the PV loop, rather than absolute measure of volume or lung capacity. PV loops were repeated in triplicate and the last 2 measurements averaged. In order to determine differences in the work expended in lung recruitment, PV-hysteresis was evaluated by integrating the PV curve to determine the area between the inflation and deflation limbs. Area measurements were compared between conditions and levels of PEEP using a 2-way ANOVA.

PV loops were also used to evaluate the pressure responsiveness of lung recruitment. Effective elastance was determined using the ratio of change in pressure to change in volume produced at each step of the PV loop. Estimated elastance trends have a parabolic relationship with pressure, whereby very low pressures have high elastance owing to derecruitment, while at higher supraphysiologic pressures elastance increases are observed as overdistension of parenchyma produces strain stiffening effects. At some pressure between these extremes a minimum elastance will be reached, reflecting the intrinsic stiffness of the lung tissue at the maximal degree of recruitment. Minimum and maximum elastance values were compared across conditions and PEEP levels using 2-way ANOVA, while the pressure step at which extreme elastance values were reached was compared across conditions by Kruskal-Wallis test at each PEEP.

Results

Monitoring of Cl₂ exposure and determination of effective dose

As Cl₂ gas is potentially reactive within the exposure system (Cheng *et al.*, 2010; Hoyle *et al.*, 2010) we have monitored the in-chamber concentration as a function of time. Concentration measurements were fit to a logarithmic regression curve and integrated over a 1-hour exposure window to quantify the Cl₂ exposure in ppm-hr (Figure 1). When no mice were placed in the container a maximal Cl₂ concentration of 202 ppm was achieved in 1 hour. The addition of 5 mice resulted in a lower rate of increase within the chamber reaching

a maximal concentration of 76 ppm. Integration of these curves throughout the measurement interval gives an effective total exposure of 160 ppm-hr for the empty chamber. With the addition of mice this value is reduced to a 60 ppm-hr exposure, reflecting the interaction of the mice with the Cl₂.

Assessment of pulmonary architecture and epithelial barrier function

High doses of Cl₂ have been shown to injure the pulmonary epithelium and produce endothelial barrier dysfunction in rodents (Tian *et al.*, 2008; Hoyle *et al.*, 2010). The effective exposure used in the current study is lower, as it was designed to avoid epithelial barrier function disruption. Tissue histology shows that the architecture was largely preserved following exposure; a transient increase in airway epithelial thickness, cellular infiltration and a slight interstitial edema were noted relative to control (Figure 2). Few inflammatory foci were observed at 24 hours, with cellular invasion and interstitial edema diminishing by 48 hours. Airway epithelial swelling and luminal irregularity remained at 48 hours, however overall median inflammatory score was not significantly elevated at any exposure time point compared to control (Table 1). In order to assess loss of barrier function resulting from Cl₂ exposure, protein content in the BAL fluid was measured (Table 1). Lack of increase in BAL protein demonstrates preservation of epithelial barrier function and suggests a lack of capillary injury and alveolar flooding.

Activation of innate inflammatory response

The innate inflammatory response within the lung involves activation of sentinel alveolar macrophages with potential recruitment of marginating cells from the blood. A hallmark of such activation is the production of NO, which can be assessed by measurement of its metabolites, NO₃⁻ and NO₂⁻, in the BAL. Neither NO metabolite was increased in the BAL fluid following exposure (Table 1). Despite a lack of epithelial barrier disruption, leukocyte transmigration may proceed in response to inflammatory mediators. To assess the extent by which cellular invasion occurred the cells from the BAL fluid were examined. Total BAL cell number was not significantly elevated at all time points following exposure (Table 1). Manual differential counting of stained cytospin slides of the BAL cells (Figure 3) demonstrated a shift from nearexclusively monocytic cells in control animals to a significant percentage of neutrophils at all time points following exposure (ANOVA, followed by t-test vs. control $p < 0.05$). The neutrophilia peaked at 24 hours following exposure but remained elevated for at least 48 hours in parallel to changes in median cell size and total cell count.

The BAL cell population was examined for markers of general inflammatory signaling, as well as classical and alternative inflammatory activity (Table 2). Transcription of the general inflammatory marker Interleukin 1- β (IL1 β) and Macrophage chemotactic factor C-C cytokine ligand 2 (CCL2) were elevated at 24 and 48 hours after exposure, while Prostaglandin Synthase 2 PTGS2 was elevated at all time points, following the time course of BAL neutrophilia. Both western blot and PCR confirmed the lack of inducible Nitric Oxide Synthase (iNOS) protein and NOS2 gene expression in BAL cells as suggested by NO₃⁻ and NO₂⁻ measurements (western blot data not shown). These data imply that there is minimal classical activation of macrophages in this exposure model. Transcription of Arginase (ARG1), a prototypical marker of alternative macrophage expression, was

dramatically increased at 24 and 48 hours. mRNA expression of the alternative activation subphenotype marker resistin like-alpha (RETNLA) was maximally induced at 24 hours, and remained elevated at 48 hours post exposure. This transcriptional profile is congruent with the induction of the alternative activation state of macrophages (Gordon, 2003; Gordon and Taylor, 2005).

Tissue response to Cl₂ mediated inflammation/oxidative stress

Transcriptional response of lung tissue to Cl₂ exposure was examined by RT-PCR on whole-lung homogenate (Table 2). Contrary to expression profiles seen in BAL cells, the relative expression of IL-1b was diminished relative to control lung tissue, with ELISA on lung tissue homogenate confirming no increase in tissue IL-1β protein (data not shown). Tissue stress may be important in producing the neutrophilia observed post Cl₂ exposure as there is a modest induction of KC, the mouse IL-8 gene analogue, 3 hours after exposure. At later time points lung tissue mRNA expression profiles are consistent with resolution response as the ratio of both NOS2 to ARG1 and CCL-20 to CCL-2 transcript are decreased. Similarly, increased tissue expression of RETNLA suggests adoption of an alternative inflammatory activation state of the respiratory epithelium.

To determine which cell populations within the lung participate in the stress response suggested by the above RT-PCR data, localization of protein expression was performed using immunohistochemical techniques. Low-level expression of the protein Resistin-like1a (RELM-a) was evident in control animals, however, prominent expression was seen in the airway epithelium and retained tissue macrophages most uniformly at 24 hours, with diminishing, heterogeneous expression observed at 48 hours (Figure 4). Mannose Receptor expression was present in the airways of control mice, with Cl₂ exposure increasing expression in both the retained inflammatory cell population and the pulmonary epithelium at both 24 and 48 hour time points (Figure 5).

Determination of surfactant composition and function

Inflammatory signaling may alter type II pneumocyte production and recycling of phospholipid and surfactant proteins. To assess the surface active and immunomodulatory capacity of the lung lining fluid, the relative content of SP-B and SP-D were measured using western blotting. SP-D in the whole BAL was significantly reduced at 3 hours following exposure, but increased at 24 hours (Figure 6). No changes in multimeric state of SP-D were detected on native gel (data not shown). Increase in SP-B content per unit phospholipid in the large aggregate fraction was observed only at 48 hours following exposure. Total phospholipid content of the large aggregate fraction of the BAL was significantly decreased by 20% 24 hours following exposure (ANOVA followed by Welch's t-test vs. control; $p < 0.05$), confirming a reduction in surface-active material within the BAL.

The effect of exposure and the alterations in lung lining fluid composition upon surfactant function were assessed by capillary surfactometry. Opening pressure was significantly increased at 3, 24 and 48 hours post Cl₂ relative to control (ANOVA followed by Welch's t-test vs. control; $p < 0.05$) with the greatest dysfunction at 24 and 48 hours.

Measurement of organ-level lung function

In order to quantify alteration in lung mechanical function, respiratory impedance measurements were obtained using the forced oscillation technique. Loss of surfactant function promotes small airway collapse and impairs reopening (Massa *et al.*, 2008), increasing tissue stiffness as measured by E_L spectra. Fitting Equation 1 to the average E_L spectra from each experimental condition at all levels of PEEP allowed comparison of the mean spectrum to that of control using a χ^2 test. At 3 hours following exposure mean E_L was not significantly elevated (Figure 7). At 24 hours E_L spectra were significantly increased only at a PEEP of 0 cmH₂O. By 48 hours, the respiratory dysfunction had worsened such that the mean E_L spectra at all levels of PEEP were significantly elevated relative to control.

Multiple studies have indicated a bronchoconstriction in response to acute Cl₂ exposure, hence respiratory resistance spectra, were examined by fitting to Equation 2. No significant changes in the overall R_L spectra were observed between groups. Constriction of central airways would be expected to increase R_L at higher frequencies, no significant changes were observed in this region of the spectra. The effect of increasing PEEP was minimal suggesting that recruitment phenomena play little role in effecting R_L in this exposure model.

To evaluate the physiologic mechanism underlying changes in E_L spectra, parameter values estimated from best fit curves were used to partition the effects of Cl₂ on airway and tissue mechanical elements (Table 3). Parameter comparisons were only made at PEEP levels and time points following exposure where overall spectra were determined significantly different from control (Figure 7). As no significant changes were observed between R_L spectra, no comparisons of parameter values between groups were performed. Both the surfactometry analysis and the PEEP dependence of the E_L spectra changes at 24 and 48 hours suggest impaired small airway stability. The parameter E_0 reflects the effective stiffness of the lung in the static limit, and thus increases with either alteration to the intrinsic mechanical properties of the lung or loss of airway stability with consequent lung derecruitment; these effects may be partially differentiated based on PEEP responsiveness. Significant elevation in the value of E_0 was detected 24 hours following exposure at PEEP of 0 and at 48 hours at all levels of PEEP, suggesting a progressive impairment of recruitment with time (Table 3). At both 24 and 48 hours following exposure there was also a significant increase in the frequency dependence factor β at low levels of PEEP, a finding consistent with mechanical heterogeneity of the parenchymal tissue. No significant change was observed in the estimates of E (values not shown). Such PEEP dependent changes in E_L parameters are consistent with those seen in progressive surfactant dysfunction.

To examine the proposal that surfactant function underlies the changes in lung mechanics, the hysteresis and effective elastance change with each step of the quasi-static PV loop were compared between conditions at varying levels of PEEP (Supplemental Figure 1). Hysteresis in the PV loop arises from the expenditure of pressure work needed to recruit collapsed airways, and the redistribution of surfactant. Therefore, alterations in these parameters of the PV loop are predicted to occur with reduced surfactant function. Figure 8 shows the mean PV loops at a PEEP of 1 cm H₂O demonstrating that such increased hysteresis is readily

apparent. Hysteresis was quantified as the area within the curve by integrating volume with respect to pressure. At 24 hours following injury significant increases in PV area were observed across all levels of PEEP, suggesting increased pressure work required to recruit the collapsed airways (Figure 8). Elastance values were estimated from the inspiratory limb at each step of the PV loop for all levels of PEEP (Supplemental figure 2); and the values of the maximal and minimum elastance were compared across exposure conditions and PEEP (Table 4). Minimum elastance trend was significantly lower from control at both 24 and 48 hours by ANOVA, while maximum elastance was lower at all times following exposure. Post-hoc testing on individual PEEP levels showed significant differences across all PEEP levels in the 24 hour condition, while defects at 3 and 48 hours were only seen at the higher levels of PEEP. Comparison of the difference between minimal and maximal elastances showed no change with exposure at any time point.

Discussion

This study uses an acute whole-body low dose exposure model for the assessment of inflammation, surfactant alteration and airway instability following Cl₂ inhalation. This exposure regimen produced transient inflammatory infiltration and mechanical dysfunction in the absence of significant tissue destruction or alveolar flooding. Prominent neutrophilia was observed early following Cl₂ inhalation; however gene expression in both the lung parenchyma and the BAL cells transitions toward a profile consistent with anti-inflammatory/pro-resolution signaling. Surfactant protein and phospholipid composition also altered with concurrent loss of surface-active function by capillary surfactometry. In parallel with these changes is a PEEP responsive increase in pulmonary elastance at 24 and 48 hours following exposure, and altered hysteresis of the PV loop. These data suggest that alteration to surfactant results in small airway stability producing mechanical dysfunction in our Cl₂ exposure model.

Prior studies have determined that whole body exposure to Cl₂ results in epithelial injury and pulmonary inflammation and further that dosing regimens are complex and do not necessarily follow Haber's Law (Hoyle *et al.*, 2010; Zarogiannis *et al.*, 2011). The dosing regimen utilized in this study is lower in both maximum-delivered concentration of Cl₂ and in total ppm-hr than examined previously. Although, it should be noted that our delivered doses are considerably lower than predicted from simple gas mixing, further demonstrating the complexity of these systems. In our study, although there are indications of inflammatory activation, airway thickening and acute neutrophilia, there is no loss of epithelial barrier function, as shown by the lack of protein influx into the BAL. The minimal extent of inflammatory change and relative lack of pulmonary injury in this study appears consistent with this being a lower exposure than those reported in the literature.

The time course of the inflammatory response observed reflects transient activation of the innate immune system, with initial neutrophil migration into the tissue, followed by transition to monocyte chemoattractant expression. Early in this response, increased expression of PTGS2 within the cell population likely reflects the preponderance of neutrophils in the invading population, while CCL2 expression at 24 hours may be responsible for the transition to macrophage recruitment. The expression profile of BAL

cells after 24 hours indicates an alternative macrophage activation state with high ARG1 expression. Increased expression of the genes RETLNA and YM1 reflect signaling which promotes the resolution of inflammation and stimulation of tissue repair (Sandler *et al.*, 2003). Immunohistochemical staining demonstrates pronounced increases in airway expression of Relm- α and the mannose receptor, markers associated with Type-2 inflammatory responses (Gordon, 2003). Such responses are typically mediated by IL-4 and IL-13 and have diverse functional roles, including the resolution of inflammation, tissue repair, fibrosis and antibody mediated and anti-fungal immunity. Recent data demonstrates that higher Cl₂ doses impair response to subsequent *Aspergillus* challenge, resulting in increased cell infiltration, but diminished production of several mediators of antimicrobial immunity: superoxide, IL-17 and IL-22 (Gessner *et al.*, 2013). A potential link between the inflammatory response seen in this exposure model and the pathophysiology of repeated exposure to chlorinated compounds may exist, as airway remodeling and hyperresponsiveness to methacholine are thought to result from repeated stimulation by M2/Th2 type cytokines (Ban and Hettich, 2005). Relm- α has an established role in airway and pulmonary vascular remodeling observed in asthma (Dong *et al.*, 2008) and pulmonary hypertension (Angelini *et al.*, 2009) through effects on recruitment of bone-marrow derived cells (Angelini *et al.*, 2010) and promotion of myofibroblast differentiation (Liu *et al.*, 2004).

Acute Cl₂ exposure was found to produce alterations in the composition and surface active function of the lung lining fluid. The observed increase in SP-D content may reflect a type II pneumocyte response to inflammatory or oxidative stress. Surfactant proteins and phospholipids undergo a complex cycle of production, secretion, reuptake and recycling by the type II cells (as well as scavenging by alveolar macrophages), which may be altered in response to Cl₂ exposure. Despite increases in the content of SP-B per unit phospholipid, the BAL fluid demonstrated loss of surface active function indicating that the lipid-aggregating ability of surfactant protein is significantly disturbed. Higher dosages of Cl₂ have been previously shown to impair BAL surface active function by bubble surfactometry, however this was observed in the setting of overt vascular leak (Leustik *et al.*, 2008). In the setting of vascular leak fibrin and albumin may interfere substantially with surface active function as demonstrated by BAL capillary surfactometry (Enhörning and Holm, 1993). Ingenito and colleagues (Mora *et al.*, 2000; Ingenito *et al.*, 2001) have observed alteration in surfactant composition, with BAL surface tension increases, correlated with significant alteration to Z_{rs} in the early inflammatory phase following acute intratracheal endotoxin exposure. In contrast to our exposure model, where pulmonary injury is limited and mechanical dysfunction subtle, the endotoxin injury demonstrated both more severe mechanical dysfunction associated with increased vascular leak as well as more dramatic alteration of surfactant content and function.

In addition to changes in surfactant composition, oxidative modification of surfactant components is known to alter their mechanical function and signaling roles. *In vitro* studies have shown that oxidation of surfactant components by hypochlorous acid reduces surface active function (Merritt *et al.*, 1993; Rodríguez-Capote *et al.*, 2006) and inhibits bacterial aggregating ability (Crouch *et al.*, 2010). Impairment of surfactant function is expected to

raise the propensity for airway collapse, thus increasing lung stiffness and the work of breathing. Resultant increases in parenchymal stresses occur in areas of collapse and in ventilated lung through generation of higher peak airway pressures; this, in turn, may result in epithelial injury and further inflammation. Oxidative/nitrosative alterations of SP-A and SP-D may also produce direct activation of the innate immune system (Matalon *et al.*, 2009). Inflammation mediated conversion of SP-D to its trimeric form reduces its anti-inflammatory function and initiates proinflammatory signaling in the alveolar macrophage through CD-91/calreticulin mediated NF- κ B activation (Gardai *et al.*, 2003). Oxidative modification in the presence of hypochlorous acid has been shown to cause aberrant SP-D crosslinking and abolish its mannose binding and lipid aggregating activity (Crouch *et al.*, 2010). In addition to altering its own signaling function, this loss of lectin binding ability may increase the fraction of other receptors bound to airway sugar moieties, influencing their signaling role. It is unclear if hypochlorite mediated oxidation of SPA alters its ability to influence macrophage phenotype, suppress phospholipid release from the type-II pneumocyte or induce SP-B and SP-C production.

Several previous studies have examined pulmonary mechanical function following acute Cl₂ exposure. These studies have principally examined methacholine induced airway constriction to examine the effect of Cl₂ exposure on hyper-responsiveness. Most reports have reported an elevated total lung resistance and elastance at a single frequency (Hoyle, 2010; Song *et al.*, 2011; Fanucchi *et al.*, 2012). As measurements made at 2.5 Hz reflect both conducting airway and viscoelastic tissue contributions to resistance, these observations are consistent with either increased airway tone or enhanced small airway collapse (Wagers *et al.*, 2004). Though often attributed to smooth muscle hyper-responsiveness, the effect of small airway closure may be particularly relevant given previous observations of normal basal airway tone (Hoyle *et al.*, 2010), epithelial hyperplasia and increased airway mucus following acute Cl₂ inhalation (Fanucchi *et al.*, 2012). Notably, Hoyle and colleagues have employed both single frequency and broad-band forced oscillation techniques, detecting an increase in total lung elastance at 2.5 Hz, that, when partitioned corresponds to an increase in tissue, but not airway resistance. To date, no study has specifically examined altered propensity for small airway collapse as a function of end-expiratory pressure as a primary endpoint.

The respiratory impedance measurement protocol employed in this study was chosen to measure the sequential responsiveness of recruitment to increasing levels of PEEP. To ensure consistent mechanics measurements across all subjects, sufficient equilibration to a mechanical steady state was allowed prior to Z_{rs} measurement. Due to the inherent path-dependence of lung recruitment on volume history, the introduction of deep inflations was omitted as they are expected to increase the steady-state fraction of open lung (Mead and Collier, 1959). Computational models applied to Z_{rs} data following intratracheal HCl instillation have predicted a parallel increase in both the pressure required to prevent small airways from collapsing as well as those required to re-recruit non-patent distal airways (Massa *et al.*, 2008). With this in mind, the effect of recruitment maneuvers in collapse-predominant injury models is not simply to normalize volume history, but also to alter the plateau elastance following transient stabilization. Accordingly, such a procedure measures

not just PEEP responsiveness but also the responsiveness to deep inflation, an effect we sought to minimize. In this light, the observed PEEP responsiveness following Cl_2 reflects progressive airway recruitment from surpassing critical opening pressures with increasing PEEP.

The constant phase model is widely used for assessment of rodent pulmonary mechanics and has been shown to be reliable in healthy mice. In the presence of pathology the adequacy of the model fit – and even the physiologic interpretation of the parameters – is often compromised (Ito *et al.*, 2004). Here, we developed a robust and sensitive empirical model-fitting technique for identifying toxicological effects on lung function. This approach allows direct comparison of R_L and E_L spectra to identify changes in respiratory impedance following Cl_2 exposure. While this strategy lacks the presumption of a mechanical-model based approach, these empirical curves allow for independent characterization of the entire frequency dependence of R_L and E_L spectra between 0.5 and 20 Hz. Directly testing the ability of the best fit curve of the control data to characterize the treatment groups allows for the features of the measured data - as opposed to populations of model-derived parameters - to determine if a difference in physiological function is present prior to comparison of parameter values. Once spectra are determined to be statistically different from control, comparison of estimated parameters can be used to support a physiological mechanism underlying dysfunction. Although the model parameters are empirically derived and have no explicit mechanical meaning, physiologic relevance can be inferred from the specific effect on the frequency response of Z_{rs} .

Differential responsiveness to recruitment with PEEP was noted in analysis of both Z_{rs} spectra and PV loops with time following exposure. The most significant mechanical alteration was an increase in both magnitude and PEEP refractoriness of E_L spectra. This increase appears to be progressive following Cl_2 exposure as it worsens with time up to a maximum at the 48-hour time point. Changes in the E_0 parameter are reflective of increased effective tissue stiffness in the static limit, a phenomenon likely related to increased airway instability and impaired lung recruitment. In each condition where E_L spectra were different from control the values of E_0 followed a parallel trend of PEEP responsiveness, with efficacy of recruitment diminishing with time following exposure. Interestingly, both maximum and minimum elastance values achieved during the PV loops were significantly reduced in the conditions with increased E_L spectra. These observations can be harmonized by considering that forced oscillation measurements are made at the first pressure beginning the PV loop, and thus represent a more derecruited state than those estimated within the PV loop. The reduction in minimal elastance following treatment may then arise from “cascading” reopening events which occur during the pressure plateau. Such events manifest as greater vertical displacement once the pressure step is achieved and thus increase the PV area, supporting this mechanism at 24 hours. Notably, this coincides with the timing where surfactant phospholipid level is significantly reduced by 20%. At 48 hours the mechanical impairment detected by forced oscillation has worsened, while the PV area is not increased, suggesting that recruitment is PEEP refractory. As recruitment phenomena depend on surfactant components, cellular release and recycling, trafficking about the hypophase and distribution within the lung, PEEP refractory regional collapse may impair global

recruitment when surfactant is sequestered into an inaccessible pool within a ventilation paradigm (Glindmeyer *et al.*, 2012; Amin *et al.*, 2013). At the lowest levels of PEEP where derecruitment is most pronounced, a significant change in the frequency dependence in E_L is observed. Increasing rate of frequency dependent change - represented by the parameter β - shifts the elastance curve to the left, and may represent an increased degree of mechanical heterogeneity.

In these studies no significant change was observed in the magnitude or frequency dependence of low frequency R_L spectra, a phenomenon observed in many lung injury models (Ingenito *et al.*, 2001; Allen *et al.*, 2007). Such changes are attributed to increases in effective energy loss modulus of the lung tissue, typically represented by changes in the parameters G and η in the constant phase model. An increase in tissue loss modulus has been correlated with airway collapse in experimental models including allergic airway hyperresponsiveness, where small airways exhibit significant luminal inflammation along with the dramatic production of mucus (Wagers *et al.*, 2004). This phenomenon has also been observed in severe lung injury models that exhibit appreciable alveolar flooding and damage to alveolar septae (Ingenito *et al.*, 2001; Allen *et al.*, 2007). In the model presented here, the alteration of low frequency E_L spectra in a manner disproportionate to R_L , may be a consequence of mild airway collapse with increased pressure required for reopening, and is indicative of the development of mechanical heterogeneity.

The acute Cl_2 exposure used in this study produced a delayed disruption of both ex-vivo surfactant function and organ-level respiratory function, potentially as a result of altered surfactant. Despite preservation of lung architecture and reduction in inflammatory infiltrate at 48 hours, the mechanical defect continued to worsen. Respiratory distress in Cl_2 exposure may be chemically mediated by TRPA1, independent of injury (Buch *et al.*, 2013), therefore, the progressive nature of lung derecruitment may be of significant clinical relevance in the patient requiring mechanical ventilation. The constellation of increased work of breathing, diminished surface active function and priming of an inflammatory response portend a complicated ventilator course, particularly as late phase mechanical dysfunction demonstrates minimal PEEP responsiveness. Mechanical ventilation can alter lung surfactant composition and function, therefore lung-protective ventilation strategies may prove critical to management of the Cl_2 exposed patient.

This study demonstrates that Cl_2 exposure produces inflammation, alters surfactant and causes mechanical lung dysfunction in the absence of overt histological injury and preserved epithelial barrier function. Changes in surfactant function and composition parallel the timing of a significantly neutrophilic pulmonary infiltrate, peaking at 24 hours post exposure while mechanical dysfunction continues to worsen for at least 48 hours. As direct chemical modification of surfactant by chlorination or oxidation is presumed to be greatest at the 3 hour time point, this is unlikely to be directly responsible for altered respiratory function at 48 hours. The PEEP responsiveness of the pulmonary impedance spectra and model estimated parameters support the theory that impairment in surfactant function produces small airway collapse as the mechanism underlying respiratory dysfunction following this Cl_2 exposure regimen.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by the following grants: NIH HL086621, ES0050022E, ES007148, ES005022, CA132624, HL074115 and AR055073. The authors would like to acknowledge contribution of Dr. Clifford Weisel in the development of the Cl₂ exposure protocol, Alejandro Ruiz for assistance with gas monitoring, as well as expert technical assistance from Dr. CJ Guo and Dr. Angela Groves.

References

- Allen GB, Leclair T, Cloutier M, Thompson-Figueroa J, Bates JHT. The response to recruitment worsens with progression of lung injury and fibrin accumulation in a mouse model of acid aspiration. *AJP - Lung Physiol.* 2007; 292:L1580–L1589.
- Amin SD, Majumdar A, Alkana P, Walkey AJ, O'Connor GT, Suki B. Modeling the effects of stretch-dependent surfactant secretion on lung recruitment during variable ventilation. *J Biomed Sci and Eng.* 2013; 6:61–70.
- Angelini DJ, Su Q, Kolosova IA, Fan C, Skinner JT, Yamaji-Kegan K, Collector M, Sharkis SJ, Johns RA. Hypoxia-Induced Mitogenic Factor (HIMF/FIZZ1/RELM α) Recruits Bone Marrow-Derived Cells to the Murine Pulmonary Vasculature. *PLoS ONE.* 2010; 5:e11251. [PubMed: 20582166]
- Angelini DJ, Su Q, Yamaji-Kegan K, Fan C, Skinner JT, Champion HC, Crow MT, Johns RA. Hypoxia-induced mitogenic factor (HIMF/FIZZ1/RELM α) induces the vascular and hemodynamic changes of pulmonary hypertension. *AJP - Lung Physiol.* 2009; 296:L582–L593.
- Ban M, Hettich D. Effect of Th2 cytokine antagonist treatments on chemical-induced allergic response in mice. *J. Appl. Toxicol.* 2005; 25:239–247. [PubMed: 15895478]
- Bherer L, Cushman R, Courteau JP, Quevillon M, Cote G, Bourbeau J, L'Archeveque J, Cartier A, Malo JL. Survey of construction workers repeatedly exposed to chlorine over a three to six month period in a pulp mill: II. Follow up of affected workers by questionnaire, spirometry, and assessment of bronchial responsiveness 18 to 24 months after exposure ended. *Occup. Environ. Med.* 1994; 51:225–228. [PubMed: 8199662]
- Bligh EG, Dyer WJ. A Rapid Method Of Total Lipid Extraction And Purification. *Can. J. Biochem. Physiol.* 1959; 37:911–917. [PubMed: 13671378]
- Buch T, Schafer E, Steinritz D, Dietrich A, Gudermann T. Chemosensory TRP Channels in the Respiratory Tract: Role in Toxic Lung Injury and Potential as "Sweet Spots" for Targeted Therapies. *Rev. Physiol. Biochem. Pharmacol.* 2013; 27:27.
- Cao Y, Tao JQ, Bates SR, Beers MF, Haczku A. IL-4 induces production of the lung collectin surfactant protein-D. *J Allergy Clin Immunol.* 2004; 113:439–444. [PubMed: 15007344]
- Chang W, Chen J, Schlueter CF, Rando RJ, Pathak YV, Hoyle GW. Inhibition of chlorine-induced lung injury by the type 4 phosphodiesterase inhibitor rolipram. *Toxicol Appl Pharmacol.* 2012; 263:251–258. Epub 2012 Jul 10. [PubMed: 22763362]
- Cheng YS, Bowen L, Rando RJ, Postlethwait EM, Squadrito GL, Matalon S. Exposing animals to oxidant gases: nose only vs. whole body. *Proc Am Thorac Soc.* 2010; 7:264–268. [PubMed: 20601630]
- Crouch EC, Hirche TO, Shao B, Boxio R, Wartelle J, Benabid R, McDonald B, Heinecke J, Matalon S, Belaaouaj A. Myeloperoxidase-dependent Inactivation of Surfactant Protein D in Vitro and in Vivo. *J. Biol. Chem.* 2010; 285:16757–16770. [PubMed: 20228064]
- Dong L, Wang SJ, Camoretti-Mercado B, Li HJ, Chen M, Bi WX. FIZZ1 plays a crucial role in early stage airway remodeling of OVA-induced asthma. *J. Asthma.* 2008; 45:648–653. [PubMed: 18951255]
- Enhorning G, Holm BA. Disruption of pulmonary surfactant's ability to maintain openness of a narrow tube. *J. Appl. Physiol.* 1993; 74:2922–2927. [PubMed: 8365993]

- Fanucchi MV, Bracher A, Doran SF, Squadrito GL, Fernandez S, Postlethwait EM, Bowen L, Matalon S. Post-exposure antioxidant treatment in rats decreases airway hyperplasia and hyperreactivity due to chlorine inhalation. *Am. J. Respir. Cell Mol. Biol.* 2012; 46:599–606. [PubMed: 22162906]
- Gardai SJ, Xiao Y-Q, Dickinson M, Nick JA, Voelker DR, Greene KE, Henson PM. By Binding SIRP α or Calreticulin/CD91, Lung Collectins Act as Dual Function Surveillance Molecules to Suppress or Enhance Inflammation. *Cell.* 2003; 115:13–23. [PubMed: 14531999]
- Gessner MA, Doran SF, Yu Z, Dunaway CW, Matalon S, Steele C. Chlorine gas exposure increases susceptibility to invasive lung fungal infection. *AJP - Lung Physiol.* 2013; 304:L765–L773.
- Glindmeyer HW, Smith BJ, Gaver DP. In situ enhancement of pulmonary surfactant function using temporary flow reversal. *J. Appl. Physiol.* 2012; 112:149–158. [PubMed: 21998268]
- Golden T, Shi JD, Guo CJ, Scott PA, Massa CB, Tu SP, Lee MJ, Yang CS, Gow AJ. γ -Tocopherol as a mechanism to inhibit acute and chronic pulmonary inflammation: Structural and functional consequences. *Nitric Oxide.* 2012; 27(Supplement):S41–S42.
- Gordon S. Alternative activation of macrophages. *Nat. Rev. Immunol.* 2003; 3:23–35. [PubMed: 12511873]
- Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. *Nat. Rev. Immunol.* 2005; 5:953–964. [PubMed: 16322748]
- Groves AM, Gow AJ, Massa CB, Hall L, Laskin JD, Laskin DL. Age-related increases in ozone-induced injury and altered pulmonary mechanics in mice with progressive lung inflammation. *AJP - Lung Physiol.* 2013; 305:L555–L568.
- Groves AM, Gow AJ, Massa CB, Laskin JD, Laskin DL. Prolonged Injury and Altered Lung Function after Ozone Inhalation in Mice with Chronic Lung Inflammation. *Am. J. Respir. Cell Mol. Biol.* 2012; 47:776–783. [PubMed: 22878412]
- Guttentag SH, Beers MF, Bieler BM, Ballard PL. Surfactant protein B processing in human fetal lung. *AJP - Lung Physiol.* 1998; 275:L559–L566.
- Hantos Z, Adamicz A, Govaerts E, Daroczy B. Mechanical impedances of lungs and chest wall in the cat. *J. Appl. Physiol.* 1992; 73:427–433. [PubMed: 1399961]
- Hedges JR, Morrissey WL. Acute chlorine gas exposure. *JACEP.* 1979; 8:59–63. [PubMed: 439544]
- Hoyle GW. Mitigation of chlorine lung injury by increasing cyclic AMP levels. *Proc Am Thorac Soc.* 2010; 7:284–289. [PubMed: 20601633]
- Hoyle GW, Chang W, Chen J, Schlueter CF, Rando RJ. Deviations from Haber's Law for multiple measures of acute lung injury in chlorine-exposed mice. *Toxicol Sci.* 2010; 118:696–703. Epub 2010 Sep 1096. [PubMed: 20819911]
- Ingenito EP, Mora R, Cullivan M, Marzan Y, Haley K, Mark L, Sonna LA. Decreased Surfactant Protein-B Expression and Surfactant Dysfunction in a Murine Model of Acute Lung Injury. *Am. J. Respir. Cell Mol. Biol.* 2001; 25:35–44. [PubMed: 11472973]
- Ito S, Ingenito EP, Arold SP, Parameswaran H, Tgavalekos NT, Lutchen KR, Suki B. Tissue heterogeneity in the mouse lung: effects of elastase treatment. *J. Appl. Physiol.* 2004; 97:204–212. [PubMed: 15020580]
- Jones R, Wills B, Kang C. Chlorine gas: an evolving hazardous material threat and unconventional weapon. *West J Emerg Med.* 2010; 11:151–156. [PubMed: 20823965]
- Kaczka D, Massa C, Simon B. Reliability of Estimating Stochastic Lung Tissue Heterogeneity from Pulmonary Impedance Spectra: A Forward-Inverse Modeling Study. *Ann. Biomed. Eng.* 2007; 35:1722–1738. [PubMed: 17558554]
- Kaczka DW, Hager DN, Hawley ML, Simon BA. Quantifying mechanical heterogeneity in canine acute lung injury: impact of mean airway pressure. *Anesthesiology.* 2005; 103:306–317. [PubMed: 16052113]
- Leroyer C, Malo JL, Infante-Rivard C, Dufour JG, Gautrin D. Changes in airway function and bronchial responsiveness after acute occupational exposure to chlorine leading to treatment in a first aid unit. *Occup. Environ. Med.* 1998; 55:356–359. [PubMed: 9764114]
- Leustik M, Doran S, Bracher A, Williams S, Squadrito GL, Schoeb TR, Postlethwait E, Matalon S. Mitigation of chlorine-induced lung injury by low-molecularweight antioxidants. *AJP - Lung Physiol.* 2008; 295:L733–L743.

- Liu MY, Wang LM, Li E, Enhorning G. Pulmonary surfactant will secure free airflow through a narrow tube. *J. Appl. Physiol.* 1991; 71:742–748. [PubMed: 1938748]
- Liu T, Dhanasekaran SM, Jin H, Hu B, Tomlins SA, Chinnaiyan AM, Phan SH. FIZZ1 stimulation of myofibroblast differentiation. *Am. J. Pathol.* 2004; 164:1315–1326. [PubMed: 15039219]
- Lutchen KR, Greenstein JL, Suki B. How inhomogeneities and airway walls affect frequency dependence and separation of airway and tissue properties. *J. Appl. Physiol.* 1996; 80:1696–1707. [PubMed: 8727557]
- Malo J-L, L'Archeveque J, Castellanos L, Lavoie K, Ghezze H, Maghni K. Long-Term Outcomes of Acute Irritant-induced Asthma. *Am. J. Respir. Crit. Care Med.* 2009; 179:923–928. [PubMed: 19234102]
- Martin JG, Campbell HR, Iijima H, Gautrin D, Malo J-L, Eidelman DH, Hamid Q, Maghni K. Chlorine-induced Injury to the Airways in Mice. *Am. J. Respir. Crit. Care Med.* 2003; 168:568–574. [PubMed: 12724121]
- Massa CB, Allen GB, Bates JHT. Modeling the dynamics of recruitment and derecruitment in mice with acute lung injury. *J. Appl. Physiol.* 2008; 105:1813–1821. [PubMed: 18948446]
- Matalon S, Shrestha K, Kirk M, Waldheuser S, McDonald B, Smith K, Gao Z, Belaaouaj A, Crouch EC. Modification of surfactant protein D by reactive oxygen-nitrogen intermediates is accompanied by loss of aggregating activity, in vitro and in vivo. *FSAEJ.* 2009; 23:1415–1430.
- Mead J, Collier C. Relation of volume history of lungs to respiratory mechanics in anesthetized dogs. *J. Appl. Physiol.* 1959; 14:669–678.
- Merritt TA, Amirkhanian JD, Helbock H, Halliwell B, Cross CE. Reduction of the surface-tension-lowering ability of surfactant after exposure to hypochlorous acid. *Biochem. J.* 1993; 295(Pt 1): 19–22. [PubMed: 8216215]
- Mora R, Arold S, Marzan Y, Suki B, Ingenito EP. Determinants of surfactant function in acute lung injury and early recovery. *AJP - Lung Physiol.* 2000; 279:L342–L349.
- Nodelman V, Ultman JS. Longitudinal distribution of chlorine absorption in human airways: comparison of nasal and oral quiet breathing. *J. Appl. Physiol.* 1999; 86:1984–1993. [PubMed: 10368365]
- Rodríguez-Capote K, Manzanares D, Haines T, Possmayer F. Reactive Oxygen Species Inactivation of Surfactant Involves Structural and Functional Alterations to Surfactant Proteins SP-B and SP-C. *Biophys. J.* 2006; 90:2808–2821. [PubMed: 16443649]
- Rouser G, Siakotos A, Fleischer S. Quantitative analysis of phospholipids by thinlayer chromatography and phosphorus analysis of spots. *Lipids.* 1966; 1:85–86. [PubMed: 17805690]
- Rudmann DG, Preston AM, Moore MW, Beck JM. Susceptibility to *Pneumocystis carinii* in Mice Is Dependent on Simultaneous Deletion of IFN- γ and Type 1 and 2 TNF Receptor Genes. *J. Immunol.* 1998; 161:360–366. [PubMed: 9647244]
- Samal AA, Honavar J, Brandon A, Bradley KM, Doran S, Liu Y, Dunaway C, Steele C, Postlethwait EM, Squadrito GL, Fanucchi MV, Matalon S, Patel RP. Administration of nitrite after chlorine gas exposure prevents lung injury: effect of administration modality. *Free Radic Biol Med.* 2012; 53:1431–1439. Epub 2012 Aug 14. [PubMed: 22917977]
- Sandler NG, Mentink-Kane MM, Cheever AW, Wynn TA. Global Gene Expression Profiles During Acute Pathogen-Induced Pulmonary Inflammation Reveal Divergent Roles for Th1 and Th2 Responses in Tissue Repair. *J. Immunol.* 2003; 171:3655–3667. [PubMed: 14500663]
- Song W, Wei S, Liu G, Yu Z, Estell K, Yadav AK, Schwiebert LM, Matalon S. Postexposure administration of a β_2 -agonist decreases chlorine-induced airway hyperreactivity in mice. *Am. J. Respir. Cell Mol. Biol.* 2011; 45:88–94. [PubMed: 20855648]
- Squadrito GL, Postlethwait EM, Matalon S. Elucidating mechanisms of chlorine toxicity: reaction kinetics, thermodynamics, and physiological implications. *Am J Physiol Lung Cell Mol Physiol.* 2010; 299:L289–L300. [PubMed: 20525917]
- Tian X, Tao H, Brisolara J, Chen J, Rando RJ, Hoyle GW. Acute Lung Injury Induced by Chlorine Inhalation in C57BL/6 and FVB/N Mice. *Inhal. Toxicol.* 2008; 20:783–793. [PubMed: 18645717]
- Wagers S, Lundblad LKA, Ekman M, Irvin CG, Bates JHT. The allergic mouse model of asthma: normal smooth muscle in an abnormal lung? *J. Appl. Physiol.* 2004; 96:2019–2027. [PubMed: 14660507]

- Wang TX, Margerum DW. Kinetics of Reversible Chlorine Hydrolysis: Temperature Dependence and General-Acid/Base-Assisted Mechanisms. *Inorg. Chem.* 1994; 33:1050–1055.
- White CW, Martin JG. Chlorine Gas Inhalation: Human Clinical Evidence of Toxicity and Experience in Animal Models. *Proc Am Thorac Soc.* 2010; 7:257–263. [PubMed: 20601629]
- Yadav AK, Bracher A, Doran SF, Leustik M, Squadrito GL, Postlethwait EM, Matalon S. Mechanisms and Modification of Chlorine-induced Lung Injury in Animals. *Proc Am Thorac Soc.* 2010; 7:278–283. [PubMed: 20601632]
- Yadav AK, Doran SF, Samal AA, Sharma R, Vedagiri K, Postlethwait EM, Squadrito GL, Fanucchi MV, Roberts LJ, Patel RP, Matalon S. Mitigation of chlorine gas lung injury in rats by postexposure administration of sodium nitrite. *AJP - Lung Physiol.* 2011; 300:L362–L369.
- Zarogiannis SG, Jurkuvenaite A, Fernandez S, Doran SF, Yadav AK, Squadrito GL, Postlethwait EM, Bowen L, Matalon S. Ascorbate and deferoxamine administration after chlorine exposure decrease mortality and lung injury in mice. *Am. J. Respir. Cell Mol. Biol.* 2011; 45:386–392. [PubMed: 21131440]

Highlights

- We examine the effects of a 60 ppm*hr Chlorine gas exposure on pulmonary inflammation and mechanical function.
- Pulmonary inflammation is transient and minor.
- Alterations in surfactant homeostasis and pulmonary mechanics are noted.
- No increase in the caliber of larger airways was suggested.
- Small airways stability appears impaired, based on PEEP response of respiratory impedance and pressure-volume relationships.

Figure 1.

Measured Cl₂ concentration differs from target concentration and depends on the presence of mice within the system. In-chamber Cl₂ concentration was monitored in real time by drawing 0.6 L/min chamber gas into an ITX-4 multigas meter. Measurements were made either with no mice or 5 mice within the chamber. Line represents the logarithmic regression of the experimental data ($R^2 = 0.9918$ for no mice, 0.8607 for 5 mice) and is used to calculate the net exposure (ppm $\times$ time) by integrating the area under the curve (160 ppm-hr in the absence of mice and 60 ppm-hr in the presence).

Figure 2.

Cl₂ exposure results in transient inflammation with preserved lung architecture. Inflation fixed lung tissue was paraffin embedded, sectioned and H+E stained. Whole lung field is shown using 8× magnification with 400× inset showing small airways and adjacent parenchyma. (A) 3 hours post exposure. (B) 24 hours post exposure. (C) 48 hours post exposure, (D) Air exposed. Note focal consolidation (arrow) at 24 hours, with inset (C) showing interstitial and airway luminal swelling and cellular infiltration. By 48 hours (D), infiltrate density was diminished and interstitial edema had waned, although airway luminal irregularity persisted.

Figure 3.

Cl₂ exposure produces transient neutrophilia. Cytospin slides were stained with modified Wright-Giemsa stain. Cell differentials were obtained by examination of 5 non-contiguous high power fields per slide. Data are mean $\pm$ SE, $n = 8$ mice per condition. * denotes statistical significance versus Air Exposed ($p < 0.05$ by Dunnet's t -test following one-way ANOVA).

Figure 4. Cl_2 exposure increases Relm- α protein in the pulmonary epithelium. Differential expression and cellular localization of the alternative-activation marker Relm- α was determined by immunohistochemical assay following Cl_2 exposure. (A) 3 Hours, (B) 24 Hours, (C) 48 Hours, (D) Air Exposed.

Figure 5. Cl₂ exposure increases Mannose Receptor protein expression in the pulmonary epithelium and alveolar macrophage. Differential expression and cellular localization of the Mannose Receptor was determined by immunohistochemical assay following Cl₂ exposure. (A) 3 Hours, (B) 24 Hours, (C) 48 Hours, (D) Air Exposed.

Figure 6.

Cl₂ exposure alters BAL surfactant content and function. Upper panel: BAL phospholipid content was determined as described. Middle panel: Surfactant function was assessed by determining initial opening pressure in a capillary surfactometer containing the large aggregate fraction of BAL using equal phospholipid loading across all samples. Lower panel: BAL content of SP-D and SP-B was assessed by western blot. Relative SP-D to SP-B content was estimated by densitometry of the band intensity, normalizing to air exposed, and

calculating the SP-D to SP-B ratio. All data are presented as mean $\pm$ SE, n = 4. * denotes statistical significance versus Air Exposed (p<0.05) using t-test following 1-way ANOVA.

Figure 7.

Cl₂ exposure produces PEEP dependent alteration to lung mechanical function. Lung Elastance (E_L – left column) and Resistance (R_L – right column) are presented between 0.5 and 20 Hz to allow independent partitioning of airway and parenchymal effects. Data points represent the mean $\pm$ SE of the experimental data, n = 8 mice per condition. Smooth lines represent the model fit to the data as described in the methods. Air exposed – filled circles and solid line; 3 hours post Cl₂ exposure – hollow circles and dotted line; 24 hours post Cl₂

exposure – filled square and dashed line; 48 hours post Cl₂ exposure – hollow square and dash and double-dotted line.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 8.

Cl₂ exposure produces PEEP dependent alteration to quasi-static pressure volume relationships. Left: Quasi-static pressure volume loops generated from a PEEP of 1 cm H₂O display increased PV-hysteresis at 24 hours following injury, a trend seen for all levels of PEEP. Curves represent the mean of 8 mice for each exposure condition; variance is not displayed for clarity. PV loops at other levels of PEEP are available in the online supplement. Right: PV loop areas were determined by integrating the volume with respect to pressure as a measure of hysteresis. PV area is significantly elevated at all levels of PEEP at 24 hours following exposure. Data are presented as mean $\pm$ SE.

Table 1

Effect of Cl₂ Exposure on Measures of Pulmonary Inflammation.

	Tissue Inflammation Score Median [25%, 75%]	BAL Protein mg/mL	BAL NO ₂ ⁻ mM	BAL NO ₃ ⁻ mM	BAL Cell Count cells × 10 ⁴
Air Exposed	1 [1,1]	160 ± 74	0.029 ± 0.009	11.31 ± 5.31	7.40 ± 2.85
3 hours post Cl ₂	1 [1,2]	193 ± 44	0.040 ± 0.014	16.60 ± 4.86	14.53 ± 6.17
24 hours post Cl ₂	2 [1,2]	164 ± 12	0.035 ± 0.006	12.41 ± 5.00	33.26 ± 26.04
48 hours post Cl ₂	2 [1,2]	211 ± 63	0.031 ± 0.007	11.48 ± 3.19	17.46 ± 8.97

Measurements of pulmonary inflammation at 3, 24, and 48 hours following Cl₂ exposure (n = 8 per treatment group). Tissue inflammation score is presented as median with 25% and 75% confidence intervals. All other values are mean ± SE. No statistically significant differences are observed following exposure.

Table 2

Effect of Cl₂ exposure on mRNA expression in BAL cells and Lung Tissue Homogenate.

Relative mRNA Expression in BAL Cells (Ct vs Air)						
	IL1b	CCL2	RETNA	YMI	PTGS2	ARG1
3 hours post Cl ₂	0.7	1.8	0.8	0.8	4.8	N.D. 1.9
24 hours post Cl ₂	5.9	402.4	14.6	1.6	33.3	5400.9
48 hours post Cl ₂	2.1	82.8	4.6	0.8	6.9	N.D. 5331.3

mRNA Expression in Lung Tissue Homogenate (Ct vs Air)						
	IL1b	CCL2	CCL20	RETNA	CxCL1(KC)	ARG1
3 hours post Cl ₂	0.48	1.37	10.54	0.57	2.19	0.39
24 hours post Cl ₂	0.57	5.61	0.72	7.68	1.40	0.67
48 hours post Cl ₂	0.23	2.39	0.53	8.75	0.81	0.71

RNA expression of inflammatory markers was assessed in both the BAL cells and in lung tissue homogenate using RT-PCR, Data are presented as fold change over Air Exposed by the Ct method. N.D. represents not detected.

Table 3

Selected Parameter Estimates for E_L Model fitting

	PEEP (cm H ₂ O)	0	1	3	6	9
E_0	Air Exposed	69.4 ± 1.58	67.8 ± 1.79	63.2 ± 1.72	55.5 ± 1.74	61.8 ± 4.09
	3 hours post Cl ₂	73.4 ± 3.85	69.5 ± 2.42	66.2 ± 2.46	56.6 ± 2.33	60.5 ± 3.94
	24 hours post Cl ₂	79.6 ± 3.85*	68.3 ± 2.82	63.3 ± 3.64	59.1 ± 5.47	64.2 ± 9.34
	48 hours post Cl ₂	81.5 ± 4.77*	74.9 ± 2.42	72.2 ± 2.51*	65.6 ± 4.49*	74.7 ± 9.48*
β	Air Exposed	0.09 ± 0.010	0.09 ± 0.009	0.27 ± 0.011	0.39 ± 0.022	0.43 ± 0.017
	3 hours post Cl ₂	0.10 ± 0.009	0.12 ± 0.014	0.27 ± 0.036	0.46 ± 0.025	0.49 ± 0.021
	24 hours post Cl ₂	0.13 ± 0.016*	0.11 ± 0.011	0.26 ± 0.025	0.36 ± 0.016	0.39 ± 0.022
	48 hours post Cl ₂	0.13 ± 0.014*	0.12 ± 0.014	0.30 ± 0.031	0.37 ± 0.009	0.43 ± 0.014

Values for E_0 and β were estimated from E_L spectra at each PEEP and are reported as mean ± SE for each treatment, n = 8 mice per condition.

* denotes statistical significance versus Air Exposed, (p<0.05), by two tailed-t test within the context of significant χ^2 test as described.

Table 4

Maximal and Minimum Elastance Values Estimated from PV Loops

	PEEP (cm H ₂ O)	0	1	3	6	9
Minimum (cm H ₂ O)	Air Exposed	42.4 ± 2.0	45.0 ± 2.5	50.9 ± 4.1	61.4 ± 6.9	96.0 ± 10.4
	3 hours post Cl ₂	42.9 ± 2.1	44.8 ± 2.4	48.6 ± 2.5	51.9 ± 3.0	66.9 ± 6.3
	24 hours post Cl ₂ #	33.9 ± 1.9*	39.3 ± 1.6*	43.8 ± 3.1*	51.1 ± 7.0	69.1 ± 10.6*
	48 hours post Cl ₂ #	42.2 ± 1.0	43.3 ± 1.3	44.8 ± 1.0*	48.1 ± 1.2*	62.4 ± 2.1*
Maximum(cm H ₂ O)	Air Exposed	67.8 ± 3.2	65.4 ± 3.4	64.6 ± 5.1	90.9 ± 5.8	152.9 ± 7.3
	3 hours post Cl ₂ #	66.8 ± 4.2	60.4 ± 4.3	58.4 ± 3.6	74.8 ± 6.1*	131.8 ± 7.6*
	24 hours post Cl ₂ #	70.4 ± 6.2	55.7 ± 3.4*	54.0 ± 5.1*	68.9 ± 7.6*	119.0 ± 7.3*
	48 hours post Cl ₂ #	65.2 ± 2.9	62.9 ± 2.9	57.0 ± 2.2*	62.5 ± 2.7*	113.2 ± 5.8*

Values for minimum and maximum elastance were derived from PV loops at each PEEP and are reported as mean ± SE for each treatment condition. # by exposure condition denotes statistical significance versus Air Exposed by 2-way ANOVA (p<0.05).

* next to data point denotes difference versus Air Exposed at given PEEP by Tukey-Kramer post-hoc test.