Formation of chlorinated lipids post-chlorine gas exposure

David A. Ford¹, Jaideep Honavar², Carolyn J. Albert¹, Mark A. Duerr¹, Joo-Yeun Oh², Stephen Doran⁴, Sadis Matalon⁴,⁵,⁶ Rakesh P. Patel²,⁵,⁶

Department of Biochemistry and Molecular Biology and Center for Cardiovascular Research¹, Saint Louis University, St. Louis, MO

Department of Pathology², Medicine³, Anesthesiology⁴, and Centers for Free Radical Biology⁵ and Lung Injury and Repair⁶, University of Alabama at Birmingham, Birmingham AL 35294

Corresponding Author:
Rakesh P. Patel, PhD
Department of Pathology
University of Alabama at Birmingham
901 19th Street South
BMR-2, room 532
Birmingham
AL 35294
Tel: 205 975 9225
Fax: 205 934 7447
E-mail: rakeshpatel@uabmc.edu

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Abbreviations: ALI (Acute Lung Injury); KC (CXCL1 chemokine); RAS (reactive airway syndrome); MNO (MahmaNonoate); PE (Phenylephrine); 2-Cl-PA (2-chloropalmitate); 2-Cl-PAld (2-chloropalmitaldehyde); 2-Cl-SA (2-chlorostearate); 2-Cl-Sald (2-chlorostearaldehyde)
Abstract

Exposure to chlorine gas can occur during accidents and intentional release scenarios. However, biomarkers that specifically indicate chlorine exposure and chlorine derived products that mediate post-exposure toxicity remain unclear. We hypothesized that chlorinated lipids formed by direct reactions between chlorine gas and plasmalogens serve as both biomarkers and mediators of post-chlorine gas exposure toxicities. 2-chloropalmitaldehyde (2-Cl-Pald), 2-chlorostearaldehyde (2-Cl-Sald) and their oxidized products, free- and esterified 2-chloropalmitic acid (2-Cl-PA) and 2-chlorostearic acid (2-Cl-SA) were detected in the lungs and plasma of mouse and chlorine gas. Levels of chlorinated lipids were highest immediately post-chlorine gas exposure, and declined during the subsequent 72h with levels remaining 20-30 fold higher at 24h compared to baseline. Glutathione adducts of 2-Cl-Pald and 2-Cl-Sald also increased peaking at 4 hours post exposure in plasma. Notably, 3-chlorotyrosine also increased after chlorine gas exposure but returned to baseline within 24h. Intranasal administration of 2-Cl-PA or 2-Cl-Pald at doses similar to those formed in the lung after chlorine gas exposure led to increased distal lung permeability and systemic endothelial dysfunction characterized by loss of eNOS dependent vasodilation. These data suggest that Cl-lipids could serve as biomarkers and mediators for chlorine gas exposure and toxicity.

Keywords: Inflammation, kinetics, lipids/oxidation, lung, nitric oxide

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Introduction

Chlorine (Cl₂) is widely used for various industrial and manufacturing applications and often transported through densely populated areas. Accidental exposure to high doses of these halogens has been reported in mass casualty scenarios worldwide (1-3). In addition, exposure to these halogens remains a concern in the military arena underscoring the need to understand detailed mechanisms of toxicity and for targeted post-exposure therapeutics.

Recent studies have elucidated the temporal toxicity profile resulting from Cl₂ gas exposure. Using murine and rodent models, we and others have shown that post-exposure injury occurs over hours to weeks characterized by acute lung injury (increased endothelial and epithelial permeability and neutrophilic inflammation), pulmonary and systemic vascular dysfunction (loss of nitric oxide homeostasis), reactive airways, pulmonary fibrosis, bronchiolitis obliterans, myocardial and dermal injury (4-13). Chlorine and its hydration product, hypochlorous acid are highly reactive limiting their direct reactions to either low molecular weight antioxidants present in the epithelial lining fluid or targets on the surfaces of epithelial and inflammatory cells (14). This suggests that Cl₂ induced injury to the hyaluronan, present mainly in the lung interstitial matrix as well as to extrapulmonary tissues and vasculature (13, 15-18) are due to the formation of secondary Cl₂-derived intermediates.

Chlorinated lipids derived from plasmalogen oxidation (Cl-lipid) are likely to be produced during chlorine exposure since the lung and surfactant are enriched with plasmalogens. Evidence for lipid chlorination is primarily derived from in vitro / ex vivo studies investigating pathogenic mechanisms of neutrophil -derived reactive chlorine species (e.g. HOCl). The strongest evidence for in vivo formation of Cl-lipid resides with the detection of 2-chloro fatty aldehydes in biological tissues (19). These species are formed by chlorination of the vinyl ether bond of plasmalogens (20) and have been detected during inflammatory diseases including atherosclerosis and myocardial infarction (21-25). Several different 2-chloro-lipids can be directly formed from plasmalogen chlorination including 2-chloropalmitaldehyde (2-Cl-Pald) or 2-chlorostearaldehyde (2-Cl-Sald), which in turn can be oxidized to the corresponding 2-
chloropalmitic acid (2-Cl-PA) and 2-chlorostearic acid (2-Cl-SA) (Figure 1) or reduced to the respective 2-chlorofatty alcohols. Additionally, recent studies have shown 2-Cl-Pald and 2-Cl-Sald are targets for nucleophilic attack by glutathione (GSH) leading to glutathionylated adducts of palmitaldehyde and stearaldehyde respectively(26). Moreover, these fatty acid species have been detected as being free or esterified. Furthermore, emerging studies indicate unique biological effects of Cl-lipid in stimulating inflammation, promoting cell-death and dysfunction in eNOS-signaling (19, 21, 23, 26-30), which are also all features of post-Cl₂ gas toxicity (4, 15). Chlorinated lipids are predicted to be formed via direct reactions of Cl₂ gas with the vinyl ether bond in plasmalogens leading us to hypothesize that these species may be mediators of post-Cl₂ gas toxicity. In this study, we show that Cl-lipid and their GSH adducts are formed after Cl₂ gas exposure, and provide evidence that they are potential mediators of post-exposure toxicity.
Materials and Methods

Materials: Unless stated otherwise all reagents were purchased from Sigma (St. Louis, MO, USA). Male (25-27g, 10-12 weeks of age) C57/bl6 mice and rats were purchased from Harlan (Indianapolis, IN, USA) and kept on 12h light-dark cycles with access to standard chow and water ad libitum prior to and post chlorine gas exposure.

Methods

Mouse or rat exposure to chlorine gas. Whole body exposures of male mice or male rats to Cl₂ gas were performed as previously described (31-33). Exposures were performed with either 2 mice or rats in the same chamber at any one time, and all exposures were performed between 8am-12pm. Exposure conditions were 400ppm Cl₂ in air for 30min using either 400ppm Cl₂ tanks or by mixing 1000ppm Cl₂ gas with compressed air. Flow meters were used to control flow rates to achieve the chamber Cl₂ target concentrations. A bubble flow meter was used to validate their performance on a weekly basis. In each case, immediately following exposure, mice were returned to room air. All experiments involving animals were conducted according to protocols approved by the UAB IACUC. Food and water were provided ad libitum.

Inducing Neutropenia: C57Bl/6 mice were rendered neutropenic as described previously (34) by intraperitoneal injection with 200µg of either anti Ly-6G (clone 1A8)(Bxcel: cat# BE0075-1) or IgG2a Isotype control (Bxcel: cat# BE0089) 24hr prior to Cl₂ gas exposure.

Cl-lipids measurement: Mice were euthanized at various times post-Cl₂ gas exposure using a mixture of ketamine/xylazine (200/10 mg/kg) administered by i.p injection. Blood was collected via cardiac puncture and lungs excised. Blood was centrifuged at 6000 rpm for 5 minutes to obtain the plasma fraction. Lungs and plasma samples were flash frozen in liquid nitrogen and stored at -80°C. Samples were shipped overnight on dry ice to Dr. Ford at St. Louis University. Free and total (i.e. free + esterified) chlorinated lipids shown in Figure 1 were measured as previously described by LC/MS following Dole extraction.
Total lipids were measured by LC/MS after base hydrolysis and esterified Cl-lipids calculated by subtracting free from total. Extractions were performed using 25µl of plasma spiked with 517 fmol of 2-chloro-[d₇,7,8,8]-palmitic acid (2-[d₇]-CIPA) as the internal standard, and for lungs, 40-50 mg of tissue was used, spiked with 20 pmol of 2-[d₆]-CIPA internal standard.

**Measurement of glutathione adducts of 2-Cl-Pald and 2-Cl-Sald:** Plasma and lung samples were analyzed as previously described (26) with modifications. Briefly, 25µL of plasma was spiked with 90 fmols of [d₆]-HDAGSH and 10mg of pulverized lung tissue was spiked with 900 fmols [d₆]-HDAGSH. Both plasma and lung were then extracted according to a similar Bligh and Dyer method as described for the Cl-lipids (35); however, the aqueous layer was saved as the GSH adducts partition to the aqueous layer. The organic layer was subsequently washed with 1 volume MeOH:H₂O (1:1 v:v) and combined with the previous aqueous layer. The combined aqueous layers were diluted with 1/3 volume of H₂O and extracted on a Strata-X followed by ESI-LC/MS/MS quantitation as previously described(26).

**3-[^13]C₉ chlorotyrosine internal standard synthesis:** The 3-[^13]C₉ chlorotyrosine internal standard was synthesized and purified as previously reported (36) with slight modification. Briefly, 2mM L-[^13]C₉ tyrosine was added to 50mM phosphoric acid (pH 2) supplemented with 100mM NaCl in H₂O. NaOCl (final concentration 2 mM) was then added dropwise to the constantly stirring solution and then incubated for 60min at 37°C. Trifluoroacetic acid (TFA) was then added to a final concentration of 0.1% and the 3-[^13]C₉ chlorotyrosine was purified by HPLC on a C18 column (Beckman Ultrasphere, 5 µm resin, 4.6x250mm). Amino acids were applied to the column equilibrated with 0.1% TFA in H₂O and eluted with 0.1% TFA in methanol. Fractions were collected monitoring at 276 nm.

**Tissue and plasma hydrolysis for Chlorotyrosine measurement:** Plasma and lung samples were collected and prepared as previously described (37) with slight modification. For lung analysis, lung tissue was prepared by homogenizing tissue in 100µM DTPA and BHT in H₂O. Approximately 0.5mg of homogenate was then diluted with 100uM DTPA and BHT in H₂O to 500uL. For plasma analysis, 25µl
of plasma was diluted with 100µM DTPA and BHT in H₂O to 500µl. Both diluted lung homogenate and plasma were delipidated twice with a single phase mixture of H₂O/methanol/H₂O-saturated diethyl ether (1:3:8 v/v/v). The pellet was dried under N₂, 10nmols [¹³C₉,¹⁵N₁]tyrosine and 10pmols 3-[¹³C₉]chlorotyrosine were added, and methane sulfonic acid (MSA) was added to a final volume and concentration of 500uL of 4N MSA in water (1% Phenol). The samples were capped, purged with argon, and incubated for 20 hours at 110ºC. Samples were then diluted with 2ml 0.1% TFA and purified on a C18 SPE column (Supelco Discovery DSC-18LT) equilibrated with 0.1% TFA. After loading, the column was washed with 2mL 0.1% TFA, and amino acids were eluted with 2 ml H₂O:methanol (7:3, vol/vol; 30%) supplemented with 0.1% TFA. Samples were then dried by vacuum.

3-chlorotyrosine mass spectrometry: Dried samples were suspended in 200µl H₂O and analyzed by LC-ESI/MS/MS using a Thermo Fisher TSQ Quantum Ultra mass spectrometer (Thermo Fisher, Waltham, MA). For experiments requiring LC/MS, a Thermo Fisher Surveyor LC system was coupled to the Quantum Ultra. LC/MS data analysis was performed using XCalibur software (Thermo Fisher). A Prodigy C-18 column (150x2.0mm ID, 5µm particle) was equilibrated with Solvent A (0.2% Formic Acid in H₂O). 20µl of sample was then injected and amino acids were eluted over a 18 minute linear gradient from 0% solvent B (0.2% Formic Acid in 8:2 ACN: H₂O) to 30% Solvent B. All analytes were analyzed using selective reaction monitoring in positive ion mode. The analyte ions measured were tyrosine (m/z 182.1 → 136.1); [¹³C₉,¹⁵N₁]tyrosine (m/z 192.1→145.1); 3-chlorotyrosine (m/z 216.1→170.1); 3-[¹³C₉]chlorotyrosine (m/z 225.1→178.1); and 3-[¹³C₉,¹⁵N₁]chlorotyrosine (m/z 226.1→179.1. The analyte 3-[¹³C₉,¹⁵N₁]chlorotyrosine was used as a marker for experimentally induced chlorination, but no significant experimentally induced chlorination was observed.

Intranasal administration of Cl-lipids. A 5mM stock of palmitic acid, palmitaldehyde, 2-Cl-PA, 2-Cl-Pald in 100% ethanol was diluted ten-fold in PBS prior to administration to mice. C57bl/6 male mice were divided into 6 groups. The mice were anesthetized using isoflurane and PBS, 10% ethanol or each fatty acid (50µl, equivalent to 25nmol) was administered into the nares (intranasal). Mice were returned to
room air and upon waking returned to their cages and then sacrificed at 6h or 24h post-administration. Mice were provided with food and water *ad libitum*.

**Airway Hyperresponsiveness.** C57bl/6 male mice were mechanically ventilated and challenged with increasing concentrations of aerosolized methacholine. Mice were sedated with xylazine (10 mg/kg i.p.) and anaesthetized with sodium pentobarbital (40 mg/kg i.p.). After the trachea was cannulated with a 16G cannula, mice were connected to ventilator (FlexiVent, Scireq, Montreal, PQ, Canada) and paralyzed using pancuronium chloride (1mg/kg i.p.). Mice were ventilated at a rate of 90 breaths per minute with a positive end-expiratory pressure of 3cm H$_2$O. Increasing concentrations of methacholine (0–20 mg/ml) were administered via aerosolization. From 20 seconds up to 3 minutes after each aerosol challenge, resistance (cmH$_2$O / mL/ s) and elastance (cmH$_2$O / mL) were recorded continuously as previously described(38).

**Acute lung injury measurements** Mice were euthanized with intraperitoneal ketamine and xylazine (100 and 10mg/kg body weight respectively). An incision was made at the neck to expose the trachea and a 3mm endotracheal cannula inserted. Lungs were lavaged with 3 X 1 ml of PBS; ∼1.8-1.9ml was recovered in all groups. Mice were then ex-sanguinated by cardiac puncture for collection of blood. Recovered aliquots of lavage fluid were kept on ice, and centrifuged immediately at 300g for 10 min to pellet cells. Supernatants were removed and stored on ice for protein analysis using the Bio-Rad Protein Assay Reagent Kit compared to BSA standards. Cells were re-suspended in 100μl PBS and counted using a Neubauer hemocytometer. Cells were then placed on slides using a cellspin (Tharmac, Germany) and stained using a two stain set consisting of EosinY and a solution of thiazine dyes (Quik-Stain, Siemens, Washington DC). Differential counts (specifically monocytes, neutrophils, and lymphocytes) were then performed on slides via light microscopy.

**Vessel studies:** Mice were euthanized and aorta excised and sectioned into 2-3mm segments and then used for vessel bioactivity assays. All vessel bioassay studies were performed in indomethacin (5μM) pre-
treated vessel segments and in bicarbonate buffered Krebs Henseleit buffer of the following composition (mM): NaCl 118; KCl 4.6; NaHCO₃ 27.2; KH₂PO₄ 1.2; MgSO₄ 1.2; CaCl₂ 1.75; Na₂ EDTA (ethylenediaminetetraacetic acid) 0.03, and glucose 11.1 and perfused with 21%O₂, 5%CO₂ balanced with N₂. A passive load of 2 grams was applied to all ring segments and maintained at this level throughout the experiments. At the beginning of each experiment ring segments were depolarized with KCl (70mM) to determine the maximal contractile capacity of the vessel. Rings were then washed extensively and allowed to equilibrate and again depolarized with KCl (70mM). The rings were then washed and allowed to re-equilibrate. Vasoconstrictor responses were tested by cumulative addition of phenylephrine (PE) doses ranging from 1nM to 3000nM. Endothelium-dependent vasodilator responses were tested by administering cumulative doses of acetylcholine (Ach), ranging from 1nM to 3000nM after tension development at maximal PE dose. In subsequent experiments, vessels were sub- maximally contracted (50% of KCl response) with PE (300nM-1000nM). When tension development reached a plateau, endothelium–independent vasodilator responses were induced by administering cumulative doses of the nitric oxide donor MAHMANONOOate (MNO).

Statistical Analysis: All results are reported as the mean ± SEM significant differences were calculated as p<0.05 by one-way ANOVA with Dunnett’s multiple comparison post-test or 2-way ANOVA with Bonferroni post test using GraphPad Prism 5. One data point from 72h group for chlorinated lipid time course measurements was excluded based on Grubb’s outlier test (P<0.05).

Results

Chlorine gas exposure increases lung levels of Cl-lipids

We first tested if Cl₂ gas exposure resulted in increases in Cl-lipid. C57bl/6 mice were exposed to Cl₂ gas 400ppm, 30min and then sacrificed at various times (0-72h) thereafter. The predicted initial products of Cl₂ reactions with plasmalogens are the 2-chlorofatty aldehydes. Figure 2 shows that both 2-ClPald and 2-
Cl-Sald are formed in the lungs after Cl₂ gas exposure with the levels being highest immediately post-exposure. Both 2-chlorofatty aldehydes decreased by ~50-75% within 30min post exposure, which remained at a steady state over the next 6hr. For 2-Cl-Pald, levels decreased further at 12h, but still remained 2-fold higher compared to baseline (P<0.05 by t-test) at 24h. Thereafter levels decreased to basal. Similar temporal changes were observed for 2-Cl-Sald.

Figure 3A shows changes in free and esterified 2-Cl-PA and 2-Cl-SA in the lungs; esterified levels representing total minus free. Both of these 2-chlorofatty acids are formed via oxidation of aldehydes shown in Figure 2. Free 2-Cl-PA levels were highest immediately post Cl₂ exposure and then decreased to basal levels over 12h. Interestingly, esterified 2-Cl-PA levels also increased to similar extent as free levels immediately post Cl₂ exposure, but remained at this high level for 6h and then slowly decreased over 72h; esterified 2-Cl-PA were still significantly elevated (8-fold) relative to basal at 72h (p<0.05 by t-test). Also, at all time points between 0.5-72h, esterified 2-Cl-PA levels were greater compared to free levels.

Figure 3B shows changes in free and esterified 2-Cl-SA. Free levels showed a similar temporal profile compared to 2-Cl-PA with levels decreasing after the cessation of exposure. Esterified 2-Cl-SA also increased initially and then decreased over 2h and then reaching a steady state that was maintained over 24h. Notably, levels of 2-Cl-SA were lower compared to 2-Cl-PA.

**Chlorine gas exposure increases circulating levels of Cl-lipids**

Figure 4A-B shows that both free and esterified 2-Cl-PA and 2-Cl-SA increase in the plasma post-Cl₂ exposure. Similar to the lung, levels of free forms were highest immediately post-exposure, decreasing over 6-12h thereafter. Esterified Cl-lipid levels immediately post-exposure trended to be higher than air (p<0.05 by t-test for t=0 and 0.5h post exposure), and increased between 2-6h (reaching significance by 1-way ANOVA), and then decreasing thereafter. Levels at 48h and 72h remained elevated (5-20 fold) compared to air (by t-test). Also, between 2-48h, esterified forms of both 2-Cl-PA and 2-Cl-SA were higher than the respective free forms, and 2-Cl-PA was typically higher than 2-Cl-SA.
Chlorine gas exposure increases glutathione-aldehyde adducts

Our recent studies demonstrate that 2-chloro-fattyaldehydes react with glutathione (GSH) to form aldehyde conjugates with the elimination of chlorine(Figure 1) (26). Fig 3C and 4C show that GSH-adducts with Pald and Sald increase in the lung and circulation respectively. Temporal changes of these adducts are similar to changes in free 2-chloro-lipids in the lung. In the plasma however, formation is slower peaking at 4h and then levels declined thereafter.

Effects of Cl2 gas on Cl-Tyr formation

Fig 3D and 4D shows that Cl2 exposure also increased lung and plasma 3-chlorotyrosine respectively, with levels being highest early post exposure then declining towards basal 12-24h thereafter.

Chlorine gas exposure increases lung and circulating Cl-lipids in rats

Figure 5 shows that Cl-lipid were also increased in rats after similar regiments of Cl2 gas exposure both in the lungs (Fig 5A-B) and the plasma (Fig C-D), with significant increases still evident 24h after exposure. These data underscore the possibility that Cl-lipid formation is uniform across different animal species after Cl2-gas exposure.

Effects of neutropenia on Cl-lipid formation

To delineate the relative contribution of chlorine-gas vs. endogenous PMN (MPO) dependent formation of chlorinated fatty acids, mice were rendered neutropenic and then exposed to Cl2 gas. Figure 6 shows neutropenia had no effect on esterified 2-Cl-fatty acid, 2-Cl-fatty acid aldehydes in the lung, nor on 2-Cl-fatty acids in the plasma. This is most likely due to the fact that levels of chlorinated fatty acids formed by activated neutrophils are orders of magnitude less than those formed by the reaction of inhaled Cl2 and HOCl with plasmalogens.

Effects of Cl-lipid on ALI indices in vivo
To test if Cl-lipids could mediate ALI in vivo, 2-Cl-PA and 2-Cl-Pald or their respective native (non-chlorinated) fatty acids / aldehydes were administered intranasally to mice and ALI assessed 6-24h thereafter. The final nominal administered dose of Cl-lipids was 12pmol / mg lung, which is comparable to maximal levels of 2-Cl-PA and 2-Cl-Pald measured in the lung after chlorine gas exposure (5pmol / mg lung), and we note that the actual concentration of applied Cl-lipid that reach the distal airways is likely to be less than 5% of the intranasal dose. Figure 7A shows that 6h after administration, 2-Cl-PA significantly increased BAL protein compared to palmitic acid alone, with trends towards increased protein for 2-Cl-Pald also noted. No differences between Cl-lipid and respective fatty acid controls nor vehicle controls was observed 24h after administration following this single intranasal administration. Figure 7B shows that both 2-Cl-PA and 2-Cl-Pald significantly increased levels of inflammatory cells in the BAL compared to non-chlorinated fatty acids at 6h, that comprised both macrophages and neutrophils (Figure 6C). No significant differences were observed at 24h, although trends towards increased inflammatory cells are noted. Figure 6D shows that there were no significant changes in the chemokine KC in the BAL 24h after 2-Cl-PA administration.

**Effects of Cl-lipid on airway reactivity**

Airway hypersensitivity is a feature of post-Cl2 gas exposure toxicity. To test if Cl-lipid play a role in this process, Cl-lipid were administered as described above and basal- and methacholine-induced airway resistance and elastance measured 24h thereafter. Figure 8A-B show that methacholine induced a dose-dependent increase in resistance. This was exacerbated with 2-Cl-PA at all methacholine doses, and with 2-Cl-Pald also, albeit only at the highest methacholine dose tested. Fig 8C-D show changes in elastance. Similar to resistance, 2-Cl-PA exaggerated methacholine effects, however 2-Cl-Pald had no effect. Finally, Figure 8E-F show basal effects of Cl-lipid treatment of reactive airways. Small but significant increases in basal resistance and elastance were observed only with 2-Cl-PA.

**Effects of Cl-lipid of systemic vascular dilation**
Our previous studies have shown that eNOS-dependent vasodilation becomes inhibited post Cl₂ gas exposure (15, 16) and other groups have shown that Cl-lipid addition to cultured endothelial cells inhibits eNOS activity (27). We therefore tested eNOS-dependent vasodilation of aorta isolated 24h after intranasal Cl-lipid administration. Fig 9A shows that PE induced contraction in a dose-dependent manner that did not differ among experimental groups. Fig 9B shows that the acetylcholine (Ach) induced vasodilation, which is eNOS-dependent, was similar in aorta isolated from native fatty acid or aldehydes treated mice. This response was significantly blunted (indicated by right shifts in dose-curves) however in aorta isolated from either 2-Cl-PA or 2-Cl-Pald treated mice. Fig 9C shows that MNO-dependent vasodilation was similar in all groups indicated that smooth muscle responsiveness to NO-donors was not altered.
Discussion

During exposure, Cl₂ gas reacts with the airways to cause acute toxicity characterized by airway cell death and activation of pathways leading to post-exposure pulmonary inflammation and increased permeability of the blood gas barrier to plasma proteins. It is likely that the post-exposure phase reflects both tissue responses to the initial insult and ongoing injury to secondary derived species. The mediators and mechanisms remain unclear underscoring the need to understand Cl₂ gas derived products. Cl₂ gas exposure also leads to tissue inflammation and microcirculatory dysfunction in extrapulmonary tissues. For example animals exposed to Cl₂ and returned to room air develop endothelial and myocardial dysfunction and failure (13, 16). This pattern of injury is observed with many chemically distinct inhaled irritants and pollutants (e.g. ozone, particulate matter) (39-42). A key question is how is the primary reactivity of the inhaled species in the lungs transduced to the periphery, with one model proposed being that the initial insult forms a secondary, longer lived species that can egress into the circulation. There are several Cl₂-specific products that may be formed including chloramines (18, 43); here we tested a role for Cl-lipid.

We used mouse and rat models of Cl₂ gas exposure that are sub-lethal but which result in post-exposure ALI, RAS and systemic inflammation. Both 16 carbon and 18 carbon chlorinated fatty acids and aldehydes were formed following Cl₂ gas exposure. For all chlorinated lipid species, the levels were greatest immediately post-exposure suggesting direct formation during the Cl₂ gas exposure. The levels of Cl-lipid decreased after exposure. The kinetics of this decay was not a simple first or second order process however, and was unique to the specific Cl-fatty acid / aldehyde species. That said, three distinct phases for the post-exposure decay could be generalized. The first was a rapid phase in which ~50-70% of the Cl-lipid formed decreased within 30min post-exposure. This was followed by a second phase where levels did not change, or changed very slightly over 6h. Whether this reflects Cl-lipid stability (lack of metabolism), or a steady-state (where formation equals decay and / or wash out) is not known. We speculate lack of metabolism, since eliminating neutrophils, the major source of endogenous chlorinating
species did not change Cl-lipid levels. The final phase was a slow (>12h) decay back to basal levels. An additional consideration is the relationship of free and esterified pools of chlorinated fatty acids. The source of 2-chlorofatty acids is 2-chlorofatty aldehyde (the product of chlorine oxidation of plasmalogens). The biological half-life of the esterified pool is longer than that of the free pool. The longer biological half-life of esterified 2-CIFA in plasma makes this a better biomarker of chlorine gas exposure compared to free 2-CIFA since it was detectable 72 h after exposure. One question that remains is to determine the complex lipid pool esterifying 2-chlorofatty acids. This requires further study but we note that esterified levels were higher than free forms likely reflecting esterified pools as a more stable, perhaps biologically-inert pool, to store bioactive 2-chlorofatty acids.

Importantly, 2-chlorofatty acids also increased in the plasma. For the free forms, levels were highest immediately post-exposure, whereas esterified forms increased post-exposure. This suggests an active esterification of free Cl-lipid and / or egress of esterified Cl-lipid from the lungs to the circulation. As with the lung levels, neutropenia had no effect on plasma Cl-lipid levels suggesting the contribution of PMN-derived myeloperoxidase activity to circulating Cl-lipid levels is minimal. This is consistent with our prior experiences where the levels of Cl-lipid formed in models of PMN-inflammation (21), are still much lower compared to levels observed here with Cl\textsubscript{2} gas formation. Collectively, these data show that plasmalogens are significant targets for Cl\textsubscript{2} gas resulting ultimately in 2-chlorofatty acid production, and also highlight our lack of understanding of how these lipid species are metabolized. In this regard, fatty aldehyde adducts with GSH increased post-chlorine exposure likely due to reactions between 2-chloroaldehydes and GSH as recently described, and confirming the concept that the redox milieu is key in controlling Cl-lipid levels and metabolism (19, 26). We also recognize that there may be a host of other Cl-lipid species formed either directly from Cl\textsubscript{2} gas or after reaction of formed Cl-lipid with biomolecules or chlorinated amino acids which have been shown to damage SERCA and lung ion channels (13, 43), measurement of these is required to fully understand metabolism and fate of formed Cl-lipid.
In addition, circulating levels of Cl-lipid over the first 6h post-exposure were several orders of magnitude higher compared to basal, and remained at least 2-fold higher at 24-48h post exposure. Notably, levels of Cl-lipid only increase by 2-3 fold in response to inflammation (e.g. after Sendai virus exposure or LPS treatment (21, 44) via myeloperoxidase-dependent mechanisms. Formation of high concentrations, ability to detect differences several hours after exposure in a readily accessible compartment i.e. plasma, stability of Cl-lipid to sample freezing, storage and transport, and the high sensitivity of LC-MS to detect Cl-lipid all suggest that Cl-lipid should be considered as selective biomarkers for Cl₂ gas exposure. A consideration in proposing Cl-lipid as biomarkers is how the levels and mechanisms of formation compare to endogenous MPO / HOCl-dependent processes. Cl₂ and HOCl are in equilibrium, and both direct reactivity of Cl₂ in aprotic solvents and HOCl can lead to Cl-lipid formation(20). While there are Cl-lipid and other oxidation products that are unique to Cl₂ and not HOCl reactions (45, 46), it is likely that both Cl₂ and Cl₂-derived HOCl mediate formation of Cl-lipids during Cl₂ gas exposure. A significant role for MPO-derived HOCl can be excluded however based on similar Cl-lipid levels in neutropenic mice exposed to Cl₂ gas. The key point for considering Cl-lipid as biomarkers for Cl₂ gas exposure is that the levels of Cl-lipid formed after Cl₂ gas exposure will be significantly higher than formed during endogenous inflammation. This concept could also be applied for other halogens. Indeed, Br-fatty acids are increased in bromine exposed mice (Matalon et al, unpublished data). We also showed formation of 3-Cl-tyrosine in the lungs and plasma of mice exposed to Cl₂ gas consistent with a recent study showing formation of both 3-Cl-Tyr and di-chloro-tyr after Cl₂ exposure of blood ex vivo (47). Similar to Cl-lipid, 3-Cl-Tyr levels declined 12-24h post exposure, although at 24h, 3-Cl-Tyr levels returned to baseline, whereas Cl-lipid remained 10-20 fold higher suggesting a greater sensitivity for the latter in detecting Cl₂ gas exposure. We also appreciate there are a myriad of other chlorine-dependent products that are likely formed after Cl₂ gas exposure that could be used as biomarkers. While some such as chloramines may mediate Cl₂ gas toxicity (18, 43), their relative short lifetimes in vivo preclude them as useful biomarkers. Future studies need to more extensively compare Cl-lipid with other chlorinated modifications for biomarker assessments.
Beyond a biomarker role, we also evaluated the potential for Cl-lipid to mediate post-Cl₂ gas exposure toxicity. Previous studies have shown that Cl-lipid can elicit cell death in endothelial and neuronal cells, promote cell permeability leading to compromised blood-brain barrier, inhibit endothelial function by inhibiting eNOS-dependent signaling and promote the inflammatory potential of endothelial cells, neutrophils and macrophages (27, 28, 30, 48, 49). The precise mechanisms linking Cl-lipid to these responses remains under investigation but modulation of MAP kinases and perturbation of redox-signaling are likely candidates. Indeed Cl-lipid will be more electrophilic compared to parent fatty acid (50) and fatty acid electrophiles are known to change redox signaling and alter cell function in various inflammatory disease settings. We therefore conducted proof of concept studies to test if intranasal administration (into the lungs) of 2-Cl-PA or 2-Cl-Pald could elicit effects similar to that observed after Cl₂ gas exposure. Changes in lung endothelial and / or epithelial permeability were indicated by increased BAL protein levels, consistent with pro-permeability effects of Cl-lipid. Also, similar to Cl₂ gas, chlorinated lipids increased levels of inflammatory cells, however these were predominantly macrophages, with smaller increases in neutrophils. Cl₂ gas increases both macrophages and neutrophils, with neutrophils being predominant. Consistent with a minimal pro-neutrophil effect, neither 2-Cl-PA nor 2-Cl-Pald had any effect on pro-neutrophilic chemokines. Ongoing studies are exploring the mechanisms by which Cl-lipid increase airway macrophage numbers and suggest regulatory roles on airway immunity. With respect to reactive airways, 2-Cl-16:0FA had a modest effect on basal airway resistance, and increased methacholine sensitivity compared to vehicle or non-chlorinated fatty acid. The 2-Cl-Pald had minimal effects of reactive airways however suggesting distinct 2-Cl-PA may elicit unique responses. More compelling were the extrapulmonary effects of intranasally administered Cl-lipid. Both 2-Cl-PA and 2-Cl-Pald inhibited eNOS-dependent vasodilation in aorta. This effect appears to be endothelial dependent, as NO (generated by the donor, MNO)-dependent vasodilation of the smooth muscle was not affected. These data are consistent with previous studies showing loss of eNOS activity in cultured endothelial cells treated with Cl-lipid and with studies showing a similar loss of eNOS-dependent vasodilation in aorta isolated from Cl₂ gas exposed animals. These data suggest that Cl-lipid can move
from the lung to the periphery to elicit extrapulmonary effects characterized by eNOS inhibition and led to the hypothesis that plasma Cl-lipid formed after Cl\textsubscript{2} gas exposure may mediate extrapulmonary toxicities to the vasculature and heart (13, 15, 16).

We do note several limitations however and appreciate that Cl-lipid alone do not duplicate all Cl\textsubscript{2} gas exposure effects. For example, mice exposed to Cl\textsubscript{2} gas develop hyper responsiveness to methacholine, which is not the case with Cl-lipid administration. The dose of Cl-lipid administered is different to that formed endogenously and other experimental limitations precluded testing of 16 and 18 carbon chlorinated species. Whether there are antagonistic, additive or synergistic interactions between 16 and 18 carbon chlorofatty acids is not known, but testing of the combination is required to better assess potential role of Cl-lipid in Cl\textsubscript{2} gas exposure toxicity. Also, intranasal administration does not only lead to airway deposition and it is possible that some effects could be mediated by direct absorption of Cl-lipid into the blood via vessels perfusing the nares and upper airways. Notwithstanding these limitations, collectively these data suggest that a single instillation of chlorinated palmitate resulted in an increase of airway resistance and alveolar permeability which are also seen in mice exposed to Cl\textsubscript{2} and returned to room air and suggest that targeting of Cl-lipid metabolism to facilitate clearance or limit reactivity may provide a new perspective on therapeutics to limit post-exposure toxicities.
References


Figure Legends

**Figure 1:** Plasmalogen derived chlorine2 and HOCl Oxidation Products. The vinyl ether bond of plasmalogens is targeted by chlorine and HOCl resulting in 2-chlorofatty aldehyde production including 2-chloropalmitaldehyde and 2-chlorostearaldehyde. 2-Chlorofatty aldehydes are either oxidized to the 2-chlorofatty acids, 2-chloropalmitic acid and 2-chlorostearic acid, or reduced to the 2-chlorofatty alcohols, 2-chloropalmitoyl alcohol and 2-chlorostearoyl alcohol. Alternatively, nucleophilic attack of 2-chlorofatty aldehydes by GSH results in either palmitaldehyde or stearaldehyde GSH adduct formation. R1 = C14H29 or C16H33.

**Figure 2:** Chlorine gas exposure increases chlorinated fatty aldehydes in the lung. C57bl/6 mice were exposed to Cl2 gas (400ppm, 30min) and then brought back to room air. At the indicated times thereafter, lungs were collected and 2-Cl-Palmaldehyde (2-Cl-Pald, Panel A) and 2-Cl-Stearaldehyde (2-Cl-Sald, Panel B) measured. Air indicates basal or air exposed mice. ‘0’ time point indicates immediately post-Cl2 exposure. Data shown are mean ± SEM, n=3-4 (except 72h n=2) and expressed relative to lung wet weight. *P<0.05 by 1-way ANOVA with Dunnett’s multiple comparison post-test relative to air.

**Figure 3:** Chlorine gas exposure increases chlorinated fatty acids, GSH-fattyaldehyde adducts and chlorotyrosine in the lung. C57bl/6 mice were exposed to Cl2 gas (400ppm, 30min) and then brought back to room air. At the indicated times thereafter, lungs were collected and free (■) and esterified (■) 2-Cl-palmitic acid (2-Cl-PA, Panel A) and 2-Cl-stearic acid (2-Cl-SA, Panel B) measured. Air indicates basal or air exposed mice. ‘0’ time point indicates immediately post-Cl2 exposure. Data shown are mean ± SEM, n=3-9 (except 72h n=2) and expressed relative to lung wet weight. *P<0.05 by 1-way ANOVA with Dunnett’s multiple comparison post-test relative to air. #P<0.05 by 2-way RM-ANOVA with Bonferroni post-test comparing free and esterified levels at each time. Panel C shows GSH adducts with palmitaldehyde (■) and stearaldehyde (□) in the lung. Data shown are mean ± SEM, n=3-4 (except 72h n=2) and expressed relative to lung wet weight. *P<0.05 by 1-way ANOVA with Dunnett’s multiple comparison post-test relative to air. #P<0.05 by 2-way RM-ANOVA with Bonferroni post-test comparing palmitaldehyde and stearaldehyde-GSH adducts at each time. Panel D shows 3-Cl-Tyr levels normalized to total Tyrosine in lungs. Maximum values at 2h correspond to 6.6 ± 2.8 pmol/mg in lung. All data are mean ± SEM, n=3-4. *P<0.05 by 1-way ANOVA with Dunnett’s multiple comparison post-test relative to air.

**Figure 4:** Chlorine gas exposure increases chlorinated fatty acids, GSH-fattyaldehyde adducts and chlorotyrosine in the circulation. C57bl/6 mice were exposed to Cl2 gas (400ppm, 30min) and then brought back to room air. At the indicated times thereafter, plasma was collected and free (■) and esterified (■) 2-Cl-palmitic acid (2-Cl-PA, Panel A) and 2-Cl-stearic acid (2-Cl-SA, Panel B) measured. Air indicates basal or air exposed mice. ‘0’ time point indicates immediately post-Cl2 exposure. Data shown are mean ± SEM, n=3-4 (except 72h n=2). *P<0.05 by 1-way ANOVA with Dunnett’s multiple comparison post-test relative to air. #P<0.05 by 2-way RM-ANOVA with Bonferroni post-test comparing free and esterified levels at each time. Panel C shows GSH adducts with palmitaldehyde (■) and stearaldehyde (□) in the plasma. Data shown are mean ± SEM, n=3-4 (except 72h n=2). *P<0.05 by 1-way ANOVA with Dunnett’s multiple comparison post-test relative to air. #P<0.05 by 2-way RM-ANOVA with Bonferroni post-test comparing palmitaldehyde and stearaldehyde-GSH adducts at each time. Panel D shows 3-Cl-Tyr levels normalized to total Tyrosine in plasma. Maximum values at 2h correspond to 0.1...
± 0.01 pmol / µl plasma. All data are mean ± SEM, n=3-4. *P<0.05 by 1-way ANOVA with Dunnett’s multiple comparison post-test relative to air.

Figure 5: Chlorine gas exposure increases chlorinated fatty acids in rats. Male Sprague Dawley rats were exposed to Cl₂ gas (400 ppm, 30 min) and then brought back to room air. At the indicated times thereafter, lungs (panels A-B) and plasma (panels C-D) were collected and free and esterified forms of 2-Cl-palmitic acid (2-Cl-PA) and 2-Cl-stearic acid (2-Cl-SA) measured. Data are mean ± SEM (n=3) *p<0.05 relative to air by t-test.

Figure 6: Effects of neutropenia on chlorine gas dependent formation of Cl fatty acids. C57bl/6 mice were treated with anti Ly-6G (clone 1A8) to induce neutropenia or IgG2a Isotype control antibody 24h prior to Cl₂ gas (400 ppm, 30 min) exposure. Mice were then brought back to room air and 2-chlorofatty aldehydes and 2-chlorofatty acids measured in the lung and plasma at the indicated times. Panels A and B show lung 2-chlorofatty aldehyde levels. Panel C-D and E-F show lung and plasma esterified levels of 2-chlorofatty acids. Control = ■, neutropenia = □. Data show mean ± SEM, n=3-4. *P<0.05 relative to respective control by 2-way ANOVA with Bonferroni post test.

Figure 7: Effects of Cl-lipid on lung permeability and inflammation. C57bl/6 male mice were exposed to saline, ethanol, PA, Pald, 2-Cl-PA or 2-Cl-Pald by intranasal administration and lung injury assessed by measuring BAL levels of protein (Panel A) and inflammatory cells (Panel B) 6-24h thereafter. Panel C shows percent of total cells that were macrophages (□) or neutrophils (■). Data are mean ±SEM (n=4-6). *P< 0.05 relative to corresponding native fatty acid by 1-way ANOVA with Tukey post-test. Panel D shows BALF levels of KC chemokine at 6h and 24h post Cl-lipid administration.

Figure 8: Effects of Cl-lipid on airway hyperresponsiveness. C57bl/6 male mice were exposed to ethanol (■), PA (△), Pald (◇), 2-Cl-PA (▼) or 2-Cl-Pald (●) by intranasal administration and basal and methacholine induced airway resistance (A,B,E) and elastance (C,D,F) determined by Flexivent. For clarity, 2-Cl-PA (A,C,E) and 2-Cl-Pald (B,D,F) are shown on separate panels; ethanol control group is the same on each panel. Data are mean ± SEM (n=3-4). For panels A-D, *p<0.05 relative to ethanol, or #p<0.05 relative to parent fatty acid by 2-way RM-ANOVA with Bonferroni post-test. For panels E-F, *p<0.05 by 1-way ANOVA with Tukey post-test.

Figure 9: Effects of Cl-lipid on aortic vasodilation. C57bl/6 male mice were exposed to PA (●), Pald (□), 2-Cl-PA (▲) or 2-Cl-Pald (◇) by intranasal administration. 24h thereafter, aorta were isolated and ex vivo responsiveness to PE-dependent contraction (Panel A); Ach (Panel B) and MNO (Panel C)-dependent relaxation assessed. Data show mean ± SEM (n=3-5). * and # p<0.05 by 2-way RM-ANOVA with Bonferroni post test for PA vs 2-Cl-PA, and Pald vs 2-Cl-Pald respectively.
Figure 1:

Figure 1
Figure 3:

A. 2-Cl-PA

B. 2-Cl-SA

C. FALD-GSH

D. 3-Cl-Tyr
Figure 4:

A. 2-Cl-PA

B. 2-Cl-SA

C. FALD-GSH

D. 3-Cl-Tyr

Figure 4
Figure 6:

A. 2-Cl-Pald

B. 2-Cl-Sald

C. 2-Cl-PA

D. 2-Cl-SA

E. 2-Cl-PA

F. 2-Cl-SA

Figure 6
Figure 7:

A. BAL Protein (μg/ml)

6 hours  24 hours

B. BALF CELLS* 10^4

6 hours  24 hours

C. Percent

6 hours  24 hours

D. pg KC/mL BALF

6 hours  24 hours

Figure 7
Figure 9:

A

B

C

Figure 9