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# 8-Isoprostane is an early biomarker for oxidative stress in chlorine-induced acute lung injury

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ABSTRACT

Inhalation of chlorine ($Cl_2$) may cause oxidative acute lung injury (ALI) characterized by pulmonary edema, pneumonitis, and hyperreactive airways. The aim of the study was to identify possible biomarkers for $Cl_2$-induced ALI.

Female BALB/c mice were exposed to $Cl_2$ for 15 min using two protocols 1) *concentration-dependent response* (25–200 ppm) and 2) *time-kinetics* (2h–14 days post-exposure).

Exposure to 50–200 ppm $Cl_2$ caused a concentration-dependent inflammatory response with increased expression of IL-1β, IL-6 and CXCL1/KC in bronchoalveolar lavage fluid 2–6 h after exposure which was followed by increased lung permeability and a neutrophilic inflammation 12–24 h post-exposure. The early inflammatory cytokine response was associated with a clear but transient increase of 8-isoprostane, a biomarker for oxidative stress, with its maximum at 2 h after exposure. An increase of 8-isoprostane could also be detected in serum 2 h after exposure to 200 ppm $Cl_2$, which was followed by increased levels of IL-6 and CXCL1/KC and signs of increased fibrinogen and PAI-1. Melphalan, a non-oxidizing mustard gas analog, did not increase the 8-isoprostane levels, indicating that 8-isoprostane is induced in airways through direct oxidation by $Cl_2$. We conclude that 8-isoprostane represents an early biomarker for oxidative stress in airways and in the blood circulation following $Cl_2$-exposure.

## 1. Introduction

Chlorine ($Cl_2$) is a powerful oxidizing gas extensively used as bleaching agent, disinfectant, and in the manufacture of industrial chemicals. Due to its high toxicity, $Cl_2$ has historically been used as a chemical weapon and is still considered a terrorist threat and used in armed conflicts globally (United Nations General Assembly 2014; Hemstrom et al., 2016). Inhalation of $Cl_2$ can produce a range of acute pulmonary effects including impaired lung function, inflammatory reactions, increase of epithelial permeability, and airway hyperresponsiveness (AHR) (Evans, 2005; Koohsari et al., 2007; Morris et al., 2005; Tuck et al., 2008; White and Martin, 2010; Williams, 1997). In some individuals the acute lung injury (ALI) evolves into long-term respiratory manifestations, collectively named reactive airway dysfunction syndrome (RADS), that are characterized by persistent cough, wheezing, chest tightness and dyspnea (Brooks et al., 1985). Not all exposed individuals develop long-standing effects like RADS and pulmonary fibrosis, and the biological mechanisms behind the pathological changes still remain unsolved (Lemiere et al., 1997). However, predicting the outcome of long-standing effects, like RADS, in a patient at an early stage is not possible. Therefore, there is a need of an early non-invasive specific diagnostic tool for ALI that enables monitoring the prognosis of delayed effects after exposure and predicting the individual need of early medical treatment to avoid severe consequences such as RADS.

We have previously demonstrated that mice and rats exposed to 200–300 ppm $Cl_2$ develop a neutrophilic pulmonary injury manifested by e.g. edema, cardiovascular effects, pulmonary fibrosis and long-standing AHR in close agreement with clinical findings in humans exposed to $Cl_2$ (Jonasson et al., 2013a, 2013b; Wigenstam et al., 2016, 2015). The acute symptoms of inhaled $Cl_2$ are generally limited to the exposed tissues (Evans, 2005; Kales and Christiani, 2004) but there is also evidence that $Cl_2$ induces systemic inflammatory responses (induced IL-8/KC and IL-6) and cardiovascular effects, e.g. alterations in blood coagulation and fibrinolysis (Luo et al., 2014; Wang et al., 2006; Wigenstam et al., 2015; Yadav et al., 2011; Zarogiannis et al., 2014). From a medical point-of-view, interference with the early response is of significance since treatment with corticosteroids within the first hours

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after a $Cl_2$-exposure is shown to reduce long-term lung injury (Jonasson et al., 2013b; Wigenstam et al., 2015).

Based on previous results, a mouse model of $Cl_2$-induced lung injury was used to investigate early markers of oxidative stress, inflammatory response, disruption of lung barrier integrity, and blood coagulation. As a marker of lipid peroxidation we used the arachidonic acid derivate 8-isoprostane (8-isoPGF$_{2\alpha}$), which is recognized to be a reliable marker of oxidative stress in airways (Roberts and Morrow, 2000). As early markers of inflammatory response the expression of cytokines associated with induction of acute inflammation was analyzed in bronchoalveolar lavage fluid (BALF) and serum (Hosseinian et al., 2015; Ware et al., 2013). The degree of ALI was determined using markers of lung barrier integrity and counting of inflammatory cells in airways. To assess effects on blood coagulation and fibrinolysis we analyzed plasminogen activator inhibitor (PAI-1) and fibrinogen, previously reported by us to be increased in serum of mice exposed for high concentrations of $Cl_2$ (Wigenstam et al., 2015). By using a concentration range of $Cl_2$ (25–200 ppm), concentration-dependencies of the biomarker expression was assessed e.g. whether the increased formation and their appearance in BALF and serum could be associated with ALI and disease severity. The high-level concentration of $Cl_2$, 200 ppm, was considered to be relevant for a scenario of a chemical disaster at an industrial plant or during transportation, resulting in life-threatening lung injuries. Such chemical disaster would also include humans exposed to lower concentrations displaying no observable acute symptoms during the first 24 h although having a potential risk to develop delayed effects.

To study the specificity of the oxidative and inflammatory markers, the responses in the $Cl_2$-induced model were compared with the responses in models of chemical-induced ALI induced by the nitrogen mustard analog, melphalan. Similar to $Cl_2$, melphalan induces an acute airway inflammation when exposed to the lung but without a prominent direct chemical-induced oxidation in target tissues. Studies have shown that melphalan is a possible surrogate for nitrogen mustard causing a neutrophilic airway inflammation with AHR in the acute-phase (1–2 days post-exposure) which turns into a lymphocytic inflammation in absence of AHR in the sub-acute phase (after 14 days) (Ekstrand-Hammarstrom et al., 2011; Wigenstam et al., 2012, 2009).

By the use of these mouse models, the present study was designed to determine the specificity of these biomarkers with the aim to identify markers in serum, BALF and exhaled breath condensate (EBC) that can potentially be used as a diagnostic tools to predict the risk of ALI and long-standing symptoms such as RADS.

## 2. Materials and methods

### 2.1. Animals

Female BALB/c mice and Sprague-Dawley rats (Envigo RMS B.V, Netherlands) were used in this study. After arrival, the animals were allowed to acclimatize for at least one week. All mice were between 10 and 11 weeks old when experiments were initiated and the rats were between 9 and 10 weeks old. The animals were housed under standard laboratory conditions (12 h daylight cycle, 22 °C, 30% relative humidity) and permitted free access to both food (R36, Lantmännen, Sweden) and water. The animals were weighted to monitor their health condition before the experiments and following exposure. The care of the animals and the experimental protocols were approved by the regional ethics committee on animal experiments in Umeå, Sweden.

### 2.2. $Cl_2$-exposure and sampling protocol

Animals were placed in a Battelle inhalation exposure tower for nose-only exposure (EMMS, UK) and were exposed to $Cl_2$ (Air Liquide, Germany; mixture crystal, 0.1 mol-% $Cl_2$, 99.9 mol-% nitrogen) according to previously described protocols (Jonasson et al., 2013a, 2013b; Wigenstam et al., 2016). Based on our previous studies we selected a range of $Cl_2$ concentrations that were believed to cause mild irritation (no evident airway inflammation) to severe sub-lethal lung injury. In the concentration-response study, the mice were exposed to 25 ppm (6.3 ppm h), 50 ppm (12.5 ppm h), 100 ppm (25 ppm h) or 200 ppm (50 ppm h) $Cl_2$ for 15 min (n = 5–7/group) and samples were collected at 2, 6 and 24 h after exposure. In the time-kinetic study the animals were exposed to 200 ppm $Cl_2$ for 15 min (n = 5-7/group) and samples were collected at 2, 6, 12, 24, 48, and 72 h up to 14 days post-exposure. Rats were exposed to 200 ppm (50 ppm h) $Cl_2$ for 15 min according to previously described protocol (Wigenstam et al., 2016) and EBC was collected 24 h after exposure (Fig. 1). Age-matched controls exposed to room air for 15 min were included at each time-point analyzed.

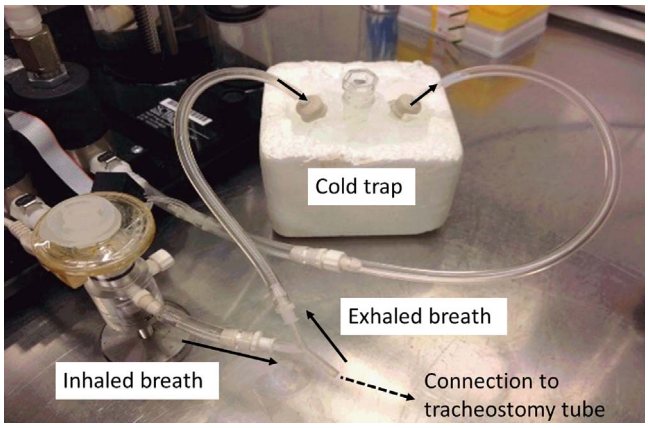


**Fig. 1.** Collection of EBC.
Experimental setup for collection of exhaled breath condensates (EBC) using a cold trap. Expired breath was sampled during 45 min from anesthetized and tracheostomized rats mechanically ventilated with a small animal ventilator.

### 2.3. Collection of EBC

Exhaled breath condensate was sampled from $Cl_2$-exposed and control rats anesthetized with pentobarbital sodium (50 mg/kg body weight (b.w)). The rats were tracheostomized with a 15-gauge cannula and mechanically ventilated with a small animal ventilator (flexiVent™, SCIREQ®) at a frequency of 90 breaths/min and a tidal volume of 10 ml/kg b.w. A positive end-expiratory pressure of 3 cm$H_2O$ was applied and the rats were paralyzed with pancuronium (0.1 mg/kg b.w). Expired air from the ventilated animal was lead through a cold trap consisting of a pear-shaped glass flask for distillation with two necks immersed in ice (Fig. 1). The connection of the ventilator tubing to the glass flask was carefully sealed and EBC was collected during 45 min, yielding approximately 150 µl of condensate from each individual rat (n = 4–5/group). The condensates were immediately frozen and stored at −80 °C until analysis.

### 2.4. Melphalan-exposure and sampling protocol

Melphalan (4-[bis(2-chloroethyl)amino]-1-phenylalanine) (Sigma-Aldrich, St Louis, MO) was dissolved in phosphate-buffered saline (PBS, Sigma-Aldrich) according to the protocol described by Wigenstam et al. (Wigenstam et al., 2012). Melphalan (50 µl) was administered by intratracheal (i.t.) instillation to anesthetized mice at a dose of 1 mg/kg b.w (n = 6–7/group). Control mice received vehicle alone (n = 5–7 at each time-point analyzed). Samples were collected and evaluated at different time-points (2, 6, 12, 24, 48, and 72 h) after exposure.

### 2.5. Serum sampling and collection of BALF

Mice were anesthetized using isoflurane and blood was collected through orbital punction. After retrieving blood samples the animals