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Acute respiratory changes and pulmonary inflammation involving a pathway of TGF-β1 induction in a rat model of chlorine-induced lung injury

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ABSTRACT

We investigated acute and delayed respiratory changes after inhalation exposure to chlorine (Cl_2) with the aim to understand the pathogenesis of the long-term sequelae of Cl_2-induced lung-injury.
In a rat model of nose-only exposure we analyzed changes in airway hyperresponsiveness (AHR), inflammatory responses in airways, expression of pro-inflammatory markers and development of lung fibrosis during a time-course from 5 h up to 90 days after a single inhalation of Cl_2. A single dose of dexamethasone (10 mg/kg) was administered 1 h following Cl_2-exposure.
A 15-min inhalation of 200 ppm Cl_2 was non-lethal in Sprague-Dawley rats. At 24 h post exposure, Cl_2-exposed rats displayed elevated numbers of leukocytes with an increase of neutrophils and eosinophils in bronchoalveolar lavage (BAL) and edema was shown both in lung tissue and the heart. At 24 h, the inflammasome-associated cytokines IL-1β and IL-18 were detected in BAL. Concomitant with the acute inflammation a significant AHR was detected. At the later time-points, a delayed inflammatory response was observed together with signs of lung fibrosis as indicated by increased pulmonary macrophages, elevated TGF-β expression in BAL and collagen deposition around airways. Dexamethasone reduced the numbers of neutrophils in BAL at 24 h but did not influence the AHR.
Inhalation of Cl_2 in rats leads to acute respiratory and cardiac changes as well as pulmonary inflammation involving induction of TGF-β1. The acute inflammatory response was followed by sustained macrophage response and lack of tissue repair. It was also found that pathways apart from the acute inflammatory response contribute to the Cl_2-induced respiratory dysfunction.

1. Introduction

Chlorine (Cl_2) is a highly reactive oxidizing toxic gas which is extensively produced in numerous industrial applications. Cl_2 is often transported as pressurized liquid which in the worst accidental case may cause large-scale exposures of toxic gas concentrations to the residents in the surrounding area. Accidental release of Cl_2 from industrial plants and during transportation may cause a large number of casualties due to the high toxicity e.g. the train derailment of a Cl_2 tanker in Graniteville in 2005 which resulted in several hundred injuries of which nine were fatal (Van Sickle et al., 2009; Mackie et al., 2014). Furthermore, Cl_2 has historically been used as a chemical weapon and is still considered a terrorist threat (Winder, 2001; Szinicz, 2005; Jones et al., 2010).

The primary cause of Cl_2 toxicity is believed to be due to direct contact with the respiratory epithelium, leading to oxidative and acidic tissue injury. Dependent on the inhaled concentration and the exposure time, there are both acute and long-term respiratory consequences of Cl_2 inhalation (Winder, 2001; White and Martin, 2010; Yadav et al., 2010a; Jonasson et al., 2013a). In contact with water, chlorine produces hydrochloric and hypochlorous acids, which can lead to a variety of respiratory injuries both in upper and lower airways. Exposure to low concentrations of Cl_2 is predominantly reported to target the upper airways while high-level exposure of Cl_2 has been associated with both airway and alveolar injuries. The injuries in lower airways are likely contributing to the observed long-term effects and decreased respiratory compliance. It is also assumed that high concentration of Cl_2 is required to produce a fibrotic repair in response to the lung injury. It should also be noted that the effects of Cl_2-inhalation are dependent not only on concentration but also on duration of e.g. exposure, respiratory rate and host susceptibility (Williams, 1997; Evans, 2005; White and Martin, 2010; Hoyle and Svendsen, 2016). The acute symptoms are generally reported to be limited to the exposed lung tissue (Kales

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and Christiani, 2004; Evans, 2005) but recent studies have demonstrated also cardiovascular effects by inhaled Cl_2, e.g. coagulation abnormalities and myocardial depression (Honavar et al., 2011; Luo et al., 2014b; Wigenstam et al., 2014; Zarogiannis et al., 2014; Zaky et al., 2015; Carlisle et al., 2016). The long-term sequelae of Cl_2-exposures are generally described as reactive airways dysfunction syndrome (RADS), bronchiectasis, decline in lung volumes and increased airways resistance (Williams, 1997; Demnati et al., 1998; Martin et al., 2003; Morris et al., 2005; Koohsari et al., 2007; Tuck et al., 2008; Chierakul et al., 2013; Clark et al., 2015). RADS is clinically characterized by signs and symptoms of asthma including cough, wheezing, chest tightness, and breathlessness. The onset of symptoms occurs within 24 h after exposure and persists for at least three months (Evans, 2005; Brooks and Bernstein, 2011).

In previous investigations, we have used mice to study acute and long-term effects by Cl_2 inhalation with and without medical treatment. Many of the toxic signs found in those studies are similar to symptoms described for RADS in humans e.g. the inflammatory response in lung tissue and regeneration of the lung epithelium during the first seven days after exposure to Cl_2 as well as the persistence of hyperreactive airways for at least 28 days together with a deposition of collagen in the lung (Jonasson et al., 2013a,b). We have also shown that the inflammatory response in the Cl_2-exposed mice is not solely limited to the lung as a systemic repercussion (coagulation and fibrinolytic abnormalities) was identified (Wigenstam et al., 2014). Administration of corticosteroids within the first hours after a Cl_2-exposure in mice significantly reduces the acute inflammatory response and the long-term lung injury (Jonasson et al., 2013b).

Lung exposure of Cl_2 induces activation of a cascade of events that may lead to excessive connective tissue formation (Kovacs and DiPietro, 1994) and in the location of severely damaged tissue fibroblast produce collagen to replace lost and damaged tissue. The recovery of a damaged lung with restoration of normal lung structure and function, depends on various factors such as the extent of the damage to lung tissue as well as the recovering capacity (Geiser, 2003; Lindsay, 2011). Previous studies performed in rats have shown both short-term and long-term effects after Cl_2-exposure with and without medical treatment (Demnati et al., 1998; Yildirim et al., 2004; Akdur et al., 2008; Yadav et al., 2010a; Fanucchi et al., 2012; Samal et al., 2012). In those studies, however, no long-term evaluation of pro-fibrotic and inflammatory mediators was performed in airways or serum.

In the present study a nose-only exposure system was used together with a small animal ventilator in order to evaluate changes in respiratory function and development of airway hyperresponsiveness (AHR) after Cl_2-exposure in rats. With the aim to investigate the role of the acute inflammation in the development of AHR and long-term lung fibrosis, treatment with the anti-inflammatory corticosteroid dexamethasone (DEX) was additionally performed 1 h after Cl_2-exposure. An extensive evaluation of inflammatory cells in airways and edema formation in lung, heart and liver tissue was performed, as well as analysis of pro-fibrotic and inflammatory mediators in BAL and serum.

2. Material and methods

2.1. Animals

Female Sprague-Dawley (SD) rats (8–9 weeks old, Envigo RMS B.V., Netherlands) were used in this study. Rats were housed in plastic cages with absorbent bedding material and were maintained on a 12 h daylight cycle, 22 °C, with a 30% relative humidity. Food (R36, Lantmännen, Sweden) and water were provided ad libitum. All rats were weighed before subjected to Cl_2, and following exposure their health condition was monitored. The experiment terminated 5 h, 24 h and 14, 28 and 90 days post exposure ($n = 5–10$ rats per group). 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 protocol

Animals were placed in individual nose-only containers (EMMS, UK) and coupled to an inhalation tower (Battelle) providing equal and simultaneous exposure to Cl_2 (Air Liquide, Germany; compressed gas in gas cylinders: 1 mol% Cl_2, 99 mol% nitrogen). The compressed gas mixture was diluted with air to the final concentration of 200 ppm. Rats were subjected to a single exposure of Cl_2-gas mixture during 15 min. The Cl_2 concentration in the inhalation tower was monitored throughout the exposure time and the experiments were conducted in a designated fume hood for toxic gas exposures. Control animals were breathing room air for 15 min.

2.3. DEX-treatment

DEX (dexamethasone 21-phosphate disodium salt, Sigma-Aldrich) was administered intraperitoneally (10 mg/kg body weight, i.p.) 1 h following exposure to Cl_2. DEX was diluted in phosphate-buffered saline (PBS, Sigma-Aldrich (St. Louis, MO, USA)) to a volume of 400 μl. The dose of DEX was determined from Luo et al. (Luo et al., 2014a). The experiment terminated 24 h or 14 days post exposure ($n = 6$ mice per group).

2.4. Respiratory mechanics

Animals were weighed and anesthetized with pentobarbital sodium (50 mg/kg body weight, intraperitoneal (i.p.) injection). Rats were tracheostomized with a 15-gauge cannula and mechanically ventilated in a quasi-sinusoidal fashion with a small animal ventilator (Flexivent ™, SCIREQ®) at a frequency of 1.5 Hz (90 breaths/min) and a tidal volume (V_T) of 10 ml/kg body weight. A positive end-expiratory pressure of 3 cmH_2O was applied. The animal's cardiac output was monitored throughout the respiratory mechanics assessment. Rats were paralyzed with pancuronium (0.1 mg/kg body weight, i.p.) before 4 sigh maneuvers at $3 \times V_T$ were performed at the beginning of the experiment to establish stable baseline respiratory mechanics and to ensure a similar volume history before the experiments. In order to measure AHR, incremental doses of inhaled methacholine (MCh, acetyl-β-methylcholine chloride, Sigma-Aldrich (St. Louis, MO, USA) were given at 5-min intervals. The MCh, diluted in saline to a volume of 20 μl, was given during 10 s as an aerosol (Aeroneb™ PRO, SCIREQ®). Each dose of MCh (0, 5, 15 and 45 mg/ml) was aerosolized without any interference with the ventilation pattern. A more detailed description of the method can be found in a previously published paper by Gustafsson et al. (Gustafsson et al., 2014). Since there were no significant changes in the long-term groups, respiratory mechanics of the 90-day group was not performed.

2.5. Differential cell count in BAL

The lungs were lavaged six times via a tracheal tube with a total volume of 2 ml + 23 ml Ca^{2+}/Mg^{2+} free Hanks' balanced salt solution (HBSS, Sigma-Aldrich (St. Louis, MO, USA)). The BAL fluid was centrifuged (10 min, 4 °C, 1500 rpm) and after removing the supernatant, the cell pellet was resuspended in 1 ml PBS. Leukocytes were counted manually in a hemacytometer and 20.000 cells were loaded onto slides using a Cytospin® centrifuge (Shandon© cytospin 3 cyto-centrifuge, cell preparation system). Cytocentrifuged preparations were stained with May-Grünwald-Giemsa reagents (Merck Millipore, VWR International, Sweden) and differential cell counts of pulmonary inflammatory cells (macrophages, neutrophils, lymphocytes, and eosinophils) were performed in duplicates using standard morphological criteria and counting 200 cells per cytospin preparation.

2.6. Inflammatory mediators in BAL and serum

Inflammatory mediators in BAL and serum were analyzed for the presence of IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p70, IL-