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Chlorobenzene induces oxidative stress in human lung epithelial cells *in vitro*

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ABSTRACT

Chlorobenzene is a volatile organic compound (VOC) that is widely used as a solvent, degreasing agent and chemical intermediate in many industrial settings. Occupational studies have shown that acute and chronic exposure to chlorobenzene can cause irritation of the mucosa of the upper respiratory tract and eyes. Using *in vitro* assays, we have shown in a previous study that human bronchial epithelial cells release inflammatory mediators such as the cytokine monocyte chemoattractant protein-1 (MCP-1) in response to chlorobenzene. This response is mediated through the NF-κB signaling pathway. Here, we investigated the effects of monochlorobenzene on human lung cells, with emphasis on potential alterations of the redox equilibrium to clarify whether the chlorobenzene-induced inflammatory response in lung epithelial cells is caused via an oxidative stress-dependent mechanism. We found that expression of cellular markers for oxidative stress, such as heme oxygenase 1 (HO-1), glutathione S-transferase π1 (GSTP1), superoxide dismutase 1 (SOD1), prostaglandin-endoperoxide synthase 2 (PTGS2) and dual specificity phosphatase 1 (DUSP1), were elevated in the presence of monochlorobenzene. Likewise, intracellular reactive oxygen species (ROS) were increased in response to exposure. However, in the presence of the antioxidants N-(2-mercaptopropionyl)-glycine (MPG) or bucillamine, chlorobenzene-induced upregulation of marker proteins and release of the inflammatory mediator MCP-1 are suppressed. These results complement our previous findings and point to an oxidative stress-mediated inflammatory response following chlorobenzene exposure.

Introduction

Chlorobenzene is an aromatic VOC which is mainly used as a degreasing agent and as an intermediate in the synthesis of other organic chemicals. In addition, chlorobenzene is employed as a solvent for adhesives, rubber, and paint, and as a fiber-swelling agent in textile processing (Merck, 1989; Willhite and Book, 1990, ATSDR, 1990). Workplace levels between 18.7 mg/m^3 and 488 mg/m^3 have been reported for different countries (Kusters and Lauwerys, 1990). Occupational studies have shown that acute and chronic exposure to chlorobenzene can cause distinct symptoms, such as headaches and irritation of the mucosa of the upper respiratory tract and eyes (U.S. EPA, 1989, 1995; von Burg, 1981; Willhite and Book, 1990; ATSDR, 1990). Indoor air concentrations of chlorobenzene resulting from usage of chlorobenzene-containing products were reported to be relatively low (BUA, 1993; Herbarth et al., 2000; Herbarth and Rehwagen, 1998). Observed indoor concentrations for this compound differ between 1 to 3.5 μg/m^3 in Leipzig, Germany (Herbarth and Rehwagen, 1998), and up to 72.2 μg/m^3 found in various cities in the USA (BUA, 1991). Although a relationship between indoor VOC exposure and respiratory diseases has been established by several epidemiological studies (Wieslander et al., 1994, 1997a, 1997b; Diez et al., 2000, 2003), no data distinguishing the different VOCs found in household settings with respect to their respiratory effects are available so far. A possible reason for this unsatisfactory situation is that due to mixed exposure situations encountered in epidemiological studies, it is difficult to attribute observable effects to a single VOC. Nevertheless, there is some evidence that chlorobenzene might contribute to the development of respiratory symptoms that were observed in correlation with indoor VOC exposure. One line of evidence comes from the reported respiratory symptoms in occupational settings. Secondly, data from our previous experimental studies provide further evidence that chlorobenzene in fact causes inflammatory effects in human lung epithelial cells and thereby could be involved in the development of respiratory symptoms. Due to the difficulty in characterizing effects of single VOCs in epidemiological studies, we have developed an experimental system allowing for an

Abbreviations: GSTP1, glutathione S-transferase pi; HO-1, heme oxygenase 1; GSH, glutathione; ROS, reactive oxygen species; VOC, volatile organic compound.

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in vivo-like exposure of lung epithelial cells at the air-liquid interface and thereby an assessment of effects of single VOCs, as well as VOC mixtures, on human cells.

As exposure to VOCs occurs predominantly by inhalation, we use airway epithelial cells to determine potential inflammatory effects of these chemicals. These cells are known immune effectors that are able to secrete pro-inflammatory mediators in response to diverse stimuli (Jany et al., 1995; Wang et al., 2008). Excessive and dysregulated production of cytokines has been proposed to play a key role in the development of chronic inflammatory conditions, such as asthma, COPD or cystic fibrosis (Rahman and MacNee, 2000; Wang et al., 2008; Kim et al., 2008). Using a human lung epithelial cell line (A549) in an experimental *in vitro* model in a previous study, we were able to show that exposure to indoor-relevant concentrations of chlorobenzene as low as 100 $\mu g/m^3$ indeed induces expression and release of inflammatory chemokines, e.g., monocyte chemoattractant protein 1 (MCP-1; Fischäder et al., 2008; Lehmann et al., 2008; Röder-Stolinski et al., 2008a). This chemokine is responsible for the recruitment of additional cells involved in lung inflammation like monocytes, lymphocytes, mast cells, eosinophiles, and basophiles (Oppenheim et al., 1991) and induces the release of inflammatory mediators by these cells such as histamine from basophiles (Kuna et al., 1992). In line with these findings, elevated MCP-1 levels were observed in patients with asthma and chronic bronchitis (Yao et al., 2004; Capelli et al., 1999).

In the present study, we have aimed at investigating in greater detail the underlying molecular mechanisms responsible for chlorobenzene-mediated induction of inflammatory effects in lung cells. As we have shown previously using the human alveolar epithelial cell line A549, the oxidative stress-responsive NF-κB and p38 MAP kinase pathways are directly involved in the induction of MCP-1 by chlorobenzene (Röder-Stolinski et al., 2008a). Thus, we have focused the present study on the question whether chlorobenzene may induce its inflammatory effects via oxidative stress. To assess a potential chlorobenzene-induced oxidative stress response, a first step was to quantify the expression of the known inducible marker enzymes for oxidative stress, heme oxygenase 1 (HO-1), superoxide dismutase 1 (SOD-1) and glutathione S-transferase π (GSTP1).

Heme oxygenase 1 oxidatively degrades heme to biliverdin, which is the rate limiting step in the synthesis of bilirubin, a potent physiological antioxidant (Stocker et al., 1987). Another reaction product is carbon monoxide, a second messenger important for maintaining cellular redox homeostasis (Bilban et al., 2008). HO-1 upregulation is correlated with powerful anti-inflammatory and antioxidant activity and is induced in different cell types in response to diverse stressful stimuli such as heat shock, cadmium or oxidative stress (Keyse and Tyrrell, 1989; Applegate et al., 1991; Koizumi et al., 2007).

SOD-1 is an important enzyme of the cellular antioxidant defence, responsible for the conversion of toxic superoxide free radicals to more stable hydrogen peroxide molecules (Bilban et al., 2008). Inhibition of SOD-1 causes protein nitration and oxidation, and SOD-1 itself appears to be a target for oxidative modification and inactivation in airway epithelial cells *in vivo* as well as *in vitro* (Comhair et al., 2005; Demicheli et al., 2007).

GSTP1, which shows a very prominent expression in lung epithelial cells and is intimately linked with the redox potential of the cell via glutathione metabolism, belongs to a family of detoxification enzymes that conjugate reduced glutathione (GSH) to various metabolites. Numbering among their substrates are secondary metabolites endogenously generated during oxidative stress, such as α,β-unsaturated aldehydes, quinones, epoxides, and hydroperoxides.

Furthermore, in order to test more directly for the presence of oxidative stress, intracellular reactive oxygen species generated by chlorobenzene-exposed A549 were monitored and the effect of the small, sulfhydryl-containing anti-inflammatory antioxidants N-(2-mercaptopropionyl)-glycine (MPG) and bucillamine on MCP-1, HO-1 and GSTP1 expression was determined.

Materials and methods

Cell culture. The human alveolar epithelial cell line A549 (ATCC No. CCL-185; LGC Promochem, Wesel, Germany) was cultured in a partially HEPES-buffered RPMI 1640 medium supplemented with 5% heat-inactivated foetal bovine serum, 2 mM L-alanyl-L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (all reagents from Biochrom, Berlin, Germany) at 37°C and 5% CO_2. Passages 3–20 were used for the exposure experiments.

Cellular assays. A549 cells were detached using trypsin-EDTA (PAA, Pasching, Austria). Depending on the type of analysis, 25 mm tissue culture inserts (transwells) with an 0.2 μm anopore membrane (Nunc, Roskilde, Denmark) or 10 mm transwells with an 0.4 μm polycarbonate membrane (TPP; Trasadingen, Switzerland) were placed into 6- or 24-well plates containing 0.4 or 1.5 ml medium/well, respectively. 5×10^4 or 4×10^5 A549 cells in 0.2 or in 2 ml were seeded into each culture insert and grown for another 1 or 3 days, respectively, until confluency.

Chlorobenzene exposure. For the sealed-exposure experiments, we used a refined version of the air-liquid cell culture model developed in our laboratory (Lehmann et al., 2008). In contrast to previous studies, the wide-neck bottles used for incubation were replaced by custom made 600 ml glass cylinders with a specially designed, narrow inlet for VOC application, thus minimizing possible loss between application and closing of the flask. In order to minimize VOC losses due to absorption, plastics were eliminated as far as possible by using glass dishes for accommodating the transwells. Lids were omitted for the same reason. Culture medium was completely removed, the cells were washed one time with PBS and the inserts placed into dishes containing 0.4 or 2 ml of exposure medium consisting of equal volumes RPMI 1640 and CO_2 independent medium (Invitrogen, Karlsruhe, Germany) supplemented with 1 ng/ml recombinant human tumor necrosis factor-alpha (rhTNF-α; ImmunoTools, Friesoythe, Germany). Cells were supplied with medium from below while being exposed apically directly to the gas phase, thus avoiding potential solubility problems and minimizing undesired interactions with medium components. Transwell-containing dishes were placed into custom manufactured steel support frames holding three to four dishes and transferred into the pre-warmed cylinders, which were sealed by a ground stopper. Immediately before exposure, 22 μl chlorobenzene (CAS 108-90-7; density 1.11 g/ml) were first diluted with 385 μl methanol (both reagents from Merck, Darmstadt, Germany), yielding 22 μl × 1.11 mg/μl = 24.42 mg in a total volume of 407 μl, which equals 60 mg/ml. With another 1:100 dilution step (in methanol), the concentration was brought down to 600 μg/ml. Using this dilution and a positive displacement pipette (transferpettor; Brand, Wertheim, Germany), 10 μl of were added to each glass cylinder (600 ml) via the small inlet which was immediately closed with a PTFE-lined screw cap. The resulting concentration was 600 μg/ml × 10 μl / 600 ml = 10 μg/l (or 10 mg/m^3). To achieve final concentrations of 1 mg/m^3 and 100 $\mu g/m^3$, the 1:100 dilution mentioned above was further diluted 1:10 or 1:100 in methanol immediately before addition of 10 μl to the incubation cylinder. For controls, 10 μl methanol were added.

Sample preparation. Depending on the subsequent sample preparation, cells were incubated for 2 h to 24 h at 37 °C. Culture medium was recovered and stored at −20 °C, and cells were harvested for Western blotting or mRNA purification by applying 200 μl or 340 μl of the respective lysis buffer directly onto the culture inserts. The lysates were recovered using a cell scraper and pipetted into reaction tubes.