Molecular targets of chloropicrin in human airway epithelial cells

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A B S T R A C T

Chloropicrin is a vaporizing, irritating compound that causes complications in the respiratory system when inhaled. In this study, we examined the effects of exposure to chloropicrin for 24 h on ultrastructure and global gene expression in primary human bronchial epithelial cells. The treatment increased the number of round and shrunken cells, which detached from culture plates more readily than the untreated control cells. Transmission electron microscopy revealed some swollen mitochondria and the appearance of autophagy/lysosome type of vacuoles in the treated cells. However, the main alteration in the ultrastructure of the treated cells was the presence of aggregated and slightly deformed cytoskeleton structures. Furthermore, confocal microscopy and immunoblotting indicated that cytoskeletal β-tubulin protein is a probable target of chloropicrin exposure. Ingenuity Pathway Analysis (IPA) of differentially expressed microarray data (fold change > ± 2 compared to controls considered) revealed that the top molecular functions were cell growth and proliferation. The main enriched top canonical pathways identified by IPA were associated with EIF2-signalling, protein ubiquitination pathway, glycolysis and mitochondrial dysfunction. Furthermore, the main upstream regulators and their target genes were involved in cell growth and proliferation and cytoskeletal organization. The alterations found here can be the core components of toxicity involved in the lung complications after chloropicrin exposure.

1. Introduction

Chloropicrin (CCl_3NO_2) is an oily, easily vaporized liquid that has strong irritating properties. It is used for disinfecting grains, synthesis of crystal violet and as a pesticide to fumigate soil against insects and other organisms (EPA-report, 2008; Ruzo, 2006). Small amounts of chloropicrin can also be formed during chlorination of water (Bond et al., 2014). The main source for human exposure is occupational i.e. when chloropicrin is being manufactured and utilized. A high acute exposure can also occur due to accidents or intentional release of chloropicrin into the environment. Exposure to a low concentration of chloropicrin (< 1 ppm) in the air causes irritation in the eyes and respiratory system. Because of its volatility, the main route of human exposure to chloropicrin is inhalation. Consequently, respiratory difficulties; cough, dyspnoea, burning, chest pain and inflammation have been reported after the exposure. Higher concentrations damage the respiratory tract and lead to emphysema and life-threatening oedema (EPA-report, 2008; Gonmori et al., 1987). In addition to the respiratory tract, chloropicrin is toxic in experimental animals in organs such as kidney, muscles and stomach (EPA-report, 2008). There is evidence

suggesting that its toxicity involves oxidative stress by increasing the amounts of reactive oxygen species (ROS), depleting cellular glutathione as well as interacting with the thiol-groups in amino acids (Pesonen et al., 2014; Sparks et al., 1997). However, the overall mechanism(s) responsible for the toxicity of chloropicrin is not well understood.

There are an increasing number of studies revealing that stress conditions caused by environmental chemicals alter the integrity of the cytoskeleton. These changes may affect cell signalling and gene expression, and thereby contribute to the pathways leading to the development of pathological, toxic conditions in tissues (Go et al., 2013; Kanda et al., 2014; Toivola and Eriksson, 1999; Toivola et al., 2010). Cytoskeleton integrity is dependent on the dynamic network of the filament systems that consist of three main components; microtubules, intermediate filaments and microfilaments. Microtubules are formed from polymerized tubulin proteins whereas microfilaments are made up of polymerized actin proteins. The proteins in the intermediate filaments are more heterogeneous and expressed in a cell type specific manners e.g. in epithelial cells, they consist of keratins (Pollard et al., 2008). The cytoskeleton and its associated proteins are very abundant

in many cell types (Pollard et al., 2008) and thus, likely targets to chemical exposures.

Our previous studies (Pesonen et al., 2012, 2014) have shown that chloropicrin increases oxidative stress, elevates the expression of the mitogen activated protein kinase (ERK1/2) and of proteins associated with endoplasmic reticulum stress. Furthermore, chloropicrin has been shown to increase cell cycle regulating proteins (p53, p27 and p21), trigger the G2/M-phase arrest and to increase a number of autophagy/ lysosome vacuoles in human cell lines (Pesonen et al., 2015). Since chloropicrin inhalation causes primary damage to the epithelium of the respiratory tract, in this study we have used primary human bronchial epithelial cells as the experimental model. These cells are the first line of defence against inhaled volatile irritants like chloropicrin. The main aim was to identify early responses and molecular pathways, which may underlie chloropicrin toxicity. The knowledge of early responses in the epithelial cells is valuable in the development of biomarkers and antidote to combat acute accidental exposure to chloropicrin. Here, we have used microscopy, microarray and immunoblotting to detect potential targets of chloropicrin in human bronchial epithelial cell.

2. Material and methods

Caution: Chloropicrin is a reactive, volatile, and toxic chemical. It must be handled carefully using protective gloves and glasses, with all procedures conducted in a laminar hood in order to avoid contamination.

2.1. Chemicals

Human primary bronchial epithelial cells (HBEpC) and Bronchial Epithelial Cell Growth Medium were obtained from European Collection of Cell Culture (ECACC, Salisbury, UK). Chloropicrin (CCl_3NO_2, CAS#: 76-06-2) was from Sigma-Aldrich (Helsinki, Finland) and DAPI from Thermo Fisher Scientific Inc. (Vantaa, Finland). Epon (LX-112-recin) was purchased from Ladd Research Industries Inc. (Williston, USA) and Uranyl acetate from Electron Microscopy Sciences (Hatfield, USA). Protein assay reagents were obtained from BioRad Laboratories Inc. (Espoo, Finland). Primary antibodies (anti-tubulin-β antibody and anti-β-actin antibody) were from Cell Signalling Technology (Danvers MA, USA) and the secondary antibody, Texas Read anti-rabbit IgG (H + L) from Vector Laboratories Inc. (Burlingame, USA). ECL primer Western blotting detection reagent was obtained from Fisher Scientific (Vantaa, Finland) and PVDF-membrane from Merck-Millipore (Espoo, Finland). ECL™ anti-mouse IgG HRP-labelled antibody was from Amersham BioSciences (Buckinghamshere, UK) and anti-rabbit IgG (goat) peroxidase conjugated antibody from CalbioChem (Darmstadt, Germany). The 10 cm culture plates were purchased from Sarstedt Inc. (Newton, USA) and Ibidi microscopy chambers from Ibidi GmBH (Martinsried, Germany). All other chemicals used were of analytical grade.

2.2. Cell culture and treatment

HBEpC-cells were cryopreserved at first passage and grown in bronchial/tracheal epithelial cell growth medium at 37 °C in a humidified incubator (with 5% CO_2 and 95% air) according to the instructions provided by ECACC. The cells were kept in culture for 2–3 days before treating them with chloropicrin (10 μM and 40 μM) for 24 h. The used concentrations of chloropicrin were chosen basing on our previous cytotoxicity assay with HBEpC cells (Pesonen et al., 2015). According to that study the low concentration (10 μM) did not have effect on cell viability and the higher concentration (40 μM) was near but still under the EC_{50}-value of chloropicrin in HBEpC cells. The vehicle control (0.1% DMSO) did not have any effects on the measured parameters when compared to the medium without DMSO.

2.3. Transmission electron microscopy (TEM)

In the TEM analysis (JEM-2100F, from Jeol, Japan) control and treated cells were fixed with 2.5% glutaraldehyde in phosphate buffer (pH 7.4) and post-fixed in 1% osmium tetraoxide ($OsO4$) for 3 h. Thereafter the cells were dehydrated with increasing concentrations of ethanol and embedded in Epon (LX-112) blocks. The blocks were sectioned into ultrathin slices and double stained with uranyl acetate and lead citrate.

2.4. Immunofluorescence and confocal microscopy

HBEpC cells grown on Ibidi plates were rinsed with DPBS and fixed with 4% paraformaldehyde at room temperature for 60 min. After fixation, the cells were permeabilized with 0.1% Triton X-100 in DPBS for 10 min and blocked with 1% BSA in DPBS for 30 min. Thereafter, the cells were incubated at 4 °C over night with the primary antibody, human anti-tubulin-β antibody, diluted 1:200 in 1% BSA. Next morning, plates were rinsed and treated for one hour at room temperature with the secondary anti-rabbit antibody (diluted 1:150). Thereafter, nuclei were stained with DAPI (1 μg/ml) at 37 °C for 15 min and images were visualized using a ZeissAxio observer inverted microscopy equipped with a Zeiss LSM 800 confocal module (Carl Zeiss microimaging GmbH, Jena, Germany). Three independent treatments with 3 replicates were performed.

2.5. Electrophoresis and immunoblotting analysis

The whole cell fractions were used in immunoblotting. Preparing of cell fractions has been described previously (Pesonen et al., 2014). Protein concentrations of the fractions were measured using Bradford method. Equal amount of cell proteins (10 μg) were loaded onto 12% acrylaminde gel and separated by electrophoresis at 200 V for 60 min (BioRad Mini-protean vertical electrophoresis) and transferred to PVDF-membrane using Trans-Blot semi-dry transfer cell. After blocking for 2 h in 5% non-fat cow milk-TBS, the membranes were incubated overnight with primary antibodies (anti-β-tubulin antibody, diluted 1:1000 and anti-β-actin antibody, diluted 1:2,000,000) at 4 °C. Thereafter the membranes were washed and treated with secondary antibodies (diluted 1:2000) for 1 h at room temperature. Protein bands were visualized with ECL + Plus system (immunoblotting detection system, Amersham BioSciences) according to the manufacturer's instructions. Densitometric analysis of protein bands was carried out using QuantityOne®-program (1-D Analysis Software, version, BioRad Laboratories Inc. USA) and the data were normalised by the loading control, β-actin. The results are expressed as a fold of the control values. Three independent experiments for each protein were performed.

2.6. Extraction of total RNA and DNA microarray analysis

After the treatment, the cells were washed with PBS-solution and the total RNA was extracted using TRI-reagent as described previously (Storvik et al., 2011). The extracted RNA was stored at − 80 °C before analysis. Three replicates were used for the further study. The microarray work was carried out at the Core Facility of the Estonian Genome Center, University of Tartu (an Illumina CSPro lab). The procedure has been described previously by Pesonen et al. (2015). In this study, we present microarray results at 24 h of exposure to chloropicrin. The other part of the study i.e. microarray results at 6 and 48 h after the exposures were published previously (Pesonen et al., 2015). The microarray data were pre-processed and normalised to median with Chipster software (CSC, Espoo, Finland). The differentially expressed gene sets were selected based on filtering to detect a fold-change > ± 2 as compared to the respective controls. The data sets of differentially expressed genes were examined according to their functional enrich-