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Chloropicrin-induced toxic responses in human lung epithelial cells

Maija Pesonen[a,b,*], Merja Häkkinen[b], Kirsi Rilla[c], Risto Juvonen[b], Tapio Kuitunen[b], Markku Pasanen[b], Kirsi Vähäkangas[b]

[a] Research and Development Department, Centre for Military Medicine, Finnish Defence Forces, Helsinki, Finland
[b] Faculty of Health Sciences, School of Pharmacy/Toxicology, University of Eastern Finland, Kuopio, Finland
[c] Institute of Biomedicine, University of Eastern Finland, Kuopio, Finland

HIGHLIGHTS

- Chloropicrin induces cell cycle regulating proteins in the lung epithelial cells.
- Chloropicrin induces cytoplasmic vacuolization and ROS-production.
- Chloropicrin modifies sulfhydryl groups of cysteine.
- *N*-acetyl-cysteine prevents chloropicrin-induced cytotoxicity and vacuolization.

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ABSTRACT

Chloropicrin is a slowly evaporating toxic irritant that is known to cause damage in the respiratory system. Here we used a lung epithelial cell line (A549) to study the molecular responses underlying chloropicrin toxicity. Glutathione (GSH), synthetic peptide and 2′-deoxyguanosine were used as *in vitro* trapping agents to identify early markers of chloropicrin toxicity. Microscopy of the cells revealed massive vacuolization by chloropicrin exposure (80–100 μM). The number of apoptotic cells increased with the chloropicrin concentration as assessed by flow cytometry. Immunoblotting analysis revealed increases in the amount of four proteins (p53, p21, p27 and phospho-Erk1/2) that are involved in DNA-damage, cell cycle regulation and apoptosis. Chloropicrin evoked a dose-dependent increase in levels of reactive oxygen species within one hour of exposure. The treatment triggered also the formation of disulphide bonds between the model thiol-containing peptides as analysed by LC/MS. Chloropicrin did not form stable adducts with the model peptides or 2′-deoxyguanosine. *N*-acetyl-cysteine (1 mM NAC) fully prevented the vacuoles and chloropicrin-induced cytotoxicity. The results suggest that an oxidative insult, particularly modification of free sulfhydryl groups in proteins is involved in the acute toxicity evoked by chloropicrin in airway epithelial cells. The protective effect of NAC as a potential antidote in chloropicrin intoxication will require further investigation.

1. Introduction

Chloropicrin is an aliphatic nitro compound (CCl3NO2) that is also known as trichloronitromethane. It is a colourless liquid that volatilizes slowly at room temperature and standard pressure. The decomposition of chloropicrin can release reactive toxic gases and vapours such as phosgene, chlorine, and oxides of nitrogen (EPA-report, 2008). Chloropicrin has strong sensory irritating and lacrimating properties and therefore it has been used as a chemical warfare agent (Sutherland, 2004). Currently it is mainly used as a pesticide to fumigate soil against insects, fungi and nematodes. The potency and relatively short half-life make it beneficial as a fumigant (Ruzo, 2006).

Due to the volatility of chloropicrin, the respiratory system, eyes and skin are the main target tissues after exposure. The main source for human exposure is occupational inhalation when chloropicrin is being manufactured and handled. In addition, accidental (Oriel et al., 2009) or intentional release of chloropicrin can be potential sources for human exposure. Individuals exposed accidentally have been reported to suffer from lacrimation, irritation cough, burnings and chest pain (O'Malley et al., 2004). The other reported clinical symptoms are nausea, vomiting, breathing difficulties and

* Corresponding author at: University of Eastern Finland, Faculty of Health Sciences, School of Pharmacy/Toxicology, PO Box 1627, 70211 Kuopio, Finland.
Tel.: +358 403552412; fax: +358 17162424.
E-mail address: Maija.Pesonen@uef.fi (M. Pesonen).

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nephritis (EPA-report, 2008; Oriel et al., 2009). Exposure to high level of chloropicrin gases or vapours damages the airways and lungs compromising respiration. The damage in the respiratory tract can lead to bronchitis and inflammation for days or even weeks after the exposure and this can result in pulmonary oedema (EPA-report, 2008; Oriel et al., 2009). Metabolism and elimination of chloropicrin are poorly understood in humans and animals and it is not known whether its metabolic products are toxic. The only *in vivo* study on the elimination of chloropicrin was carried out in mice and indicated that the main elimination route was *via* urine and to a minor extent through the lungs after intraperitoneal exposure. Some metabolic products were found in urine *i.e.* nitromethane, raphanusamic acid as well as numerous unidentified polar products (Sparks et al., 1997, 2000).

Although there is evidence of chloropicrin-caused adverse health effects in humans, there are no analytical methods to measure chloropicrin in human fluids because of its reactive and unstable properties. Specific biomarkers are not available to assess exposures, either. In addition, the mechanism of its action in the respiratory system or other tissues is poorly understood. Chloropicrin has previously been shown to be mutagenic in a bacterial testing-system in the presence of exogenous liver post-mitochondrial fraction (Schneider et al., 1999). In addition, DNA strand-breaks and increase of sister chromatid exchanges have been reported in chloropicrin exposed human lymphocytes (Garry et al., 1990; Liviac et al., 2009). Reports from Mackworth (1948) and Sparks et al. (2000) indicated that chloropicrin may act by inhibiting thiol-containing enzymes such as succinate dehydrogenase and pyruvic oxidase, enzymes with important roles in energy metabolism.

We have previously shown that chloropicrin increases the expression levels of ER-stress-related proteins as well as the amount of reactive oxygen species (ROS) in an epithelial cell line originating from human retina (Pesonen et al., 2012). Since exposure to chloropicrin is predominantly *via* inhalation, in this study we used a human lung epithelial cell line (A549) as an experimental model to investigate molecular responses to chloropicrin. Glutathione (GSH), 2′-deoxyguanosine, and a synthetic thiol-containing peptide were used as trapping agents to identify early biomarkers for chloropicrin exposure. Potential of ROS-production and expression of stress involved proteins, p53, p21, p27 and MAP-kinase, phospho-Erk1/2 were studied. Changes of the expression of these proteins by ROS have been linked to various signalling pathways, associated with cell survival or cell death (O'Reilly, 2005; Lu and Xu, 2006; Abukhdeir and Park, 2009; Kim et al., 2011).

2. Materials and methods

2.1. Chemicals

3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA), dimethyl sulfoxide (DMSO), 2′deoxyguanosine, *N*-acetyl-L-cysteine (NAC) and reduced L-glutathione (GSH, 99%) were from Sigma-Aldrich (Helsinki, Finland). Chloropicrin (CAS no.: 76-06-2) was provided by the Defence Forces Technical Research Centre (Lakiala, Finland). Dulbecco modified Eagle medium (DMEM), fetal bovine serum (FBS), and gentamicin were from Lonza (Verviers, Belgium). ECL Prime Western blotting detection reagent from Fisher Scientific (Vantaa, Finland) and PVDF-membrane from Millipore (Espoo, Finland). ER-trackerTM Blue-White DPX was from Invitrogen Ltd. (Carlsbad CA, USA). Anti-p53, anti-p21WAF1/Cip1 (12D1) and anti-p27Kip1 antibodies, phospho(p44/p42)MAPK(Erk1/2) antibodies, anti-β-actin, and anti-rabbit-IgG were all from Cell Signaling Technology (Danvers MA, USA). ECL™ anti-mouse IgG HRP-labelled antibody was from Amersham BioSciences (Buckinghamshere, UK), anti-rabbit IgG (goat) peroxidase conjugated antibody from CalbioChem (Darmstadt, Germany) and peptide (gly-D-tyr-D-pro-D-cys-D-pro-D-his-D-pro, ≥95%) was synthesized in GL Biochem (Shanghai, China).

2.2. Cell culture and treatment

Human lung epithelial cell line (A549) was from American Type Culture Collection (ATCC CRL-2302TM, USA). The cells were maintained in Dulbecco's modified Eagle's medium supplemented with heat-inactivated fetal bovine serum (9%), and gentamicin (10 μg/ml), at 37 °C in a 5% CO_2-95% air-humidified incubator. In the experiments, the cells were seeded at densities that allowed the untreated cells to reach a nearly confluent state at the end of the experiments. The cells were treated with increasing concentrations (1, 10, 50, 80, 100 or 200 μM) of chloropicrin in DMSO or 0.1% DMSO controls 24 h after plating the cells in fresh medium. The cells were harvested at 6, 24 or 48 h after the treatment. The vehicle control (0.1% DMSO) did not have any effect on cell viability, ROS or protein expression when compared to the medium control without DMSO. Antioxidants were added into the culture medium 24 h before or at the same time as chloropicrin. Different final concentrations of 0.5, 1, 10 and 50 mM for NAC in the culture medium were used in the preliminary study. One millimolar NAC was effective, it was used in subsequent experiments. Higher NAC-concentrations than 1 mM tended to increase toxicity when used in combination with chloropicrin. The corresponding concentrations for ascorbic acid were 0.1, 1, and 10 mM.

In staining experiments, the cells grown on 8-well Ibidi plates were washed with phosphate buffer (pH 7.4). Thereafter the probe (ER-trackerTM) at a concentration of 100 nM was added into the culture medium of control and chloropicrin-treated cells and incubated for 30 min at 37 °C. Thereafter the medium was changed to fresh probe-free medium and cells were imaged with a ZeissAxio Observer inverted microscopy (40× NA 1.3 oil objective) equipped with Zeiss LSM 700 confocal module (Carl Zeiss Microimaging GmbH, Jena, Germany).

2.3. Cell viability and determination of ROS-production

The cytotoxicity of chloropicrin was measured using MTT-reduction assay as described previously by Pesonen et al. (2012). The concentration of chloropicrin that reduced cell viability by 50% (EC50-value) was estimated by using the four parameter logistic regression fitted with Prism (version 5). The ROS-production was assessed by using a fluorescent indicator 2′,7-dichlorodihydrofluorescein diacetate (H2DCFDA) according to Loikkanen et al. (2003) and modified as described by Pesonen et al. (2012).

2.4. Electrophoresis and immunoblotting analysis

Immunoblotting was carried out by using the whole cell fractions. In the isolation of the fractions, the cells were washed with ice-cold PBS and scraped from the plates (on ice) in cold PBS, centrifuged (1300 rpm for 10 min) and the pellets were suspended in RIPA-like lysis-buffer (50 mM Tris–HCl, pH 7.4, 250 mM NaCl, 0,1% SDS, 0.5% NP40, 2 mM dithiothreitol, and protease and phosphatase inhibitors). The suspensions were incubated on ice for 30 min and centrifuged (13,000 rpm for 15 min). Supernatants were collected and used for analysis. Protein concentrations of the fractions were measured with the Bradford method using protein assay reagent obtained from BioRad Laboratories Inc. (Helsinki, Finland).

The cell protein fractions were subjected to 10–15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred