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Effects of acute *in vitro* exposure of murine precision-cut lung slices to gaseous nitrogen dioxide and ozone in an air–liquid interface (ALI) culture

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ABSTRACT

The aim of this study was to establish an air–liquid interface (ALI) culture of precision-cut lung slices (PCLS) for direct exposure of lung cells to gaseous contaminants. Nitrogen dioxide (NO_2) and ozone (O_3) were selected as model gas compounds. Acute pro-inflammatory and toxic effects of NO_2 and O_3 on live lung tissue were investigated. Murine PCLS were exposed to different flow rates (3–30 mL/min) of synthetic air, O_3 (3.5–8.5 ppm), or NO_2 (1–80 ppm). Tissue survived *ex vivo* in ALI culture and resisted exposure to NO_2 (1–10 ppm) and O_3 (3.5–8.5 ppm) for 1 h. Longer exposure to NO_2 resulted in a clear loss of viability, whereas exposure to O_3 was less effective. Exposure to NO_2 dose-dependently induced release of the pro-inflammatory IL-1α (40%), whereas RANTES, IL-12, and eotaxin remained unchanged. Early secretion of IL-1α (80%), RANTES (>800%), MIP-1β (44%), and MCP-1 (60%) was already detected after 1 h of exposure to O_3. The obtained data showed that direct exposure to O_3 and NO_2 induced cytotoxicity and pro-inflammatory responses in PCLS with ALI culture. This provides a model that more closely resembles *in vivo* exposure of airborne contaminants, and thus should be appropriate for toxicity testing.

1. Introduction

The lung is a major conduit for inhalable toxic substances. Inhalation toxicology serves the purpose of assessing the health hazard for humans, mostly by animal experiments (as described e.g. in the OECD guideline for testing of chemicals 403/433/436, acute inhalation toxicology, adopted May 12, 1981). The health risks have to be determined by establishment of dose–effect relationships to estimate the toxic potency of compounds. The imperative to develop alternative methods in the field of acute inhalation toxicology in the context of REACH and the 3Rs (Bakand et al., 2007; Russell and Burch, 1959, reprinted 1992) is currently the basis for the employment of live lung tissue (also called precision-cut lung slices, PCLS). With the use of PCLS as an *ex vivo* model of "acute inhalation injury" chemicals can be tested for respiratory toxicity without animal experiments.

Characteristic features of chemically induced injury to the lung include cellular changes and respiratory inflammation. Both, cellular alterations and release of inflammatory cytokines as quantifiable parameters can be reproduced in the PCLS model (Henjakovic et al., 2008; Nassimi et al., 2009). Other features of the human disease, such as changes in the histopathological picture due to chronic inflammation, cannot be studied in organotypic cultures of lung tissue. This includes the trafficking of cells from blood into the lungs during injury that cannot be assessed due to the lack of circulatory systems. Nevertheless, a model based on PCLS offers the possibility to study nearly all naturally occurring cell types of the respiratory tract situated in their physiological environment, without animal experimentation. This fact has attracted the use of the technique over the past years for purposes such as calcium signaling, analysis of processes of detoxification or bronchoconstriction (Bergner and Sanderson, 2002; De Kanter et al., 2004; Martin et al., 1996; Wohlsen et al., 2003). A common obstacle to all these *ex vivo* models is the necessity to apply soluble chemical fractions to the submerged organotypic cultures. Hence, analyzing the characteristics and mechanisms of the toxicity induced by gaseous compounds or other airborne material using an *in vitro* or *ex vivo* technique requires an air–liquid interface (ALI) exposure technique to enable free contact between native atmospheres and the biological test system. A great advantage of PCLS compared to cell lines is the chance to expose alveolar ducts and alveoli containing many cell types required for immune responses, such as macrophages, dendritic cells, endothelial and epithelial cells, directly to the gaseous compound. Contrasting with submerged treatment a wide range of insoluble chemicals can be further analyzed. Moreover, particulate matter (PM), a component of urban air pollution consisting of

Abbreviations: ALI, air–liquid interface; BCA, bicinchoninic acid; COPD, chronic obstructive pulmonary disease; CV, coefficient of variation; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; LPS, lipopolysaccharide; NO_2, nitrogen dioxide; O_3, ozone; OD, optical density; PBS, phosphate-buffered saline; PCLS, precision-cut lung slices.

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solid and liquid particles, can be analyzed prospectively by deposition from the aerosol phase. First experiments with diesel PM were performed in PCLS by Morin et al. and Bion et al. in a rolling system supplying slices with culture medium and additionally exposing them to complex gases alternately (Bion et al., 2002; Morin et al., 1999). These complex rolling systems can be substituted by an ALI exposure which offers the opportunity of an improved comparability to *in vivo* exposures.

Ozone (O_3) and nitrogen dioxide (NO_2) were selected as model compounds for direct exposure of lung tissue to gaseous airborne contaminants. Both gases are well known environmental oxidant air pollutants to which humans can be exposed. Exposure to high levels of O_3 (1–5 ppm) and NO_2 (100–500 ppm) is associated with lung injury and toxication. NO_2 is a precursor of photochemical smog and also leads to generation of O_3. It facilitates sensitization at least at high exposure concentrations in animal studies (Moldeus, 1993), and consequences of inhalation of lower doses (<0.5 ppm) are associated with exacerbation of asthma, COPD, or pneumonia in humans (Belanger et al., 2006; Bernstein et al., 2004; Cheng et al., 2007; Lee et al., 2007; Schelegle et al., 2003; Strand et al., 1997). Modulation of airway inflammation in asthma patients is ascribed to elevated neutrophil levels (Scannell et al., 1996), increasing epithelial permeability after exposure to those gaseous compounds, and the release of pro-inflammatory mediators IL-1α, IL-8, and TNF-α *in vitro* (Bayram et al., 2001). Exposure to O_3 leads to deciliation and disruption of epithelial cells with increased transmucosal permeability (Bhalla, 1999). Acute effects of O_3 and NO_2 at relatively high concentrations of 5 ppm are observed within 4 h in studies with mouse and rat lung alveolar type II cells, while an exposure to NO_2 induced morphological changes like shedding of epithelial cells into the airways, proliferation of these cells, or pulmonary edema, as shown *in vivo* (Hajela et al., 1990; Persinger et al., 2001).

The purpose of this study was to reproduce known biological effects of the irritant gases NO_2 and O_3 in PCLS in a gas-phase exposure system using an ALI technique. Special emphasis was placed on establishing the *in vitro* exposure of organotypic lung cultures to gaseous compounds without inducing harm to the tissue by the procedure itself. It could thus be shown that PCLS can be adapted for air–liquid interface culture. Furthermore, our results conclusively confirmed that acute exposure of live lung tissue to single high doses of NO_2 and O_3 was associated with tissue injury and inflammation, offering the chance to use lung slices in a testing approach that more closely reflects natural conditions and responses.

2. Materials and methods

2.1. Animals and husbandry conditions

Female mice (BALB/cAnNCrl, 8–10 weeks) were obtained from Charles River (Sulzfeld, Germany). Animals were kept under conventional housing conditions (22 °C, 55% humidity, and 12-h day/night cycle).

2.2. Preparation of PCLS and tissue culture

Lung slices were prepared as previously described (Held et al., 1999; Henjakovic et al., 2008; Nassimi et al., 2009; Ressmeyer et al., 2006). Briefly, animals were sacrificed with an i.p. overdose of pentobarbital-Na. Extraction of lung tissue was performed directly *post-mortem* to conserve vitality of the tissue. Lungs were filled *in situ* with 1.5% low-melting agarose medium solution. Lungs were cooled *in situ* with ice, lung lobes were separated and cut in EBSS into approximately 250-μm thick slices using a special microtome (Krumdieck tissue slicer; Alabama Research and Development, Munford, AL, USA). Tissue slices were incubated in Dulbecco's modified eagle's medium/nutrient mixture F-12 Ham (DMEM) with L-glutamine and 15 mM HEPES. PCLS were washed with DMEM for 1 h. Medium for incubation contained 100 units/mL penicillin and 100 μg/mL streptomycin. PCLS were cultured for 1 day at 37 °C, 5% CO_2, and 100% air humidity under cell culture conditions.

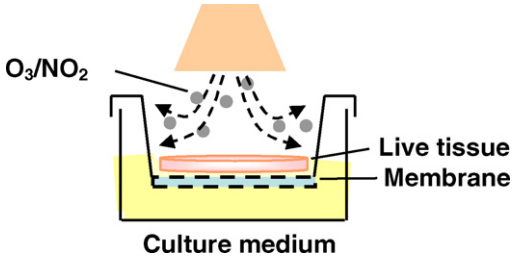

Fig. 1. Experimental set up of exposure conditions using air–liquid interface culture. Vacuum was generated to direct synthetic air, O_3, or NO_2 over the slices. PCLS were supplied with medium from below the membrane.

2.3. Media, reagents and chemicals

Pentobarbital-Na was purchased from Merial (Hallbergmoos, Germany). PBS (0.1 M sodium phosphate and 0.15 M NaCl, without Ca^{2+} and Mg^{2+}) was obtained from Lonza (Verviers, Belgium). Dulbecco's modified eagle's medium/nutrient mixture F-12 HAM (DMEM) with L-glutamine, 15 mM HEPES, and 7.5% (w/v) sodium bicarbonate, pH 7.2–7.4 was supplied by Sigma–Aldrich (Munich, Germany). Medium for cultivation was prepared with penicillin and streptomycin (Sigma–Aldrich, Munich, Germany). Low-melting agarose, Earle's Balanced Salt Solution (EBSS), and Triton X-100 were also purchased from Sigma–Aldrich (Munich, Germany). LPS (*Escherichia coli*, serotype 0111:B4) was supplied lyophilized by Sigma–Aldrich (Munich, Germany) and dissolved in PBS, pH 7.4. WST-1 was purchased from Roche (Mannheim, Germany).

2.4. In vitro exposure of PCLS using air–liquid interface (ALI) conditions

Immediately before exposure, lung tissue slices (250-μm thick) were washed with DMEM and placed onto polyethylene terephthalate (PET) membranes with pore sizes of 3 μm, 1.6×10^2 pores/cm^2, and an area of approximately 1 cm^2 (3181, Becton Dickinson, Germany).

Tissue was exposed to synthetic air (20.5% O_2 in N_2, Messer Griesheim, Germany), O_3, or NO_2 (see below) using gas flow rates of 10 mL/min per exposed PCLS. During exposure the tissue slices were fed from beneath the membrane with pre-warmed (37 °C) DMEM medium supplemented with L-glutamine, HEPES, and penicillin/streptomycin alone (Fig. 1). Changes of pH-value after exposure to gaseous compounds were not observed. The air–liquid interface exposure lasted either for 1 h or 3 h. After exposure membranes were transferred to companion plates. Fresh medium was added on top of the slices and PCLS were post-incubated for 21 h or 23 h.

Following incubation, culture supernatants were collected for determination of extracellular cytokine levels. PCLS were lysed with 1% Triton X-100 in PBS for measurement of intracellular cytokine levels. Samples were stored at −80 °C after addition of 0.2% protease inhibitor cocktail (Sigma–Aldrich, Munich, Germany), and cytokines were measured either by Luminex technology or by ELISA.

2.4.1. Exposure to nitrogen dioxide (NO_2) and ozone (O_3)

NO_2 concentrations were diluted from a stock of 100 ppm NO_2 in synthetic air using mass flow controllers for different flow ranges (Analyt, Germany) in a gas flow system. O3 was generated in situ by photolysis of synthetic air using a PenRay-lamp (Oriel Sarl, Paris) and diluted with synthetic air. Neither carbon dioxide nor any humidification was added to the test atmospheres.

2.5. Incubation of PCLS with LPS

PCLS were incubated with 5 ng/mL LPS in DMEM using standard submerged cell culture conditions. As negative control tissue slices were incubated without addition of LPS. Medium was replaced after 1 h and tissue was further incubated for 23 h in DMEM without LPS. Culture supernatant was collected, PCLS were lysed as described above, and samples were stored at −80 °C for further analysis.

After incubation, culture supernatants were collected for determination of extracellular cytokine levels. PCLS were lysed with 1% Triton X-100 in PBS for measurement of intracellular cytokine levels. Samples were stored at −80 °C after addition of 0.2% proteinase inhibitor cocktail (Sigma–Aldrich, Munich, Germany), and cytokines were measured either by Luminex technology or by ELISA.

2.6. WST-1 reduction

The viability reagent WST-1 is a tetrazolium salt commonly used for spectrophotometric quantification of cellular viability. After incubation of PCLS, the medium was removed and PCLS were incubated for 1 h at 37 °C with 0.125 mL WST-1 solution per slice (diluted 1:10 in culture medium, prepared freshly). Absorbance of the formazan solution was determined at 420–480 nm with a reference wavelength of 690 nm.