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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

S. Switalla, J. Knebel, D. Ritter, N. Krug, A. Braun, K. Sewald*

Fraunhofer Institute for Toxicology and Experimental Medicine, Nikolai-Fuchs-Str. 1, 30625 Hannover, Germany

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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.

* Corresponding author at: Fraunhofer Institute for Toxicology and Experimental Medicine, Department of Immunology, Allergology and Immunotoxicology, Nikolai-Fuchs-Str. 1, 30625 Hannover, Germany. Tel.: +49 511 5350 323.
E-mail address: katherina.sewald@item.fraunhofer.de (K. Sewald).

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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.

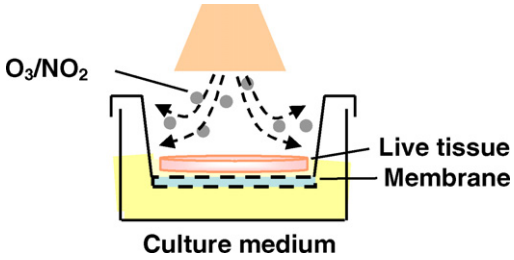

Fig. 1. Experimental set up of exposure conditions using air–liquid interface culture. Vacuum was generated to direct synthetic air, O_3, or NO_2 over the slices. PCLS were supplied with medium from below the membrane.

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.

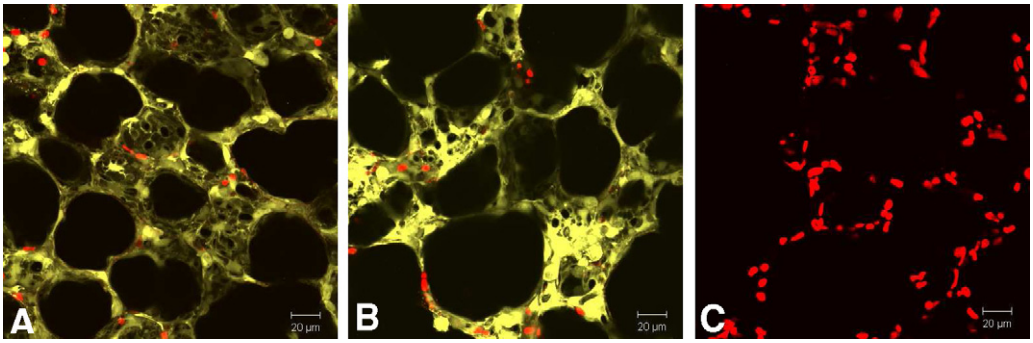

Fig. 2. Image analysis of PCLS after cultivation with different culturing systems. Tissue slices were stained with 4 μM calcein AM and 4 μM EthD-1 after 24 h of submerged cultivation (A), 24 h of air–liquid interface cultivation (B), and after cell lysis with Triton X-100 (C). The images were examined by confocal laser scanning microscopy and analyzed with IMARIS 5.5.3. Red colour shows cell nuclei (⌀ 5 μm) of dead cells and green colour the cytoplasm of viable cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

2.7. Calcein AM/ethidium homodimer-1 staining

Vitality of the tissue slices was also checked by calcein acetoxymethyl/ethidium homodimer-1 (calcein AM/EthD-1) staining (Invitrogen, Karlsruhe, Germany) using a confocal laser scanning microscope Meta 510 (Zeiss, Jena, Germany). Live cells were distinguished by enzymatic conversion of calcein AM to intensely green fluorescent calcein. EthD-1 binds to DNA and therefore produces intracellular orange/red fluorescence in nuclei of dead cells. Lung slices were incubated with 4 μM calcein AM and 4 μM EthD-1 for 45 min at room temperature. PCLS were washed in DMEM and investigated by confocal laser scanning microscopy (40× water immersion objective, excitation wavelengths 488 nm and 543 nm, emission filters BP 505–550 nm and LP 560 nm, thickness 20 μm). Image stacks of a defined volume were analyzed with Bitplane IMARIS 5.5.3 (Henjakovic et al., 2008).

2.8. Quantitative image analysis with IMARIS 5.5.3

Image stacks of PCLS were quantitatively analyzed with IMARIS 5.5.3 software. In a defined volume the ratio of numbers of EthD-1-labeled cell nuclei to volume of calcein in cytoplasm of live cells was determined. Cell nuclei of dead cells were counted as spots ≥5 μm diameter, and volumes of cytoplasm of live cells stained with calcein were determined. Thresholds were set once for each channel and used for all datasets. Viability of PCLS is expressed as quantity of spots in 10^5 μm^3 green tissue volume.

2.9. Measurement of cytokines and chemokines by multiplex bead array assay

Levels of the cytokines IL-1α, TNFα, RANTES, eotaxin, MCP-1, MIP-1β, IL-6, IL-10, IL-12p40, KC, and G-CSF in supernatants and lyses extracts of PCLS exposed to O_3 and NO_2 were determined using Luminex technology. The cytokine multiplex bead array assay kit was purchased from BioRad (Bioplex cytokine assay). This kit was used according to the manufacturer's specifications. Lower limits of detection were 6 pg/mL for TNFα, 2 pg/mL for IL-1α, 5 pg/mL for RANTES, 148 pg/mL for eotaxin, 2 pg/mL for MIP-1β, 2 pg/mL for IL-10, 2 pg/mL for IL-12p40, 2 pg/mL for IL-6, 1 pg/mL for G-CSF, 3 pg/mL for KC, and 14 pg/mL for MCP-1. The measured cytokine levels of TNF-α and IL-10 were below detection limits.

2.10. ELISA: quantification of IL-1α and MIP-1β

IL-1α and MIP-1β were measured in tissue culture supernatants and lyses extracts of PCLS using commercially available enzyme-linked immunosorbent assay kits (ELISA DuoSet, R&D Systems, Wiesbaden-Nordenstadt, Germany). ELISA was performed according to the manufacturer's specifications. The lower limit of detection was 31.25 pg/mL for both cytokines.

2.11. Protein determination

Protein concentrations were determined by the BCA method, with bovine serum albumin (BSA) as standard (BCA Protein Assay Kit, Pierce, Rockford, IL, USA). Twenty-five microliters of sample or BSA were incubated with 200 μL BCA reagent for 30 min at 37 °C. Absorbance was measured at a wavelength of 592 nm.

2.12. Statistical analysis

Data in the figures are given as means ± SEM. Statistical analysis was performed by unpaired *t*-test, by One-way ANOVA Dunnett's test or by One-way ANOVA Tukey test (software: GraphPad Prism 4, version 4.03). Differences between treated samples and control were considered statistically significant at the level of $p < 0.05$.

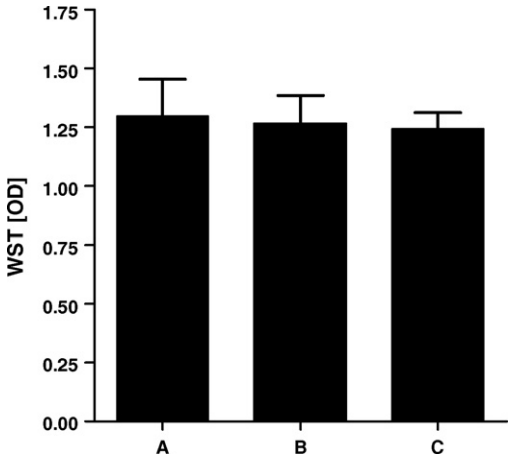

Fig. 3. Metabolic activity of lung tissue after submerged culture, and permanent ALI culture versus lung tissue exposed to synthetic air. Enzymatic activity in PCLS was measured via WST-1 assay after 24 h of submerged cultivation (A), 24 h of permanent ALI cultivation (B), and after 1 h exposure to synthetic air with 23 h submerged post-incubation (C). $n = 9$. OD = optical density at 420–480 nm.

3. Results

3.1. Air–liquid interface culture of PCLS versus submerged culture

In a first set of experiments, conditions for an ALI cultivation of murine lung slices on microporous membranes were defined for further use in exposures to gaseous compounds. Viability of lung tissue should not be affected by the procedure itself. Therefore, a membrane with a defined pore size and density was chosen which allowed a humidified microclimate around the tissue and enabled nutrification from the basal side of the membrane alone (Fig. 1).

According to quantitative image analysis, viability of ALI-cultivated PCLS remained stable at about 100% compared to standard submerged cultivation, indicating no progressive cellular damage during the first 24 h of exposure. Calcein AM/EthD-1 staining demonstrated tissue slices with alveolar walls remaining intact, whereas cytotoxic Triton X-100 clearly increased the number of dead cells (Fig. 2). Similar results were obtained for metabolic activity of lung tissue after submerse and ALI culture (Fig. 3). Here, WST-1 assay showed no differences of mitochondrial enzyme activity between tested culture conditions. Hence, live lung tissue tolerated ALI cultivation using these conditions and was compatible with a culture situation needed for an *in vitro* exposure of lung parenchyma to gaseous compounds.

While viability was unaffected by the mode of cultivation, it needed to be demonstrated that the tissue was still able to release

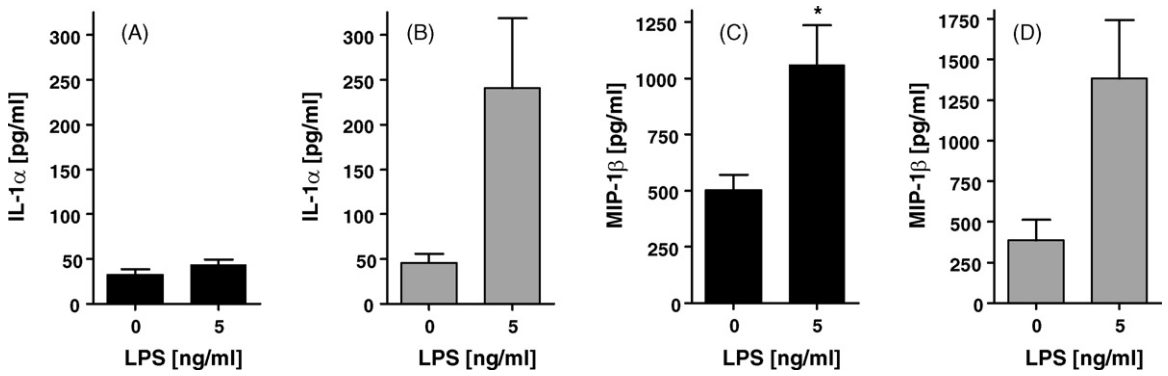

Fig. 4. Extracellular and intracellular accumulation of IL-1α (A and B, respectively) and MIP-1β (C and D, respectively) in PCLS after 1 h air–liquid interface cultivation followed by 23 h post-incubation with 5 ng/mL LPS (one PCLS/insert). Cytokine levels in culture supernatants were determined by ELISA. Data are presented as mean ± S.E.M. IL-1α: 0 ng/mL LPS: $n = 5$, 5 ng/mL LPS: $n = 8$, MIP-1β: $n = 4$, $^*p < 0.05$ (unpaired t-test).

soluble mediators such as pro-inflammatory cytokines. Therefore, PCLS were ALI-cultivated and post-incubated in the presence of LPS. Treatment with 5 ng/mL LPS induced a strong increase in the intracellular pro-inflammatory cytokine IL-1α and the chemokine MIP-1β (Fig. 4). Extracellular MIP-1β was also elevated, whereas the extracellular concentration of IL-1α remained constantly low. After continuous air–liquid cultivation of PCLS in the presence of LPS, cytokine concentrations in the basal medium were below the lower limit of detection.

3.2. *In vitro exposure to synthetic air: influence of the gas flow rate on tissue viability*

PCLS were exposed to synthetic air (20.5% O_2 in N_2) using flow rates between 3 and 30 mL/min to investigate the effects of the gas flow rates on tissue viability. The test atmospheres were not humidified and no carbon dioxide was added. Control tissue was either not exposed ("submerged control") or exposed for the same time under the same conditions but without gas flow ("air–liquid interface").

Viability of the exposed tissue was unaffected at gas flow rates from 3 to 10 mL/min. The number of dead cells (EthD-1$^+$ cells) and the volume of live cells (calcein$^+$ cells) were similar to those observed with no flow, that is 'air–liquid interface' culture (Fig. 5A–C). However, by using a flow rate of 30 mL/min per microporous membrane the ratio between stained cell nuclei of dead cells and stained living cytoplasm doubled after 1 h of exposure from 5.5 to 13.1 compared to a flow rate of 3 mL/min (Fig. 5D). Production of MIP-1β and IL-1α remained absolutely unchanged over four experiments at all flow rates. Furthermore, variability of repeated experiments regarding the production of cytokines was between 14% and 30%. These results show that a constant flow exposure of PCLS with direct contact of tissue and test atmosphere is possible. A gas flow rate of 10 mL/min was used for each of the subsequent experiments.

3.3. *Effect of in vitro exposure to NO_2 and O_3 on PCLS*

Live lung tissue of mice was exposed to synthetic air (exposure control), NO_2, or O_3 (both in synthetic air) using the ALI technique. Quantitative analysis of live/dead stained tissue revealed that 1 h of exposure resulted in no changes of viability. But exposure to 80 ppm NO_2 for 3 h, however, resulted in decreasing amounts of calcein stained parenchyma and more necrotic cell death (as evidenced by bright nuclear fluorescence of orange/red Eth-D1 stain). For O_3 we tested concentrations between 3.3 and 8.5 ppm. An effect on viability of the tissue could be observed after 3 h of exposure to 8.5 ppm O_3 (Fig. 6, Figs. S1 and S2).

Exposure to 10 ppm of NO_2 modulated the inflammatory response of live lung tissue and induced a dose-dependent trend for release of the pro-inflammatory cytokines IL-1α (40% at 10 ppm/1 h), whereas RANTES (Fig. 7), IL-12, eotaxin, and MCP-1 remained more or less unchanged at approximately 25 pg/mg for IL-12, 300 pg/mg for eotaxin, and 25 ng/mg for MCP-1. Changes between the control and the highest concentration lay at a maximum of approximately 30% for all cytokines. Brief exposure to O_3 also induced a significant increase in the pro-inflammatory cytokine IL-1α and a more than 8-fold increased expression for RANTES (Fig. 8). MIP-1β and MCP-1 were elevated dose-dependently (MIP-1β 44% and MCP-1 60%), while IL-12 and eotaxin remained nearly unchanged at approximately 25 pg/mg for IL-12 and 1030 pg/mg for eotaxin.

4. Discussion

The application of an *in vitro* exposure model was developed and evaluated for testing of respiratory inflammatory mediators and toxicity induced by airborne contaminates. Gaseous compounds have been subject to various toxicological studies *in vitro* using different experimental setups. Some setups included submerged cultures with the test gas directed over the adherent cells by shaking them at certain angles so that the cells oscillated between exposure and immersion in a culture medium (Bion et al., 2002; Bombick et al., 1998; Rusznak et al., 1996). Others passed the gas over the cell suspension (Ayyagari et al., 2007). In our studies exposure of PCLS to NO_2 and O_3 was performed on microporous membranes using ALI conditions combined with a continuous flow of the test atmosphere to each individual slice to favor an unobstructed contact of PCLS and the gas phase. In contrast, a submerged exposure situation always leads to intensive mixing of the test gas and the culture medium. Thus, the gaseous compound reacts with components of the culture medium and direct contact with cells is hindered. Therefore, such a testing strategy would document the toxicity of the locally generated reaction products of the test gas and the culture medium, rather than represent the toxic properties of the gas itself (Devalia et al., 1993; Tarkington et al., 1994). Moreover, the use of isolated or cultured cells includes several disadvantages such as changing phenotypes (e.g. activation of dendritic cells) as a result of cultivation (Swanson et al., 2004) and a lack of connective tissue that allows interaction and communication between cells. PCLS possess these capabilities and represent a unique and promising biological test system providing a useful tool to study the complexity of gas-induced lung alterations *in vitro*.

In the present study, particular attention was paid to efficient adjustment of the exposure conditions for complete preservation of the nature of living tissue. The lung tissue stayed viable dur-

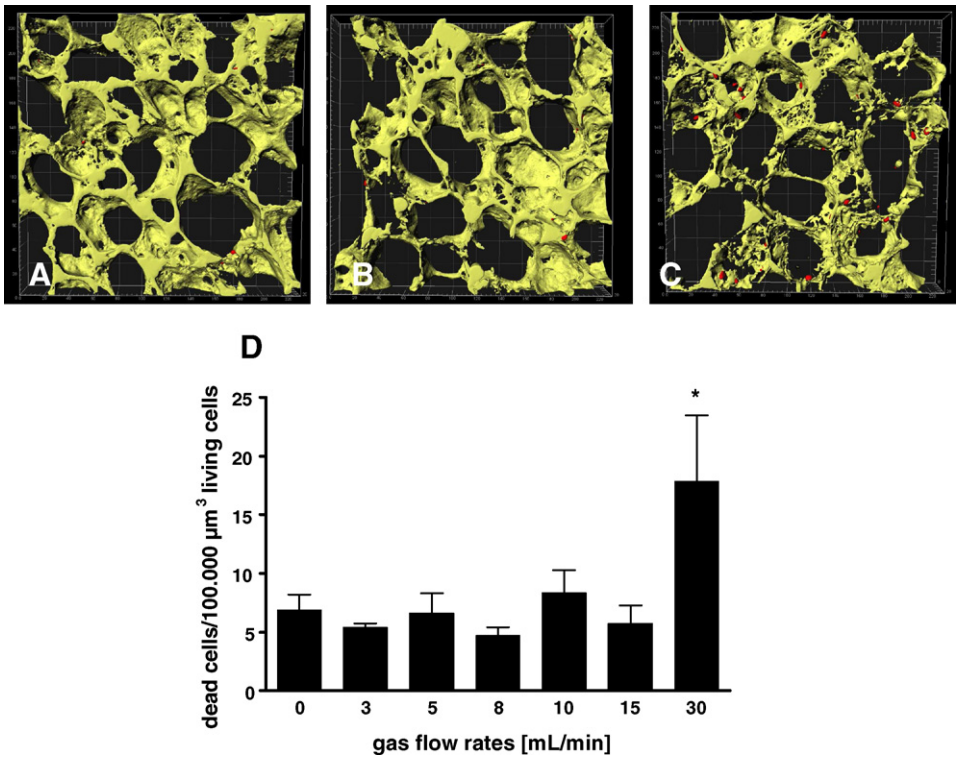

Fig. 5. Image analysis of PCLS after exposure to synthetic air at gas flow rates of 10 mL/min (B) and 30 mL/min (C) for 1 h followed by 23 h post-incubation under submerged conditions. Control tissue (A) was not exposed but air–liquid interface cultivated. Tissue slices were stained with 4 μM calcein AM and 4 μM EthD-1. The images were examined by two-colour confocal laser scanning microscopy and analyzed with IMARIS 5.5.3 (D). Red colour shows cell nuclei (⌀ 5 μm) of dead cells and green colour the cytoplasm of viable cells (grid spacing = 20 μm). $n = 4$, $^{*}p < 0.05$ (One-way ANOVA Dunnett's test). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

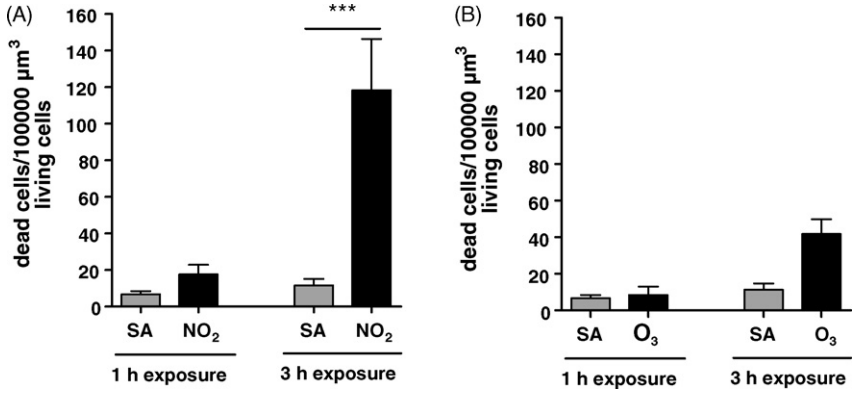

Fig. 6. Quantitative image analysis of murine PCLS exposed to 80 ppm NO_2 and 8.5 ppm O_3. The quantitative analysis with IMARIS was performed after 1 and 3 h of exposure to NO_2 and O_3 followed by 23 h of submerged post-incubation versus exposure to synthetic air (SA). $n = 4$; $^{***}p < 0.005$ (One-way ANOVA Tukey).

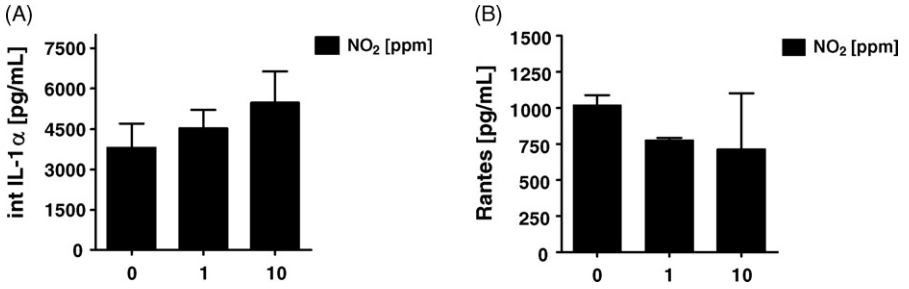

Fig. 7. Intracellular increase of IL-1α (A) and extracellular increase of RANTES (B) in PCLS exposed in air–liquid interface culture to NO_2 for 1 h followed by 23 h submerged post-incubation. Cytokine levels in culture supernatants and tissue lysates were determined by ELISA or Luminex technology. Data are presented as mean ± S.E.M. int IL-1α: $n = 8$, RANTES: $n = 3$ (One-way ANOVA Dunnett's test).

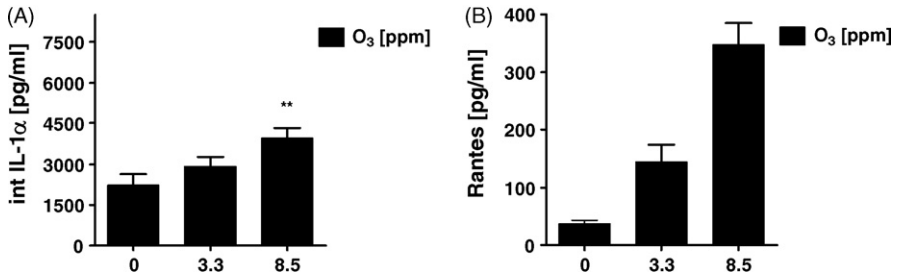

Fig. 8. Intracellular increase of IL-1α (A) and extracellular increase of RANTES (B) in PCLS exposed to O_3 in air–liquid interface culture for 1 h followed by 23 h submerged culture post-incubation. Cytokine levels in culture supernatants and tissue lysates were determined by ELISA or Luminex technology. Data are presented as mean ± S.E.M. int IL-1α: $n = 5$, RANTES: $n = 3$, ** $p < 0.01$ (One-way ANOVA Dunnett's test).

ing ALI exposure with nutrification from the basolateral side of the microporous membrane for at least 24 h, which until now could be shown only for submerged cultures or roller systems (Held et al., 1999; Martin et al., 1996; Richter et al., 2000). Live slices showed high metabolic enzyme activity in the WST-1 assay, and the morphology remained unaltered as indicated by fluorescence staining, as previously shown (Henjakovic et al., 2008). Comparable viability was achieved after exposure of murine PCLS to synthetic air at flow rates below 15 mL/min, which did not seem to induce a drying and therefore dying of the tissue. Higher flow rates, however, induced a decrease in cell viability. These results correlate with data shown in studies using *in vitro* single cell systems (human lung fibroblasts and human bronchial epithelial cells) under ALI conditions (Ritter et al., 2001). Therefore, both cell lines and PCLS resist physical forces that occur during exposure to gaseous compounds. Moreover, since the exposure flow rates tolerated by PCLS (about 10 mL/min/cm^2) are even higher compared to *in vitro* single-cell systems (about 3–8 mL/min/cm^2) they can be characterized as a robust and reliable biological testing material under these conditions (Ritter et al., 2001). After adjustment of the exposure conditions the model was used to expose live lung tissue to gaseous compounds. O_3 and NO_2 were selected as model gases for unobstructed exposure of lung tissue to gaseous compounds. Both gases are well-known ubiquitous indoor and outdoor oxidant pollutants.

The toxicant NO_2 induces respiratory irritation, acute inflammation, pulmonary edema, and pneumonia (Winder, 2004). Its poor water solubility and high reactivity cause cytotoxicity and inflammation in the terminal conducting airways, the alveolar ducts, and the alveoli (Tu et al., 1995). Cell types such as ciliated cells of the bronchial epithelium, types I and II cells of the alveolar epithelium, and alveolar macrophages seem to be very sensitive to NO_2 exposure. NO_2 is hydrolyzed to give nitric acid and reaches concentrations of about 65% in the culture medium (Devalia et al., 1993; Postlethwait and Mustafa, 1989). Brief high-dose exposure to NO_2 induced cell death in murine PCLS. Such a NO_2-mediated cell injury in lung parenchyma may be causally related to the early generation of pro-inflammatory cytokines. Indeed, cytokine expression after exposure to NO_2 was previously reported in human bronchial epithelial cells or alveolar macrophages (Dandrea et al., 1997; Devalia et al., 1993). Exposure to 45 ppb to 45 ppm NO_2 for 30 min to 6 h led to an upregulation of TNF-α, IL-1β, IL-8, and GM-CSF (Ayyagari et al., 2004; Dandrea et al., 1997; Devalia et al., 1993). Pre-existing inflammation in atopic and asthmatic individuals make cells more susceptible to injury upon exposure to NO_2, after which significant upregulation of the chemokine RANTES was observed (Bayram et al., 2001). But contrary to our expectations, although there was a trend, we did not observe a significant initial increase in the pro-inflammatory cytokine IL-1α in live lung tissue, whereas RANTES was not changed at all after short-term exposure to NO_2. Long-term exposure and subsequently continuous damage of lung cells can lead to chronic inflammation which cannot be observed in the experimental set up in PCLS due to limited exposure and culturing time. But both cytokines are known to be increased and therefore also to be involved in chronic inflammatory lung diseases (Greally et al., 1993; Zhu et al., 2001). They belong to the IL-1 gene family, but in contrast to IL-1β IL-1α is an intracellular regulator and mediator of local inflammation (Dinarello, 1996). Even if the release of cytokines is commonly thought to be one of the earliest events, our data suggest that IL-1α and RANTES in non-inflamed lung tissue under normal basal conditions may not be involved to the same extent as with other environmental oxidants such as O_3.

O_3 is a very reactive gas and penetrates deeply into the lungs. It induces acute inflammation by stimulating cytokine secretion by macrophages and epithelial cells (Manzer et al., 2008). Damage of ciliated and alveolar type I cells occurs, and squamous metaplasia as well as rapid influx of neutrophils were observed in humans (Bascom, 1996; Uysal and Schapira, 2003). Single acute exposure to O_3 did not induce immediate toxicity in parenchyma, but survival of tissue *ex vivo* was reduced after prolonged exposure to 8.5 ppm O_3. O_3 is highly reactive with a variety of organic biomolecules such as proteins, unsaturated fatty acids, or nucleic acids. It is known that the time of diffusion of O_3 into the tissue is 3-fold higher than its half-life (Pryor, 1992). Hence, most of the O_3 gas cannot reach deeper areas of the lung tissue and subsequently does not induce a clear cytotoxic effect, as observed for NO_2. Early substantial secretion of the pro-inflammatory cytokine IL-1α and a trend for the chemokine RANTES could already be seen after 1 h of exposure. Similar results after exposure to up to 100 ppb O_3, including upregulation of RANTES and IL-1α, were obtained in bronchial epithelial cell cultures (Bayram et al., 2001) or rat NR8383 alveolar macrophage culture (Manzer et al., 2008). These data are supported by the description of the early phase which is initiated during 2–24 h after acute exposure to O_3. Here, chemotactic factors like RANTES are synthesized and released to direct the migration and activation of neutrophils (Leikauf et al., 1995). Influx of inflammatory cells like neutrophils cannot be quantified in PCLS because of the lack of cell migration from the circulation, but was shown in *in vivo* studies with patients exposed to 0.25 ppm O_3 for 3 h (Alexis et al., 2008).

Toxicity data of several inhalation toxicity studies after acute, subacute, and chronic exposure to NO_2 and O_3 are available from the Registry of Toxic Effects of Chemical Substances (RTECS) (http://www.cdc.gov/niosh/). For example, the LC_{50} for NO_2 in rats was reported to be about 220 mg/m^3 (117 ppm) following 1 h of exposure to NO_2 (NIOSH, 2008). For human beings, first symptoms of toxication have been reported after acute exposure to NO_2 (40–70 min) at doses between 56 mg/m^3 and 169 mg/m^3 (30–90 ppm) (NIOSH, 2008). In comparison, 1-h exposure to 11 ppm NO_2 induced loss of cell viability by up to 50% in both A549 and fibroblasts (Bakand et al., 2006, 2007). In our study, the range of exposure doses was selected from 10 to 80 ppm NO_2 to cover concentrations that can occur in scenarios where human beings are exposed to brief high doses of NO_2. Our results demonstrated significantly more necrotic cells at 80 ppm NO_2, indicating that

acute NO_2 exposure leads to cell damage. This suggests that a high sensitivity of single cell type-based *in vitro* test methods may not entirely reflect the *in vivo* situation after exposure to brief high doses of NO_2. Similarly, the LC_{50} for O_3 in mice has been shown to be about 12.6 ppm (23.6 mg/m^3/3 h) and for inhalation in humans was 50 ppm (98 mg/m^3/30 min). In a single monocyte cell culture 0.5 ppm O_3 (0.98 mg/m^3) was lethal to at least 60% of the cells (Klestadt et al., 2002). This discrepancy between *in vivo* and *in vitro* studies is a subject of debate and controversy in the scientific community (Bakand et al., 2005; Blaauboer, 2002); although according to our understanding most of the mentioned models have advantages in certain applications. Moreover, historical *in vivo* data that are available vary strongly from study to study and can hardly be used as reference for the validation of *in vitro* models.

In summary, our studies demonstrated that PCLS offer a suitable model to study the cytotoxic and pro-inflammatory effects of inhaled irritant compounds such as O_3 and NO_2 under direct ALI conditions. It can be used to study the effects of gaseous compounds on lung cells in organotypic cultures. The advantage of combining of PCLS with ALI conditions is it should decrease the need for animal experiments for risk assessment. It also more closely reflects real life inhalation of airborne contaminants, and may be more suitable for toxicity testing in this context.

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.toxlet.2010.04.004.

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