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# Inflammatory effects of acrolein, crotonaldehyde and hexanal vapors on human primary bronchial epithelial cells cultured at air-liquid interface

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ABSTRACT

The cytotoxicity of aldehydes was studied using human primary bronchial epithelial cells (PBEC) cultured at the air-liquid interface (ALI) or under submerged conditions. PBEC were exposed for 30 min *via* the air phase to acrolein (0.1–1 mg/m$^3$), crotonaldehyde (1.5–15 mg/m$^3$) or hexanal (22–221 mg/m$^3$) or under submerged conditions to acrolein (0.1 and 0.2 mg/L), crotonaldehyde (1 and 2 mg/L) or hexanal (10 and 20 mg/L). Cell culture medium was collected 8 h and 24 h post-exposure and analyzed for interleukin-8 (IL-8) and matrix metalloprotein-9 (MMP-9). The gene expression of inflammatory and oxidative stress markers were measured 6 h post-exposure. In the ALI setup, all three aldehydes caused increased secretion of IL-8, acrolein and crotonaldehyde also increased the gene expression of inflammatory and oxidative stress markers. In contrast, exposure under submerged conditions resulted in significantly reduced IL-8 secretion. The inflammatory response seen in the air phase exposures correspond well with previous *in vivo* studies. This indicates that lung models cultured at ALI are more suitable than submerged cell cultures in toxicity assessment studies of inhaled agents.

## 1. Introduction

Acrolein and crotonaldehyde are two α, β unsaturated aldehydes mainly formed during incomplete combustion and both are present in relatively large amount in cigarette smoke (acrolein content in cigarettes is about 25–140 μg/cigarette (Witz, 1989) and crotonaldehyde content in cigarettes is about 10–228 μg/cigarette (Kuwata et al., 1979). n-Hexanal (hereafter called hexanal) is present in volatile organic chemicals, used as flavoring additives in both conventional and e-cigarettes.

In tobacco smoke acrolein is regarded as one of the largest contributor to respiratory irritation (Fowles and Dybing, 2003). Acrolein has been shown to increase *MUC5AC* gene expression in NCI-H292 cells (Borchers et al., 1999). MUC5AC are one of the major gel-forming secreted mucins in human airways, and is usually expressed by goblet cells (Haswell et al., 2010). Also, treatment with non-cytotoxic doses of acrolein resulted in a dose dependent increase in MUC5AC positive cells (Haswell et al., 2010).

The appearance of the three aldehydes in cigarette smoke is of particular concern, as smoking is the major environmental risk factor for chronic obstructive pulmonary disease (COPD) (Bein and Leikauf, 2011). COPD is a growing global public health problem and is predicted to be the third leading cause of death by 2020 (Murray and Lopez, 1997). Humans may also be exposed to acrolein, and crotonaldehyde, in a variety of environmental settings like, in restaurant kitchen, automobile exhaust, forest fires and to hexanal from emissions of stored wood pellets and fiber boards.

Both acrolein and crotonaldehyde are highly irritating to the mucous membrane, eyes, nose, and upper respiratory tract. Due to their reactivity with respiratory-lining fluid or cellular macromolecules, these aldehydes may alter gene expression, inflammation, mucociliary transport, and the alveolar–capillary barrier integrity (Bein and Leikauf, 2011). Animal *in vivo* studies in mice, rodents and guinea pigs have shown that acute exposure to both acrolein and crotonaldehyde are able to induce inflammation in the lung (Andre et al., 2008; Cichocki et al., 2014; IARC, 1995; Sun et al., 2014).

There are only a few published human studies including lung effects of the three aldehydes. However, we have previously performed a chamber exposure study where 18 healthy volunteers inhaled vapor of acrolein at 0 (control), 0.05 ppm and 0.1 ppm (0.1 and 0.2 mg/m$^3$) for 2 h. Measurements of the inflammatory markers interleukin-6 (IL-6), C-reactive protein (CRP), serum amyloid A, and Clara-cell protein in plasma were performed. Further, interleukin-8 (IL-8) and IL-6 were analyzed in induced sputum. We found no exposure related

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inflammatory effect at these exposure levels (Dwivedi et al., 2015). We have also performed a chamber exposure study where 12 healthy volunteers inhaled vapors of hexanal at 0 (control), 2 and 10 ppm (8.8 and 44.1 mg/m$^3$) for 2 h. CRP and IL-6 were measured in plasma. A decrease in CRP after exposure to 2 and 10 ppm of hexanal but no effect on IL-6 levels were found (Ernstgard et al., 2006). The exposure levels in the chamber studies, together with the occupational exposure limits (8 h) of 0.1 ppm (0.2 mg/m$^3$) for acrolein (Swedish Work Environment Authority, 2005); American Conference of Government Industrial Hygienists, ACGIH (ACGIH, 2003) and 2 ppm (6.2 mg/m$^3$) for crotonaldehyde (ACGIH) (ACGIH, 2004) were used to select air phase concentrations in the present study.

In the lung, *in vivo* exposure occurs at an air-liquid interface (ALI) close to the apical cell surfaces of the epithelial cells, while *in vitro* submerged exposure poorly represents a likely route of exposure. Therefore reliable human airway wall models cultured at ALI mimic realistic exposures of the human airway wall and may replace animal experiments in future toxicity assessment studies of inhaled agents. The human bronchial epithelium is pseudo-stratified and consists of different types of cells. When culturing human primary bronchial epithelial cells (PBEC) at ALI leads to the development of a respiratory epithelium including ciliated cells, goblet cells, club cells and basal cells. However, exposure to both cigarette smoke and environmental pollutants leads to structural and functional abnormality of lung epithelium (Schamberger et al., 2015).

The aim of this study was to use our airway mucosa models (including differentiated human PBEC together with fibroblasts) cultured at air-liquid interface in combination with our newly developed exposure system and expose them to vapors of acrolein, crotonaldehyde and hexanal. For comparison, PBEC were also exposed to the three aldehydes under submerged conditions. This exposure system represents a novel approach to assess respiratory toxicity effects by vapors present in air pollution.

The inflammatory response includes analyses of IL-8 and Matrix metalloproteinase-9 (MMP-9) at protein levels. IL-8 is an important chemoattractant for neutrophils that is produced by airway epithelial cells (Harada et al., 1994). MMPs are a large family of endopeptidases that proteolytically degrade extracellular matrices and are also involved in the inflammatory response to environmental pollutants (McMillan et al., 2004).

In addition to the above proteins, we measured the gene expression of the pro-inflammatory markers *IL8*, *MMP9*, nuclear factor kappa-light-chain-enhancer of activated B cells (*NFKB*), tumor necrosis factor alpha (*TNFA*), *IL6*, and the oxidative stress marker hemeoxygenase1 (*HMOX1*). *NFKB* controls transcription of DNA, cytokine production and cell survival and is also involved in cellular responses to stimuli such as stress, cytokines and free radicals. *TNFA* is involved in systemic inflammation and acute phase reactions. *IL6* is involved in pro-inflammation and infection response, whereas *HMOX1* is induced by oxidative stress and hypoxia. Thus, *NFKB* and *HMOX1* are expected to play important roles in acrolein and crotonaldehyde toxicity.

## 2. Material and methods

### 2.1. Culture of primary bronchial epithelial cells (PBEC)

PBEC were originally obtained from a piece of a central, macroscopically normal bronchus of patients who underwent lobectomy at the Karolinska University Hospital in Stockholm. The collection of PBEC was ethically approved by Ethical Review Board north, Karolinska Institutet (Dnr 99-357). The cells were established as previously described by Strandberg and colleague (Strandberg et al., 2007) and used at passage 3 in our experimental set-ups. In brief, PBEC were grown submerged in cell culture plasticware (Petri dish, Thermofisher Scientific Massachusetts, USA) precoated for 2 h with 30 μg/ml vitrogen 100 (Cohesion Technologies, Palo Alto, CA, USA), 10 μg/ml fibronectin (Gibco, Paisley, Scotland, UK), 10 μg/ml bovine serum albumin (BSA, Boeringer Mannheim, Mannheim, Germany) and 20 U/ml penicillin/streptomycin (pen/strep, BioWhittaker, Walkersville, MD, USA) in phosphate-buffered saline buffer without calcium and magnesium (PBS, Gibco). The PBEC were cultured to 80% confluency at 37 °C in humidified atmosphere of 5% $CO_2$ in complete keratinocyte serum-free medium (KSFM, Gibco). Complete KSFM consists of KSFM supplemented with 5 ng/ml epidermal growth factor (EGF, Gibco), 50 μg/ml bovine pituitary extract (BPE, Gibco), and 20 U/ml pen/strep. The medium was changed every second day. The PBEC were transferred to transwell inserts and to 24-well plates for ALI and submerged conditions, respectively.

### 2.2. Culture of human lung fibroblasts (MRC-5)

MRC-5 (Medical Research Council cell strain 5) cells are lung fibroblasts originally derived from a 14-week male fetus. MRC-5 cells obtained from American Type Cell Culture (passage 27 was used) were grown submerged in a petri dish with Dulbecco's Modified Eagle Media (DMEM, Gibco Life Technologies) supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Gibco) and 20 U/ml pen/strep. When co-cultured with bronchial epithelial cells, the KFSM medium was used instead of DMEM, since MRC-5 cells tolerate environmental changes better than PBEC.

### 2.3. Establishment of an airlifted bilayer model of airway mucosa

Generation of the airway mucosa model takes 21–30 days including cell propagation, cell culture on transwell inserts, airlifting and cell differentiation as earlier described (Ji et al., 2017). First, PBEC were cultured in pre-coated semiporous 0.4 μm transwell inserts in 12-well plates with a seeding density of $10^5$ cells/insert. After attaining confluency around day 7, the inserts were turned upside down and put in a sterile petri dish to add MRC-5 cells in complete DMEM to the other side of the insert membrane. The petri dish was covered and incubated for 30 min at 37 °C; 50 μl complete DMEM was added every 10 min to prevent draining. After 30 min of incubation the inserts were again placed in the plate with 1 ml of complete KSFM per well, enough to cover the cell cultures with medium both at the apical and basal side of the inserts. The next day, the models were airlifted by removing the medium and adding 870 μl co-culture medium (*i.e.* complete KSFM with 6 μg/ml $CaCl_2$ in double distilled $H_2O$, 15 ng/ml ethanolamine in $ddH_2O$, and $10^{-5}$ M retinoic acid) to the basal side of the insert. The differentiation of PBEC to ciliated and mucous producing cells takes about two weeks in the incubator where airway mucosa models are maintained at 37 °C and 5% $CO_2$ and changing medium at basal side every second day. Thus, the aldehyde exposures were performed after two weeks of ALI culturing.

### 2.4. Airlifted exposures

Models including PBEC from at least three different donors in triplicates were used. Inserts were washed with phosphate buffer saline (PBS) the day before the aldehyde exposure to remove dead cells and excess mucus. Basal medium was changed immediately before the start of the exposure. The exposure to aldehyde vapors in the exposure chamber lasted for 30 min (Fig. 1), followed by incubation without aldehydes up to 24 h. Control exposure was performed with an equivalent exposure to clean air for 30 min. The apical side was lavaged by adding 180 μl KSFM medium. The apical medium was collected after 15 min of incubation at 37 °C. Basal and apical media were collected at 8 h and 24 h post exposure and stored at − 80 °C for later analysis of IL-8 and MMP-9 levels by ELISA.