Toxicology in Vitro 27 (2013) 632–640

Contents lists available at SciVerse ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

A dynamic system for single and repeated exposure of airway epithelial cells to gaseous pollutants

Pierre Edouard Kastner [a,b], Stéphane Le Calvé [b], Wuyin Zheng [b], Anne Casset [a], Françoise Pons [a,*]

[a] Laboratoire de Conception et Application de Molécules Bioactives (LCAMB), UMR 7199 CNRS – Université de Strasbourg, Faculté de Pharmacie, 74 route du Rhin, BP 60024, 67401 Illkirch Cedex, France

[b] Laboratoire des Matériaux, Surfaces et Procédés pour la Catalyse (LMSPC), UMR 7515 CNRS – Université de Strasbourg, 25 rue Becquerel, 67087 Strasbourg Cedex 02, France

ARTICLE INFO

Article history:
Received 9 March 2012
Accepted 8 November 2012
Available online 17 November 2012

Keywords:
Airway epithelium
Gaseous pollutants
Exposure system
Repeated exposures

ABSTRACT

In vitro models are promising approaches to investigate the adverse effects and the mode of action of air pollutants on the respiratory tract. We designed a dynamic system that allows the single or repeated exposure of cultured cells to two major indoor air gaseous pollutants, formaldehyde (HCHO) and nitrogen dioxide (NO_2), alone or as a mixture. In this system, the Calu-3 human bronchial epithelial cell line was exposed at the air–liquid interface (ALI) or submerged by culture medium to synthetic air or to target concentrations of HCHO and/or NO_2 once or on 4 consecutive days before assessment of cell viability and necrosis, IL-6 and IL-8 release and trans-epithelial electrical resistance. Our data showed that whereas the ALI method can be used for single short-term exposures only, the submerged method provides the possibility to expose Calu-3 cells in a repeated manner. As well, we found that repeated exposures of the cells to HCHO and NO_2 at concentrations that can be found indoors triggered a significant decrease in cell metabolism and an increase in IL-8 release that were not evoked by a single exposure. Thus, our work highlights the fact that the development of systems and methods that allow repeated exposures of cultured cells to gaseous compounds in mixtures is of major interest to evaluate the impact of air pollution on the respiratory tract.

1. Introduction

In developed countries, air pollution is of major concern because of its potential adverse effects on human health. In modern societies people spend 80–90% of their time in closed environments. Studies addressing indoor air quality have shown that a huge variety of pollutants may be found indoors, among which gaseous compounds such as formaldehyde (HCHO) and nitrogen dioxide (NO_2) (Dales et al., 2008; Marchand et al., 2006; Sakai et al., 2004; World Health Organization, 2010). Epidemiological studies have suggested an association between indoor air pollution and human respiratory health, especially asthma (Bernstein et al., 2008; Burr, 1999; Hulin et al., 2010; Roda et al., 2011; World Health Organization, 2010). In addition, animal studies have demonstrated deleterious effects of indoor pollutants on the respiratory tract (Kawano et al., 2012; Larsen et al., 2007; Lino dos Santos Franco et al., 2006; Poynter et al., 2006). However, these studies concerned a small number of pollutants among the huge variety that can be found indoors and sometimes gave conflicting results. Therefore, beside epidemiological and animal studies, *in vitro* models are promising approaches to better investigate the adverse effects and the mode of action of indoor air pollutants on the respiratory tract (Aufderheide et al., 2003; Bakand et al., 2006b; Berube et al., 2010; Klein et al., 2011).

In the respiratory tract, the epithelium acts as a protective physical barrier, allowing the mechanical clearance of noxious substances and is involved in the host defense by producing a wide range of pro-inflammatory and immune mediators (Holgate, 2008). It plays thus a crucial role in the defense against noxious air pollutants. Airway epithelium is also the first structure that inhaled environmental agents encounter upon their entry in the respiratory tract. Integrity and functions of the airway epithelium have been shown to be impaired in response to several air pollutants including indoor gaseous compounds such as HCHO and NO_2 (Bakand et al., 2007; Baulig et al., 2003; Garcon et al., 2006; Kastner et al., 2011; Pariselli et al., 2009; Persoz et al., 2010). These effects could contribute to the development and severity of lung diseases (Mills et al., 1999). Thus, *in vitro* models based on airway epithelial cells are currently developed to investigate the adverse effects and the mode of action of air pollutants on the respiratory tract. The cellular composition of the epithelium changes all along the airways to fulfill multiple functions (hydration, host defense,

* Corresponding author. Address: UMR 7199, Faculté de Pharmacie, 74 route du Rhin, 67400 Illkirch Cedex, France. Tel.: +33 3 68 85 42 03; fax: +33 3 68 85 43 06.
E-mail address: pons@unistra.fr (F. Pons).

0887-2333/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tiv.2012.11.011

clearance, gas exchange, etc.). Nearly a dozen epithelial cell types have been identified in the whole respiratory tract, among which nasal, olfactory, ciliated and alveolar (Berube et al., 2010). Different models may be used to study the airway epithelium response to air pollutants, according to the area of interest in the respiratory tract (Verstraelen et al., 2008). Among these models, the Calu-3 human epithelial cell line is considered as a good model to study airway epithelium integrity and functions (Grainger et al., 2006; Wan et al., 2000; Zhu et al., 2010). It is one of the few respiratory cell lines that form tight junctions *in vitro*. Besides, the Calu-3 cell line is derived from bronchial epithelium, which plays a central role in the physiopathology of asthma (Holgate, 2008). It is thus a model of interest to analyze the role of air pollutants in the development and severity of this lung disease.

Cells in culture can be exposed to airborne gaseous pollutants by different manners. Agents commercially available under a liquid form, such as HCHO or crotonaldehyde can be diluted in the culture medium before their addition on submerged cells (Kastner et al., 2011; Liu et al., 2010; Lovschall et al., 2002). However, this method is often judged as poorly representative of the *in vivo* situation. The air–liquid interface (ALI) method that consists in culturing cells on inserts and in exposing them to a gaseous pollutant flow after removal of the culture medium is considered to be more relevant (Aufderheide et al., 2003; Bayram et al., 1999; Haswell et al., 2010; Pariselli et al., 2009; Persoz et al., 2010). However, Pariselli et al. reported a significant decrease in cell viability that was accompanied by a significant necrosis in the HaCaT keratinocyte cell line after exposure to air at the ALI for a period as short as 1 h. Although such changes did not occur in the alveolar epithelial cell line A549 (Pariselli et al., 2006), it is reasonable to think that exposure at the ALI could be too stressful to carry out repeated cell exposures to gaseous pollutants. A method where cells cultured in plates and maintained submerged by a thin layer of culture medium are exposed to a gas flow by means of a rocking table has been used by several groups to expose cells to gaseous species (Persinger et al., 2001; Rusznak et al., 1999). This method assuring humidification of the cultures during their exposure could represent an interesting way to limit cell stress during exposure of airway cells to gaseous pollutants, particularly when exposures have to be repeated.

Indoor air pollution is most often composed of a mixture of pollutants and exposure to this pollution generally occurs in a repeated manner. However, studies carried out so far on airway epithelial models concerned essentially single short-term exposures to individual pollutants at levels far higher than environmental concentrations. In this context, we undertook to design a dynamic system that allows: (1) the controlled generation of two major gaseous indoor air pollutants, HCHO and NO_2 at concentrations found indoors, (2) the exposure of cultured airway epithelial cells to these compounds, separately and as a mixture, at the ALI or while submerged by a thin layer of culture medium and maintained under rocking, and (3) the real-time analysis of pollutant concentrations during the course of cell exposures in order to ensure that cultures are actually exposed to the desired atmosphere. Our hypotheses were on the one hand, that exposure of submerged cells might allow repeating exposures with limited stress, and on the other hand, that repeated exposures or association of pollutants might trigger some toxicological effects that would not be observed in response to single exposure or to individual pollutants. To test these hypotheses, the Calu-3 cell line, on which we previously described the effects of solubilized HCHO (Kastner et al., 2011), was exposed once or on 4 consecutive days to synthetic air or HCHO and/or NO_2 before assessment of cell viability and necrosis, IL-6 and IL-8 release and trans-epithelial electrical resistance (TEER).

2. Material and methods

2.1. Chemicals and reagents

HCHO (37% in water), 2,4-dinitrophenylhydrazine (2,4-DNPH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and ammonium acetate were purchased from Sigma–Aldrich (Saint-Quentin-Fallavier, France). Acetylacetone and acetic acid were obtained from Merck (Darmstadt, Germany). DMEM/F-12 culture medium, fetal bovine serum (FBS), fungizone (250 $\mu g\ mL^{-1}$), penicillin–streptomycin mix (10,000 U mL^{-1} and 10,000 $\mu g\ mL^{-1}$, respectively), trypsin, HEPES (1 M) and phosphate buffered saline (PBS) were manufactured by Gibco and obtained from Invitrogen Corp (Cergy Pontoise, France). NO_2 (16.4 ppmv) diluted in air was obtained from Messer (Puteaux, France). Synthetic air (20 ± 1% O_2 in N_2, global purity 99.999%) was purchased from Air Liquide (Paris, France).

2.2. Design of the exposure system

As shown in Fig. 1, the exposure system we designed is divided in three devices: a gas generation device, a cell exposure device and a gas analysis device, as detailed below.

2.2.1. Gas generation device – generation of controlled atmospheres
2.2.1.1. Generation of gaseous HCHO concentrations. Gaseous HCHO was obtained from an HCHO aqueous solution using a permeation tube. To do so, synthetic air was passed through a microporous PTFE membrane tube (180 cm × 0.8 cm i.d., Sumitomo Corporation, Osaka, Japan) at a highly controlled flow rate (F_{HCHO}) of 25–100 mL min^{-1} (Brooks Instrument, 5850S, Abrest, France). The tube was surrounded by 100 mL of a 0.10% HCHO aqueous solution thermostated at 20.0 ± 0.2 °C. Phase equilibrium was achieved, for the dissolved HCHO, at the gas/water interface along the inner surface of the tube, similarly to the technique widely used to measure the Henry's law constant in our laboratory (Feigenbrugel et al., 2004). At the exit of this small reactor, the gas phase containing HCHO was diluted with synthetic air (F_{AIR}). The target HCHO concentrations (up to 250 $\mu g\ m^{-3}$ including the average concentrations found indoors and concentrations representative of pollution peaks (Salthammer et al., 2010; Sarigiannis et al., 2011)) were reached by varying the gas flow (F_{HCHO}) through the microporous tube as shown on Supplementary Fig. S1A.

2.2.1.2. Generation of NO_2 concentrations. The target NO_2 concentrations (up to 1200 $\mu g\ m^{-3}$ including the average concentrations found indoors and concentrations representative of pollution peaks (Sakai et al., 2004; Speizer et al., 1980; Tunnicliffe et al., 1994)) were obtained from a 16.4 ppmv commercial gas cylinder by varying the NO_2 flow (F_{NO2}), as shown on Supplementary Fig. S1B.

The dilution air flow (F_{AIR}) was humidified by passing through a water bubbler. The resulting relative humidity was typically 40 ± 5%. The total gas flow ($F_T = F_{HCHO} + F_{NO2} + F_{AIR}$) was set at 3500 mL min^{-1} to allow sampling of sufficient volumes of gaseous mixture for the simultaneous real-time monitoring of the two pollutants.

2.2.1.3. Generation of HCHO and NO_2 mixture. Before cell exposure to pollutants, we verified that the two pollutants could be generated as a mixture and that the target concentrations of each one were stable during all the course of cell exposures. Fig. 2 shows the record of a real-time measurement of the two pollutants during the generation of a mixture of 200 $\mu g\ m^{-3}$ HCHO and 800 $\mu g\ m^{-3}$ NO_2, which are the highest target concentrations of each pollutant in our study. These concentrations were reached