Environmental Research 156 (2017) 148–157

Contents lists available at ScienceDirect

Environmental Research

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

Particulate metal bioaccessibility in physiological fluids and cell culture media: Toxicological perspectives

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ARTICLE INFO

Keywords:
Bioaccessibility
Trace metals
Mucus
Cytotoxicity
Inflammation

ABSTRACT

According to the literature, tiny amounts of transition metals in airborne fine particles ($PM_{2.5}$) may induce proinflammatory cell response through reactive oxygen species production. The solubility of particle-bound metals in physiological fluids, i.e. the metal bioaccessibility is driven by factors such as the solution chemical composition, the contact time with the particles, and the solid-to-liquid phase ratio (S/L). In this work, $PM_{2.5}$-bound metal bioaccessibility was assessed in various physiological-like solutions including cell culture media in order to evidence the potential impact on normal human bronchial epithelial cells (NHBE) when studying the cytotoxicity and inflammatory responses of $PM_{2.5}$ towards the target bronchial compartment. Different fluids (H_2O, PBS, LHC-9 culture medium, Gamble and human respiratory mucus collected from COPD patients), various S/L conditions (from 1/6000 to 1/100,000) and exposure times (6, 24 and 72 h) were tested on urban $PM_{2.5}$ samples. In addition, metals' total, soluble and insoluble fractions from $PM_{2.5}$ in LHC-9 were deposited on NHBE cells (BEAS-2B) to measure their cytotoxicity and inflammatory potential (i.e., G6PDH activity, secretion of IL-6 and IL-8). The bioaccessibility is solution-dependent. A higher salinity or organic content may increase or inhibit the bioaccessibiliy according to the element, as observed in the complex mucus matrix. Decreasing the S/L ratio also affect the bioaccessibility depending on the solution tested while the exposure time appears less critical. The LHC-9 culture medium appears to be a good physiological proxy as it induces metal bioaccessibilities close to the mucus values and is little affected by S/L ratios or exposure time. Only the insoluble fraction can be linked to the $PM_{2.5}$-induced cytotoxicity. By contrast, both soluble and insoluble fractions can be related to the secretion of cytokines. The metal bioaccessibility in LHC-9 of the total, soluble, and insoluble fractions of the $PM_{2.5}$ under study did not explain alone, the cytotoxicity nor the inflammatory response observed in BEAS-2B cells. These findings confirm the urgent need to perform further toxicological studies to better evaluate the synergistic effect of both bioaccessible particle-bound metals and organic species.

1. Introduction

In vitro toxicological assays performed on normal human bronchial epithelial cells (NHBE) are powerful tools to evaluate various health impacts or to study specific biological response mechanisms initiated by inhaled pollutants such as fine particles ($PM_{2.5}$). The World Health Organization (WHO) estimates that $PM_{2.5}$ exposure contributes to 3% of cardiopulmonary and 5% of lung cancer deaths yearly, making it the 13th cause of mortality worldwide (WHO, 2014). When exposed to PM, the population may develop both respiratory and cardiovascular diseases linked to complex chemical and biological interactions between particle components, physiological fluids covering the respiratory tract, and defense mechanisms of pulmonary epithelial cells (Goix et al., 2016; Guney et al., 2016; Wiseman, 2015). The solubility of chemical compounds present on airborne particles in those physiological fluids is the bioaccessibility, measured through an *in vitro* test generally performed with synthetic fluids. This is a critical and complex parameter that controls the release and potential toxicity of pollutants on a local scale or on a more systemic scale, by becoming bioavailable and transferred into the blood circulation, allowing them to reach other target organs (Guney et al., 2016). Pollutant bioaccessibility, associated with particle lung deposition, has been studied for the past 20 years,

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http://dx.doi.org/10.1016/j.envres.2017.03.029
Received 12 December 2016; Received in revised form 21 February 2017; Accepted 17 March 2017

testing *in vitro* a variety of solutions simulating more or less the lung environment (Dean et al., 2016; Mukhtar et al., 2015; Wiseman, 2015).

The insoluble fraction directly in contact with the epithelial cells may locally affect signal transmissions, cell-surface receptor sites and other mechanisms. Indeed, a recent study suggests that molecular mechanisms involved in proinflammatory gene activation linked to insoluble or soluble particle components can induced the direct formation of Reactive Oxygen Species (ROS), interactions with the lipid layer of cellular membranes, activation of cell surface receptors, and direct interactions with intracellular molecular targets (Øvrevik et al., 2015).

Many of these works have demonstrated that the close correspondence of the *in vitro* physiological conditions matching real life characteristics is necessary to evaluate the actual bioaccessibility of pollutants. Parameters such as body temperature, pH, contact time, solid-to-liquid ratio (S/L), finely-tuned composition of the extraction solution, including buffer, bioorganic compounds and complexing agents are also important factors that need to be considered (Caboche et al., 2011; Mukhtar et al., 2015). Recent reviews have highlighted the lack of standardized methods to estimate the elemental solubility within the human lungs (Henderson et al., 2014; Hillwalker and Anderson, 2014; Mukhtar and Limbeck, 2013; Wiseman, 2015; Kastury et al., 2017). Transition metals (e.g., Fe, Cu) are mainly considered in these works as they are potentially harmful (Ayres et al., 2008; Kelly, 2013) through ROS production such as the hydroxyl radical (˙OH), which represents an important mechanism underlying the particle-induced health effects (Delfino et al., 2005).

Considering that only 18–20 mL of bronchial and alveolar fluids are present in the human lungs, Boisa et al. (2014) and others have shown that the ratio between the amount of particles and the volume of extracting solution is a key parameter to avoid saturation of the solution or competition/complexation between constituents that could underestimate the bioaccessibility of some critical elements. Likewise, the extraction time (i.e., defined as the contact time between the particles and the solution) has been investigated, from a few minutes to hundreds of hours, in various studies (Twining et al., 2005; Caboche et al., 2011). These previous works also concluded that short extraction times could limit the complete dissolution of some elements while long periods could lead to precipitation of some compounds. For example, Caboche et al. (2011) obtained an optimum bioaccessibility after 24 h of extraction on various particle airborne-like standard reference materials while Wragg and Klinck (2007) suggested that a 100 h duration test could lead to conservative results for Pb in the PM_{10} size fraction of contaminated soils.

Most toxicologists working on cell lines or primary cultures of human epithelial cells exposed to atmospheric particles are typically using specific physiological conditions compatible with the cell culture conditions (e.g., adherent or suspension cells or even air-liquid interface) and the toxicological endpoints under study (Gray et al., 1996; Mitschik et al., 2008; Ross et al., 2007). Indeed, cells are cultured in nutritive media (e.g., amino acids, carbohydrates, vitamins, minerals) containing balanced salt solutions (e.g., PBS, DPBS, HBSS), favoring their survival or growth. In addition, the quantity of airborne particles deposited on the cells, often expressed in $\mu g\,mL^{-1}$ or $\mu g\,cm^{-2}$, is usually large enough to promote short-term toxicological effects by producing a cell stress response while avoiding any major death cells (i.e., non or low-cytotoxic doses) (Boublil et al., 2013; Dergham et al., 2012, 2015; Gualtieri et al., 2011; Leclercq et al., 2016; Longhin et al., 2013). In such *in vitro* studies, the contact time between particles and cells may vary from a few hours to a few days after single or repeated exposures. For example, Gray et al. (2010) evaluated the bioaccessible fraction of mercury from mines waste calcine using water and physiological fluids including a modified Gamble solution and a cell culture medium (RPMI-1640). They evidenced significant differences in the solubility of Hg depending on the solution and a lower bioaccessibility with the cell culture medium. Few studies were focused on metals bioaccessibility in cell culture media and none of them took into consideration the cell exposure time, the S/L ratio or the presence of cellular secretions.

In order keep a sufficient dose to study underlying mechanisms while contributing to the effort to be as close as possible to human airborne particles exposure levels, toxicologists must apply non or low-cytotoxic doses (Boublil et al., 2013; Dergham et al., 2012, 2015; Gualtieri et al., 2011; Leclercq et al., 2016; Longhin et al., 2013). As argued by our previous works, after single short-term exposure of human lung epithelial cells to such non or low cytotoxic doses of atmospheric particles, oxidative stress and inflammation were among the main adverse cell outcomes (Dagher et al., 2005, 2007; Dergham et al., 2012, 2015; Diémé et al., 2012; Garçon et al., 2006; Kouassi et al., 2010; Leclercq et al., 2016). Both these processes have also an important role in eliciting the induction and/or the exacerbation of lung chronic inflammatory diseases, such as asthma, allergy, and chronic obstructive pulmonary diseases (COPD). The course of the proinflammatory response is generally characterized by studying the release of proinflammatory cytokines (i.e., interleukin-6, IL-6; interleukin-8, IL-8). Indeed, Barnes (2009) indicated that multiple cytokines, such as IL-6 and IL-8, play a key role in inflammation orchestration within inflammatory airway diseases; IL-6, which often works in concert with other cytokines, provides a link between innate and acquired immunity, whereas IL-8, like other chemokines play a key role in the inflammatory cell recruitment from the circulation.

In the current investigation, we developed an original strategy to better evaluate the bioaccessibility of metals contained in airborne particles while remaining close to the specific physiological conditions. To this purpose, $PM_{2.5}$ were collected during winter time in an urban environment and submitted to six different physiological-like solutions at given S/L ratios and contact times, from water to human respiratory mucus. More precisely, metals bioaccessibility was performed in: (1) water, (2) PBS, (3) LHC-9 culture medium and (4) LHC-9 culture medium consumed by BEAS-2B cells which were compared to (5) Gamble (see Section 2.3 for definitions), or the more realistic (6) mucus-containing respiratory fluids collected from patients suffering from COPD. To demonstrate the need to take into account this concept and fully apprehend the toxicological results and the mechanisms behind, the total, soluble and insoluble $PM_{2.5}$ fractions, were tested on human bronchial epithelial BEAS-2B cells for various toxicological endpoints. Cytotoxicity was evaluated through extracellular glucose-6-phosphate dehydrogenase activity (G6PD), and inflammatory response through the secretion of interleukin-6 (IL-6) and interleukin-8 (IL-8) in cell-free culture supernatants (LHC-9). A critical evaluation regarding the limits of the cell culture generally used is discussed according to the measured bioaccessible fractions.

2. Material and methods

2.1. Site descriptions and sampling protocol

The city of Lille (in the Nord-Pas-de-Calais region, NPdC, North of France) has a population of about 230,000 inhabitants (density: 6600 inhab/km^2) and constitutes the heart of a regional metropolis of more than 1 million inhabitants. The sampling site located in the center of Lille is surrounded by medium-size buildings and located less than 500 m from a high traffic express line (N265) and a large train station. The NPdC region is frequently exceeding the yearly $PM_{2.5}$ target value ($20\,\mu g\,m^{-3}$) and the PM_{10} limit value ($40\,\mu g\,m^{-3}$) set by the EU air quality directive (2008/50/EC).

Fine particles ($PM_{2.5}$) were collected on the roof-top of a building at about 15 m above ground using a High Volume Tish cascade impactor (Tish Environmental) during the cold season from November 2013 to April 2014. The air sampler operates at $35\,m^3\,h^{-1}$ on 6 stages from 0.39 µm to 10.2 µm, but only the last 3 stages collecting particles with a median aerodynamic diameter below 2.1 µm (hereafter called $PM_{2.5}$)