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Differential immunomodulatory responses to nine polycyclic aromatic hydrocarbons applied by passive dosing

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ABSTRACT

Studying the effects of hydrophobic chemicals using *in vitro* cell based methods is hindered by the difficulty in bringing and keeping these chemicals in solution. Their effective concentrations are often lower than their nominal concentrations. Passive dosing is one approach that provides defined and stable dissolved concentrations during *in vitro* testing, and was applied to control and maintain freely dissolved concentrations of polycyclic aromatic hydrocarbons (PAHs) at levels up to their aqueous solubility limit. The immunomodulatory effects of 9 different PAHs at aqueous solubility on human bronchial epithelial cells were determined by analysing the cytokine promoter expression of 4 different inflammatory cytokines using stably transfected recombinant A549 cell lines. Diverse immunomodulatory responses were found with the highest induction observed for the most hydrophobic PAHs chrysene, benzo(a)antracene and benzo(a)pyrene. Cytokine promoter expression was then studied in dose response experiments with acenaphthene, phenanthrene and benzo(a)anthracene. The strongest induction was observed for benzo(a)anthracene. Cell viability analysis was performed and showed that none of the PAHs induced cytotoxicity at any of the concentrations tested. Overall, this study shows that (1) immunomodulatory effects of PAHs can be studied *in vitro* at controlled freely dissolved concentrations, (2) the most hydrophobic PAHs were the strongest inducers and (3) induction was often higher at lower exposure levels and decreased then with concentration despite the apparent absence of cytotoxicity.

1. Introduction

More than 100 different polyaromatic hydrocarbons (PAHs) have been identified in the environment and working place (Srogi, 2007). Low level exposure to PAHs mainly occurs via food ingestion, whereas increased exposure levels are observed when people are occupationally exposed (Srogi, 2007). The latter group includes asphalt workers, coke oven workers and professional drivers, with exposure mainly taking place via inhalation (Karakaya et al., 2004, 1999; Srogi, 2007). Exposure to PAHs has been linked to cancer development, and the mechanisms behind the carcinogenicity of these compounds are well-described. Benzo(a)pyrene is the most carcinogenic PAH, particularly when taking into account the potentially high exposure to this compound (Petry et al., 1996). A meta-analysis has shown that the risk of lung cancer is also increased upon inhalation of PAHs (Armstrong et al., 2004). The potential of PAHs to induce carcinogenesis in the lung has also been shown using *in vitro* systems (Mollerup et al., 2001). Furthermore, a recent study has shown that the exposure of human airway cells to benzo(a)pyrene results in mitochondrial dysfunction and an altered oxidation status, leading to cellular toxicity (Min et al., 2011). Oxidative cellular stress induced by PAHs can lead to a number of different responses, including those related to the immune system (Sorensen et al., 2003). For example, exposure of the human alveolar cell line A549 to benzo(a)pyrene or 1-nitropyrene resulted in the activation of the NF-κB pathway, which led to an increased IL-8 expression (Pei et al., 2002). Immunomodulatory effects due to cellular stress were also found after exposure of A549 cells to fluoranthene when directly added to the cell culture (Oostingh et al., 2008).

Although the lung epithelium has long been regarded to function only as a physical barrier for foreign compounds, more recently the role of the lung epithelium in innate and adaptive

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immunological responses has become increasingly recognised. Alveolar and bronchial epithelial cells can secrete a range of different cytokines upon exposure to foreign compounds that can promote an innate immune response, and in certain situations also specifically direct adaptive immune responses (Lambrecht and Hammad, 2008, 2010).

Therefore, the current study investigated the immunomodulatory response of lung epithelial cells to different PAHs using the alveolar epithelial cell line A549. The immunomodulation of several different environmental chemicals, single walled carbon nanotubes and nanoparticles have already been described in previous publications, which have shown the suitability of this approach for screening for immunomodulatory effects (Herzog et al., 2009; Oostingh et al., 2008; Pfaller et al., 2010).

PAHs are poorly soluble and hydrophobic compounds that are susceptible to sorptive and in some cases evaporative losses. Such losses can be dramatic, particularly when testing in open plastic cell culture plates at elevated temperatures, which are the typical test conditions for *in vitro* assays. To date, most human immunomodulation studies have involved first dissolving the PAHs in dimethyl sulfoxide (DMSO), followed by direct or indirect dosing of the aqueous solutions used for the *in vitro* tests (Davila et al., 1996; Machala et al., 2001; Min et al., 2011; Murahashi et al., 2007; Oostingh et al., 2008; Pei et al., 2002). This approach is practical, but the dissolved concentrations of the PAHs are poorly defined since sorption to the plastic or serum components, evaporative losses and precipitation are unaccounted for. This often leads to freely dissolved concentrations that are much lower than the nominal added concentrations and that also decrease during the test (Schreiber et al., 2008; Riedl and Altenburger, 2007). Freely dissolved concentrations are generally perceived as the effective exposure concentration in *in vitro* tests, and reductions in freely dissolved concentrations can thus directly affect both the *in vitro* response and furthermore the apparent sensitivity of the test (Heringa et al., 2004; Gülden and Seibert, 2005). In addition, protein-chemical interactions are also an issue for *in vivo* exposures, since pulmonary surfactants lining the lung epithelium bind PAHs and interact with other binding constituents (Zhao et al., 2012). Indirect dosing involves the initial dissolution of the test chemical in DMSO in the cell culture medium, followed by addition of this medium to the cells. This has been shown to give more reliable data compared to the direct spiking of test chemical in DMSO to the cells (Tanneberger et al., 2010). However, even with indirect spiking, losses are not compensated for, and in any case the effective freely dissolved concentrations are still unknown. Moreover, direct or indirect spiking also result in the addition of DMSO to the cells in culture. Although DMSO might not affect the cells when administered alone, spiking inevitably results in the analysis of mixture toxicity since the DMSO could interact or modify the effect of the test chemicals. Therefore, in order to produce reliable, defined and constant exposure of hydrophobic compounds in cell culture systems new dosing approaches have been developed; with one being passive dosing (Booij et al., 2011; Kramer et al., 2010; Kwon et al., 2009; Smith et al., 2010). Therefore, in the presented study the PAHs were administered via passive dosing using silicone, which ensured a well-controlled dosing regimen (Smith et al., 2010, 2013). Passive dosing involves a reservoir of test chemical dissolved in a biocompatible polymer, such as silicone, acting as a partitioning source to the cell culture medium, giving stable dissolved concentrations at well-defined levels up to and including aqueous solubility. Stably transfected promoter cell lines, containing the interleukin (IL)-8 promoter sequence or NF-κB binding sequences, were used to determine the effects of the different PAHs at the promoter level. The advantages when using these cell lines are multiple. The luciferase reporter system is very sensitive and small changes in promoter activation can be detected. Moreover, previous studies performed with these cell lines have shown that alterations in cytokine and chemokine promoter induction occur at earlier stages than apoptosis or cell death (Oostingh et al., 2008; Röder-Stolinski et al., 2008). Initially, the immunomodulatory responses upon exposure to 9 PAHs at their respective aqueous solubilities were determined, and for 3 of these PAHs full concentration–response testing was additionally performed. Cell viability was always analysed in parallel, to determine whether the observed responses were due to cell death as such. The results were analysed in relation to a number of different factors, including the properties of the PAHs and their concentrations.

2. Materials and methods

2.1. Chemicals and materials

Passive dosing was performed using food-grade silicone O-rings (outer diameter of 14.4 mm, inner diameter of 9.6 mm, mass of 231 mg (C.V. 1%, $n = 10$), calculated volume of 0.171 ml (Order no. ORS-0096-24, Altec, Cornwall, United Kingdom). Cell culture was performed in Costar 24-well flat bottom cell culture-treated polystyrene plates (Corning Inc., Corning, NY). Nine PAHs were selected as model hydrophobic compounds as listed in Table 1. The chemicals were obtained from the following companies: acenaphthene (99%, Sigma), fluorene (99%, Aldrich, Germany), phenanthrene (99.5%, Aldrich), anthracene (99%, Acros, Belgium), fluoranthene (99%, Aldrich), pyrene (>99% Fluka, Germany), benz(a)anthracene (99%, Aldrich), chrysene (99% Cerilliant, TX, USA) and benzo(a)pyrene (98%, Cerilliant). Ethylacetate (p.a. grade) and methanol (HPLC grade) were used as solvents (Merck, Darmstadt, Germany), and super Q treated Milli-Q water was used for cleaning (Millipore, MA). Unless stated differently, the cells were cultured in RPMI 1640 medium supplemented with 10% v/v foetal calf serum (FCS), penicillin (end concentration 100 U ml^{-1}), streptomycin (end concentration 100 μg ml^{-1}) and L-glutamine (end concentration 2 mM) as previously described (Oostingh et al., 2008). Cell culture medium reagents were obtained from PAA Laboratories (Pasching, Austria). IL-8 -specific antibodies and the IL-8 standard were obtained from BD Biosciences (Schwechat, Austria). The CellTiterBlue test was purchased from Promega (Madison, WI). D-Luciferin was obtained from Sigma–Aldrich Co. (St. Louis, USA, Order Number L9504) and was used at a concentration of 66.7 mg l^{-1}.

2.2. Loading of the silicone O-rings with PAHs

O-rings were cleaned and loaded with PAHs as previously described (Smith et al., 2010). In brief, O-rings were first

Table 1
Relevant characteristics of the PAHs used.

PAH	MW	Aqueous solubility at 37 °C (μg L^{-1})[a]	AhR agonist[b]
Acenaphthene	154.2	7315	Poor
Fluorene	166.2	3662	Poor
Phenanthrene	178.2	1617	Weak
Anthracene	178.2	85.8	Poor
Fluoranthene	202.3	381.6	Weak
Pyrene	202.3	223.9	Weak
Chrysene	228.3	n.a.	Strong
Benzo(a)anthracene	228.3	18.6	Strong
Benzo(a)pyrene	252.3	3.5	Strong

[a] The aqueous solubility was calculated using the aqueous solubility versus temperature regressions given in Mackay et al. (2006).
[b] Capacity of the PAH to function as an AhR agonist is taken from Machala et al. (2001) and Murahashi et al. (2007).