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Mono-2-ethylhexylphthalate (MEHP) induces TNF-α release and macrophage differentiation through different signalling pathways in RAW264.7 cells

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ABSTRACT

Epidemiological studies have associated indoor phthalate exposure with increased incidences and severity of asthma in children and adults, and inflammatory effects have been suggested as a possible mechanism. Recent studies report that phthalates may activate mitogen-activated protein (MAP) kinase p38 and various peroxisome proliferator-activated receptor (PPAR) isoforms. Here we confirm and extend these findings by investigating possible signalling pathways activated in the murine monocyte-macrophage cell line RAW264.7, using mono-2-ethylhexylphthalate (MEHP) as a model compound. MEHP exposure (0.3–1.0 mM) for 3 h increased tumour necrosis factor (TNF)-α release and changed the cellular morphology into elongated spindle-like appearance, resembling more differentiated anti-inflammatory macrophages (M2). This was accompanied by increased expression of the macrophage differentiation marker CD163. Western analysis showed phosphorylation of p38 and Akt after 30 min exposure. Experiments using specific inhibitors suggested that MEHP-induced activation of both p38 and the phosphoinositide-3 (PI3) kinase/Akt pathway were involved in the release of TNF-α; whereas only PI3kinase seemed to be involved in differentiation. In contrast, inhibitors of PPARα and γ reduced differentiation, but did not affect TNF-α release. In conclusion, MEHP induced cytokine release and triggered differentiation of RAW264.7 cells, possibly into M2-like macrophages, but different signalling pathways appear to be involved in these responses.

1. Introduction

The incidence of childhood asthma has increased during the last decades, and it is now one of the most common chronic diseases among children and adolescents in the western world (Pearce et al., 2007). This increase is believed to be associated with a combination of environmental changes and genetic factors affecting the immune system (Dietert, 2011). Interestingly, several epidemiological studies have indicated a relationship between phthalates in the indoor environment and airway diseases in children (reviewed in Bornehag and Nanberg, 2010).

Phthalates are used as plasticizers in numerous consumer products. They are not covalently bound to the plastic, and leakage into the environment may cause human exposure through diet, skin and inhalation (Schettler, 2006). Diet and skin are considered to be major exposure routes for phthalates, but exposure through inhalation also seems to contribute to the total exposure, since recent studies have reported that phthalate concentrations in indoor air were associated with the corresponding urinary metabolites in adults (Adibi et al., 2008). A range of phthalates have been detected in indoor dust and air, including di(2-ethylhexyl)phthalate (DEHP) (Rakkestad et al., 2007; Bergh et al., 2011). Although the use of DEHP has been reduced the last 10 years due to potentially negative effects on human health (European Chemicals Agency, 2009), it is still one of the most common phthalates found in indoor dust (Bergh et al., 2011).

When DEHP enters the human body it is rapidly hydrolysed to its primary metabolite MEHP, and then further oxidized into various metabolites (Koch and Calafat, 2009). The hydrolytic cleavage of DEHP is achieved by lipases. These enzymes have been identified in a number of organs including lungs, where they can be found in several cell types such as alveolar macrophages and type 2 cells as well as in lung lining fluid (Albro and Thomas, 1973; Mahoney et al., 1982; Coonrod et al., 1989). This suggests that DEHP could be hydrolysed in the lung and eventually cause cellular exposure to MEHP. However, to our knowledge, the kinetics of DEHP metabolism after inhalation exposure and the sites for the

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hydrolysis of DEHP into MEHP has not been well described for humans. MEHP is considered to account for the majority of the toxic effects induced by DEHP (Li et al., 1998), and many studies use MEHP as a model compound to investigate the toxicity of phthalates.

The mechanisms underlying possible phthalate-induced effects on the respiratory system are not known. Oie et al. (1997) hypothesized that MEHP could mimic prostaglandins and thromboxanes in the lungs, and thereby increasing airway inflammation. *In vitro* studies have confirmed that mono-phthalates can induce a release of inflammatory mediators from lung cells, although at high concentrations (Jepsen et al., 2004; Rakkestad et al., 2010). Phthalates have also been shown to cause airway irritation and exert adjuvant effects on immune responses in mice (Larsen et al., 2004, 2007; Hansen et al., 2007). More specifically, Hansen and co-workers found that MEHP increased the level of eosinophils in bronchoalveolar lavage and ovalbumin-specific IgG1, but not IgE, in serum. Furthermore levels of the type 2 cytokines IL-5 and IL-10 were elevated in cultures of local lymph node cells, suggesting that MEHP may modulate the immune response towards a type 2 response, indicative of activation of the adaptive immune system (Hansen et al., 2007).

Pulmonary macrophages are resident immune cells that are likely to be involved in the pathogenesis of asthma (Hamid et al., 2003). Whereas some studies suggest a suppressive role of macrophages, others suggest that their release of pro-inflammatory mediators may contribute to aggravation of asthma symptoms (Hamid et al., 2003; Peters-Golden, 2004). Circulating monocytes differentiate into activated macrophages, and the local environment such as the cytokine milieu plays an essential role in this activation process. Macrophages are classified into pro- and anti-inflammatory macrophages, also known respectively as classically and alternatively activated macrophages (AAMs) (Laskin, 2009; Mantovani et al., 2004). Classical activation is characterised by high release of pro-inflammatory cytokines, cytotoxicity and tissue injury, whereas alternative activation is characterised by the release of anti-inflammatory cytokines, immune suppression and tissue repair (Laskin, 2009). AAMs are also referred to as anti-inflammatory macrophages, MØ2 or M2 (Mantovani et al., 2004). Increased numbers of alternatively activated IL-13 producing macrophages have been detected in lungs of patients with chronic obstructive pulmonary disease (COPD), and AAMs were recently postulated to be involved in the sex-difference in asthma prevalence (Melgert et al., 2010; Kim et al., 2008). A role of AAMs in chronic lung disease like asthma and COPD has been suggested, but it is still unclear whether these macrophages are the cause or the effect of these lung diseases (Byers and Holtzman, 2010).

Several phthalate metabolites, including MEHP, have been reported to activate peroxisome proliferator-activated receptors (PPARs) (Hurst and Waxman, 2003; Feige et al., 2007). PPAR is a family of nuclear receptors that function as ligand-activated transcription factors, and the known PPAR isotypes α, γ and δ/β can be activated by fatty acids, fatty acid derivatives, but also synthetic compounds like phthalates (Yessoufou and Wahli, 2010). PPARs participate in a range of cellular processes including lipid metabolism, glucose homeostasis, proliferation and differentiation, but also in positive and negative regulation of inflammation (Yessoufou and Wahli, 2010). PPARγ is known to activate phosphate and tensin homologue deleted on chromosome 10 (PTEN), which is a negative regulator of the phosphoinositide-3 (PI3) kinase/Akt pathway (Lee et al., 2005). Akt is involved in cellular proliferation, differentiation, protein synthesis and survival (Matheny and Adamo, 2009) and some studies also indicate that activation of Akt promotes production of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α (Rajaram et al., 2006). Thus PTEN could indirectly act as a negative regulator of TNF-α release through inactivation of the PI3K/Akt pathway. Another group of signalling molecules that are known to regulate the production of many pro-inflammatory cytokines, including TNF-α, is the mitogen activated protein (MAP) kinases, that are activated in response to stress stimuli (Raman et al., 2007; Saklatvala, 2004). Accordingly, both p38 and ERK have been reported to be involved in phthalate induced cytokine release (Oh et al., 2010).

TNF-α is a pro-inflammatory cytokine that has been implicated in many aspects of the airway pathology of asthma, including dysregulation of the inflammatory response and development of airway hyperresponsiveness (Berry et al., 2007). It has previously been demonstrated that exposure to MEHP caused increased release of TNF-α from primary rat lung macrophages. Both the 5-lipooxygenase pathway and the p38 MAP kinase were found to be involved in this response, whereas PPARα had a modulating effect limiting the release of TNF-α. MEHP also induced an altered cellular morphology which was suggested to be associated with cellular differentiation (Rakkestad et al., 2010). Here, the monocyte-macrophage cell line RAW264.7 was used to further investigate signalling pathways involved in differentiation and in the release of the pro-inflammatory cytokine TNF-α.

2. Materials and methods

2.1. Cell culture and exposure conditions

The partially differentiated monocyte-macrophage cell line RAW264.7 (American Type Tissue Collection, Rockville, MD) was used to investigate the cellular effects of MEHP (TCI Europe, Belgium). The cells were grown in a humidified atmosphere at 37 °C and 5% CO_2 in DMEM (Lonza BioWhittaker, Verviers, Belgium) supplemented with 100 μg/ml penicillin/streptomycin (BioWhitaker™, MD, USA) and 10% heat-inactivated fetal bovine serum (FBS) (EuroClone, Italy). The cells were seeded at 27,000 cells/cm^2 (equivalent to 200,000 cells/ml) and incubated for one day. The medium was replaced 1 h before exposure. After 3 h exposure the medium was collected and centrifuged for 10 min to remove cells (300 × *g*). Supernatants were stored at −70 °C for cytokine or lactate dehydrogenase (LDH) analysis.

Cells were exposed to MEHP concentrations between 0.05 and 1.0 mM, by application of stock solutions of MEHP in dimethyl sulfoxide (DMSO; Sigma–Aldrich, St. Louis, MO, USA). The final concentration of DMSO did not exceed 0.5%. Cells grown in cell culture medium with DMSO were used as controls.

To investigate the influence of various signalling molecules on the MEHP-induced release of TNF-α, the cells were incubated with inhibitors for 30 min prior to MEHP exposure. The applied inhibitors were; PPARα inhibitor (MK886, 10 μM, Enzo Life Sciences, Farmingdale, NY, USA), PPARγ inhibitor (GW9662, 10 μM, Enzo Life Sciences), p38 inhibitor (SB202190, 1 μM, CalBiochem, Darmstadt, Germany), PI3K inhibitor (Wortmannin, 1 μM, CalBiochem) and Akt inhibitor IV (10 nm, CalBiochem). Ascorbic acid (Sigma–Aldrich) was used as an anti-oxidant (100 μM).

2.2. Cytotoxicity

Fluorescence microscopy after staining cells (~0.5 × 10^6 cells) with propidium iodide (PI; 10 μg/mL) and Hoechst 33342 (5 μg/mL) (both from Sigma–Aldrich) for 30 min was used to investigate MEHP-induced plasma membrane damage and changes in nuclear morphology associated with necrosis and apoptosis of the RAW 246.7 cells. A minimum of 300 cells was counted for each sample. In addition, the release of LDH from the cytosol of damaged cells into the cell culture medium was used as a measure for cytotoxicity, using a colorimetric assay (Cytotoxicity detection kit, Roche, Switzerland).

2.3. Detection of reactive oxygen species (ROS)

ROS measurements were performed by means of a dichlorofluorescein (DCF) assay. Stock solution of dichlorofluorescein diacetate (DCF-DA, Sigma–Aldrich) was dissolved in DMSO to a concentration of 125 mM. The cells were plated on 12- or 24-well plastic dishes and incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 30 min with DCF-DA added to a final concentration of 125 μM. Excess DCF-DA was removed after 30 min and the cells washed and incubated with buffer containing 0.05–1.0 mM MEHP. The fluorescence was measured in a Fluostar Optima fluorescence plate reader (BMG-labtech, Offenburg, Germany)/Galaxy Instrument (Nerliens Meszansky AS, Oslo, Norway) with the excitation filter set at 488 nm and the emission filter at 525 nm. The fluorescence from each well was captured, digitised, and stored. ROS formation was calculated as percent increase in fluorescence compared to unexposed control cells, after subtraction of the background, i.e. fluorescence signal measured in wells containing medium only.