



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

Anette Kocbach Bølling^{a,*}, Johan Ovrevik^a, Jan Tore Samuelsen^b, Jørn A. Holme^a, Kirsten E. Rakkestad^c, Gro H. Mathisen^c, Ragnhild E. Paulsen^c, Mónica Suárez Korsnes^a, Rune Becher^a

^a Division of Environmental Medicine, Norwegian Institute of Public Health, P.O. Box 4404 Nydalen, N-0403 Oslo, Norway

^b Nordic Institute of Dental Materials AS, PO Box 3874 Ullevaal Stadion, N-0805 Oslo, Norway

^c Department of Pharmaceutical Biosciences, School of Pharmacy, P.O. Box 1068 Blindern, 0316 Oslo, Norway

ARTICLE INFO

Article history:

Received 13 September 2011

Received in revised form

18 November 2011

Accepted 19 November 2011

Available online xxx

Keywords:

Phthalates

Asthma

Inflammation

Macrophage differentiation

Intracellular signalling

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 PPAR α 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.

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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

* Corresponding author. Tel.: +47 21076670; fax: +47 21076686.

E-mail addresses: Anette.Kocbach@fhi.no, anette.kocbach@gmail.com (A. Kocbach Bølling).

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). PPAR γ 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-lipoxygenase pathway and the p38 MAP kinase were found to be involved in this response, whereas PPAR α 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% CO₂ in DMEM (Lonza BioWhittaker, Verviers, Belgium) supplemented with 100 µg/ml penicillin/streptomycin (BioWhittaker™, MD, USA) and 10% heat-inactivated fetal bovine serum (FBS) (EuroClone, Italy). The cells were seeded at 27,000 cells/cm² (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; PPAR α inhibitor (MK886, 10 µM, Enzo Life Sciences, Farmingdale, NY, USA), PPAR γ 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 ($\sim 0.5 \times 10^6$ 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 264.7 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% CO₂ 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.

2.4. Cytokine analysis

The levels of the pro-inflammatory cytokine TNF- α in the supernatants were analysed by an enzyme-linked immunosorbent assay (ELISA) kit used according to the manufacturer's manual (DuoSet ELISA kit, R&D Systems Inc., MN, USA). The increase in colour intensity was quantified using a plate reader (Revelation Version 4.22, Thermo Labsystems, VA, USA).

2.5. Differentiation

Macrophage morphology was assessed by phase contrast microscopy. The relative number of cells with altered morphology compared to unexposed cells was counted in images to quantify the observed effects. Expression of CD163, a marker for the anti-inflammatory M2 cells (van der Plas et al., 2009; Mantovani et al., 2004), was assessed by immunocytochemistry. After 3 h, unexposed and MEHP exposed cells were washed in PBS and fixed in methanol for 3 min before incubation for 20 h at room temperature with a goat anti-mouse CD163 primary antibody (sc18796, Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:400). After washing and incubation for 3 h with Alexa fluor 488 conjugated anti-goat IgG as secondary antibody (Invitrogen, CA, USA; dilution 1:500) the preparations were mounted and visualized using a Nikon Eclipse E400 microscope and a SPOT diagnostic instruments digital camera. As a control for unspecific binding, the primary antibody was omitted from this procedure. Staining is visualized in green. All antibodies were diluted in PBS containing 1% bovine serum albumin.

The presence of the macrophage surface markers CD11b, F4/80 and CD23 was investigated by flow cytometry. Exposed cells and controls were washed 2 times in wash buffer (PBS with 1% FCS and 0.1% sodium azide), followed by a 2 h incubation at 4 °C with either PE (phycoerythrin) conjugated rat anti-mouse CD11b (cat. Nr. 01715B, PharMingen, San Diego, CA), FITC conjugated rat anti-mouse CD23 (cat nr. 553138, BD Pharmingen) or biotin conjugated rat anti-mouse F4/80 (MCA 497B, Serotech), all diluted 1:100 in wash buffer. For F4/80, cells were washed 2 times in wash buffer and incubated with the secondary antibody, streptavidin-PE-Cy5 (cat nr. 554062, BD Pharmingen, diluted 1:100) for 2 h. All cells were then washed 2 times in wash buffer and analyzed with a LSRII flow cytometer (BD Biosciences). The number of MEHP exposed cells expressing surface markers was compared with the number of cells expressing these proteins in the unexposed control group.

2.6. Western blotting

Cells were scraped of the dish after 30 min exposure, and a total protein of 25 μ g was prepared, electrophoresed, blotted onto nitrocellulose filters, and analyzed using specific antibodies (Rakkestad et al., 2010). Ponceau-staining was used for loading control. The filters were blocked with TBS-Tween containing 3% BSA (Sigma–Aldrich), and then incubated with primary antibodies towards p-p38, p-Akt, PTEN, pPTEN and β -actin (all from Cell Signalling, MA, USA) diluted in TBS-Tween containing 1% BSA. Working dilutions of 1:1000 (p-p38, p-Akt, p-PTEN and PTEN) and 1:5000 (β -actin) were found appropriate. Immunoreactive protein was detected using a chemiluminescence system according to the manufacturer's instructions (Super-Signal® West Dura chemiluminescence system, Thermo Scientific, IL, USA) after incubation with horseradish peroxidase-conjugated secondary antibody. Optical quantification of the protein bands was performed by using the KODAK 1D Image Analysis Software.

2.7. Statistical analysis

The statistical analysis was performed in GraphPad Prism (GraphPad Software, CA, USA). One- or two-way ANOVA was used to analyse the data sets, as specified in the figure legends. Dunnett's or Bonferroni post-tests were used to compare groups for one- and two-way ANOVA, respectively. As indicated in the figure legends, some data were log transformed before performing ANOVA to fulfil the assumption of equal standard deviations of all sets of replicates, as recommended by the program developers. Moreover, repeated-measures ANOVA was applied if cytokine levels varied too much between experiments. For the ROS measurements we used one sample *t*-test since this data set is not suitable for ANOVA analysis. Generally, *p* values <0.05 were considered as significant. In all figures, bars represent mean $\pm$ standard error of the mean (SEM).

3. Results

3.1. Cytotoxicity and ROS

The effect of MEHP on cell toxicity was assessed by counting the number of apoptotic and necrotic cells following PI/Hoechst staining as well as measuring the leakage of LDH. Three hours exposure to MEHP did not cause a significant increase in the toxicity (data not shown). Exposure to MEHP increased the levels of ROS in a concentration dependent manner (Fig. 1a). Preliminary experiments

showed a slight increase in ROS levels with longer exposure times, but 30 min exposure was chosen to determine the concentration effect in Fig. 1a. The ROS levels were markedly reduced in the presence of ascorbic acid during exposure to 0.5 mM MEHP (Fig. 1b), but were not influenced by the inhibitors of the investigated cell signalling pathways (data not shown; PPAR α , PPAR γ , PI3K, Akt, p38).

3.2. Effects of MEHP on TNF- α release

MEHP exposure caused a concentration dependent increase in the release of the pro-inflammatory cytokine TNF- α after 3 h exposure (Fig. 1c). The TNF- α release increased with increasing exposure time, with an approximately 10-fold increase after 48 h exposure (data not shown). The MEHP-induced TNF- α release was not affected by ascorbic acid (Fig. 1d), suggesting that the increased ROS levels observed during MEHP exposure were not involved in this response.

3.3. Cell signalling pathways involved in the TNF- α release

To investigate cellular signalling pathways that could be involved in the MEHP-induced release of TNF- α , cells were pre-incubated with various inhibitors for 30 min and then exposed to 0.5 mM MEHP for 3 h. Phthalates are well known ligands for the PPARs. The PPAR α and - γ inhibitors MK886 and GW9662 did, however, not significantly affect the MEHP-induced TNF- α release, suggesting that these PPARs were not involved in this response (Fig. 2a). In contrast, inhibitors of the PI3kinase (Wortmannin) and one of its down-stream kinases Akt (Akt inhibitor IV) significantly decreased the release of TNF- α after 3 h MEHP exposure (Fig. 2b). Furthermore, the p38-inhibitor SB202190 also caused a partial decrease in the TNF- α release.

To further explore the possible involvement of these pathways, their activity was examined by their relative degree of phosphorylation. Exposure to 0.1 and 0.5 mM MEHP for 30 min markedly increased the levels of phosphorylated p38 and Akt in the RAW cells, although the increase was not statistically significant for p-Akt (Fig. 3). These data support that the p38 and PI3kinase/Akt pathways were activated during MEHP exposure. The PTEN levels were not increased during MEHP exposure, but there was a slightly increased phosphorylation at the Ser380 site in some experiments (data not shown). This increased phosphorylation would however suggest an inactivation of PTEN, since it causes covering of its active site.

3.4. Effects of MEHP on differentiation

Exposure to MEHP changed the morphology of the RAW267.4 cells in a concentration dependent manner (Fig. 4a–d). The unexposed cells were dominated by round, spherical cells, while the exposed cells more often showed an elongated and flattened morphology, as indicated by arrows in the figure. The MEHP induced differentiation, with respect to changed morphology, was concentration dependent, and after 3 h exposure to 1.0 mM MEHP approximately 50% of the cells showed an elongated morphology (Fig. 4e).

The RAW267.4 is a partially differentiated macrophage-like monocytic cell line (Yagnik et al., 2000), and the altered morphology may suggest an increased degree of differentiation or maturation of the cells. Cellular differentiation is commonly characterised by changes in surface markers, we therefore used immunocytochemistry and flow cytometry to investigate if MEHP exposure affected the expression of various macrophage surface markers including CD11b, F4/80, CD23 and CD163. CD163, which is a marker for the M2 anti-inflammatory macrophage phenotype (van der Plas et al.,

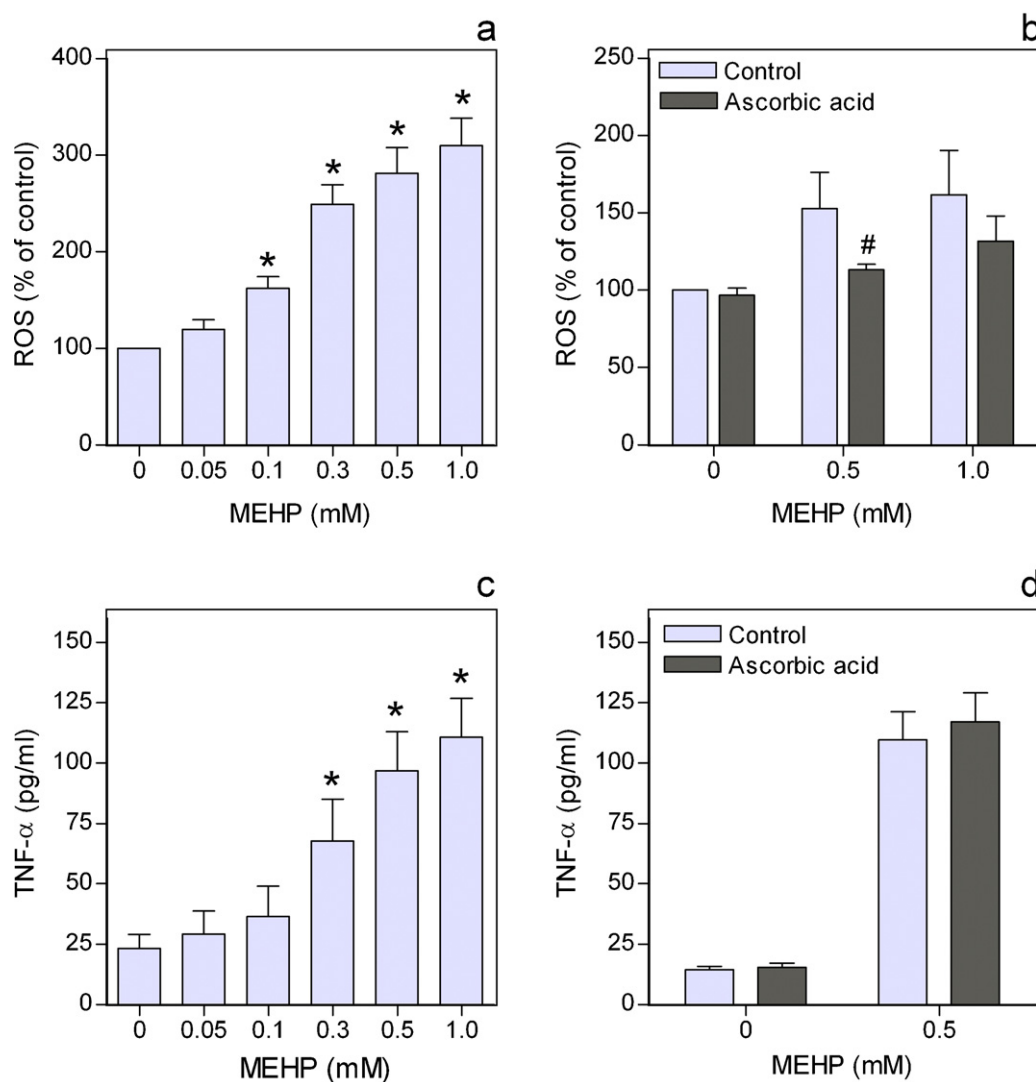


Fig. 1. Levels of reactive oxygen species (ROS) and TNF- α . ROS levels were measured in RAW267.4 cells after 30 min exposure to (a) increasing concentrations of MEHP or (b) MEHP in presence or absence of the anti-oxidant ascorbic acid (100 μ M, 30 min pre-incubation). Release of TNF- α was measured after 3 h exposure to (c) increasing concentrations of MEHP or (d) in the presence or absence of ascorbic acid. *Significant increase compared to control, #significant decrease compared to MEHP-exposed control.

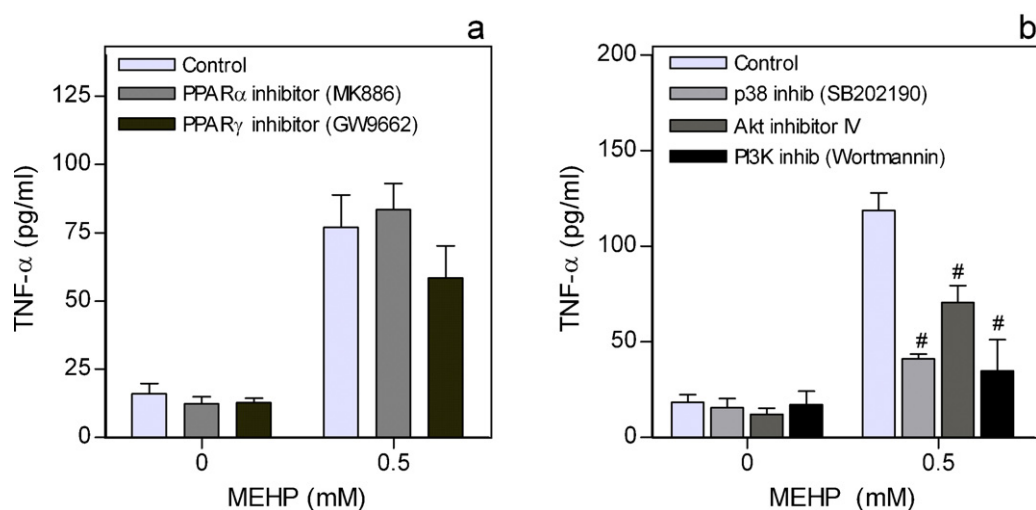


Fig. 2. Effect of inhibitors on release of TNF- α . Release of TNF- α from RAW267.4 cells after 3 h exposure to MEHP in the presence or absence of (a) the PPAR inhibitors MK886 (10 μ M) and GW9662 (10 μ M) and (b) the inhibitors of p38 (SB202190; 1 μ M), Akt (Akt inhibitor IV; 10 nM) and PI3K (Wortmannin; 1 μ M). #Significant decrease compared to MEHP-exposed control (two-way ANOVA with Bonferroni post-test on log transformed data; $N=6$ in (a) and $N=4$ in (b)).

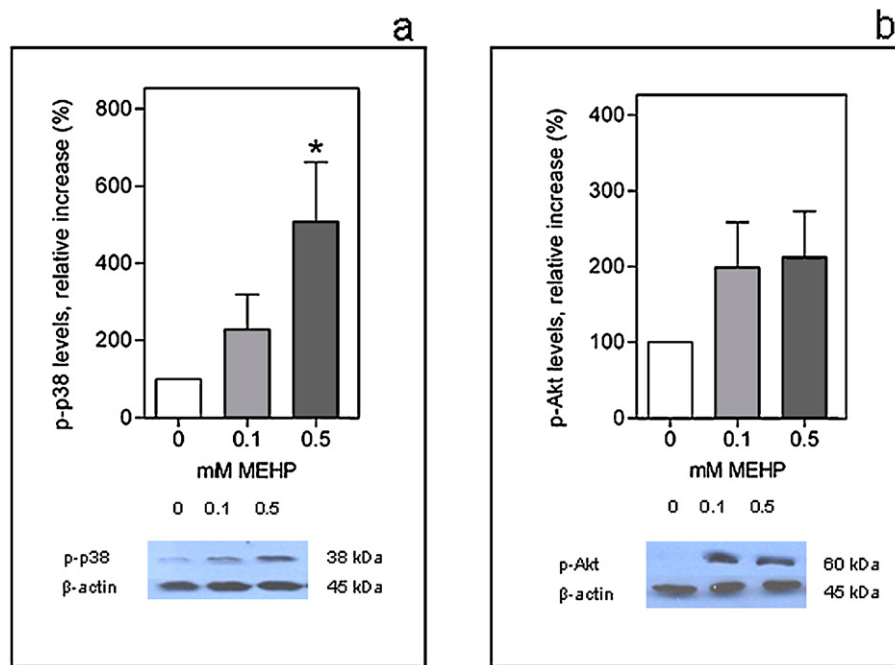


Fig. 3. Western blotting for phospho-p38 and phospho-Akt. Lysates were prepared from RAW267.4 cells exposed to 0.1 or 0.5 mM MEHP for 30 min. The figures show quantification of protein levels by densitometry normalised to the corresponding β -actin blots, and representative examples of western blots with corresponding β actin blots to control for protein loading for (a) p-p38 and (b) p-Akt. *Significant increase compared to control (one-way ANOVA with Dunnett's post-test, $N = 4$).

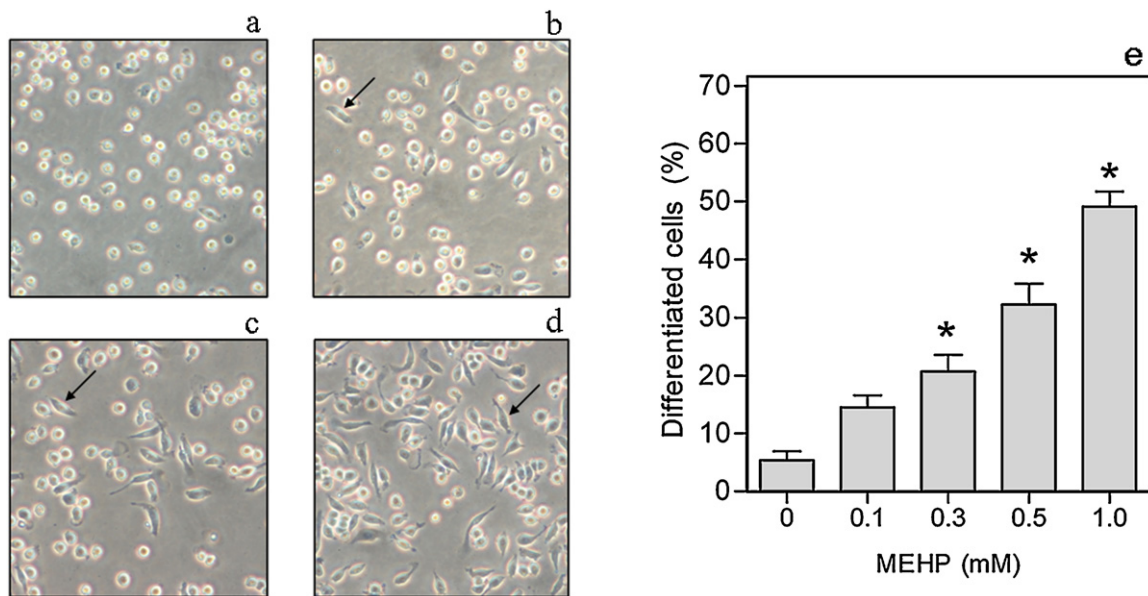


Fig. 4. Morphological differentiation of RAW267.4 cells. Light microscopy images of cells after 3 h exposure to (a) DMSO, (b) 0.3 mM MEHP, (c) 0.5 mM MEHP and (d) 1.0 mM MEHP. (e) Percentage of cells with altered morphology counted in light microscopy images. *Significant increase compared to unexposed control (one-way ANOVA with Dunnett's post-test ($N = 5$)).

2009; Mantovani et al., 2004), was the only surface marker found to be affected by MEHP exposure. As illustrated in Fig. 5, immunocytochemistry revealed that the amount of CD163 antibodies on the cell surface increased due to MEHP exposure. This may suggest that the altered morphology represents a differentiation into AAMs.

The MEHP-induced morphological differentiation was significantly reduced in the presence of both PPAR inhibitors (Fig. 6a). Furthermore, the PI3kinase inhibitor almost completely blocked the changes in morphology, whereas the Akt and p38 inhibitors had no effect in our experiments (Fig. 6b).

4. Discussion

Phthalates are ubiquitous environmental contaminants, and epidemiological studies have reported an association between the levels of DEHP in house dust and childhood asthma and wheezing (Bornehag et al., 2004; Kolarik et al., 2008). Inflammatory responses in the lung have been suggested as a possible mechanism for these phthalate-induced effects. We have previously shown that MEHP, the primary metabolite of DEHP, induced pro-inflammatory responses in primary cultures of rat lung alveolar macrophages

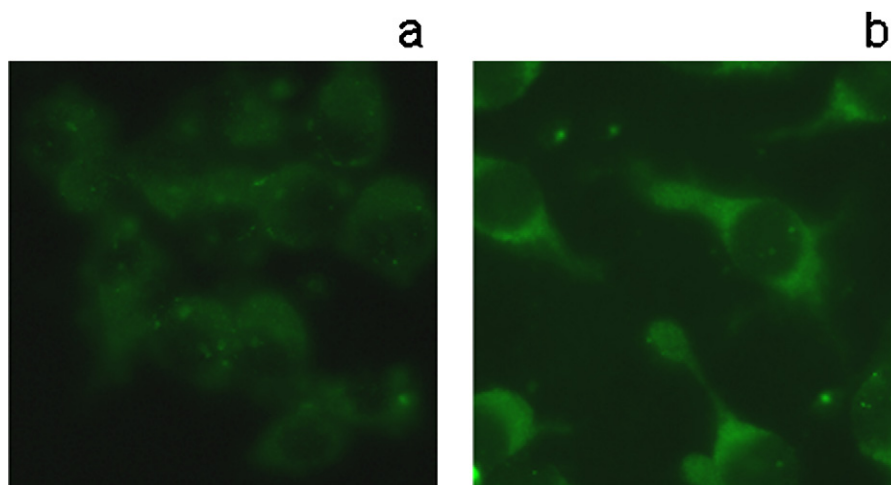


Fig. 5. Immunocytochemical staining with CD163 antibody. RAW267.4 cells were stained with CD163 antibody, a surface marker for alternatively activated M2 macrophages. Fluorescence microscopy images of (a) unexposed control cells and (b) cells exposed to 1.0 mM MEHP for 3 h, indicating increased expression of CD163 after exposure to MEHP.

(Rakkestad et al., 2010). In the partially differentiated monocyte-macrophage cell line RAW264.7, we found that MEHP induced a concentration dependent increase in cell death and a release of the pro-inflammatory cytokine TNF- α . Furthermore, exposure to MEHP increased the number of RAW cells with an elongated and flattened morphology, as well as staining for surface marker CD163, indicative of differentiation into M2-like macrophages. The MEHP-induced changes seen in RAW cells were in the same concentration range as that previously reported in the primary alveolar macrophages *in vitro* (Rakkestad et al., 2010).

Lung macrophages produce the pro-inflammatory cytokine TNF- α , an important mediator of immune regulation and inflammatory responses implicated in the pathology in asthma (Berry et al., 2007; Hamid et al., 2003). MEHP concentrations above 0.3 mM induced a rapid release of TNF- α from RAW cells, which is in accordance with data from other cell lines that show increased levels of pro-inflammatory markers like IL-6, IL-8, IL-1 β and VEGF (Jepsen et al., 2004; Yao et al., 2007; Vetrano et al., 2010). DEHP has been reported to mediate inflammatory responses in a similar concentration range (100 μ M) in mast cells (Oh and Lim, 2010). To our knowledge calculations to determine relevant concentrations

of DEHP in the lung lining fluid due to inhalation exposure to DEHP have not been published. The present responses were observed at rather high concentrations with arguable relevance for *in vivo* inhalation exposure. However, it is a well-known problem in mechanistic toxicology that *in vitro* cell lines are less sensitive and often demand higher concentration of a foreign compound to detect responses. Interestingly, in a study using primary lung macrophages from exudates, that may be considered as primed or activated cells, DEHP concentrations as low as 10 nM induced release of inflammatory mediators like TNF- α , IL-1 β and IL-6 (Yamashita et al., 2005). This illustrates that phthalates may induce inflammatory responses at lower concentrations, when more sensitive cell models are used.

Release of TNF- α can be mediated through activation of MAP kinases and in particular p38 (Saklatvala, 2004). In agreement with this we found increased phosphorylation of p38 after MEHP exposure and reduced TNF- α release after preincubation with a p38 inhibitor. This implies a role for the p38 pathway in the MEHP-induced TNF- α release from the RAW cells similar to our previous findings in rat alveolar macrophages (Rakkestad et al., 2010). Our data also suggest involvement of the PI3K/Akt pathway in the

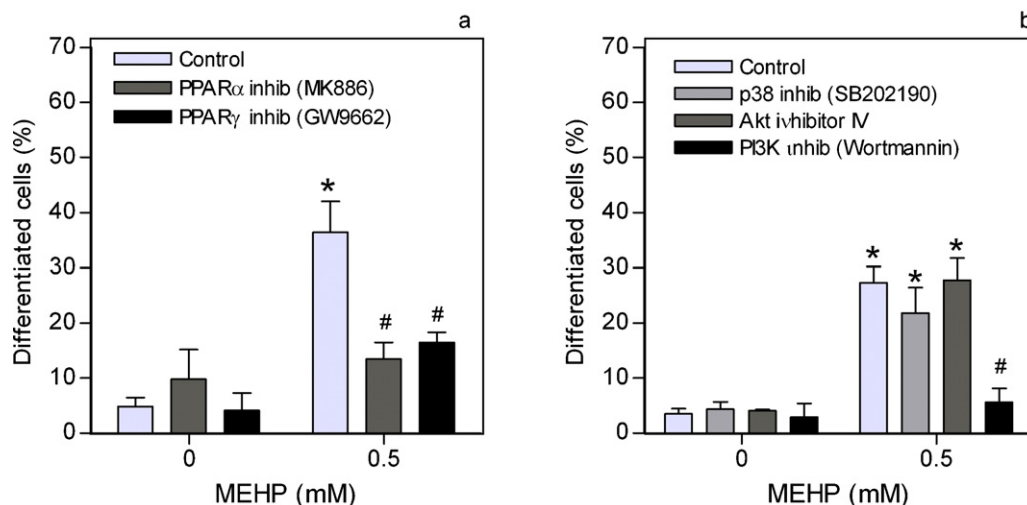


Fig. 6. Effect of inhibitors on morphological differentiation. Percentage of cells with altered morphology after 3 h exposure to MEHP in the presence or absence of (a) the PPAR inhibitors MK886 (10 μ M) and GW9662 (10 μ M) or (b) the inhibitors of p38 (SB202190; 1 μ M), Akt (Akt inhibitor IV; 10 nM) and PI3K (Wortmannin; 1 μ M). *Significant increase compared to unexposed control, #significant decrease compared to MEHP-exposed control (two-way ANOVA with Bonferroni post-test on log transformed data, N=3–6).

release of TNF- α . A possible downstream effect of MEHP-induced p38 and PI3K/Akt signalling might be increased activity of transcription factors such as AP-1 and NF- κ B, as reported in DEHP- or MEHP-exposed mast cells and germ cells (Oh and Lim, 2011; Oh et al., 2010; Rogers et al., 2008). Both AP-1 and NF- κ B are reported to be involved in TNF- α regulation in RAW cells (Lee et al., 2003; Cho et al., 2004).

ROS function as cell signalling molecules for normal biological processes, but excessive generation of ROS may cause damage to cellular molecules and organelles which can ultimately disrupt normal physiology (Auten and Davis, 2009). In line with studies in other cell types (Kasahara et al., 2002; Rakkestad et al., 2010), we find that MEHP induces a concentration dependent increase in ROS levels in RAW cells. Anti-oxidant treatment reduced the MEHP-induced ROS levels, but did not affect the TNF- α release. Thus the cytokine response did not appear to result from MEHP-induced oxidative stress in the RAW cells.

In primary rat alveolar macrophages MEHP induced morphological changes including increased numbers and size of vacuole-like structures and cellular flattening (Rakkestad et al., 2010). In accordance, exposure to MEHP induced morphological changes in RAW cells, and quantification of this differentiation revealed a concentration dependent effect. The elongated spindle-like appearance after MEHP exposure resembles the morphology of anti-inflammatory macrophages M2 (van der Plas et al., 2009; Mantovani et al., 2004). MEHP exposure also increased expression of the M2-marker CD163 (van der Plas et al., 2009; Mantovani et al., 2004), further supporting the notion that the MEHP promotes M2-like differentiation in RAW cells. However, CD163 expression and morphology alone may not be sufficient to characterise M2 differentiation. As pointed out by Mantovani et al. (2004), fully polarized M1 and M2 cells represent the extremes of a continuum of functional macrophage phenotypes. Thus, other proteins associated with M2 macrophages need to be measured to determine the degree of differentiation induced by MEHP.

Inhibitors of PI3kinase and PPAR α and γ partly blocked the MEHP-induced morphological changes, suggesting that these signalling pathways were involved in the differentiation process. PI3kinase activity has been linked to diverse cellular processes including cell growth, proliferation and differentiation (Ito et al., 2007). The involvement of the PI3kinase signalling pathway in regulating MEHP-induced differentiation of RAW cells is in agreement with studies in adipocytes showing that PI3kinase lipid products are signalling intermediates promoting cellular differentiation (Saad et al., 1994). PPAR γ plays an immunomodulatory role in alveolar macrophages, and acts as a limiting factor for possible injurious inflammation in the lung through negative regulation of expression of pro-inflammatory cytokines such as TNF- α , increasing phagocytic activity and stimulating monocyte differentiation into macrophages (Reddy, 2008). PPAR α and γ are both expressed in differentiated macrophages, and PPAR γ has been suggested to act as a negative regulator of macrophage activation (Ricote et al., 1998). A recent study reported that PPAR γ , but not PPAR α and δ , promoted monocyte differentiation towards alternative macrophages M2 (Bouhlef et al., 2009). This is partly in contrast to what we found in the present study, where both PPAR α and γ seemed to be involved in the differentiation process, since inhibitors of both isoforms reduced the morphological changes in the RAW cells.

In line with the more recent classification into pro- and anti-inflammatory macrophages (Mantovani et al., 2004), some studies report that differentiation of monocytes into mature macrophages is accompanied by an increased release of TNF- α (Wewers and Herzyk, 1989), whereas others report a reduced TNF- α release (Yagnik et al., 2000; Smythe et al., 2003). In the present study, exposure to MEHP increased the release of TNF- α from RAW cells, and altered the cell morphology suggesting a possible

differentiation towards anti-inflammatory M2-like macrophages. Since the M2 phenotype is generally associated with a low release of TNF- α , a concomitant release of TNF- α and a possible differentiation into anti-inflammatory M2 was not expected. Although differentiation into M2 macrophages is generally associated with reduced release of proinflammatory cytokines such as TNF- α , M2 macrophages have been reported to release TNF- α depending on stimuli (Mantovani et al., 2004). Furthermore, in line with the anti-inflammatory role of M2 macrophages, it has been suggested that low release of TNF- α could have an anti-inflammatory function and be involved in tissue repair (Kuroki et al., 2003). The release of TNF- α and cellular differentiation seem to involve different cellular pathways in the RAW cells, but further studies are necessary to determine whether these two processes are independent.

The phthalate monoester MEHP is known to function as a ligand for PPARs, and has been found to activate PPAR α , γ and δ/β in various cell types (Feige et al., 2007; Hurst and Waxman, 2003). Although PPARs are often suggested to be involved in phthalate-induced effects in the lung (Magliozzi et al., 2003; Rosicarelli and Stefanini, 2009), a plausible link between PPAR activation and the pulmonary effects of phthalates has not been established. Our experiments suggested that neither PPAR γ nor α were involved in the MEHP-induced release of TNF- α from RAW cells, instead both inhibitors blocked the MEHP-induced differentiation. Thus, modulation of macrophage differentiation into different phenotypes might be a possible role for PPARs in phthalate-induced effects in the lung. Interestingly, alternatively activated (or anti-inflammatory) macrophages have been suggested to be involved in chronic lung diseases like asthma, but to what extent these macrophages influence the development of lung diseases is still unclear (Byers and Holtzman, 2010).

In conclusion, we show that MEHP triggers release of the pro-inflammatory cytokine TNF- α and initiate a possible differentiation towards M2-like (anti-inflammatory) macrophages. Moreover, these two responses appear to involve different signalling pathways.

Conflict of interest

None.

Acknowledgements

We thank H. Skoglund, L. Ekeren and H.J. Dahlman for excellent technical assistance throughout the study. Financial support from the Norwegian research council (NFR) is gratefully acknowledged.

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