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Endoplasmic reticulum stress as a novel cellular response to di (2-ethylhexyl) phthalate exposure

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ABSTRACT

Di (2-ethylhexyl) phthalate is a high-production chemical widely used as a plasticizer for polyvinyl chloride products. Due to its ubiquitous presence in environmental compartments and the constant exposure of the general population through ingestion, inhalation, and dermal absorption, this compound has been subjected to extensive in vivo and in vitro toxicological studies. Despite the available information, research on the cytotoxicity of di (2-ethylhexyl) phthalate in mammalian cells is relatively limited.

In this paper, an in vitro multi-parametric approach was used to provide further mechanistic data on the toxic activity of this chemical in Vero and HaCaT cells. Our results reveal that a 24 h exposure to di (2-ethylhexyl) phthalate causes, in both cell lines, an inhibition of cell proliferation that was linked to cell cycle delay at the G1 phase. Concomitantly, the tested compound induces mild endoplasmic reticulum stress which leads to an adaptive rather than a pro-apoptotic response in mammalian cells. These findings demonstrate that there are multiple potential cellular targets of di (2-ethylhexyl) phthalate-induced toxicity and the need to develop further experimental studies for the risk assessment of this ubiquitous plasticizer.

1. Introduction

Di (2-ethylhexyl) phthalate (DEHP) is a high-production chemical widely used as a plasticizer for polyvinyl chloride (PVC) products which have a number of applications such as building materials, food packaging, cosmetics, and medical devices. The presence of DEHP and its metabolites in human body fluids, as a result of the constant exposure to the compound through ingestion, inhalation, and dermal absorption (Wittassek et al., 2011), has raised scientific and public concerns about possible detrimental health effects. Although to date there is no convincing evidence of public health hazards, different regulations to limit the use of certain phthalates, including DEHP, have been enacted and/or proposed in the USA, Europe, and Canada (Kamrin, 2009).

DEHP has been subjected to extensive in vivo and in vitro toxicological studies, as summarized in recent reviews (Caldwell, 2012; Magdouli et al., 2013). In particular, much interest has focused on the possible associations between repeated exposure to DEHP and harmful reproductive and/or developmental outcomes (Swan, 2008). Moreover, cytotoxicity studies conducted in diverse mammalian cell lines showed that the compound causes lysosomal destabilization (Peropadre et al., 2013) at relatively low concentrations (≥10 μM), exhibits anti-apoptotic properties (Maire et al., 2005), interferes with fatty acid homeostasis (Xu et al., 2005), decreases cell growth (Martinasso et al., 2006) and alters gene expression profiles (Hokanson et al., 2006) at moderate concentrations (50–100 μM), and impairs insulin binding (Rengarajan et al., 2007) at high concentrations (≥200 μM). The carcinogenic hazard of the compound has also received considerable attention, and DEHP has been reclassified by the International Agency for Research on Cancer (IARC) as possibly carcinogenic to humans (Group 2B) (IARC, 2012).

In the past few years, a number of epidemiological and experimental findings have documented that this plasticizer, like other environmental pollutants, may have an effect on metabolic homeostasis increasing the risk for obesity, insulin resistance, and type 2 diabetes (Tang-Péronard et al., 2011). Although not yet fully understood, evidence is accumulating that interaction with ligand-activated nuclear receptors (NRs) may be a critical step in the onset and/or treatment of these interrelated metabolic disorders (Mauvais-Jarvis, 2011). The activation of the three isotypes (α, β, γ) of proliferator-activated receptors (PPARs) by DEHP and its metabolites as well as the resulting metabolic consequences has already been well established (Desvergne et al., 2009). Nevertheless, given the extensive crosstalk between transcription factors, coregulators, and signaling pathways involved in the control of energy homeostasis, much work is still needed to fully understand the etiology of metabolic diseases. Noteworthy, endoplasmic reticulum

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(ER) stress that activates a conserved network of transcriptional events, termed the unfolded protein response (UPR) (Hetz, 2012), is increasingly recognized as an emerging mechanism in the pathogenesis and progression of metabolic diseases and other human disorders (Ozcan and Tabas, 2012). Furthermore, there is recent evidence that a causal relationship may exist between exposure to different environmental pollutants and ER stress (Kitamura, 2013), but no sufficient data are available in this respect concerning DEHP, except for a recent study which reports that the compound causes apoptosis in rat INS-1 cells as a consequence of UPR activation (Sun et al., 2015).

The aim of this study was to provide new mechanistic insights into the cytotoxic activity of DEHP, using Vero and HaCaT cells. It is well established that, because of pronounced interspecies variations, non-human and human primate cell lines may provide more suitable and relevant results for human health. Cell proliferation rates as well as the possible activation of critical UPR markers, including the chaperone GRP78, the pro-apoptotic transcription factor CHOP, and the anti-apoptotic protein Bcl-2, were assessed. Our findings, although simplified with respect to the in vivo situations, provide evidence that DEHP triggers an ER stress response in non-rodent cell lines and highlight the importance of establishing more precisely the causal relationships between broadly used industrial chemicals and human diseases.

2. Materials and methods

2.1. Cell culture and DEHP treatments

Vero cells (ATCC No. CCL-81) and HaCaT cells (CLS-Cell Lines Service, Eppelheim, Germany), derived from monkey kidney and human keratinocytes, respectively, were routinely cultured at 37 °C in 25 cm^2 flasks (Falcon, Becton Dickinson, USA) under a 5% CO_2 humidified atmosphere, using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine (all from Lonza, Switzerland). Exponentially growing cells were seeded at a density of 10^5 cells/ml in different cell culture surfaces (Falcon, Becton Dickinson, USA), depending on the experimental requirements. After an overnight incubation, Vero and HaCaT cells were exposed to increasing concentrations of di (2-ethylhexyl) phthalate (DEHP) ranging from 1 to 100 μM (0.39–39.05 μg/ml). At given times during the experimental period, both treated and untreated cells were gently washed with phosphate-buffered saline (PBS) and processed according to the different procedures.

Stock solutions of 10^{-2} M and 10^{-3} M of DEHP (CAS No 117–81-7, Sigma, USA) were prepared in ethanol and maintained in darkness at room temperature. The working solutions were prepared before use in DMEM supplemented with 2% (*v*/*v*) FCS and sterilized by filtration through a 0.22 μm Millipore® filter. Ethanol concentrations in medium did not exceed 1% (*v*/*v*) including the control groups.

2.2. Cell proliferation assays

Cell density was determined using the sulforhodamine B-based TOX6 kit (SRB assay, Sigma) adapted to 24-well plates, according to the manufacturer's instructions. In brief, following treatments with 1–100 μM DEHP for 24 h, cells were fixed in cold 50% trichloroacetic acid for 1 h, air dried, and stored. After 20–30 min staining with sulforhodamine B solution (0.4%), stain was removed, cultures were quickly washed with 1% acetic acid several times and air dried. The incorporated dye was then solubilized in Tris base solution (10 mM), and absorbance at 565 nm was measured using a Spectrafluor microplate reader (Tecan, Austria).

To analyze cell-cycle distribution by flow cytometry, Vero and HaCaT cells were seeded in 25 cm^2 culture flasks and exposed to 100 μM DEHP during 24 h. The cells were collected by trypsinization and fixed in 70% (*v*/*v*) ice-cold ethanol during at least 18 h. After gentle washing with PBS, the cells were incubated for 30 min in a solution containing 50 μg/ml RNase A and 50 μg/ml propidium iodide in 0.1% (*w*/*v*) sodium citrate buffer (all from Sigma). Nuclei were then analyzed using a FACSCalibur flow cytometer and the WinMDI 2.8 free software (Becton Dickinson, USA).

The determination of mitotic index (MI) was performed in cells grown on glass coverslips into 6-well culture plates. After a 24 h exposure to DEHP, cells were fixed with cold methanol and stained with 5 μg/ml Hoechst 33258 (Riedel de Haen, Germany). Three thousand cells were counted at each concentration point, and the MI was calculated as the ratio between the number of cells in mitosis and the total number of cells and expressed as percentage of controls.

2.3. Western blotting

Whole lysates of Vero and HaCaT cells, treated for 4 or 24 h with 100 μM DEHP, were prepared with RIPA buffer (50 mM Tris–HCl, pH 8 + 1% Nonidet P-40 + 150 mM NaCl) and protease inhibitor cocktail (Roche, Germany). After incubation on ice for 30 min, lysates were centrifuged for 30 min (13,000 rpm at 4 °C) and supernatant was collected. An aliquot of the total cell extracts was used to measure protein concentration with the Pierce® BCA protein assay kit (ThermoScientific, USA). Protein samples (50 μg per lane) were mixed with Laemmli buffer, boiled for 5 min at 100 °C, and subjected to SDS-PAGE on 10% gel at 100 V for 1–2 h. After protein transfer onto nitrocellulose membranes (Bio-Rad, USA), the membranes were blocked for 1 h with 5% non-fat dry milk in Tris buffered saline (TBS) containing 0.05% Tween-20 (TTBS) and then incubated with antibodies anti-GRP78, anti-GADD153, anti-Bcl-2 (Santa Cruz Biotechnology, USA) and anti-γ-tubulin (Sigma) overnight at 4 °C, at dilutions recommended by the manufacturer. After washing three times with TTBS, the membranes were incubated at room temperature for 1 h with horseradish peroxidase-conjugated anti-mouse, anti-rabbit, or anti-goat antibodies (Santa Cruz Biotechnology, USA). The membranes were washed three times in TTBS and then probed with the Immun-Star HRP chemiluminescent kit (Bio-Rad). Band intensities were quantified using Image Lab 3.0.1. software (Bio-Rad) and data were expressed as the relative density of the protein normalized to γ-tubulin and respective time controls.

2.4. Morphological studies

Complementary morphological studies to evaluate the presence of abnormal intracellular accumulations as well as the integrity of key subcellular structures were conducted using different microscopic techniques. To visualize intracellular lipid droplets, Vero and HaCaT cells grown on sterile glass coverslips into 6-well culture plates and treated with DEHP for 24 h, were fixed with 4% (*v*/*v*) paraformaldehyde for 15 min and stained with Nile red (1 μg/ml; Sigma) in the dark for 5 min at room temperature. Afterwards, the cells were examined by fluorescence microscopy under blue exciting light (λ = 450–500 nm). Microscopy observations were carried out using a Leica DMI 3000B microscope (Germany), equipped with an EL6000 compact light source and appropriate excitation filters. The images were acquired with a CCD camera Leica DFC310FX and processed using the software Leica Aplication Suite 3.5.0 and Adobe Photoshop 9.0 (Adobe Systems Inc., USA). All comparative images (treated vs. untreated samples) were obtained under identical microscope and camera settings. For quantification, integrated density after thresholding images from three independent experiments was analyzed according to Mehlem et al. (2013), using the free image-processing software Fiji (Schindelin et al., 2012).

To further investigate the intracellular targets of the compound, an ultrastructural analysis was conducted in Vero and HaCaT cells treated for 24 h with 100 μM DEHP. The cells were washed in PBS, briefly trypsinized and centrifuged at 1000 rpm for 20 min. The pellet was