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

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A B S T R A C T

Di (2-ethylhexyl) phthalate (DEHP) 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.

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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 and DEHP has been reclassified, 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 inter-related 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...
(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 cm² flasks (Falcon, Becton Dickinson, USA) under a 5% CO2 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 10³ 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 DEHP for 24 h, cells were washed in 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 FACScan® 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 Application 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
fixed in 2.5% (v/v) glutaraldehyde (Panreac) in 0.05 M sodium cacodylate (Panreac) buffer (pH 7.5) for 30 min at room temperature. After three rapid rinses in sodium cacodylate solution (SC), the cells were post-fixed for 1 h with 1% (w/v) osmium tetroxide (Electron Microscopy Sciences, USA) in 0.05 M SC, washed in SC buffer, and then dehydrated through a graded series of ethanol and embedded in Araldite (Electron Microscopy Sciences). Ultrathin sections were cut with a diamond knife on a Reichert Ultracut S ultramicrotome and collected on 200 mesh copper grids (Electron Microscopy Sciences). The sections were contrasted by uranyl acetate and lead citrate (both from Sigma) and examined in a JEM 1010 (JEOL, Japan) transmission electron microscope, operated at an accelerating voltage of 80 kV.

2.5. Statistical analysis

Statistical evaluations were carried out using the SPSS-11.5 software (SPSS Inc., USA). The results were analyzed by Student’s t-test for simple comparisons or by analysis of variance (ANOVA) followed by Bonferroni post hoc test for multiple comparisons. Unless otherwise stated, each data point represents the arithmetic mean ± standard deviation of at least three independent experiments. EC50 (50% effective concentration) values were obtained with the program GraphPad Prism 4.0 (GraphPad software, USA) using non-linear regression. The level of statistical significance was p ≤ 0.05 (*) or p ≤ 0.01 (**).

3. Results

3.1. Effects on cell growth induced by DEHP

The SRB assay, based on the measurement of cellular protein content, showed that a 24 h exposure to the compound results in a concentration-dependent decline in cell number with a similar behavior for both cell lines (Fig. 1). Even though the effects on HaCaT cells resulted in lower protein values, statistically significant differences with control cells appeared in both cell lines after 10 μM DEHP (p < 0.05) and 100 μM DEHP (p < 0.01) treatments. No EC50 values were obtained with the DEHP concentration range tested in the present study. On the basis of these data, the highest concentration of the compound (100 μM) was selected for subsequent studies.

In the same experimental conditions, flow cytometry analysis of DNA content revealed a significant accumulation of Vero cells in G0/G1 phase with a concomitant decrease in the proportion of cells in the S and G2/M phases (Fig. 2A). Likewise, significant differences in mitotic index values were found between controls and treatments with DEHP 100 μM (Fig. 2A). A comparable cell cycle profile was obtained for HaCaT cells (Fig. 2B). However, while the MI and the percentage of cells in G2/M phase were significantly decreased, changes in the fraction of cells in G0/G1 and S phases appeared not statistically significant in comparison with control values. Furthermore, a slight but significant increase in the sub-G1 apoptotic fraction was observed in DEHP-treated HaCaT cells.

3.2. Induction of UPR markers after DEHP exposure

To determine the possible induction of ER stress by DEHP, we analyzed by Western blot the protein expression levels of two critical UPR markers, the chaperone GRP78/Bip and the pro-apoptotic transcriptional factor CHOP/Gadd153. GRP78 protein expression showed a tendency to rise at 4 h in Vero cells, reaching a significant 2.1-fold increase at 24 h, whereas the CHOP levels remained unchanged at 4 h and slightly reduced at 24 h, as compared with controls (Fig. 3A, C). In HaCaT cells, DEHP stimulated transient increases in GRP78 and CHOP expression (2.8- and 1.5-fold respectively) at 4 h and returned to values comparable to that of controls by 24 h. We next investigated whether DEHP-treated cells exhibited altered expression of the anti-apoptotic Bcl-2 protein, a crucial modulator of cell death signaling. As shown in Fig. 3A and C, Vero cells exhibited a 2.8-fold increase in Bcl-2 expression at 24 h. In HaCaT cells, DEHP treatment for 4 h resulted in elevated Bcl-2 protein expression, while longer incubation times (24 h) led to reduced levels of the anti-apoptotic protein Bcl-2 (Fig. 3B, C).

3.3. Morphological changes in cells exposed to DEHP

Treatment with 100 μM DEHP for 24 h resulted in an accumulation of cytosolic lipid droplets (CLDs) in both Vero and HaCaT cells, as evidenced by Nile red staining (Fig. 4). Quantification of CLDs, which appeared as bright yellow-gold dots distributed throughout the cytoplasm and occasionally clustered around the nucleus, revealed that relative lipid content was significantly increased in DEHP-treated cultures.

Fig. 1. Effects of DEHP on cell number. Concentration-response curves obtained in the Sulforhodamine B assay after treatment for 24 h of Vero and HaCaT cells with increasing DEHP concentrations. The values represent the mean ± SD from three independent determinations with 4 replicates each, and the symbols indicate statistically different values in treated cells compared to untreated control cultures (ANOVA post hoc Bonferroni; * p ≤ 0.05; ** p ≤ 0.01).
A deeper insight into the subcellular alterations observed in treated Vero and HaCaT cells was acquired by TEM (Fig. 5). Control Vero cells showed a normal ultrastructure with uncondensed chromatin and well-preserved organelles. Following treatments with 100 μM DEHP, virtually all examined cells exhibited pleiomorphic autolysosomes containing amorphous cellular debris and/or multilamellar structures, suggesting the induction of autophagy. Moreover, we noted marked excess of glycogen in the forms of single β-particles and clusters or rosettes (α-particles) in the cytoplasm. Control HaCaT cells displayed a regular ultrastructure with abundant mitochondria and RER that was also disrupted by DEHP treatments. The most striking features included severely damaged mitochondria, showing pale matrix and loss of cristae, and endoplasmic reticulum dilation and/or vesiculation.

4. Discussion

Evidence of detrimental health effects in humans associated with exposure to DEHP is still quite limited, and risk assessments have been based primarily on results of animal studies. Likewise, the cellular basis of the pronounced toxicity of this broadly used plasticizer is poorly understood. A previous work from this laboratory showed that DEHP causes sublethal cytotoxic effects, including lysosomal destabilization and plasma membrane damage, in cultured mammalian cells (Peropadre et al., 2013). In this paper, we provide further mechanistic data on the toxic activity of this controversial chemical in Vero and HaCaT cells.

Under our experimental conditions, exposure to the tested compound resulted in a concentration-dependent loss of cell density and...
caused a reduction in cell proliferation rates at the highest concentration tested (100 μM). Attenuation of cell cycle progression represents a common initial event during the adaptive response in mammalian cells, to repair or minimize damage at the subcellular or molecular level, and to restore cell homeostasis. The cell cycle machinery in eukaryotes is highly coordinated by the master regulatory proteins cyclins and cyclin-dependent kinases (CDKs). Regulation of G1-S transition depends initially on D-type cyclins (D1, D2, and/or D3) associated with CDK4/6 and later on cyclin E-CDK2 complexes (Sherr and Roberts, 2004). Interestingly, several in vitro studies showed that a treatment with 100 μM DEHP for 24 h decreases the protein levels of cyclin D1 and CDK2 in rat calvarial osteoblasts (Bhat et al., 2013). Likewise, a 24 h exposure to higher concentrations of the compound (277 μM) significantly reduces mRNA expression of cyclin D2 and CDK4 in mouse antral follicles (Gupta et al., 2010), although the underlying mechanisms have not yet been fully elucidated.

In a subsequent set of experiments, we examined the possibility that DEHP could induce ER stress and UPR activation in Vero and HaCaT cells.

Fig. 4. Appearance of CLDs in Vero and HaCaT cells exposed to DEHP for 24 h. Left panel: Representative images of control and DEHP-treated Vero and HaCaT cells fixed in paraformaldehyde and stained with Nile red to visualize intracellular accumulation of cytosolic lipid droplets (CLDs). Bar, 10 μm. Right panel: Quantification of the relative lipid content from a set of randomly acquired images after Nile red staining. Values represent mean ± SD of three independent experiments. Asterisks indicate statistically significant differences (Student’s t-test, p ≤ 0.05) from control untreated cells.

Fig. 5. Ultrastructural changes caused by DEHP in Vero and HaCaT cells. Representative transmission electron micrographs of control and DEHP-treated Vero and HaCaT cells. Arrowheads indicate intracellular glycogen deposits (α-particles) in Vero cells and asterisks show dilation of endoplasmic reticulum in HaCaT cells. Bar, 0.5 μm.
The mammalian UPR, mediated by the activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1) and double-stranded RNA activated protein kinase (PKR)-like ER kinase (PERK), promotes cell survival through upregulation of chaperones and global attenuation of protein synthesis (Walter and Ron, 2011). However, under severe or prolonged ER stress, UPR eventually induces cell death by activating apoptotic pathways (Gorman et al., 2012). To assess the effects of DEHP exposure on the ER stress response, we first examined by Western blot analysis the expression of the ER chaperone GRP78/Bip, which represents a major hallmark of UPR induction (Lee, 2005). GRP78 protein levels were increased by more than 2-fold in both cell lines tested, suggesting that the compound can activate efficiently the UPR under our experimental conditions. It should be noted, however, that the representative markers examined, namely, the chaperone GRP78, the pro-apoptotic transcription factor CHOP, and the anti-apoptotic protein Bcl-2, exhibited different temporal expression patterns in Vero and HaCaT cells, thereby demonstrating distinct kinetics of UPR induction and recovery. These cell-type differences are in agreement with previous studies showing that the ability to manage and cope with ER stress varies between cell lines (Murray et al., 2004). UPR activation in Vero cells produced persistent overexpression of GRP78 protein, a key feature of an adaptive response to mild chronic ER stress resulting in cell survival (Rutkowski et al., 2006). In addition, we found that whereas CHOP levels remained stable at 24 h, Bcl-2 protein expression was clearly induced at this time point. Taken together, these data are consistent with the notion that increased GRP78 protein levels attenuate CHOP underexpression and/or reparative autophagy might also explain the anti-apoptotic effects of DEHP, at least in some types of ER stressed cells.

In contrast with Vero cells, ER stress markers were simultaneously overexpressed in HaCaT cells and also readily deactivated within 24 h. Furthermore, although a slight but significant increase in the sub-G1 apoptotic fraction was detected by flow cytometry, the majority of HaCaT cells ultimately survive after a 24 h exposure to DEHP. These findings are compatible with a transient UPR induction in which CHOP levels were insufficient to trigger massive apoptosis, likely due to concomitant overexpression of GRP78 and Bcl-2 that could prevent or markedly delay cell death to facilitate adaptation. In support of this assumption, it has been reported that cell fate is dependent not only on the severity or duration of ER stress but also on the balance between pro-death and pro-survival factors (Rutkowski et al., 2006). It should be noted also that no signs of autophagy were detectable by TEM in HaCaT cells after a 24 h exposure to DEHP. These results suggest that a causal relationship might exist between duration of UPR and induction of cytotoxic/protective autophagy. Besides, it is tempting to speculate that the different expression kinetics of GRP78 in Vero and HaCaT cells may underlie these discrepant results since it is well known that this chaperone, through maintenance of ER homeostasis, facilitates autophagy in mammalian cells (Li et al., 2008).

ER stress, which occurs as a result of protein misfolding, may be induced by different stimuli such as alterations in redox homeostasis, nutrient deprivation, or failure of post-translational modifications (Hayer-Hansen and Jäättelä, 2007). Although it was not the aim of this study to assess the mechanism leading to ER dysfunction in DEHP-treated cells, it should be noted that the compound is known to reduce dolichol phosphate levels and therefore the rates of protein glycosylation in rat liver microsomes (Ganning et al., 1987). This suggests that DEHP may share a common mechanism of action with tunicamycin, an inhibitor of N-linked glycosylation, considered as one of the most potent inducers of the UPR (Bull and Thiede, 2012).

Another remarkable finding in this study was the appearance of cytosolic lipid droplets (CLDs) in both cell lines, following exposure to DEHP. CLDs are known to accumulate under cellular stress functionally linked to pathological conditions, including obesity and related metabolic diseases (Walther and Farese, 2012). In agreement with our findings, a number of studies have recently demonstrated that ER stress consistently stimulates lipid droplet formation in mammalian cells (Zhang and Zhang, 2012). More importantly, there is now compelling evidence that CLDs are present in cells to sequester misfolded proteins at the early stage of ER stress (Hapala et al., 2011), being considered as key mediators in ER-associated degradation (ERAD) of proteins (Klemm et al., 2011). It is also worth mentioning that kidney epithelial Vero cells exhibited increased glycogen deposits, as a result of DEHP exposure. Interestingly, while caution should be exercised in extrapolating in vitro observations to the in vivo situations, our findings closely resemble some previously observed in animal studies. It has been reported that accumulation of glycogen granules (the so-called Armanni–Ebshtein lesion) is the most frequent alteration in the kidney tubular epithelial cells of experimentally diabetic rats (Kang et al., 2005), although the pathological significance remains unclear.

It is worth mentioning that, under experimental conditions, comparable to those used in this study, other authors have recently reported that DEHP causes UPR activation and subsequent apoptosis in rat insulinoma INS-1 cells, suggesting that ER stress partially contributes to β-cell dysfunction (Sun et al., 2015). However, we demonstrated that the compound induces mild ER stress which leads to an adaptive rather than a pro-apoptotic response in Vero and HaCaT cells. It is now well established that while severe ER stress is involved in the pathophysiology of a number of human diseases, ER preconditioning protects cells against subsequent toxic insults and might therefore cause resistance to pharmacological treatments (Rutkowski and Kaufman, 2007).

In conclusion, our present data that deserve further investigation confirm and extend previous findings in rodent cells and support the hypothesis that ER stress is a common cellular response to DEHP exposure in different types of mammalian cells.

Conflict of interest statement

The authors declare no competing financial interest.

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Transparency document

The Transparency document associated with this article can be found in online version.

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