

Short Communication

Phthalates Deregulate Cell Proliferation, but Not Neuroendocrine Transdifferentiation, in Human LNCaP Prostate Cancer Cell Model

(phthalates / prostate cancer cells / cell cycle modulation / neuroendocrine transdifferentiation / androgen receptor)

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Abstract. Phthalate esters are ubiquitous environmental pollutants widely used as plasticizers, which have been shown to interfere with both endocrine regulation and development of reproductive organs. In the present study, we examined the impact of diethylhexyl phthalate (DEHP) and dibutyl phthalate (DBP) on the proliferation of androgen-sensitive human prostate carcinoma LNCaP cells and related events. The results showed that both compounds were able to inhibit cell cycle progression in a dose-dependent manner. However, only DEHP was found to weakly reduce androgen receptor (AR) protein levels after long-term exposure, while only DBP partially inhibited expression of the prostate-specific antigen (*KLK3*) gene, a model AR transcriptional target. This indicated that inhibition of cell proliferation was likely independent of any AR modulations. Both phthalates induced suppression of cell proliferation, but none of them affected the levels of markers as-

sociated with neuroendocrine transdifferentiation (NED) in LNCaP cells. Taken together, the presented data indicate that phthalates may exert long-term negative effects on the proliferation of prostate epithelial cells derived from the carcinoma model, which are, nevertheless, largely independent of the modulation of AR expression/activity, and which do not alter further processes associated with NED.

Introduction

Phthalates are widely used plasticizers, which are ubiquitously distributed in the environment, and which are considered to pose a potential health risk to both animals and humans. Two frequently encountered phthalates are diethylhexyl phthalate (DEHP), a plasticizer used in manufacturing articles made of polyvinyl compounds, and dibutyl phthalate (DBP), which is used as a component in latex adhesives, cosmetics, in cellulose plastics, or as a dye solvent (Hauser and Calafat, 2005). The non-occupational human exposure to these compounds mainly results from their widespread use in personal care and consumer products. Both compounds have been demonstrated to produce reproductive and developmental toxicity (Hauser and Calafat, 2005; Lyche et al., 2009). Upon exposure, dialkyl phthalates are rapidly metabolized into monoesters, and some toxic effects of phthalates are caused by these metabolites rather than by the parent compounds (Hauser and Calafat, 2005). The toxic effects of phthalates within the male reproductive system have been attributed e.g. to their anti-androgenic properties, generation of oxidative stress, or to modulation of activities of oestrogen and/or peroxisome proliferator-activated receptors (Gazouli et al., 2002; Lovekamp-Swan and Davis, 2003; Bility et al., 2004; Thompson et al., 2004; O'Brien et al., 2005;

Received June 27, 2014. Accepted July 21, 2014.

This work was supported by projects CELLBIOL (CZ.1.07/2.3.00/30.0030), FNUSA-ICRC (CZ.1.05/1.1.00/02.0123) and HistoPARK (CZ.1.07/2.3.00/20.0185) from the European Regional Development Fund.

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Abbreviations: AR – androgen receptor, CS – dextran/charcoal-stripped FBS, DBP – dibutyl phthalate, DEHP – diethylhexyl phthalate, DHT – 4,5 α -dihydrotestosterone, DMSO – dimethyl sulphoxide, FBS – foetal bovine serum, NED – neuroendocrine transdifferentiation, PBS – phosphate-buffered saline, PSA – prostate-specific antigen.

Noriega et al., 2009). Phthalates have also been documented to modulate activity of further nuclear receptors, such as the pregnane X receptor and the constitutive androstane receptor, through a variety of mechanisms, which may consequently lead to deregulation of the metabolism of steroid hormones as well as lipids (Hurst and Waxman, 2004; Wyde et al., 2005; Cooper et al., 2008; DeKeyser et al., 2011). Increased phthalate exposure has been suggested to be associated with an increased cancer risk (Lopez-Carrillo et al., 2010) or to induce transgenerational epigenetic changes associated with obesity or reproductive disease (Manikkam et al., 2013).

Prostate carcinoma is presently one of the most prevalent forms of cancer in the developed world, and environmental factors, including diet, have been shown to contribute to its aetiology (American Cancer Society, 2009). Developmental exposure to phthalates has been shown to contribute to proliferative and inflammatory disorders of the prostate *in vivo* (Scarano et al., 2009). Phthalates could thus deregulate growth of prostate tumour cells via a range of mechanisms described above and contribute to altered proliferation/survival of prostate carcinoma cells. Nevertheless, in other studies, no tumour-promoting effect of single phthalate exposure on chemical-induced carcinogenesis has been observed (Kohno et al., 2004). Interestingly, recent studies seem to indicate that phthalates may, at least *in vitro*, also contribute to cancer progression, and this has also been observed in cellular models derived from prostate cancer cells (Hsieh et al., 2012; Lee et al., 2014). Although it has been shown that DEHP may negatively affect prostate carcinoma cell survival in culture (Erkekoglu et al., 2010), possible involvement of androgen receptor (AR) or further impact of phthalates on phenotypic changes in prostate cancer cells, such as modulation of neuroendocrine transdifferentiation (NED), are presently unknown. NED is one of the central events contributing to the development of anti-androgen resistance during prostate cancer progression (Cindolo et al., 2007). The cells with neuroendocrine-like properties, which may arise from prostate epithelial cells through NED, are androgen-independent, they are able to secrete several neuropeptides and promote prostate cancer progression. NED can be induced *in vitro* by various stimuli, including androgen depletion (Yuan et al., 2006) or high cell density and cell cycle modulation (Pernicová et al., 2014). Therefore, we hypothesized that compounds affecting AR activity and/or cell cycle progression, such as phthalates, might also potentially alter NED.

In the present study, we examined the impact of DEHP and DBP on androgen-sensitive cells derived from prostate carcinoma. We particularly focused on the effects associated with long-term disruption of cell proliferation control, modulation of androgen receptor (AR) responsiveness and NED. We used LNCaP cells as a primary model, since this cell line has been previously observed to be responsive to a wide array of environmental pollutants, including polychlorinated compounds, polycyclic aromatic hydrocarbons or phthalates (Endo

et al., 2003; Kizu et al., 2003; Barnes-Ellerbe et al., 2004; Morrow et al., 2004; Hrubá et al., 2010; Erkekoglu et al., 2011). Our present results indicate that phthalate esters may exert long-term negative effects on the proliferation of prostate cancer epithelial cells, which are probably independent of the modulation of AR expression and/or its transcription activity; however, these effects do not appear to be linked with the modulation of NED.

Material and Methods

Chemicals

DBP (CAS No. 84-74-2, analytical standard), DEHP (CAS No. 117-81-7, analytical standard) and 4,5 α -dihydrotestosterone (DHT) (CAS No. 521-18-6, purity $\geq 99.0\%$) were purchased from Fluka (Buchs, Switzerland). Stock solutions were prepared in dimethyl sulphoxide (DMSO) (Merck, Darmstadt, Germany) and stored in the dark. Propidium iodide was from AppliChem GmbH (Darmstadt, Germany). Mouse monoclonal antibody against β -actin was obtained from Sigma-Aldrich (Prague, Czech Republic). Rabbit polyclonal antibody against AR, and mouse monoclonal antibody against γ -enolase, were from Santa Cruz (Santa Cruz, CA). Mouse monoclonal antibody against tubulin β -III was from Promega (Madison, WI). Secondary anti-mouse and anti-rabbit IgG antibodies were from GE Healthcare Life Sciences (Little Chalfont, UK). All other specific reagents and media supplements are indicated below.

Cells

LNCaP cells (human prostate carcinoma cells; DSMZ, Braunschweig, Germany) were cultivated in RPMI 1640 media (Invitrogen, Carlsbad, CA) supplemented with NaHCO₃ (Sigma-Aldrich), penicillin/streptomycin, and 10% foetal bovine serum (FBS; Sigma-Aldrich). Cells were cultivated in a humidified incubator (37 °C, 5% CO₂). For the long-term cultivation (8 days) and androgen depletion studies, LNCaP cells were cultivated in phenol-red free media supplemented with 5% FBS or dextran/charcoal-stripped FBS (CS). To evoke NED, LNCaP cells were cultivated as follows: cells were seeded at a density of 20,000/cm² in the appropriate complete medium with FBS (day -1). After 24 h, the medium was exchanged for medium with 5% FBS or CS (day 0). Cells were then continuously cultivated for 8 days without splitting, but with exchange of the medium for fresh medium twice a week. Cells were exposed to test compounds on day 0, and both phthalates were then freshly added to the cells at indicated concentrations with each medium exchange (days 3 and 6). Cells were then collected for counting and Western blot analysis on day 8.

Cell cycle/cell proliferation analysis

For cell cycle analysis, LNCaP cells were grown for 48 h in RPMI 1640 medium with 10% FBS and exposed

to test compounds dissolved in DMSO (maximal concentration, 0.1%, v/v) for another 24 h. Following the treatment, the medium was removed, cells were harvested by trypsinization, and then suspended in phosphate-buffered saline (PBS) with 10% FBS. Cells were then centrifuged and fixed in 70% ethanol at 4 °C overnight. Fixed cells were washed once with PBS and stained with propidium iodide as described previously (Hrubá et al., 2010). Cells were then analysed in a FACSCalibur flow cytometer using a 488-nm (15 mW) air-cooled argon-ion laser for excitation of propidium iodide, and CELLQuest software version 5.1.1 (Becton Dickinson, San Jose, CA) for data acquisition. A minimum of 15,000 events was collected per sample. Data were analysed using ModFit LT version 3.0 software (Verity Software House, Topsham, ME). For proliferation analysis, cells were cultivated for 8 days in either 5% FBS or 5% CS medium, as summarized above. Cells were then washed with ethylenediaminetetraacetic acid/PBS, harvested by trypsinization and centrifuged. After that, cells were resuspended in PBS and cell numbers were assessed using Coulter Counter (Beckman Coulter, Brea, CA).

Real-time reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated from cells using the NucleoSpin RNA II kit (Macherey-Nagel). The amplifications of samples were carried out using a QuantiTect Probe RT-PCR kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's specifications. Amplifications were run in a LightCycler machine (Roche Diagnostics GmbH, Mannheim, Germany) using the following programme: reverse transcription at 50 °C for 20 min and initial activation step at 95 °C for 15 min, followed by 40 cycles at 95 °C for 0 s and 60 °C for 60 s. The primers and probes for the PSA/KLK3 mRNA were designed and synthesized in Generi Biotech (Hradec Králové, Czech Republic), (F: 5'-CCTGAGCACCCCT-ATCAAC-3'; R: 5'-CCTGGACCTCACACCTAAG-3'; probe: 5'-CTCAAGCCTCCCCAGTTCTACTGACC-3'). The changes in gene expression were calculated using the comparative threshold cycle method (Schmittgen and Livak, 2008) with POLR2A (primers and probe were purchased as a kit from Generi Biotech) as a normalizing gene.

Western blotting

Following the treatment, cells were washed once with ice-cold PBS and lysed with 1% SDS lysis buffer (10% glycerol; 100 mM Tris, pH 7.4). Protein concentrations were determined using bicinchoninic acid and copper sulphate (Sigma-Aldrich). For analysis of NED markers, cells were lysed with RIPA buffer containing inhibitors of proteases/phosphatases, and proteins were quantified with Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA). Equal amounts of protein were separated in SDS-polyacrylamide gels and

transferred onto a polyvinylidene difluoride Hybond-P membrane (GE Healthcare). After incubation with the respective primary and secondary antibodies, detection was performed using an ECLPlus Western blotting detection system (GE Healthcare) or Immobilon Western HRP substrate (Millipore, Billerica, MA).

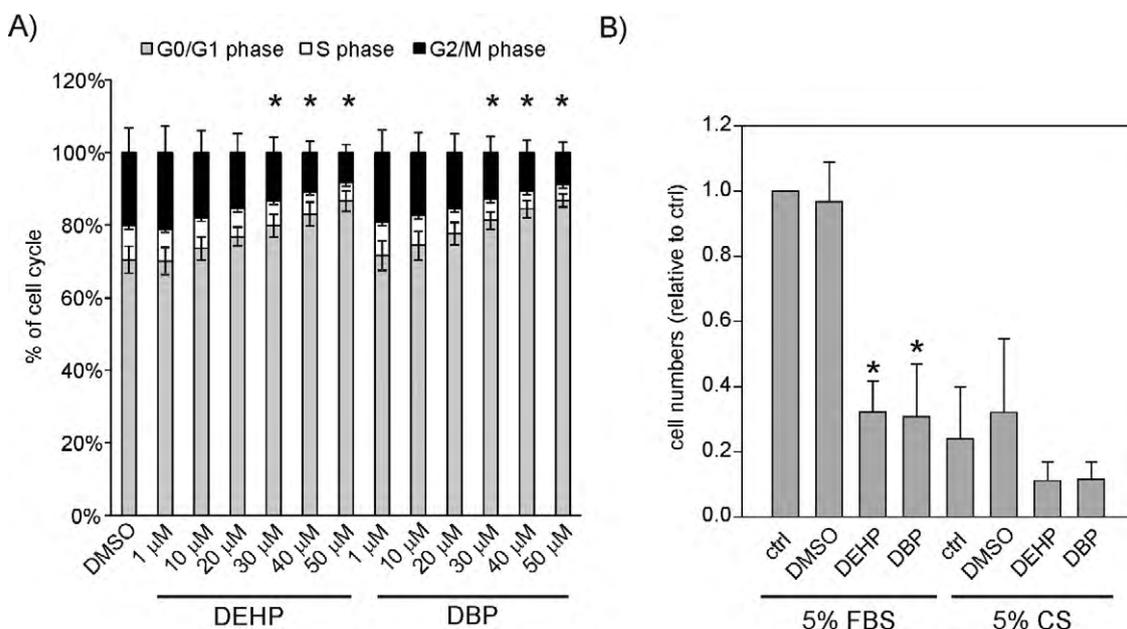
Results and Discussion

First, we investigated the effects of DEHP and DBP on cell cycle progression in LNCaP cells. As shown in Fig. 1A, both compounds blocked cell cycle progression in a dose-dependent manner and they primarily induced accumulation of cells within G1 phase of the cell cycle. The suppression of cell cycle progression occurred at lower concentrations than the previously reported toxic effects of DEHP in LNCaP cells, which were associated with production of reactive oxygen species, activation of p53 tumour suppressor and induction of p21^{WAF1/Cip1} cyclin-dependent kinase inhibitor (Erkekoglu et al., 2011). The effects of DBP have not been previously examined in the LNCaP cell model. Nevertheless, we did not observe any significant accumulation of p21^{WAF1/Cip1} in LNCaP cells upon 24-h treatment with phthalates (data not shown), which seems to suggest that induction of p21^{WAF1/Cip1} was not responsible for the observed cell cycle progression inhibition. Similarly, we did not observe any significant effects of phthalates on retinoblastoma protein phosphorylation or expression of p27^{Kip1}, another cyclin-dependent kinase inhibitor (data not shown).

We next examined the effects of phthalates on cell numbers after a prolonged exposure. The 8-day exposure time was selected to match the length of experiments examining NED markers. The results suggest that both compounds exerted long-term growth-inhibitory effects on LNCaP cells, as we observed significantly lower numbers of cells upon 8-day treatment with phthalates as compared to control cells (Fig. 1B). The decrease of the cell numbers was significant in the group cultivated in FBS medium, and the trend was also similar in cells cultivated in the CS medium, although there it was not statistically significant due to a higher control group variation. Together, the experiments depicted in Fig. 1 suggested that both DEHP and DBP inhibit proliferation of LNCaP cells and this leads to substantially reduced cell numbers upon long-term incubation of the cells with either compound.

LNCaP cells are androgen-sensitive cells and their proliferation is sensitive to the presence of androgens (Horoszewicz et al., 1983). Phthalates have been reported to exert anti-androgenic effects on the prostate and male reproductive organs *in vivo* (Lee and Koo, 2007; Vo et al., 2009), and this has been related e.g. to reduction of circulating androgens (Meeker et al., 2009) or reduced local production of testosterone and/or additional factors required for the proper development of male reproductive organs (Thompson et al., 2004; David, 2006). Nevertheless, their direct impact on AR

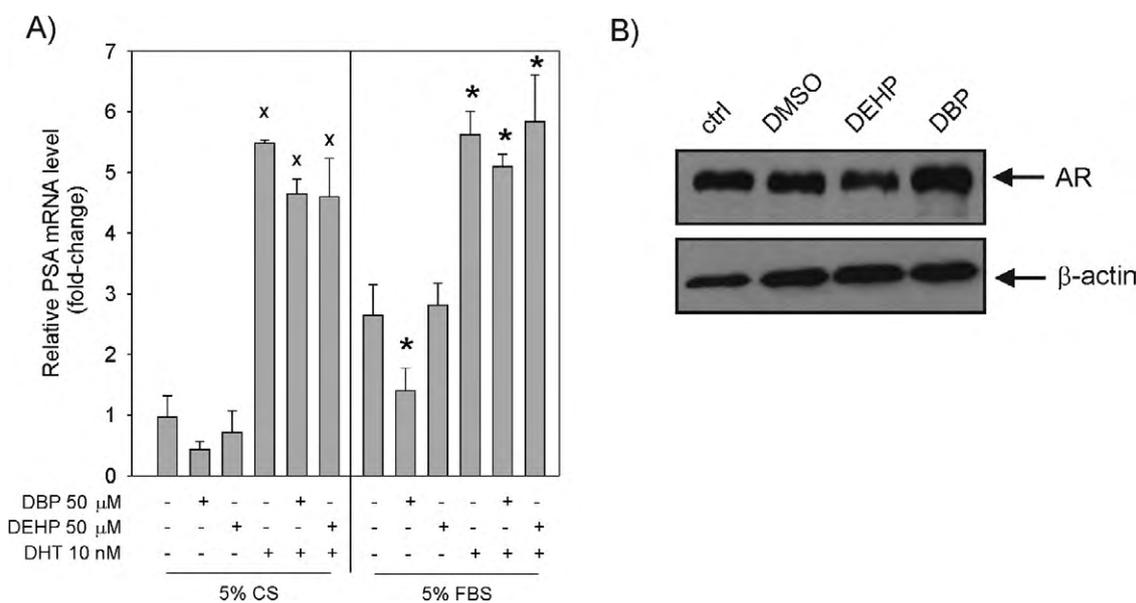
Fig. 1. DEHP and DBP inhibit cell cycle progression and reduce the number of LNCaP cells.



A. LNCaP cells were exposed to a given concentration range of either DEHP or DBP (or DMSO as a negative control) for 24 h. Cells were collected, stained and analysed for cell cycle distribution by flow cytometry, as described in Material and Methods. The data are expressed as mean \pm S.D. of three independent experiments. *A significant difference between control (DMSO) and respective treatments ($P < 0.05$).

B. LNCaP cells were exposed to DMSO, 50 μ M DEHP or 50 μ M DBP for 8 days in either cultivation medium or medium supplemented with CS. Cells were counted using Counter Center. The data are expressed as mean \pm S.D. of three independent experiments. *A significant difference between control and respective treatments ($P < 0.05$).

Fig. 2. DBP and DEHP exert weak inhibitory effects on PSA mRNA levels and AR expression, respectively, in LNCaP cells.



A. The effects on basal and DHT-induced PSA mRNA levels were examined in cells exposed to DEHP, DBP and/or DHT in either cultivation medium (5% FBS), or in CS medium (5% CS). The levels of PSA mRNA were determined after 24-h exposure to compounds at indicated concentrations. Isolation of total RNA and qRT-PCR was performed as described in Material and Methods. The results are expressed as mean \pm S.D. of three independent experiments. ^x A significant difference between control cells cultivated in CS medium and treated samples ($P < 0.05$). * A significant difference between control cells cultivated in cultivation medium and treated samples ($P < 0.05$).

B. LNCaP cells were exposed to DMSO, 50 μ M DEHP or 50 μ M DBP for 8 days in cultivation medium. The levels of AR and β -actin (loading control) were analysed by Western blotting. The results are representative of three independent experiments.

remains unclear, since e.g. in reporter gene assays carried out in MDA-kb2 cells, DEHP, and to a lesser extent also DBP, both increased the AR-dependent transcriptional activity at high concentrations (Shen et al., 2009). Therefore, we next evaluated the impact of DEHP and DBP on both basal and DHT-induced levels of PSA mRNA, a model transcriptional target of the AR (Fig. 2A). We used 50 μ M concentration, which was found to be effective in suppression of cell cycle progression. Only DBP was found to reduce the basal levels of PSA in LNCaP cells; DEHP exerted no effect. This suggested that the inhibition of cell cycle progression was not due to their anti-androgenic effects, since both compounds exerted similar effects on cell cycle progression. Neither DEHP nor DBP had a major effect on the DHT-induced PSA expression. Nevertheless, since we could not exclude that DBP and DEHP may affect AR after long-term exposure, we also examined their effect on AR protein levels upon prolonged exposure (8 days) used for NED estimation. As shown in Fig. 2B, DEHP, but not DBP, reduced the AR protein levels, which indicated that a significant reduction in cell numbers induced by both compounds was not related to the modulation of AR expression.

The above data indicated that both DEHP and DBP may have negative effects on the proliferation of LNCaP cells, which are independent of AR modulations. Modulation of cell cycle progression and its regulators is a factor contributing to NED (Pernicová et al., 2014). Therefore, in final experiments, we evaluated the levels of two markers of NED – γ -enolase (a specific isoform of enolase found in the cells of neuronal origin) and tubulin β -III, a class III member of the β tubulin protein family, whose expression is associated with prostate tumour aggressiveness, and which also modulates survival of prostate carcinoma cells (Ploussard et al., 2010). As shown in Fig. 3, neither DBP nor DEHP substantially modulated γ -enolase or tubulin β -III levels. This indicated that the inhibitory effects of phthalates on the

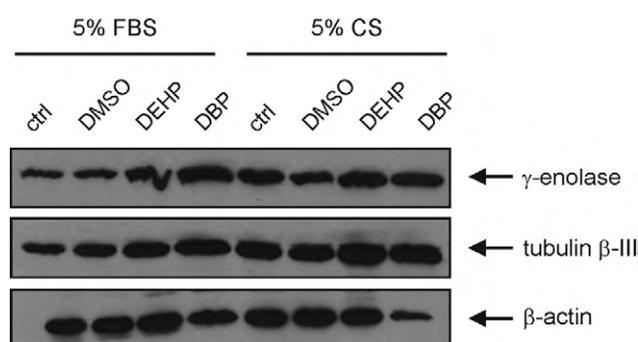


Fig. 3. DEHP or DBP do not modulate levels of NED markers in LNCaP cells.

LNCaP cells were exposed to DMSO, 50 μ M DEHP or 50 μ M DBP for 8 days in either cultivation medium or medium supplemented with CS. The levels of γ -enolase, tubulin β -III and β -actin (loading control) were analysed by Western blotting. The results are representative of three independent experiments.

proliferation of LNCaP cells did not translate into modulation of NED in this cell model.

In conclusion, the results of the present study indicate that phthalates may exert long-term negative effects on the proliferation of prostate epithelial cells derived from the carcinoma model, which are, nevertheless, independent of the modulation of AR expression/activity, and which do not alter further processes associated with NED. Future studies should more precisely establish the mechanisms associated with anti-proliferative effects of phthalates in this particular cell model, which is being used to analyse the impact of environmental pollutants on prostate cells.

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