EXPERIMENTAL STUDY

Phthalate induced toxicity in prostate cancer cell lines and effects of alpha lipoic acid

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ABSTRACT
OBJECTIVE: The effects of dimethyl phthalate, diethyl phthalate, diisobutyl phthalate, di-n-butyl phthalate, benzylbutyl phthalate, di-2-ethylhexyl phthalate were investigated on human prostate cancer cell lines DU145 and PC3 in vitro.
MATERIALS AND METHODS: Standards of dimethyl phthalate, diethyl phthalate, di-isobutyl phthalate, dibutyl phthalate, benzyl butyl phthalate, and di-ethyl hexyl phthalate were used. Alpha lipoic acid was used as an antioxidant compound. DU145 and PC3 human prostate carcinoma cells were used. MTT assay were used for cytotoxicity assay.
RESULTS: A low dose proliferative effect of phthalates in vitro was observed. With the hypothesis of the inhibition of aerobic glycolysis activity in cancer treatment, α-lipoic acid was applied to cells; where as a contrary to previous studies, no change in the cell proliferation was observed. In combination with ALA, at IC50 and lower doses, an increase of the cytotoxic effect was found for DIBP, DBP and BBP; while for DMP, DEP and DEHP, a decrease was observed for DU145 cells. In PC3 cells, a decrease was observed for DMP, DEP and DBPs; while no significant difference were observed for DEHP, DIBP and BBP.
CONCLUSION: The present study demonstrates preliminary information regarding the low dose proliferative effects of phthalates in prostate cancer in vitro (Tab. 2, Fig. 2, Ref. 65). Text in PDF www.elis.sk.
KEY WORDS: phthalate, prostate cancer, DU145, PC3, alpha lipoic acid, in vitro.

Introduction

Prostate cancer, as among the most commonly diagnosed cancer in men, has no effective treatment; while steroid hormones such as androgen were found to influence their growth and survival. Prostate cancer was found to be an ideal candidate for exogenous preventive measures, such as dietary and pharmacological prevention, due to the high prevalence, long latency, endocrine dependency, availability of serum markers (e.g. prostate-specific antigen) and the histological precursor lesions. However, there is currently no strong evidence to suggest that dietary interventions can reduce/induce the risk of prostate cancer (1).

Alpha lipoic acid (ALA), a naturally occurring cofactor, is important in the assortment of enzyme complexes controlling metabolism, including the conversion of pyruvate to energy in the mitochondrion. This compound is shown to be included in a variety of biological process associated with oxidative stress, including cancer (2, 3). ALA was found to generate reactive oxygen species (ROS), triggering the mitochondrial pathway of apoptosis in cancer cells, which contributes ALA-dependent cell death in various types of cancer cells in experimental studies, including lung (4, 5) colon, (6) breast (7, 8), leukemia (9, 10) and liver (11). Due to its powerful antioxidant capacity and importance in glycose metabolism by supporting pyruvate dehydrogenase reaction and oxidation of glycose, ALA has turned into a promising complementary therapeutic agent in the eradication of tumor cells. The mechanism of action of ALA is complex and differs according to the cancerous cell type (12). ALA was found to inhibit the second messenger NF-κB (nuclear factor κB), leading to decreased proliferation, metastasis, invasion, chemo/radio resistance and inflammation of cancer cells (13–15). ALA was found to induce the hyperacetylation of histones related to the proliferation of many types of cancer cells, which would eventually lead to apoptosis (16). As mentioned previously, the increased uptake of oxidizable substrates into the mitochondrion of cancerous cells also stimulate apoptosis. Antimitagenic and anticlastogenic effects of this compound has also been studied (17, 18). Due to its anti-inflammatory property, protective effects on nerve damage and neuropathy from chemo drugs like the platinum, related to its anti-inflammatory property were also described previously (19, 20). Epidemiology and experimental research indicate discordance for the relationship between ALA and prostate cancer. Increased risk has been associated previously (21–23). Azrad et al (23) defined the genetic variation related to ALA metabolism,
where the prostatic ALA, independent of diet, was found to be significantly and positively associated with biomarkers of aggressive disease affecting the tumor proliferation rates. Meanwhile, these studies show drawbacks such as collection of the accurate dietary data, inter-individual differences in the metabolism of ALA or the sampling sizes (24).

Phthalates, known as the plasticizers (making plastics more flexible or soft), have a variety of commercial uses, including personal-care products (e.g. perfumes, lotions, cosmetics), paints, food, construction industry, and certain medical devices and pharmaceuticals (25). These ubiquitous environmental, endocrine disrupting contaminants, were found to have adverse effects on male reproductive health (26, 27). Irreversible changes in the male reproductive tract due to phthalate exposure, even in the prenatal period, is shown to interfere with the androgen signaling pathway, causing permanent adverse effects on reproductive development corresponding a decline in male fertility due to changes in sperm concentration and semen quality. These compounds are also associated with an impaired development and alter the regular function of prostate (26). Exposure to DEHP (di-2-ethylhexyl phthalate), DEHA (di(2-ethylhexyl) adipate), (28) and DBP (diisobutyl phthalate) (29) in the diet, were found to result in decreased weight of the prostate.

The effects of phthalates on prostate cancer cells were studied extensively especially in LNCaP cells; since this cell line was found to express estrogen receptor-α, estrogen receptor-β and androgen receptors (ARs), which were linked to the endocrine disrupting property of phthalates. DBP was found to promote LNCaP prostate cancer proliferation through the crosstalk between TGF-β and ER signaling pathway (30). Meanwhile, Hruaba et al (31) showed that, at lower concentrations, DEHP (50 μM) and DBP (50 μM) were found to suppress cell cycle proliferation in a dose-dependent manner through induction of accumulation of cells within G1 phase of the cell cycle. Previously, DEHP (3 mM) and its main metabolite MEHP (mono(2-ethylhexyl)phthalate-3 μM) caused production of reactive oxygen species, activation of p53 tumor suppressor and induction of p21WAF/Cip1 cyclin-dependent kinase inhibitor; where this effect was inhibited by selenium (32). DBP was also shown to promote LNCaP cell proliferation by upregulating the gene expression of c-myc and cyclin D1 and by downregulating the expression of p21 (15). DEHP was also found to weakly reduce AR protein levels after long-term exposure (8 days), while only DBP partially inhibited expression of the prostate-specific antigen (KLK3) gene, a model AR transcriptional target. Overall, it was stated that DEHP and DBP may have negative effects on the proliferation of LNCaP cells, independent of AR modulations. Possible involvement of AR or phenotypic changes such as modulation of neuroendocrine trans differentiation (NED) due to phthalate exposure are still unknown (31). The relationship between phthalate/alpha lipoic acid and male reproduction has recently been studied in an in vivo model. Bi-n-butyl phthalate (BNBBP) was found to cause testicular toxicity through testosterone, follicule stimulating hormones (FSH) and antioxidant enzymes in Wistar rats; where ALA was found to mitigate BNBP-induced testicular toxicity through antioxidant mechanism and by direct free radical scavenging activity (33).

While the majority of the prostatic cancers are adenocarcinomas characterized by the expression of luminal differentiation markers AR and prostate-specific antigen (PSA), where LNCaP cells are used as the main in vitro model; androgen independent models DU-145 and PC3 (as a model for small cell neuroendocrine carcinoma) are used in studies for the evaluation of the effects of chemicals independent of AR and more aggressive phenotypes (34, 35). Therefore, the aim of the current study was to evaluate the effects of phthalates on androgen independent cell lines DU-145 and PC3 and to assess the possible interaction with the antioxidant ALA.

Materials and method

Chemicals

Standards of dimethyl phthalate (DMP), diethyl phthalate (DEP), di-isobutyl phthalate (DIBP), dibutyl phthalate (DBP), benzyl butyl phthalate (BBP), and di-ethyl hexyl phthalate (DEHP) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Alpha lipoic acid (DL-Thioccic acid, 98+ %) was purchased from Acros Organics (New Jersey, USA).

Cell culture conditions

DU145 (HTB-81) human prostate carcinoma cells derived from the brain metastatic site and PC3 (CRL-1435), grade IV human prostate adenocarcinoma cells derived from the bone metastatic site used in the study were acquired from the American Type Culture Collection (ATCC™). All cell culture procedures were performed under strict sterile conditions and kept inside a 5 % CO2 incubator at 37 °C. Cells were cultivated using RPMI 1640 medium (Gibco®) supplemented with 10 % fetal bovine serum (Thermo Fisher Scientific) and penicillin-streptomycin (100 U/ml, Thermo Fisher Scientific). Cell culture medium was replaced every other day. Cell growth was checked using phase-contrast microscopy.

Cytotoxicity

Sub-culturing and/or cell cultivation was carried out when a confluent monolayer of cells was observed over the majority of growth surface via Juli FL software (Seoul, Korea). For the cytotoxicity assays, cells were seeded in 96-well microplates at a density of 3 x 10^4 cells/mL in 100 μL. The microplates were incubated for 24 h to allow for cell attachment and growth in the plates while the following day 20 μL phthalate was added to the media for another 24 h incubation for cytotoxicity assays of MTT ((3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide). Based on the preliminary assays, half dilutions between 0.0061–12.5 μg/ml for DMP, DIBP and BBP; 0.0002–0.5 for DEP and DBP; 0.0006–1.25 μg/ml were applied. Concentration of alpha lipoic acid was decided according to preliminary cytotoxicity studies and our previous study (10). Treatments at each dose were conducted at three replicates in the same plate and all the experiments were repeated four times. Medium only and 0.1 % Triton
X-100 served as negative and positive controls, respectively. MTT assays were performed soon after the incubation. Cell viability was quantified using SpectraMax i3/i3x Multi-Mode Detection Platform (Molecular Devices, Sunnyvale, CA) at 540 nm for MTT.

**Statistical analysis**

Percent cytotoxicity were calculated for each concentration using Microsoft Excel computer program. Regression analysis was done using the plotted values against the corresponding doses by SPSS 17.0 where the highest correlation coefficient (R2) is selected for the fit and IC50 values are calculated. Results for ALA and phthalate combination were presented as the means ± SDs. Statistical analysis was done using one way analysis of variance (ANOVA) for multiple samples and Student’s t-test for comparing paired sample sets. p values less than 0.05 were considered statistically significant.

**Results**

**IC50 Values for the Tested Phthalates:** Among the tested phthalates, DEHP induced the highest cytotoxicity on DU 145 cells; where the least cytotoxic compound was DMP in the same cell line. PC3 cells were more susceptible to DMP, DEP and BBP than DU 145 cells (p > 0.05). Among average IC50 values for both cells, the most cytotoxic compound was DEHP followed by DBP. PC3 cells were found to be more susceptible to the tested phthalates compared to DU 145 cells (Tab. 1).

**Phthalate Combination with Alpha Lipoic Acid:** Following the co-administration of ALA with IC50 doses of pyrethroids, an increase of the cytotoxic effect were found for DIBP, DBP and BBP (13.09, 25.22, 5.36 %, respectively); while for DMP, DEP and DEHP, a decrease (9.27, 8.12, 7.76 %, respectively) was observed for DU145 cells (Fig. 1 a, Tab. 2). In PC3 cells, a decrease was observed for DMP, DEP and DBPs (26.58, 17.01 and 16.02 %, respectively); while no significant difference were observed for DEHP, DIBP and BBP (p > 0.05) (Fig. 2, Tab. 2).

**Discussion**

Endocrine disrupting compounds were found to influence the development and progression of prostate cancer mainly through estrogen reprogramming of the prostate gland resulting permanent alterations and gene expression for prostatic lesions with aging (36, 37). Epidemiologic evidence linked prostate cancer and environmental contaminants with endocrine disrupting potential such as pesticides (chlorpyrifos, fonofos, coumaphos, phorate, permethrin) (38–40) bisphenol A (41), PCBs (42), dioxin (43), cadmium (44), and arsenic (45) which are known to have estrogenic activities. Estrogens have been implicated as potential agents in the development and progression of prostate cancer through hormonal dysregulation, hyperprolactinemia, inflammation, which would lead mutations and DNA damage and epigenotoxigenic pathways (46, 47).
In order to study the genetic and molecular changes of prostate cancer development and progression, in vitro culture models such as LNCaP, DU145, PC3 and TSU-Pv1 were developed. In the nuclear compartment of PC3 cells, the presence of high affinity estrogen binding sites were reported; indicating the evidence for a specific estrogen receptor; where significant proliferative activity was inhibited (48). This was supported by Matsumura et al. (49) where phytoestrogen genistein was found to inhibit the proliferative activity and induced the expression of p21, a regulator of cell cycle progression and ERβ in the PC-3 cells. Lau et al (50) tested the receptor-mediated estrogenic and antiestrogenic action of normal and malignant prostatic epithelial cells; where LNCaP cells (androgen-sensitive human prostate adenocarcinoma cells) were found to express Erβ, and estrogen responsive genes (progesterone receptor and pS2), DU145 expressed ER-β and PR, and PC-3 cells exhibited ER-α, ER-β, and pS2 mRNA. Relative potencies of their estrogenic activities of the phthalate compounds tested in the current study descended in the order BBP > DiBP > DBP > DEHP > DEP > DMP; where BBP showed its estrogenic activity mainly through Erβ. DMP and DEP did not induce Erα-β agonism or Erβ/AR antagonism (51). In the current study, the least toxic compounds on both cells were DEP and DMP, this would suggest a possible estrogen receptor dependency for the toxic effects of these two compounds. Also in the current study, DMP, DEP and BBP induced less cytotoxic effects on DU145 cells than PC-3 and vice versa for DiBP, DBP and DEHP cells. Even though DU145 and PC3 were reported to be AR negative (52), both cells were found to express detectable levels of AR mRNA and protein, where levels of AR protein were found to increase after the androgen ligand (dihydrotestosterone) treatment (53). The expression of AR in PC3 and DU145 cell line were found to inhibit the cell proliferation; through upregulation of p21 by androgen signaling through AR (53, 54). From this point, androgen antagonist phthalates such as DiBP, DBP and BBP would be expected to have lower cytotoxic activity; meanwhile DMP and DEP (not AR antagonism) were found to have the least cytotoxicity. For DiBP, DBP and BBP, relative inhibitory concentration (RIC20) for AR antagonistic activity were found as 6.2x10^-6, 4.8x10^-6, 2.9x10^-6 M (51) respectively; while IC50 values in the current study for the same compounds were 1.44x10^-6, 1.23x10^-7 and 3.3x10^-7 M for DU-145 cells; 2.51x10^-6, 3.47x10^-7, 1.59x10^-7 for PC-3 cells. Since the concentration for the cytotoxic effects of DBP and BBP are lower than the levels causing possible antiandrogenic effects, AR pathway could not be attributed directly. AR are linked to different phosphorylation sites, which are expected to induce different functions and phosphorylation process is cell type specific,(51) the differences in the cytotoxic effects between DU-145 and PC-3 along with the different types of phthalates, might be related to the AR, ER receptor affinity and phosphorylation of these receptors.

Erfαs, a mediator of epithelial differentiation and as an anti-proliferative molecule, regulating many molecular pathways including upregulation of apoptotic genes (55) is expressed in both DU145 and PC3 cells (50). Among phthalates, BBP, which effects directly as an agonist for Erβ (51), is expected to have higher tumor-suppressing function (55). This was confirmed in the current study for BBP, being the most cytotoxic compound in PC-3 cells, which express both Erβ and Erα. Interestingly, BBP was found to be the third cytotoxic compound in DU145 cells, which express Erβ only. Recently, the opposing roles of ERs and ERβ in prostate cancer are under discussion; (56) since the tumor-promoting roles of ERβ2 and ERβ5 isoforms were identified. Since these isoforms play an important role in tumor progression and currently, no information is available for phthalates, future studies are required to understand estrogen receptor mediated effects of phthalates in prostate cancer.

Neuroendocrine differentiation (NED) as a structural and functional feature of prostate cancer, appears during advanced stages, and found to be responsible for treatment resistance and poor prognosis.(57) Androgen depletion is also correlated to the induction of NED in prostate cancer cells in vitro (54, 58). Meanwhile, androgen-deprivation conditions did not induce NED in PC3 and DU145 cells (59). Therefore, the results of the current study could not be discussed within NED perspective; while neuron-specific enolase and chromogenin A expression could be studied in future.

Contradictory results in the previous studies with LNCaP cell lines and phthalates, raise concerns over more complicated molecular mechanisms behind the mechanism of action of these compounds. DBP at 1 μM treatment induced cell proliferation; (30) while at 50 μM decreased cell proliferation independent from AR expression and activity (31). DEHP induced cytotoxicity at 3 mM concentration through induction of reactive oxygen species (ROS) and activation of nuclear p53 and p21 proteins; (60) while this effect was found at much lower concentrations (50 μM) in the study by Hrubá et al (31) Experimental and epidemiological evidence for the non-monotonic dose response relationship of endocrine disrupting compounds reveal a need for different strategic methods for the risk assessment of these substance in human health (61). Among these compounds, phthalates were found to induce adverse effects at low concentrations (62). Low dose exposure to DEHP (100 μg DEHP/kg/day) was found to alter sperm morphology and chromatin DNA damage leading sperm toxicity in rats (63), and increase susceptibility to testicular autoimmunity (increase in IFN-γ positive cells) and damage to blood testis barrier in mice (64).

The use of the powerful antioxidant, ALA; which is involved in many important biological and biochemical cellular processes, is used in the ancillary treatment of many diseases, such as diabetes, cardiovascular, neurodegenerative, autoimmune diseases, cancer and AIDS (3, 11, 12). Meanwhile, their use as a potential anti-cancer agent is discussed for prostatic cancer patients where epidemiologic and experimental researches indicate discordance (24). Recently, prostatic ALA, was significantly and positively associated with biomarkers of aggressive prostatic cancer progression and tumor proliferation rates (21–23). Choi et al (65) studied the effects of ALA on the antioxidant system in prostatic cancer cells PC-3, LNCaP, and RWPE-2 cell lines where the expression of Ref-1 protein was increased with 125, 250, and 500 μM of ALA in PC-3 significantly. Treatment of LN-
CaP cells with increasing concentrations of ALA (0, 0.125, 0.5, 1, 10, 125, 250, 500 μM, 1 mM, and 2 mM) resulted in a dose-dependent decrease in cell viability, where significant induction of cell loss was observed at 250 μM; whereas no information is available for PC-3 cells. In our study, we used a similar dose 200 μM for ALA, a slightly lower dose than the IC50. The mRNA expressions of SOD-1, SOD-2, catalase, and GSH-Px were also found to be decreased by ALA in PC-3 with 125, 50 and 500 μM treatment along with an increase of Ref-1 protein, which has multifunctional roles involved in oxidative DNA damage repair (65). In the current study, ALA were found to increase the cytotoxicity of the estrogen receptor agonist phthalates (51), BBP, DIBP and DBP significantly. According to current literature, information regarding ALA and estrogen receptor is missing. Therefore, current study might provide a preliminary information for the mechanism of action of ALA through estrogen receptor (especially Erα).

Conclusion

Future directions on the development of effective therapeutic strategy for the prostate cancer would be linked to the effective control on the hormonal and neuroendocrine transdifferentiation pathways. Meanwhile, various molecular differences of the tumor type and epigenetic factors including endocrine disrupting compounds, like phthalates, makes the accurate treatment difficult and the progression more aggressive. Combination therapies to reduce the resistance of chemotherapeutics, such as antioxidants would be directed. In the current study, the responses of two different cell lines DU-145 and PC3 on exposure to phthalates were found to be different and the cytotoxic effects of estrogen receptor agonist phthalates (DIBP, DBP and BBP) were found to increase the cytotoxic effects in PC3 cells, which are known to be a more aggressive tumor type than DU145 cells. Even though the current study has the limitation of providing in vitro data that might not carry over to in vivo conditions, it could be suggested that the combination ALA upon exposure to estrogenic environmental contaminants might be beneficial for the progression of the prostatic tumor.

References

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