Toxicology Letters 217 (2013) 50–58

Contents lists available at SciVerse ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Biological impact of phthalates

Rishikesh Mankidy[a,*], Steve Wiseman[a], Hong Ma[a], John P. Giesy[a,b,c,d]

[a] Toxicology Center, University of Saskatchewan, Saskatoon, SK, Canada
[b] Department of Veterinary Biomedical Sciences, University of Saskatchewan, Saskatoon, SK, Canada
[c] Zoology Department, Center for Integrative Toxicology, Michigan State University, East Lansing, MI, USA
[d] Department of Biology and Chemistry and State Key Laboratory for Marine Pollution research, City University of Hong Kong, Kowloon, Hong Kong, China

HIGHLIGHTS

- Investigated the biological impact of phthalates DEHP, DEP, DBP and BBP.
- Phthalates differing in physicochemical properties have similar endpoints.
- Phthalates simultaneously affect multiple cellular targets.
- Demonstrated the need for the simultaneous assessment of multiple endpoints.

ARTICLE INFO

Article history:
Received 19 October 2012
Received in revised form
27 November 2012
Accepted 28 November 2012
Available online 7 December 2012

Keywords:
Phthalate
Oxidative stress
Caspase
Embryotoxicity

ABSTRACT

Esters of phthalic acid are chemical agents used to improve the plasticity of industrial polymers. Their ubiquitous use in multiple commercial products results in extensive exposure to humans and the environment. This study investigated cytotoxicity, endocrine disruption, effects mediated *via* AhR, lipid peroxidation and effects on expression of enzymes of xenobiotic metabolism caused by di-(2-ethy hexyl) phthalate (DEHP), diethyl phthalate (DEP), dibutyl phthalate (DBP) and benzyl butyl phthalate (BBP) in developing fish embryos. Oxidative stress was identified as the critical mechanism of toxicity (CMTA) in the case of DEHP and DEP, while the efficient removal of DBP and BBP by phase 1 enzymes resulted in lesser toxicity. DEHP and DEP did not mimic estradiol (E_2) in transactivation studies, but at concentrations of 10 mg/L synthesis of sex steroid hormones was affected. Exposure to 10 mg BBP/L resulted in weak transactivation of the estrogen receptor (ER). All phthalates exhibited weak potency as agonists of the aryl hydrocarbon receptor (AhR). The order of potency of the 4 phthalates studied was; DEHP > DEP > BBP » DBP. The study highlights the need for simultaneous assessment of: (1) multiple cellular targets affected by phthalates and (2) phthalate mixtures to account for additive effects when multiple phthalates modulate the same pathway. Such cumulative assessment of multiple biological parameters is more realistic, and offers the possibility of more accurately identifying the CMTA.

1. Introduction

Phthalates, which are esters of phthalic acid are primarily used to enhance plasticity of industrial polymers (Sears and Darby, 1982). They are used in a number of consumer end products such as toys, paints, adhesives, lubricants, packaging and building materials, personal care items, electronics, medical devices, and are an unavoidable part of modern life (Horn et al., 2004; Shea, 2003). A recent study estimated that 11 billion pounds of phthalates were produced worldwide every year (Lowell Center for Sustainable Prroduction, 2011). While these plasticizing agents impart beneficial properties to plastics, they are not bound to the polymer by a covalent linkage which makes them susceptible to leaching from the matrix (Fromme et al., 2012). Once released into the atmosphere, they have the potential for long-range transport, eventually entering the food chain (Federal Environmental Agency, 2007).

Structures and physical properties vary among phthalates, which influences their chemodynamics in the environment (Staples, 1997). Phthalates with lesser molecular weights, such as diethyl phthalate (DEP) have greater bioaccumulation factors (BAFs), while larger phthalates such as di-(2-ethy hexyl) phthalate (DEHP) tend to have lesser BAFs (Staples, 1997). Despite their greater water–octanol partitioning coefficients (K_{ow}), phthalate esters do not have greater biomagnification factors (BMFs) such that concentrations of phthalates are not greater in higher trophic levels of aquatic food webs (Gobas F 2003). This is probably due

* Corresponding author at: Toxicology Center, University of Saskatchewan, 44 Campus Drive, Saskatoon, S7N 5B3 Canada. Tel.: +1 306 966 8733/4680; fax: +306 966 4796.
E-mail address: mankidy@gmail.com (R. Mankidy).

0378-4274/$ – see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.toxlet.2012.11.025

to the fact that phthalates have a fairly short half-life in the environment with greater than 50% degradation occurring within 28 days (Staples, 1997), primarily *via* photo-degradation. Furthermore, phthalates such as DEHP, are readily bio-transformed and excreted (Barron, 1995), which results in lesser bioaccumulation.

In humans, phthalates have been detected in matrices such as blood, urine, saliva, amniotic fluid, breast milk and cord blood (Latini et al., 2003b; Main et al., 2006; Silva et al., 2004a,b, 2005). The major pathway of exposure to phthalates is the oral route, though inhalation and dermal absorption may play a significant role in exposure (Adibi et al., 2003; Rudel et al., 2003) Infants and toddlers are the most vulnerable receptors because: (1) they exhibit more hand-to-mouth activity, and (2) consume the most food as a percent of their body weight (wargo et al., 2008). The situation is exaggerated by the fact that ubiquitous phthalates such as DEHP, which have been classified as endocrine disrupting chemicals (EDCs), exhibit an oral absorption factor of 0.55 (Rhodes et al., 1986), and affect the most vulnerable receptors at critical stages of development.

Phthalates have been reported to affect multiple biochemical processes in humans and wildlife. These include effects on reproduction, damage to sperm (Rozati et al., 2002), early onset of puberty in females (Wolff et al., 2010), anomalies of reproductive tract (Desdoits-Lethimonier et al., 2012), infertility (Rozati et al., 2002; Tranfo et al., 2012) and adverse outcomes of pregnancy (Latini et al., 2003a; Whyatt et al., 2009), to neurodevelopment (Engel et al., 2010; Miodovnik et al., 2011) and allergies (Bornehag et al., 2004; Jaakkola et al., 2000). Because humans and wildlife can be exposed simultaneously to several phthalates any assessment of the risks posed by phthalates needs to consider combined effects of all of the phthalates in mixtures. To do this requires knowledge of the critical mechanisms of toxic action (CMTA) of each phthalate. It is only by this knowledge that it can be determined how to aggregate the exposures. While effects of phthalates have been observed and described, few studies have been conducted to determine the CMTA. The CMTA is the endpoint that is not only most severe but that which occurs at the least concentration. For instance, if individual phthalates had similar mechanisms of toxic action, with different potencies, a toxic units approach could be applied. If the mechanisms are different, then the effects of the various phthalates would be better assessed by considering them individually.

In the study presented here, phthalates of different molecular sizes were investigated. A greater molecular weight phthalate, DEHP, a lesser molecular weight phthalate, DEP, and two phthalates with intermediate molecular weights, dibutyl phthalate (DBP) and butyl benzyl phthalate (BBP) were studied. The chemical and physical properties of these phthalates vary and they also have different uses in manufacturing and consumer products. Effects of these four phthalates were assessed both *in vitro* and *in vivo* to elucidate CMTAs such as endocrine disruption, oxidative stress, aryl hydrocarbon receptor (AhR) receptor-mediated effects, and the expression enzymes of phase I xenobiotic metabolism. In conclusion we present a scheme which grades the 4 phthalates on the overall potential effects on biological systems and allows for comparison between phthalates with respect to each individual biological end point.

2. Materials and methods

2.1. Cytotoxicity

MVLN cells were propagated in DMEM/F-12 media containing 10% FBS at 37 °C, 5% CO_2. Cytotoxicities of phthalates were determined by exposing 8×10^4 MVLN cells to DEHP, DEP, DBP or BBP (Sigma–Adlrich, St. Louis, MO) for a period of 24 h. WST-1 reagent (Roche Applied Science, Indianapolis, IN) was used to determine metabolically active cells at the end of the incubation period according to the manufacturer's recommendations.

2.2. Caspase-3 assay

Active caspase-3 was assayed in MVLN cells at concentrations that resulted in cell death. 1.5×10^6 cells were exposed to phthalates for 3 h in a 6-well plate at concentrations which exhibited cytotoxicity at 24 h. Cells were harvested, lysed, and active Caspase-3 was quantified using EnzChek Caspase-3 Assay Kit (Life Technologies, Carslbad, CA) according to the manufacturer's recommendations. Amount of fluorescence generated was normalized by the amount of protein (μg) in the extract.

2.3. Endocrine disruption

2.3.1. H295R steroidogenesis assay

The H295R Steroidogenesis assay has been validated and is an established system routinely used as a tier-I screen for steroidogenic effects of test chemicals (Hilscherova et al., 2004; Sanderson et al., 2000; Zhang et al., 2005). This cell line has the full complement of enzymes required sex steroid biosynthesis (Gracia et al., 2006; Hecker et al., 2006, 2007, 2011), and hence is a good model to study disruptions in steroidogenesis. H295R cells, purchased from ATCC (Manassas, VA), were propagated in DMEM/Hams F-12 medium containing 10%FBS at 37 °C, 5% CO_2. Cells were exposed to phthalates under conditions previously described (Hecker et al., 2006). Following exposure to phthalates for 48 h, conditioned media was collected and concentrations of 17-β estradiol (E_2) and testosterone (T) in culture media were determined by use of ELISA (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's recommendations.

2.3.2. Estrogen receptor transactivation assay

MVLN cells, derived from the MCF-7 breast cancer cell line, are engineered to express luciferase under the control of estrogen responsive elements (Demirpence et al., 1993; Pons et al., 1990). Transactivation of the estrogen receptor (ER) by phthalates was determined by exposing 3×10^4 cells to individual phthalates for 48 h in a 96-well plate. Following exposure, cells were lysed and luminescence quantified by use of SteadylitePlus reagent (Perkin-Elmer, Waltham, MA). Potencies of individual phthalates as agonists of the ER were determined by lumniscence caused by standard concentrations of E_2 (Sigma–Aldrich, St. Louis, MO)

2.4. Aryl hydrocarbon receptor transactivation assay

The assay to determine the potential for phthalates to activate the AhR was conducted as described (Garrison et al., 1996) with a few modifications. H4IIE cells were propagated in DMEM containing 10%FBS at 37 °C, 5% CO_2. 5×10^4 H4IIE cells were exposed to the phthalates for 24 h. Cells were harvested and luminescence quantified using SteadylitePlus reagent (Perkin-Elmer, Waltham, MA) according to the manufacturer's recommendations. Luminescence derived from the exposure to phthalates was compared with that obtained from a standard curve for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; Wellington Laboratory, Guelph, ON) to determine TCDD equivalents.

2.5. Fathead minnow experiments

2.5.1. Embryotoxicity

Fathead minnows (*Pimephales promelis*) were cultured in 200 L tanks in the Aquatic Toxicology Research Facility (ATRF) in the Toxicology Center at the University of Saskatchewan. Tanks for breeding fish were maintained as described previously (He et al., 2012). Fertilized embryos were collected within 1 h post fertilization (hpf) and were pooled in a Petri-dish containing control water. Eggs were rinsed 3 times and unfertilized eggs discarded. One exposure replicate consisted of 10–15 eggs in each well of a 6-well plate containing 2 mL of control water or containing phthalates. 50% of the volume (1 mL) was replaced daily with fresh test solutions. Exposures were performed at 25 ± 1 °C with a 16/8 h light/dark photoperiod. Daily enumeration of live and dead embryos was made prior to the 50% water renewal and dead eggs were discarded. Exposures were terminated 96 hpf and cumulative percent mortality determined. Live embryos were collected, flash frozen in liquid nitrogen, and stored at −80 °C until needed for determination of lipid peroxidation and abundances of transcripts of target genes. All exposures were conducted on 5 separate batches of eggs.

2.5.2. Lipid peroxidation

Fertilized eggs exposed to phthalates for 96 h were used to determine the degree of lipid peroxidation by use of the Lipid hydroperoxide assay kit (Cayman Chemical, Ann Arbor, MI). Wet mass of pooled embryos from each replicate was determined prior to extraction of lipids. Lipids were extracted with 500 μL chloroform containing 1% triton X-100 and directly used in the assay according to recommendations of the manufacturer. Amount of lipid peroxide (nmol) was quantified by reading the absorbance at 500 nm. Data were normalized by the wet weight (mg) of tissue.

2.5.3. Molecular studies

Total RNA was extracted from 5 embryos in each treatment group by use of the Qiagen RNeasy Plus Mini Kit according to the manufacturer's protocol (Qiagen, Mississauga, ON, Canada). The concentration of RNA was determined with a