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Phthalates modulate steroid 5-reductase transcripts in the Western clawed frog embryo

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ABSTRACT

Phthalates are used worldwide in the manufacturing of plastics, added to cosmetic products, personal care products, pharmaceuticals, medical devices, and paints; and are widely detected in soil, surface water, and organism tissues. Phthalate esters have been previously shown to interfere with the endocrine system in vertebrates. However, few studies have investigated the effects of phthalates on testosterone-converting enzymes that affect hormone levels and reproduction. In the present study, we exposed the Western clawed frog (*Silurana tropicalis*) to 0.1, 1, and 10 μM diethylhexyl phthalate (DEHP), dibutyl phthalate (DBP), and diethyl phthalate (DEP) during early amphibian embryonic development. Additional DBP exposures were conducted *ex vivo* using mature frog testes. Malformations and mRNA levels of genes associated to reproduction and oxidative stress were evaluated. 0.1 μM DEHP, DBP, and DEP induced an array of malformations, including incomplete gut coiling, edemas, and eye malformations. Moreover, all three phthalates increased the expression of androgen-related genes, such as steroid-5α-reductase 1, 2, 3, steroid-5β-reductase, and androgen receptor at concentrations ranging from 0.1 to 10 μM depending on the phthalate and gene. Data suggest that the phthalate esters tested are teratogens to the amphibian embryo and that these phthalates exhibit an androgenic activity in amphibians.

1. Introduction

Phthalates are used worldwide in the manufacturing of plastics (Daniels, 2009). Leaching due to the non-covalent bonding of phthalates to polymers leads to the introduction of phthalates into our ecosystems. Phthalates are also used as additives in various cosmetic products, medical devices, personal care products, pharmaceuticals, and paints (reviewed in Magdouli et al., 2013). Due to their wide use, phthalates are commonly detected in soil, surface water, and organism tissues (Bauer and Herrmann, 1997; Blair et al., 2009). Diethylhexyl phthalate (DEHP) is one of the most used plasticizers, and was detected in various environmental compartments (reviewed in Magdouli et al., 2013). For example, DEHP was detected at concentrations ranging between 0.01 and 25 μg/L in rivers in Japan (Suzuki et al., 2001; Yuwatini et al., 2006) and reported in the influent of a wastewater treatment plant in France at concentrations up to 44 μg/L (Dargnat et al., 2009). Dibutyl phthalate (DBP) and diethyl phthalate (DEP) are two other plasticizers that have been widely detected in waters. These phthalate esters were detected in the Tama River in Japan at concentrations ranging from 0.088 to 0.54 μg/L DBP and 0.004 to 0.31 μg/L DEP (Suzuki et al., 2001). The False Creek in Vancouver, BC, Canada also showed concentrations in seawater of ~0.1 μg/L DEP (Blair et al., 2009).

Phthalate esters have been shown to interfere with vertebrate development on different levels. The main mechanism of action behind phthalate induced transcriptional changes has been reported to be the peroxisome proliferation-activated receptors (PPARs, Gazouli et al., 2002). In addition, heat shock proteins have been shown to be modulated after phthalate exposure in different species and are attributed to early warning signs of cellular stress (reviewed in S.C. Gupta et al., 2010). Cellular oxidative stress is caused by the presence of reactive oxygen species, which can lead to DNA damage in cells and result in abnormalities. For example, proteins such as glutathione transferase, glutathione peroxidase, and heat shock protein 70 have been previously reported to be altered when exposed to the phthalate DEHP (reviewed in Mathieu-Denoncourt et al., 2015a).

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In addition, research has shown that phthalates can have androgenic and/or anti-androgenic properties and can adversely affect development and reproduction of male vertebrates (Latini et al., 2006; Kay et al., 2014). For example, feminization of gonads by exposure to DBP was found in juvenile Murray rainbowfish (Bhatia et al., 2015). In addition, disrupted spermatogenesis was observed in the African clawed frog after DBP exposure (Lee and Veeramachaneni, 2005). The mechanism of action by which phthalates mediate their action is still not completely understood (Mathieu-Denoncourt et al., 2015a). Previous research suggested that phthalates interfere with hormone synthesis by modulating the expression of sex steroid-related genes (Wong and Gill, 2002; Lehmann et al., 2004; Thompson et al., 2004). For example, decreased mRNA and protein levels of StAR have been observed in rat testis after DEHP exposure (Borch et al., 2006). StAR is responsible to transport cholesterol to the inner mitochondria in order to synthesize steroids, including androgens. Decreased expression levels of *star* have also been correlated with reduced levels of the androgen testosterone (T) (Borch et al., 2006), suggesting that T metabolism could be directly affected by phthalate exposure.

Testosterone is converted to the potent androgen 5α-dihydrotestosterone (5α-DHT) by steroid-5α-reductase (Srd5α) and to 5β-dihydrotestosterone (5β-DHT) by steroid-5β-reductase (Srd5β). Few mammalian studies have investigated if phthalates are capable of modulating Srd5α and Srd5β. Exposure to mono-ethylhexyl phthalate (MEHP) decreased Srd5α protein levels in a primary cell culture of immature rat Leydig cells (Svechnikov et al., 2008). Similarly, exposure to DBP in rats significantly decreased Srd5α2 protein in the proximal penis (Kim et al., 2010). In contrast, a concentration dependent increase of Srd5α activity was detected in testis after DEHP exposure of pubertal rat (Kim et al., 2003). These studies show that phthalates can modulate Srd5α. However, no studies have addressed the effects of phthalates on Srd5β expression.

Srd5 are involved in vital biological functions (reviewed in Langlois et al., 2010a) and their dysregulation leads to a variety of diseases in humans, in particular in the male reproductive system and liver (reviewed in Azzouni et al., 2012). Thus, there is a need to determine how phthalates with androgenic and/or anti-androgenic properties affect Srd5α and Srd5β in lower vertebrates, such as amphibians.

The overall objective of the present study was to understand the effect of the three phthalates DEHP, DBP, and DEP in the Western clawed frog (*Silurana tropicalis*). Specifically, we exposed the Western clawed frog to DEHP, DBP, and DEP during early embryonic development and investigated malformations and mRNA levels of genes involved in oxidative stress and reproduction. As the frog embryos responded to DBP, we further chemically-challenged mature frog testes *ex vivo* in order to analyze if DBP could interfere with normal testis regulation in males. Thus, this study presents novel insights in regards to interactions between phthalates and *srd5* during two critical periods of *S. tropicalis*.

2. Materials and methods

2.1. Experimental design

Maintenance of male and female *S. tropicalis* occurred in dechlorinated and aerated water at the Queen's University Animal Care facilities (Kingston, ON, Canada) in accordance with guidelines of the Institution's animal care protocols and the Canadian Council on Animal Care. Animals were kept in a 12:12 h light:dark cycle with a water temperature of 26 ± 1 °C.

In vivo exposure was executed by exposing eggs of *S. tropicalis* to phthalates. Breeding procedure was performed as described in Langlois et al. (2010b). Briefly, eggs were collected and kept in Frog Embryo Teratogenesis Assay-Xenopus (FETAX) solution consisting of 625 mg NaCl, 96 mg $NaHCO_3$, 75 mg $MgSO_4$, 60 mg $CaSO_4{\cdot}2H_2O$, 30 mg KCl, 15 mg $CaCl_2$/L, and 0.04 ppm gentamycin sulphate. The fertilized eggs were dejellied using 2% (w/v) L-cysteine. 200 embryos (divided in 5 jars) were exposed to 0.1, 1, and 10 μM DEHP (Sigma, Oakville, ON, Canada), DBP (Sigma, Oakville, ON, Canada), or DEP (Sigma, Oakville, ON, Canada) once the eggs reached Nieuwkoop and Faber (NF) stage 11 (Nieuwkoop, 1994). NF 11 embryos were also exposed to two negative controls: a water only control and a solvent control (0.05% Ethanol) and to a positive control to test alteration of *srd5* mRNA levels; finasteride (100 μM; a known Srd5 inhibitor; Langlois et al., 2010c). The FETAX solution was kept at 26 °C and changed every 24 h. Daily water change also ensured a steady source of phthalate exposure, as phthalates are known to degrade over time. Dead embryos were discarded once a day and recorded. Embryos were sampled in pools (n = 10) at stage NF 46 (after an exposure time of 72 h) and flash frozen on dry ice until RNA isolation.

The *ex vivo* assay used has been previously optimized for frogs and described in Bissegger et al. (2014). Briefly, six male adult frogs were anesthetized in 0.1% MS-222 (ethyl 3-aminobenzoate methanesulfonate, Sigma, Oakville, ON, Canada). Testes were carefully dissected from each animal. Each testis (n = 6 per treatment group, one testis per frog was used as a control sample and the other testis was exposed to DBP) was weighed (to correct for steroid production) and placed in a separate 1.5 mL centrifuge tube filled with 500 μL ice cold Lebovitz (L-15 media, Sigma, Oakville, ON, Canada) containing 10 mM HEPES, 50 μg/mL gentamicin (Fisher Scientific, Ottawa, ON, Canada) and 2% synthetic serum replacement (Sigma, Oakville, ON, Canada) at pH 7.4. Once all animals were dissected, the testes were transferred into designated wells in a 24-well plate containing 500 μL ice cold L-15 media. Prior to the start of the incubation, the media in each well of the 24-well plate was replaced by 500 μL of L-15 media containing 0.05% ethanol (control samples) or L-15 media containing 10 μM of DBP. The 24-well plates were incubated for 6 h at 26 °C using an orbital shaker at 100 rpm. After 6 h, the organs were snap-frozen on dry ice and stored at −80 °C for subsequent RNA isolation. The media of each sample was also collected and stored at −80 °C for steroid analysis.

2.2. Malformation analysis

After the embryonic exposure, a subset (n = 46–103) of randomly collected animals at NF 46 was fixed in 10% formalin for each treatment in order to conduct morphological analysis. Malformation analysis was performed based on the Atlas of Abnormalities (Bantle et al., 1998). A Nikon SMZ18 microscope (Nikon, Mississauga, ON, Canada) was used to observe malformations in eyes (reduction in size, asymmetric formation, incomplete separation from the brain and cyclops), tails (shortening and flexure), hearts (failure to coil in an 'S' shape), guts (failure to coil), gills (shredded appearance), and head and face (reduction in size and unusual shape).

2.3. Analysis of phthalate concentration

Phthalate concentrations present in the water during the embryo exposure were measured using liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) optimized for DEHP, DBP, and DEP. Chromatographic separation was performed using an Accela 600 LC system (Thermo Scientific, Waltham, MA, USA) with a Zorbax HDHR Eclipse plus C18 column combined with C18 Eclipse plus (12.5 × 2.1 mm ID., 1.8 μm) guard column (Agilent Technologies, Santa Clara, CA, USA) and using a gradient of two mobile phases. Initial mobile phase conditions consisted of 2 mM ammonium formate in 0.1% formic acid buffer and 0.1% formic acid in methanol ran at a ratio of 60:40, respectively. From 1 to 6 min, the gradient was changed gradually to 1:99 followed by a hold at 1:99 for 4.75 min. The initial buffer composition was then held for 4.25 min until the next sample was started. The flow rate was 0.3 mL/min. Ten μL of the sample or its dilution were injected using an autosampler kept at 4 °C. As an internal standard in each sample, dimethyl-d6 phthalate (CDN Isotopes, Pointe-