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Maternal *in utero* exposure to the endocrine disruptor di-(2-ethylhexyl) phthalate affects the blood pressure of adult male offspring

D.B. Martinez–Arguelles [a,b], M. McIntosh [a,c], C.V. Rohlicek [a,c], M. Culty [a,b,d], B.R. Zirkin [e], V. Papadopoulos [a,b,d,e,*]

[a] The Research Institute of the McGill University Health Centre, Montreal, Quebec, Canada H3G 1A4
[b] Department of Medicine, McGill University, Montreal, Quebec, Canada H3G 1A4
[c] Department of Pediatrics, McGill University, Montreal, Quebec, Canada H3G 1A4
[d] Pharmacology & Therapeutics, McGill University, Montreal, Quebec, Canada H3G 1A4
[e] Department of Biochemistry and Molecular Biology, Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD 21205, USA

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ABSTRACT

Di-(2-ethylhexyl) phthalate (DEHP) is used industrially to add flexibility to polyvinyl chloride (PVC) polymers and is ubiquitously found in the environment, with evidence of prenatal, perinatal and early infant exposure in humans. *In utero* exposure to DEHP decreases circulating testosterone levels in the adult rat. In addition, DEHP reduces the expression of the angiotensin II receptors in the adrenal gland, resulting in decreased circulating aldosterone levels. The latter may have important effects on water and electrolyte balance as well as systemic arterial blood pressure. Therefore, we determined the effects of *in utero* exposure to DEHP on systemic arterial blood pressure in the young (2 month-old) and older (6.5 month-old) adult rats. Sprague-Dawley pregnant dams were exposed from gestational day 14 until birth to 300 mg DEHP/kg/day. Blood pressure, heart rate, and activity data were collected using an intra-aortal transmitter in the male offspring at postnatal day (PND) 60 and PND200. A low (0.01%) and high-salt (8%) diet was used to challenge the animals at PND200. *In utero* exposure to DEHP resulted in reduced activity at PND60. At PND200, systolic and diastolic systemic arterial pressures as well as activity were reduced in response to DEHP exposure. This is the first evidence showing that *in utero* exposure to DEHP has cardiovascular and behavioral effects in the adult male offspring.

Introduction

Di-(2-ethylhexyl) phthalate is a plasticizer used industrially to add flexibility to polyvinyl chloride (PVC) polymers. Multiple studies have identified DEHP as an endocrine disruptor with antiandrogenic activity (Shelby, 2006). Endocrine disruptors are chemicals found in the environment that can interfere with the normal function of the endocrine system (Latini et al., 2004). Since DEHP is not part of the covalent structure of the PVC, it can leach out from the polymer material and, in humans, can be absorbed through skin contact, oral exposure (Frederiksen et al., 2007), and inhalation (Heudorf et al., 2007; Huang et al., 2011). Once absorbed, DEHP is metabolized into mono-2-ethylhexyl phthalate (MEHP) by lipases in the lining of the gut. MEHP has antiandrogenic activity 10-times greater than DEHP (Frederiksen et al., 2007). The typical human exposure is 3–30 μg/kg/day (Koch et al., 2003; McKee et al., 2004) and is mainly from contact with consumer products. However, DEHP exposures of 20 mg/kg/day and higher have been described in workers at a PVC plant (Huang et al., 2011). DEHP and its metabolites have been found in semen (Phillips and Tanphaichitr, 2008), saliva (Silva et al., 2005), amniotic fluid (Huang et al., 2007; Silva et al., 2004), umbilical cord blood (Latini et al., 2003), human milk and baby formula (Frederiksen et al., 2007; Huang et al., 2009). This extensive biodistribution of DEHP and its metabolites presents a particular threat to prenatal, neonatal, and infant development due to the high exposures and low body mass during early life (McKee et al., 2004). In this regard, enteral and parenteral infant feeding procedures using PVC containers and lines are sources of DEHP (Calafat et al., 2004). It has also been estimated that patients undergoing hemodialysis receive from 36–457 μg DEHP/kg/day (Doull et al., 1999), and that neonates receiving blood products may be exposed to 10–20 mg DEHP/kg/day (Loff et al., 2000). Higher levels of exposure in medical settings have been observed in neonatal intensive care unit patients (Green et al., 2005; Weuve et al., 2006) likely due to blood bags, tubing, and other medical equipment leaching large amounts of DEHP (Loff et al., 2000).

Abbreviations: ACTH, Adrenocorticotropic hormone; ALT, Alanine transaminase; ATIIR, Angiotensin II receptor; BUN, Blood urea nitrogen; CO_2, Carbon dioxide; DBP, Di (n-butyl) phthalate; DEHP, Di-(2-ethylhexyl) phthalate; GD, Gestational day; MEHP, Mono-2-ethylhexyl phthalate; MR, Mineralocorticoid receptor; PND, Post-natal day; PVC, Polyvinyl chloride.

* Corresponding author at: The Research Institute of the McGill University Health Centre, Montreal General Hospital, 1650 Cedar Avenue, Room C10-148, Montréal, Québec, Canada H3G 1A4. Fax: +1 514 934 8439.
E-mail address: vassilios.papadopoulos@mcgill.ca (V. Papadopoulos).

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The antiandrogenic effects of DEHP following *in utero* exposure to DEHP have been well-established in animal models (Culty et al., 2008; Martinez-Arguelles et al., 2009, 2011). In addition, we have identified anti-aldosterogenic effects after such exposure (Martinez-Arguelles et al., 2011). Acute exposure to DEHP *in utero* has been shown to disrupt the organogenesis of androgen-dependent tissues by inhibiting testosterone production by fetal-type Leydig cells in a dose-dependent manner (Culty et al., 2008). Such exposure also affects the function of the adult-type Leydig cells, which develop after puberty, resulting in decreased levels of testosterone formation (Culty et al., 2008; Martinez-Arguelles et al., 2009, 2011). In search of the mechanisms that would explain the decrease in androgen formation, we previously reported that mineralocorticoid receptor (MR) expression, a regulator of androgen biosynthesis, was reduced in the adult Leydig cells (Ge et al., 2005; Martinez-Arguelles et al., 2009). The decrease in MR was in addition to reduced circulating levels of aldosterone in the young adult (PND60). The latter finding suggested that the adrenal gland was a long-term target of DEHP and opened the possibility of aldosterone-mediated long-term effects of DEHP (Martinez-Arguelles et al., 2011). The decrease in aldosterone levels was associated with reduced expression of angiotensin II receptors (ATIIRs), particularly ATIIR-1b which is selectively expressed in the rat zona glomerulosa (Martinez-Arguelles et al., 2011) where aldosterone is produced. Aldosterone is involved in the regulation of water and electrolyte homeostasis, and its deregulation leads to changes in systemic arterial blood pressure. Aldosterone production is stimulated by angiotensin II, increased levels of potassium, and in extreme events such as profuse blood loss, the adrenocorticotropic hormone (ACTH).

Based on these observations, we hypothesized that the reduction in aldosterone formation induced by *in utero* exposure to DEHP affects systemic arterial blood pressure in the adult male offspring. The results obtained demonstrate that *in utero* exposure to DEHP indeed has cardiovascular and behavioral effects which manifest in the young adult and older male offspring. These observations support the fetal origin of disease hypothesis, which contends that exposure to an environmental agent during development may affect the physiology later in life.

Material and methods

DEHP treatment and animal care. Timed pregnant Sprague–Dawley rats were purchased from Charles River Laboratories (Senneville, Quebec) and kept on a 12 L/12D day cycle with lights on at 7 AM and access to food and water ad libitum. The pregnant dams were gavaged daily with corn oil or 300 mg/kg/day DEHP (Sigma-Aldrich, St. Louis, MO) from gestational day (GD) 14 until parturition [postnatal day (PND) 0]. This window of exposure corresponds to the first peak of fetal testosterone production and, when dams are exposed to 300 mg DEHP/kg/day, results in decreased circulating levels of testosterone and aldosterone at PND60 (Culty et al., 2008; Martinez-Arguelles et al., 2009, 2011). The animals were weighed every two days, and the doses were adjusted accordingly. The male offspring were weaned at PND21, and housed 2 animals per cage until transmitter implantation. Animals were handled according to protocols approved by the McGill University Animal Care and Use Committees.

Blood pressure measurements and salt restriction/increase. The male offspring of DEHP exposed mothers received intravascular telemetric blood pressure transmitter probes (TA11PA-C40 model; Data Sciences International, St Paul, MN) to monitor abdominal aortic pressure and locomotor activity at young adult PND60 age, and PND200, representative of old age, as previously described (Ross et al., 2010). One week following placement of the instrument, systemic arterial pressure and locomotor activity were continuously monitored by telemetry for 48 h. Locomotor activity was determined from changes in received signal strength resulting from movement of the animals. The average systolic, diastolic, and mean pressures as well as average heart rate and locomotor activity were recorded every 5 seconds using Dataquest ART 4.1 software (Data Sciences International, St Paul, MN).

Sodium intake. Chow containing normal-salt (0.2%, T.201815), high-salt (8%; TD.92012), or low-salt (0.01%; TD.90228) were obtained from Harlan Laboratories and were administered for 7 days before data collection.

Aldosterone measurements. Male offspring were euthanized at PND60 or PND200 by CO_2. Blood was collected by percutaneous cardiac puncture and shipped to Analytics Incorporated (Gaithersburg, MD) for serum measurements. Aldosterone was measured using a solid-phase coated tube radioimmunoassay obtained from Siemens Healthcare Diagnostics (Deerfield, IL), according to the manufacturer's instruction.

Statistics. For all experiments, the experimental unit was the pregnant dam and the number of male offspring examined is indicated under each figure legend. All data are expressed as mean ± SEM. Comparisons between groups were made using an independent *t*-test. The null hypothesis of no effect was rejected at $P<0.05$.

Results

In utero exposure to DEHP affects activity at PND60

We previously reported that circulating aldosterone levels were decreased at PND60 after *in utero* exposure to DEHP (Martinez-Arguelles et al., 2011). To determine whether the decrease in aldosterone levels had an impact on blood pressure, we treated pregnant dams with oil or 300 mg DEHP/kg/day and implanted blood pressure transmitters in their male offspring. Seven days after surgery, telemetric blood pressure data were collected in the unrestrained male offspring at PND60. Fig. 1a shows that the systolic, diastolic systemic arterial pressures and heart rate at PND60 were not affected by prior *in utero* DEHP exposure (Fig. 1a, b, c). However, locomotor activity during the daytime and nighttime periods was decreased in rats exposed *in utero* to DEHP (Fig. 1d).

In utero exposure to DEHP affects systemic blood pressure at PND200

To determine if *in utero* exposure to DEHP had an effect on blood pressure in the older rat (PND200), we treated pregnant dams with oil or 300 mg DEHP/kg/day and measured systemic blood pressure at PND200. Seven days after surgical insertion of blood pressure transmitters in the male offspring, a first round of measurements was collected for 48 h while the animals were fed a normal 0.2% sodium diet. After the first data collection, the animals were switched to a high salt or low salt diet for seven days, followed by a second 48-hr round of telemetric data collection. Fig. 2a shows that *in utero* exposure to DEHP decreased the systolic blood pressure during the nighttime period in the normal salt diet group. This decrease in systemic blood pressure was also observed in the low sodium diet during the nighttime readings. In addition, the animals fed the low sodium diet exhibited decreased diastolic pressures during the daytime and nighttime (Fig. 2b). Male offspring exposed to the high sodium diet had no significant changes in systolic or diastolic blood pressure. Fig. 2c shows that at PND200, there was a significant decrease in nighttime activity of animals that had been exposed *in utero* to DEHP. This decrease was not present in the group placed in the high salt group. The low salt group showed a trend of decreased activity, but the decrease was not significant ($P<0.1133$).

In utero exposure to DEHP decreased aldosterone levels in the PND200 rat

To determine if aldosterone levels remained decreased in the PND200 rats, we collected blood samples from the male offspring and