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Antiandrogenic effect of perinatal exposure to the endocrine disruptor di-(2-ethylhexyl) phthalate increases anxiety-like behavior in male rats during sexual maturation

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ABSTRACT

Di-2-ethylhexyl phthalate (DEHP) is the most widely used phthalate to convey flexibility and transparency to plastic products made of polyvinyl chloride. It has been recognized as endocrine disruptor and associated with reproductive toxic effects. We examined the effects of perinatal exposure to DEHP on anxiety-like behavior, using the Elevated Plus Maze (EPM) test, in male and female rats at different stages of sexual development. Anxiety-like behavior was expressed as a) frequency of open arm entries over the total arm entries (% FEO); b) time spent in them compared with total time the animal stayed in the EPM (% TSO) and c) time spent in closed arms (TSC). Because DEHP has anti-androgenic action we also tested control and exposed immature male rats pretreated with testosterone. We found sex differences in behavior induced by DEHP; while male rats of 45 and 60 days of age showed a significant decrease in FEO and TSO percentages, as well as an increase in TSC, no changes were observed in anxiety-like behavior in perinatal DEHP exposed females at these ages of sexual maturation. In 60-day-old male rats, DEHP exposure produced a significant decrease in serum testosterone levels. Testosterone replacement was able to antagonize the adverse effects of DEHP exposure on LH, activating the negative feed-back mechanism of this steroid on reproductive axis, as well as increasing FEO and TSO percentages to similar values observed in the control group. These findings suggest that the anti-androgenic action of this chemical could be one possible mechanism underlie anxiogenic-like behavior produced by perinatal DEHP exposure in 60-day-old male rats.

Introduction

The plasticizer di-(2-ethylhexyl) phthalate (DEHP) has been identified as endocrine disruptor (EDC) and associated with developmental and reproductive toxic effects (NTP-CERHR, 2006) in laboratory animals (Akingbemi et al., 2001, 2004; Borch et al., 2006; Foster et al., 2001; Gray et al., 2000) and in humans (Latini et al., 2003; Skakkebaek et al., 2001; Swan, 2008; Swan et al., 2005). DEHP is widely used in every day consumer products and medical devices; however the potential risk of the exposure to this substance is high because it can leach from plastic products readily into foods, beverages or directly into body fluids (Gartner et al., 2009; Latini, 2000; Lovekamp-Swan and Davis, 2003).

DEHP induces anti-androgenic action by an androgen receptor-independent mechanism (Akingbemi et al., 2004; Howdeshell et al., 2008; Moore et al., 2001; Parks et al., 2000), as well as reduction in the expression of steroidogenesis related-factors and interaction with peroxisome proliferator-activated receptors (Borch et al., 2006; Latini et al., 2006; Kambia et al., 2008). The toxicity of DEHP can begin during gestation and lactation periods and it is attributable to the action of its primary metabolite, mono-(2-ethylhexyl) phthalate (MEHP), which crosses the placental barrier and passes into breast milk (Latini et al., 2003; Main et al., 2006; Stroheker et al., 2005). Pre and perinatal exposure to DEHP produces reproductive abnormalities in androgen-dependent processes (Akingbemi et al., 2001; Albro, 1987; Borch et al., 2005; Gray et al., 2000; Parks et al., 2000), alters sexual differentiation (Andrade et al., 2006), suppresses testosterone production (Culty et al., 2008) and affects sexual behavior (Dalsenter et al., 2006) in male rats offspring. In this critical stage of development, DEHP exposure induces an increase in the number of ovarian atretic tertiary follicles, (Grande et al., 2007) hypoestrogenic anovulatory cycles and polycystic ovaries (Davis et al., 1994; Lovekamp-Swan and Davis, 2003) in adult female offspring rats. Recently, we have demonstrated that gestational and postnatal DEHP exposure modify serum gonadotropin levels and

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the hypothalamic content of amino acid neurotransmitters in male and female rats during sexual maturation (Carbone et al., 2010, 2012).

There is growing concern that, endocrine-active compounds may disrupt hormone-dependent events during nervous system development, affecting a variety of sexually dimorphic behaviors (Patisaul and Polston, 2008). However, experimental studies of DEHP effects on behavior are limited, they have shown significant associations between prenatal exposure to phthalates and adverse effects on pup learning, memory, and behavior in rodents (Arcadi et al., 1998; Li et al., 2009; Tanaka, 2002, 2005). Epidemiological studies have associated the concentrations of phthalates in maternal urine during pregnancy with adverse child cognitive, motor and behavioral development (Engel et al., 2009, 2010; Miodovnik et al., 2011; Swan et al., 2010; Whyatt et al., 2012). Detrimental effect on behavior and possible sex differences in the sensitivity of the infants to prenatal phthalates has been suggested (Kim et al., 2011). Also, clinical symptoms of attention deficit hyperactivity disorder (ADHD) have been identified in school-aged children exposed to environmental phthalates (Kim et al., 2009) and a significant association between this behavioral disorder and the severity of anxiety symptoms in children, and adolescents has been reported (Liu et al., in press). However, little information concerning the effects of perinatal exposure to DEHP in anxiety-like behavior is presently available.

On the other hand, a reduction of testosterone (T) production by phthalates during development has been hypothesized as a probable mechanism to explain the changes in play behavior observed in boys (Swan et al., 2010). This androgenic steroid is well known for its function in reproduction, sexual differentiation and sexual behavior as well as for its modulating effect on anxiety. Animal studies have demonstrated that testosterone's mediation of anxiety-like behavior and cognitive processes may be through the actions of its metabolites. It has been reported that T and its 5α reduced metabolite, dihydrotestosterone, as well as the systemic administration of anabolic steroids reduce anxiety-like behavior in rodents (Bing et al., 1998; Bitran et al., 1993; Edinger and Frye, 2005; Frye and Edinger, 2004; Frye and Seliga, 2001; Osborne et al., 2009; Rojas-Ortiz et al., 2006). While removal of testes – the primary source of endogenous androgens – through gonadectomy results in increased anxiety-like behavior (Bitran et al., 1993; Frye and Seliga, 2001), T replacement is able to abolish this effect (Edinger and Frye, 2004, 2005; Frye and Seliga, 2001).

The purpose of the present work was to study the effect of perinatal exposure to DEHP on the anxiety-like behavior, in male and female rats at different stages of sexual maturation. In order to ascertain if the antiandrogenic action of DEHP could be involved in probable changes in the behavior induced by this chemical, we also examined the effect of the pretreatment with T in DEHP-exposed immature male rats. An oral route in DEHP administration was chosen for this study to mimic the most likely route.

Materials and methods

Animals and drug

Wistar rats used for this work were provided by the Department of Physiology, School of Medicine, Universidad de Buenos Aires, Argentina. Animals were maintained under a controlled environment (temperature 22°–24°; lights on from 7.00 am to 7.00 pm) and had free access to food and filtered water, until time of sacrifice. All animals were fed with balanced food for laboratory rodents (Cooperation, ACA-16014007, Argentine Cooperative Association, Animal Nutrition Division, Argentina Industry). The diet contains 15% of soy, but as the food used and the quantity of food intake by control and DEHP treated groups were similar, we assumed that all animals were exposed to equivalent levels of food-borne phytoestrogens. Moreover, the same lots of diet were provided to animals from all groups at the same time during the course of the study to control across groups for possible variation in the content of the diet. We used ultrapure filtered water (obtained from EDS-Pack, Millipore Merck, installed in the Milli-Q water system) that was presumed to be free of phthalates and other EDC. To minimize additional exposures to endocrine-disrupting chemicals, rats were housed in stainless steel cages with wood bedding and water was supplied in glass bottles. All animal procedures were performed in accordance with protocols of the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals. Approval to conduct the study was granted by the Animal Care and Ethics Committee of the School of Medicine-UBA (CICUAL).

DEHP (99% pure, Cat D 20,115-4, Aldrich Chemical Company, Inc. Milwaukee, Wisconsin, USA) was administered in drinking water. The estimated average dose of exposure was 30 mg/kg/bw/day of DEHP. The amount of DEHP added to each glass bottle was adjusted daily to reach this dose, according to the body weight and the volume of liquid consumed by the corresponding animal. Surrogate dams and pups receiving DEHP drank the same amount of liquid as those which did not receive this chemical. It was assumed that all DEHP solution missing in the bottle had been consumed by the animals. Assessments do not account for possible leakage or evaporation of the solution or for potential loss of DEHP activity during the 24-hour period. DEHP solution was made up fresh daily by sonicating for 30 min, which ensures a permanent and homogenized solution. Similarly to what was observed by other authors (Vandenberg et al., 2012), we found no reference in DEHP dose administered to animals, which may cause similar blood levels in humans. For this reason, we administered a dose of DEHP previously tested that is capable of producing neuroendocrine and reproductive effects in rat (Carbone et al., 2010, 2012; Tanaka, 2002, 2005). This dose is in the range of the no-observable-adverse-effect level (NOAEL 28.9–36.1 mg/kg/day) (David et al., 2000).

Testosterone propionate (Sigma Chemical Co., Saint Louis Mo.) diluted in sesame oil (vehicle) was injected subcutaneously (1 mg/0.1 ml). This is the minimal dose which is capable to return gonadotropin levels to normal levels in antiandrogenized male rats (Justo et al., 1989).

Experimental design

After acclimatization for 1 week, female (250–300 g) and male (300–350 g) were co-housed (1:1). The animals were examined daily for copulatory plug. Mating was confirmed by the presence of a copulatory plug, and this day was recorded as first day of gestation (GD1). Pregnant dams (n = 10) were each placed in an individual metallic cage. Upon delivery, pups were sexed according to anogenital distance and were cross fostered, distributing four males and four females pups per cage with one surrogate dam. These actions allowed us to minimize the use of siblings to avoid potential litter effects. Surrogate dams (n = 10) with their pups were randomly assigned into Control (C) and DEHP exposure groups (n = 5 dams per group), receiving water DEHP free or water containing DEHP (30 mg/kg/bw/day) from postnatal day (PND) 1 to weaning, respectively. Pups from control and DEHP groups were weaned at 21 days of age and housed by sex (n = 8 male or female per cage) according to the treatment they had received during breastfeeding. Pups of DEHP groups continued to receive the same dose from PND 21 to the day of sacrifice. The exposure to endocrine disruptors (e.g. bisphenol-A) during fetal and perinatal periods is an important determining factor of their effects on behavior (Xu et al., 2012). In the present study, we chose the postnatal exposure from birth to pubertal development to test the hypothesis chronic exposure to DEHP during this period could cause behavioral changes in different stages of sexual maturation in which there are also important modifications in the levels of gonadal steroids and gonadotropins (Dohler and Wuttke, 1974), as well as in brain concentration of inhibitory and excitatory neurotransmitters and its receptors (Moguilevsky et al., 1995).

The pups were randomly divided for two experiments. In the first experiment, we studied the anxiety-like behavior in C and DEHP male and female pups of 30, 45 and 60 days of age (n = 8 males or females for C and DEHP groups of each age). In the second one, we evaluated anxiety-like behavior in C and DEHP males which were injected