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Neonatal xenoestrogen exposure alters growth hormone-dependent liver proteins and genes in adult female rats

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HIGHLIGHTS

- GH secretion and action on liver gene expression are sexually dimorphic.
- Bisphenol is an endocrine disruptor which may alter brain sexual differentiation.
- Neonatal administration of bisphenol increased pituitary GH and liver IGFI content in adult female rats.
- Neonatal bisphenol defeminized liver *Cyp2c12*, *Adh1* and *Hnf6* expression.
- Early exposure to BPA may compromise liver capacity for metabolic detoxification.

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ABSTRACT

The hypothalamic-growth hormone (GH)–liver axis represents a new concept in endocrine regulation of drug toxicity. Preponderant sex differences are found in liver gene expression, mostly dependent on the sexually dimorphic pattern of GH secretion which is set during the neonatal period by gonadal steroids. We tested if GH-dependent sexually dimorphic liver enzymes and proteins was perturbed by neonatal Bisphenol A (BPA) treatment in female rats. Female rats were sc injected with BPA (50 or 500 μg/50 μl) or castor oil vehicle from postnatal day 1 to 10. At five months serum prolactin, pituitary GH, and serum and liver insulin growth factor-I (IGF-I) were measured by RIA. Major urinary proteins (MUPs) were determined by electrophoresis. Liver *Cyp2c11*, *Cyp2c12*, *Adh1*, *Hnf6*, and *Prlr* mRNA levels were determined by real time PCR. Pituitary GH content and liver IGF-I concentration were increased by neonatal BPA treatment, indicating partial masculinization of the GH axis in treated females. GH-dependent female predominant liver enzyme genes (*Cyp2c12* and *Adh1*) and a transcription factor (*Hnf6*) were downregulated or defeminized, while there were no changes in a male predominant gene (*Cyp2c11*) or protein (MUP). Our findings indicate that perinatal exposure to BPA may compromise the sexually dimorphic capacity of the liver to metabolize drugs and steroids.

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1. Introduction

Bisphenol A (4,4′-dihydroxy-2,2-diphenylpropane, BPA) is a known endocrine disruptor present in polycarbonate plastic, epoxy resins, CDs, DVDs, electrical and electronic equipment, carbonless paper, and a wide range of household products. Considered to be a weak estrogen (Fernandez et al., 2009; Taylor et al., 2011; Zsarnovszky et al., 2005), BPA can leach from polycarbonate plastics, epoxy resins, and other products in contact with food and drink, and recent measurements by the Centers for Disease Control revealed detectable levels of BPA in the urine samples of 92.6% of more than 2500 participants of the cross sectional NHANES (National Health and Nutrition Examination Survey) study (Calafat et al., 2008). Furthermore, BPA has been detected in amniotic fluid, neonatal blood, placenta, cord blood and human breast milk (Rubin and Soto, 2009). Nevertheless, the question of whether or not the

Abbreviations: ADH, alcohol dehydrogenase; AU, arbitrary units; BPA, bisphenol A; CIS, cytokine-inducible SH2-containing protein; CYP, cytochrome P450; *Cyp2c11*, cytochrome P450 gene 2C11; *Cyp2c12*, cytochrome P450 gene 2C12; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GH, growth hormone; GHRH, growth-hormone-releasing hormone; HNF6, hepatocyte nuclear factor 6; IGF-I, insulin-like growth factor; LOAEL, lowest-observed-adverse-effect-level; MUP, major urinary protein; RIA, radioimmunoassay; PRLR, prolactin receptor; PCR, polymerase chain reaction; PND, postnatal day; SOCS2, suppressor of cytokine signaling 2.

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endocrine disruptor BPA poses a threat to human neuroendocrine and reproductive development remains under intense scrutiny.

Critical periods of fetal and infant development are more sensitive to low doses of hormones than adult tissues and thus more vulnerable to endocrine toxicity. Research focused on neonatal critical windows of development in rodents (prior to, and immediately after birth) has shown that perinatal exposure to low, environmentally relevant doses of BPA can advance puberty and reproductive senescence, alter estrous cyclicity, disrupt ovarian, testicular and mammary gland development, alter brain sexual dimorphism, embryo implantation, and masculinize behavior in the open field (Adewale et al., 2009; Fernandez et al., 2009; Frye et al., 2012; Rubin and Soto, 2009; Rubin, 2011; Taylor et al., 2011; Varayoud et al., 2011).

Sex specific organization of the developing central nervous system depends on the effects exerted by gonadal hormones during very restricted or critical periods of neural differentiation (Arnold and Gorski, 1984; Becu-Villalobos et al., 1997). In newborn male rodents testosterone from the testis enters the bloodstream and is carried to the brain where it is aromatized to estradiol. Estradiol then masculinizes the developing central nervous system (Becu-Villalobos et al., 1997; Dorner, 1981). The female brain, on the other hand, develops largely in the absence of estradiol (ovaries begin to secrete free estrogen after the critical window of sexual differentiation of the hypothalamus) and is considered to be feminine. This process results in the organization of a broad spectrum of responses which are congruent with the genotype, and assures adequate behavioral and neuroendocrine responses in males and females which ultimately tend to reproductive success. Therefore, in females if testosterone, abnormal estrogen levels or endocrine disruptors are present in the blood during an early critical period, female traits may be permanently *masculinized* or *defeminized*. Masculinization refers to the organization of male characteristics, and defeminization refers to the lack of development of feminine traits (Arnold and Gorski, 1984). Alterations in the sexual organization of the hypothalamus may result in anomalies in fitness and reproduction, and unleash endocrine adaptations that permanently alter metabolism, increasing the susceptibility to develop later disease (Fernandez et al., 2009; Gore, 2008).

In a previous work we demonstrated organizational effects of neonatal testosterone on the growth hormone releasing hormone (GHRH)–growth hormone (GH) axis, and sexually dimorphic GH-dependent liver enzymes in female mice (Ramirez et al., 2010). In rodents, GH regulates the sexually dimorphic expression patterns of a large number of liver-expressed genes, including many receptors, signaling molecules, and enzymes of steroid and drug metabolism, especially cytochrome P450s (*Cyps*) (Waxman and Holloway, 2009). These patterns are dictated by the sex dimorphism of plasma GH profiles, which is especially prominent in rats and mice, but also evidenced in humans. In rodents, GH secretion patterns are characterized by a marked pulsatility in males, in which elevated GH peaks occur every 3.5–4 h, interrupted by periods of no measurable hormone, whereas in females there are more frequent and overlapping plasma GH peaks, resulting in a nearly constant presence of GH in circulation (Wehrenberg and Giustina, 1992). Adult patterns of pituitary GH secretion are set during the neonatal period by exposure to testosterone, or estrogen, which program the hypothalamic regulation of GH secretion (Chowen et al., 2004; Dorner, 1981; Ramirez et al., 2010), and underlie the sex dimorphism in liver metabolism of lipids, steroid hormones, drugs and xenobiotics. Sex dimorphism in the liver response to GH is involved in body growth, pheromone communication pathways, energy homeostasis, inflammatory responses, predisposition to alcohol-induced liver disease and steroid metabolism during pregnancy (Waxman and Holloway, 2009).

Approximately 90% of more than 1000 sex-specific liver genes were shown to be under pituitary control, demonstrating a near-global dependence of sex-specific gene expression on the pituitary gland, consistent with the widespread regulatory role of GH (Wauthier and Waxman, 2008; Waxman and Holloway, 2009). In particular, liver cytochrome P450 genes 2C11 and 2C12 (*Cyp2c11* and *Cyp2c12*) have served as important models for investigation of the effects of GH on liver gene expression. They have a key role in hepatic drug and steroid metabolism and detoxification, and striking responsiveness to sex-dependent temporal patterns of plasma GH stimulation: *Cyp2c11* is stimulated by the male pattern of intermittent GH pulses, while *Cyp2c12* is positively regulated by the continuous plasma GH female pattern (Wauthier and Waxman, 2008).

While most studies on the effect of neonatal exposure to BPA and other endocrine mimetics have concentrated on their toxic effect on the reproductive axis, in the present work we test the hypothesis that neonatal BPA may perturb the GH axis and modify sexually dimorphic GH-dependent liver enzymes in female rats. Our results identify a toxic effect of an endocrine disrupting chemical on the growth axis and GH-dependent liver gene expression in rats.

2. Methods

2.1. Animals

Studies were performed according to protocols for animal use (Institute of Laboratory Animal Resources 1996) and approved by the institutional animal care and use committee of the Instituto de Biología y Medicina Experimental, Consejo Nacional de Investigaciones Científicas y Técnicas (IByME-CONICET). Sprague-Dawley rats (200–250 g) were maintained under a controlled 12-h light/dark cycle and temperature conditions. They were housed in steel cages, given free access to commercial laboratory chow and tap water in glass bottles.

Pregnant females were housed individually; on the day of birth [postnatal day (PND) 1], litters were reduced to eight pups. From PND1 to PND10 pups received a daily subcutaneous injection of BPA (Aldrich, Milwaukee, WI, USA) in castor oil [50 μg/50 μl (BPA50); 500 μg/50 μl (BPA500)], or castor oil vehicle (a phytoestrogen-free diluent for steroids, control females and males). The lowest observed adverse effect level (LOAEL) for BPA is currently set in the US at 50 mg/kg body weight per day (U.S.EPA, 1993), therefore the highest BPA dose in this study (500 μg/50 μl), equivalent to 25–62.5 mg/kg, used in this and other strains of rats (Ishido et al., 2007; Khurana et al., 2000; Patisaul et al., 2006, 2007; Salian et al., 2009; Taylor et al., 2008) is considered a "high dose"; while the 50 μg/50 μl dose (equivalent to 2.5–6.25 mg/kg) is below the LOAEL. Each litter included one vehicle injected female, one BPA50 and one BPA500 female, and one male; this allowed us to control for possible effects due to dam variability. The four remaining pups in each litter were untreated males or females not used for this study.

Rats were euthanized at 5 months of age.

2.2. Reagents

Unless otherwise specified, all chemicals were purchased from Sigma (St. Louis, MO).

2.3. RIAs

Prolactin and GH were measured by RIA using kits provided by the National Institute of Diabetes and Digestive and Kidney Diseases [NIDDK; Dr. A.F. Parlow, National Hormone and Pituitary Program (NHPP), Torrance, CA]. Results are expressed in terms of mouse prolactin standard RP3 or mouse GH standard AFP-10783B. Intra- and interassay coefficients of variation were 7.2% and 12.8%, and 8.4% and 13.2%, for prolactin and GH, respectively. IGF-I was determined by RIA, in serum samples subjected to the acid–ethanol cryoprecipitation method as previously described (Ramirez et al., 2010). Antibody used was UB2-495 provided by Drs. L. Underwood and J.J. Van Wyk, Hormone Distribution Program of the NIDDK.

Pituitaries (1–1.5 mg) or liver samples (50 mg) were homogenized in ice-cold PBS and centrifuged at 3000 rpm for 5 min. Supernatant protein contents were measured with the QUBIT Fluorometer and the QUANT-IT protein Assay Kit (Invitrogen, Buenos Aires, Argentina). Aliquots of equal quantity of protein were used to assay pituitary GH, or liver IGF-I content.

2.4. Urine dosage of major urinary proteins (MUPs)

Urine collected from 5-month-rats between 1500 and 1700 h was centrifuged briefly for 3 min at 8800 × *g*. Five microliters of the supernatant were boiled in SDS

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