ORIGINAL ARTICLE

Evidence to suggest glutamic acid involvement in Bisphenol A effect at the hypothalamic level in prepubertal male rats

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Abstract

OBJECTIVES: The aim of present paper was to study the probable role of glutamic acid (GLU) as a mediator of bisphenol A (BPA) effect at the hypothalamic level and its effects on the reproductive axis of prepubertal male rats.

METHODS: Mated Wistar rats were treated with either 0.1% ethanol (control group, n=10) or BPA (BPA group, n=10) in their drinking water until their offspring were weaned at the age of 21 days. The estimated average dose of exposure to dams was approximately 2.5 mg/kg body weight/day of BPA. At the prepubertal stage (35 days of age), the male rats were sacrificed and Gn-RH and glutamic acid (GLU) release, an amino acid involved in Gn-RH secretion, were measured in hypothalamic samples containing medio basal and anterior preoptic area (MBH-APOA), by RIA and HPLC respectively. LH, FSH serum levels were measured by RIA and testosterone by EQLIA.

RESULTS: Gn-RH and GLU release decreased significantly in animals exposed to BPA ($p<0.001$, $p<0.01$). LH, FSH and testosterone serum levels were also decreased by treatment ($p<0.0001$).

CONCLUSION: Present results provide evidence that BPA may act at the hypothalamic level to decrease GLU release which in turn may modify Gn-RH secretion altering the normal function of the axis.

Abbreviations:

BPA	- Bisphenol A	ERβ	- Estrogen receptor β
EDs	- Endocrine disruptors	APOA-MBH	- Anterior preoptic area - Medio basal hypothalamus
GLU	- Glutamic acid	LH	- Luteinizing hormone
Gn-RH	- Gonadotropin releasing hormone	FSH	- Follicle stimulating hormone
ERα	- Estrogen receptor α	NMDAR	- N-Methyl-D-aspartate receptor

INTRODUCTION

There is growing interest in the role of estrogens in male reproductive development and function, which has emerged from the potential adverse effects of synthetic environmental estrogens, also called xenoestrogens or endocrine disruptors (EDs) (Sharpe 1998). EDs cause adverse effects in target organs by interfering with the interaction of endogenous hormones and their receptors (Akingbemi *et al.* 2004). Male exposure to EDs has been associated with high incidence of reproductive disorders such as decreased of sperm counts, erectile dysfunction and cryptorchidism (Mostafa *et al.* 2007; Toppari *et al.* 1996; Crisp *et al.* 1998).

BPA is a xenoestrogen used in the manufacture of polycarbonate plastics and epoxy resins from which a variety of products are made, (Brotons *et al.* 1995; Krishnan *et al.* 1993). BPA is reported to be an ED with weak estrogenic activity, binding to both estrogen receptor ERα and ERβ with low affinity (vom Saal *et al.* 1998). In rodents, developmental exposure to BPA increased prostate weight, decreased epididymal weight (Pflieger *et al.* 2004; Prins 1992) and daily sperm production (Nelson 2003). Moreover, it has been reported that early exposure to BPA, during fetal life and/or the lactational period may alter the normal function of the hypothalamus-pituitary-testicular axis (Kubo *et al.* 2003, Mathews *et al.* 2001).

It is well established that Gn-RH is the primary hypothalamic hormone involved in the activation of pituitary gonadotropins during sexual maturation and the onset of puberty. Gn-RH secretion is under the control of different neurotransmitter systems, like the excitatory amino acid GLU, among others (Moguilevsky & Wutke 2001; Moschos *et al.* 2002; Rettori *et al.* 1994). Estrogens regulate the gonadal axis acting on peripheral organs, but also change the release of Gn-RH and gonadotropins (Herbison 1998), being one of the mediators of these changes the hypothalamic amino acid system (Brann & Mahesh 1992; Jarry *et al.* 1992).

Considering these information, the aim of the present study was to determine the possible role of GLU as a mediator of BPA effect at the hypothalamic level and its effects on pituitary and testis in prepubertal male rats. An oral route of BPA administration was chosen for this study to mimic the most likely route of exposure to the compound in humans and wildlife.

MATERIALS AND METHODS

Animals and treatments

Wistar female rats (weighing 250–300 g) from the Department of Physiology, School of Medicine, University of Buenos Aires, were singly housed in metal cages. The animals were kept in a light and temperature controlled environment (lights on from 07.00 to 19.00 h, T: 22–24 °C), and had free access to a pellet diet and tap water. The diet contains soybean meal, but as all animals were exposed to the same levels of phytoestrogen the feed intake was equivalent for control and BPA treated rats.

After acclimatization to the light/dark cycle for one week, the experiment was started. Male Wistar rats (weighing 300–350 g) and female rats were co-housed (1:1) until mating was confirmed by observation of a copulatory plug. The day the mating was confirmed was recorded as gestation day 0 (GD 0). At this moment mated female rats were separated and singly housed in metal cages. They were treated with either 0.1% ethanol (control group, n=4) (Funashabi *et al.* 2004) or BPA (4, 4` Isopropylidene-Diphenol, MP Biomedicals LLC, Germany) dissolved in ethanol (BPA group, n=4) in their drinking water until their offsprings were weaned at the age of 21 days. The number of suckling male pups (n=10) was equalized for each mother. BPA was dissolved in 100% ethanol at the concentration of 25 mg/ml and further diluted 1:1 000 with drinking water to make a final concentration of 25 mg/l BPA. The estimated average dose of exposure to dams was approximately 2.5 mg/kg body weight/day of BPA, which was 20 times less than no-observed-adverse-effect level (NOAEL: 50 mg/kg body weight/day), (Cagen *et al.* 1999; Funashabi *et al.* 2004). These estimates were based on the measurements of the difference in the amount of water placed in the water bottle each day and the amount remaining on the following day. The assessments assume that all the water lost from the bottle was consumed. They do not account for possible leakage or evaporation of the water or for potential loss of BPA activity during the 24 hr period. It is very important to note that the amount of BPA used in the present study was similar to the highest dose of human exposure reported in some previous studies (Olea *et al.* 1996; Brotons *et al.* 1995; Krishnan *et al.* 1993; Tohei *et al.* 2001). The actual level of BPA affecting the fetuses during gestation or that was ingested postnatally by the offspring during the period of lactation was not estimated in this study (Rubin *et al.* 2001).

On 21 day of life the male pups (n=10) were separated from the mother and housed in metal cages until their sacrifice on PND 35 when the different endpoints were studied. This age group of rats was used for the experiment because the prepuebrtal period is a time of active reproductive tract development and hormonally active chemicals are known to exhibit greater potency during sexual differentiation in rodents and humans (Colborn *et al.* 1993).

Animal care was carried out according to the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (1996, published by National Academy Press, 2101 Constitution Ave. NW, Washington DC 2005, and USA).

Tissue processing and hypothalamic incubation

The animals were killed by decapitation. Hypothalamic samples containing APOA-MBH were dissected with

the help of a stereomicroscope. The hypothalamic samples were cut to a depth of 3–4 mm and were laterally bordered by the hypothalamic sulci, rostrally, 3 mm anterior to the optic chiasma and caudally, by the mammillary bodies. The thickness of each sample was less than 2 mm. After dissection, the APOA-MBH (2 APOA-MBH/tube, n=10 incubations) were put into plastic chambers containing 500 µl of Earle's medium with glucose (1 mg/ml) and bacitracin (20 mM). The pH was adjusted to 7.4. The chambers were incubated in a Dubnoff shaker at 37 °C under constant shaking (60 cycles/min) in an atmosphere 95% O_2, 5% CO_2. After 30 minutes of preincubation, the medium was discarded and APOA-MBH were incubated in fresh medium for 60 minutes. The medium was collected and immediately frozen at –80 °C for Gn-RH and amino acids determinations.

Gn-RH determination

Gn-RH was measured in the medium, in duplicate by RIA using a highly specific antibody generously provided by Dr. Hubertus Jarry (University of Göttingen, Germany). The intra and inter assay coefficients of variation were lower than 9% and 10% respectively, the sensitivity was 0.2 pg/tube and the curve was linear up to 100 pg. The results were expressed as pg/APOA-MBH.

Amino acid determination

The concentration of the amino acid GLU, in the medium was determined by HPLC after derivatization with phenylisothiocyanate and UV detection at 254 nm, as previously described (Jarry *et al.* 1992). The drugs used did not interfere with the derivatization process. Mean inter- and intra-assay coefficients of variation were 4.0 and 5.6% respectively. The detection limit was 10 pmoles for GLU. The mobile phase consisted of 0.57 M sodium acetate buffer (pH 6.5) containing 10% acetonitrile (Sintorgan, Buenos Aires, Argentina). Amino acid used as standard was from Sigma Chemical Co St Louis, Mo, USA. Results were expressed as nmoles/APOA-MBH.

LH, FSH and testosterone determination

LH serum concentrations were measured in duplicate using a double antibody radio immunoassay (RIA). The material for the assay was kindly provided by the NIAMDD Rat Pituitary Program. Intra and inter assay coefficients of variation were 8% and 10%, respectively. Values were expressed as ng/ml in terms of the reference preparation (rat LH-IRP 1).

FSH serum concentrations were measured in duplicate using a double antibody radio immunoassay (RIA). The material for the assay was kindly provided by the NIAMDD Rat Pituitary Program. Intra and inter assay coefficient of variation were 8% and 10%, respectively. Values were expressed as ng/ml in terms of the reference preparation (rat FSH-IRP 1). Testosterone serum concentrations were measured by a competitive immunoassay provided by VITROS, (Immunodiagnostic Products Testosterone Reagent Pack, Ortho Clinical Diagnostics by Johnson & Johnson Company). Intra and inter assay coefficients of variation were 3.1% and 7.0% respectively. Values were expressed as nmol/l.

Statistical Analysis

The differences between the means of the two experimental groups were calculated by Student's t-test, using the program STATISTICA for windows (StatSoft, Inc., Tulsa, OK). $p<0.05$ was considered significant.

RESULTS

As it can be seen in figure 1 and 2 Gn-RH and GLU release decreased significantly in treated animals, ($p<0.001$, and $p<0.01$). Serum concentrations of LH, FSH and testosterone were also decreased by administration of BPA ($p<0.0001$), (Figures 3, 4 and 5).

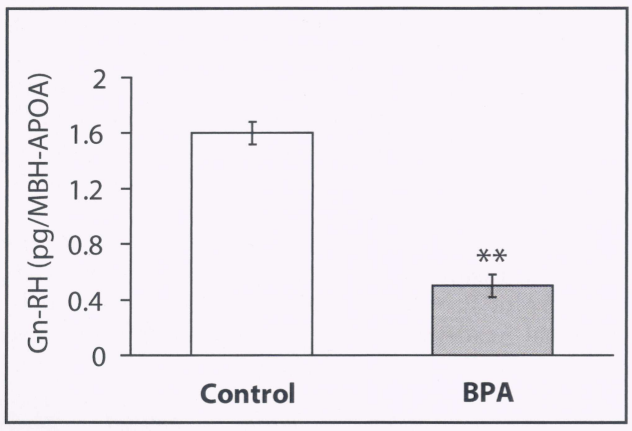

Fig. 1. Effect of BPA administration on Gn-RH release in prepubertal male rats. Each column represents the mean ± SEM of 10 incubations, (2 MBH-APOA/tube). **$p<0.001$ vs. control.

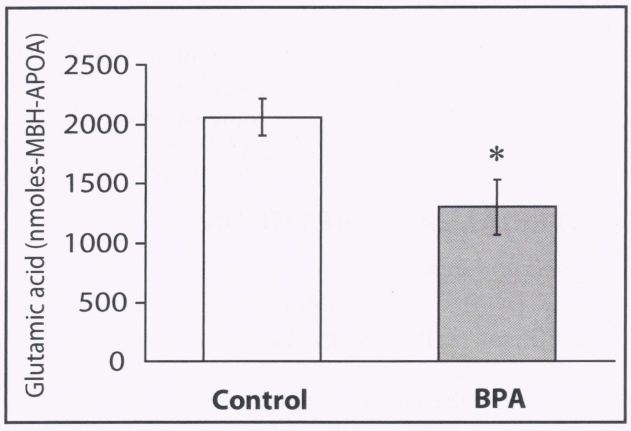

Fig. 2. Effect of BPA administration on Glu release in prepubertal male rats. Each column represents the mean ± SEM of 10 incubations, (2 MBH-APOA/tube). *$p<0.01$ vs. control.

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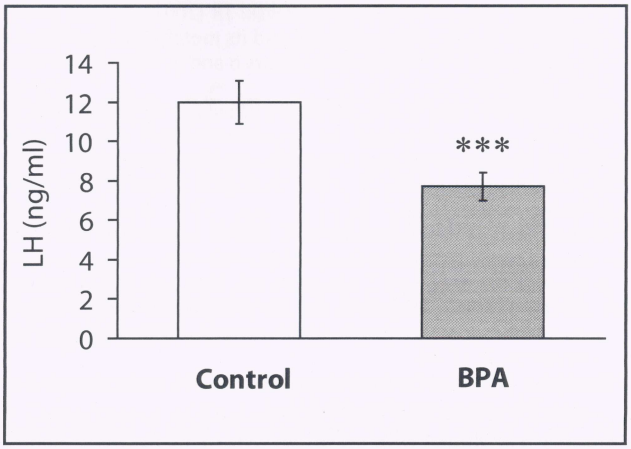

Fig. 3. Effect of BPA administration on serum LH levels in prepubertal male rats. Each column represents the mean ± SEM of 10 determinations. ***$p<0.0001$ vs. control.

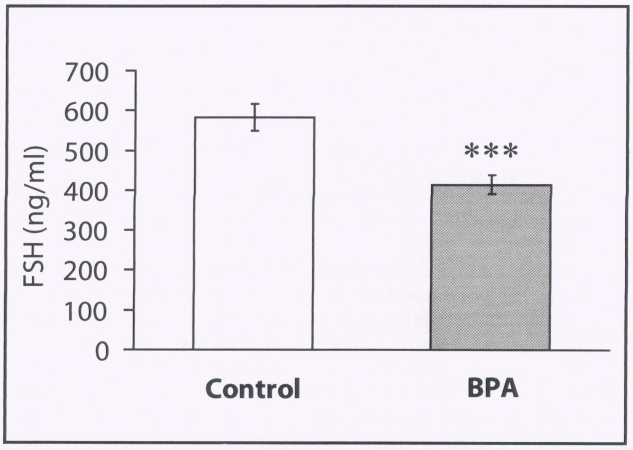

Fig. 4. Effect of BPA administration on serum FSH levels in prepubertal male rats. Each column represents the mean ± SEM of 10 determinations. ***$p<0.0001$ vs. control.

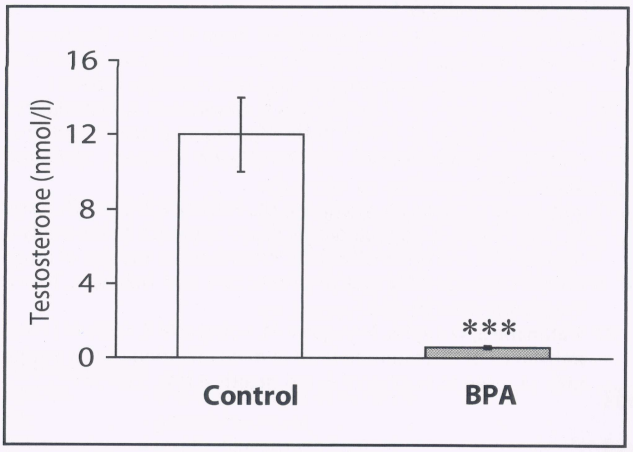

Fig. 5. Effect of BPA administration on serum testosterone levels in prepubertal male rats. Each column represents the mean ± SEM of 10 determinations. ***$p<0.0001$ vs. control.

DISCUSSION

As was mentioned above, the main hormone involved in the regulation of puberty onset is Gn-RH in the hypothalamus. Gn-RH neurons are affected by inputs from neurotransmitters such as glutamate, acting via the NMDA receptor (NMDAR) and NMDAR itself is subject to estrogen regulation (Gore 2001). Moreover, the presence of estrogen receptor (ER) on glutamatergic neurons in the hypothalamus of pubertal female monkeys has been previously reported by Thind & Goldsmith (1997). In males, estrogens take part in the negative feedback mechanism at the hypothalamic level (Mooradian & Morley 1987; Rochira *et al.* 2006) and it is known that in prepubertal stages the hypothalamic-pituitary unit is highly sensitive to the effect of this steroid. Since it has been stated that BPA has an estrogenic effect, (vom Saal *et al.* 1998) a probable mechanism of this ED would be the facilitation of the negative feedback mechanism produced by estrogen action in the hypothalamus. Considering these previous findings, it seems probable that in our male offspring, BPA may bind to ER present on GLU neurons leading to a decrease in its release which in turn may induce a decrease of Gn-RH secretion. However a direct effect on Gn-RH neurons may not be discarded, consistent with this idea is the repression of Gn-RH promoter by estradiol in Gn-RH producing GT1 cells (Rey *et al.* 1999). Hypothetically, interaction of ER-β with Gn-RH gene could inhibit the transcription of Gn-RH mRNA, as observed during episodes of negative estrogen feedback. BPA binding to ER-β on Gn-RH neuron may be another mechanism involved in the decrease of its release as has been previously reported (Hrabovszky *et al.* 2000).

Our results also showed a decrease in LH, FSH and testosterone serum levels. These changes may be explained by the decrease in Gn-RH release or by a direct effect of BPA on the pituitary and testis. It has been shown that after exposure to diethylstilbestrol (DES) during fetal life and/or suckling period FSH and LH levels were reduced as was demonstrated by Kubo *et al.* (2003). Akingbemi *et al.* (2004) have also reported that BPA treatment decreases LHβ chain expression and increases ERβ mRNA levels in BPA-treated rat pituitaries, suggesting that BPA-induced suppression of LHβ expression is ER mediated. Moreover, they and other authors have also shown that BPA acts directly on Leydig cells to decrease testosterone production after treatment of these cells with BPA in vitro (Akingbemi *et al.* 2004; Watanabe *et al.* 2003).

It is clear that pre, perinatal and early postnatal overexposure to BPA results in adverse effects on the male reproductive axis delaying the onset of puberty. Our results provide evidence of BPA effect on the axis, when it is administered in drinking water, being the dams exposed to a dose 20 times less than no-observed-adverse-effect level. Moreover, our findings showed that BPA inhibitory effect at the hypothalamic level may be

mediated by GLU. These findings suggest that GLU may be involved in BPA effect at the hypothalamic level, being one of the neuroendocrine mechanisms responsible of Gn-RH decrease in the animals exposed to this ED.

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