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Environmental obesogen tributyltin chloride leads to abnormal hypothalamic-pituitary-gonadal axis function by disruption in kisspeptin/leptin signaling in female rats

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ABSTRACT

Tributyltin chloride (TBT) is a xenobiotic used as a biocide in antifouling paints that has been demonstrated to induce endocrine-disrupting effects, such as obesity and reproductive abnormalities. An integrative metabolic control in the hypothalamus-pituitary-gonadal (HPG) axis was exerted by leptin. However, studies that have investigated the obesogenic TBT effects on the HPG axis are especially rare. We investigated whether metabolic disorders as a result of TBT are correlated with abnormal hypothalamus-pituitary-gonadal (HPG) axis function, as well as kisspeptin (Kiss) action. Female Wistar rats were administered vehicle and TBT (100 ng/kg/day) for 15 days via gavage. We analyzed their effects on the tin serum and ovary accumulation (as biomarker of TBT exposure), estrous cyclicity, surge LH levels, GnRH expression, Kiss action, fertility, testosterone levels, ovarian apoptosis, uterine inflammation, fibrosis, estrogen negative feedback, body weight gain, insulin, leptin, adiponectin levels, as well as the glucose tolerance (GTT) and insulin sensitivity tests (IST). TBT led to increased serum and ovary tin levels, irregular estrous cyclicity, and decreased surge LH levels, GnRH expression and Kiss responsiveness. A strong negative correlation between the serum and ovary tin levels with lower Kiss responsiveness and GnRH mRNA expression was observed in TBT rats. An increase in the testosterone levels, ovarian and uterine fibrosis, ovarian apoptosis, and uterine inflammation and a decrease in fertility and estrogen negative feedback were demonstrated in the TBT rats. We also identified an increase in the body weight gain and abnormal GTT and IST tests, which were associated with hyperinsulinemia, hyperleptinemia and hypoadiponectinemia, in the TBT rats. TBT disrupted proper functioning of the HPG axis as a result of abnormal Kiss action. The metabolic dysfunctions co-occur with the HPG axis abnormalities. Hyperleptinemia as a result of obesity induced by TBT may be associated with abnormal HPG function. A strong negative correlation between the hyperleptinemia and lower Kiss responsiveness was observed in the TBT rats. These findings provide evidence that TBT leads to toxic effects direct on the HPG axis and/or indirectly by abnormal metabolic regulation of the HPG axis.

1. Introduction

The hypothalamic-pituitary-gonadal (HPG) axis is the principal modulator of reproductive function. In females, the hypothalamic gonadotropin-releasing hormone (GnRH) neuron plays a pivotal role in the regulation of the cascade of hormonal events necessary for normal reproduction (Bauer-Dantoin et al., 1995). GnRH is a peptide

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synthesised and released by GnRH neurons that stimulates the production and secretion of luteinising hormone (LH) and follicle-stimulating hormone (FSH) in gonadotrophs (Wildt et al., 1981). LH and FSH act on their respective receptors to stimulate ovary maturation and estrogen production (Legan and Karsch, 1975; Richards et al., 1980).

Estrogens are a steroid hormone synthesised in females within the ovarian granulosa cells, and many of their actions are mediated by nuclear estrogen receptors (ERs) (Katzenellenbogen et al., 1993; Mangelsdorf et al., 1995; Hanley et al., 2000). The ERs are composed of ER alpha (ERα) and ER beta (ERβ) (Kuiper et al., 1996; Tremblay et al., 1997). The ERα and ERβ are encoded from separate genes (*ESR1* and *ESR2*) with variable tissue distributions and functions (Couse et al., 1997). ERα expression is predominately present in the hypothalamus, pituitary and uterus (Kuiper et al., 1996). ERβ expression is mainly present in the hypothalamus, ovary and lung (Tremblay et al., 1997). Also, the non-classic estrogen signaling involves different membrane receptors, such as GPR30 and STX-R associated with others intracellular pathways (Qiu et al., 2003; Thomas et al., 2010; Terasawa and Kenealy, 2012).

In the female brain, estrogen plays a critical role in the regulation of GnRH neuron activity and gonadotrophs, with bimodal effects on the hypothalamus, including inhibitory and stimulatory influences on GnRH secretion (estrogen negative and positive-feedback) (Petersen et al., 2003; Radovick et al., 2012). Recently, our understanding of the HPG axis control was advanced by the identification of the role of kisspeptin (Kiss), the peptide product of the Kiss-1 gene, and its receptor, G-protein-coupled receptor 54 (GPR54/Kiss-1R) (de Roux et al., 2003; Seminara et al., 2003). This neuropeptide has shown to be powerful stimulators of GnRH synthesis and secretion in mammals (Gottsch et al., 2004; Messager et al., 2005; Dhillo et al., 2005, 2007). Kiss neurons from the arcuate (ARC) and anteroventral periventricular (AVPV) nucleus have been proposed as the anatomical loci of estrogen negative and positive feedback to control GnRH secretion (Roa et al., 2008; Novaira et al., 2012). Previous studies have demonstrated the general concept that Kiss-secreting neurons were expressed and activated GnRH neurons to advance the process of reproductive axis control (Roa et al., 2008; Novaira et al., 2012).

Recent studies have indicated that the HPG axis function is affected by environmental factors (Walker et al., 2013; Topper et al., 2015; Skinner, 2016). Organotins (OTs) comprise endocrine-disrupting chemicals (EDCs) associated with abnormal function and steroidogenesis of rodent gamete cells (Kishta et al., 2007; Shen et al., 2014; Si et al., 2015). Grote et al. (2009) reported that OTs lead to irregular sexual development in offspring rats. Podratz et al. (2012) reported that tributyltin (TBT) impairs estrous cyclicity and ovary morphophysiology. TBT has been identified as an organometallic pollutant that was once used as a naval anti-fouling agent and tends to accumulate in seafood; consequently, human exposure primarily occurs through TBT-contaminated seafood (Fent, 1996; HELCOM, 2010; Graceli et al., 2013). OTs are detected in human blood at levels that range from 64 to 155 ng/mL, which leads to TBT tissue accumulation and dysfunction (Whalen et al., 1999). TBT exposure may lead to neural, metabolic and adrenal dysfunctions in in vivo and in vitro models, thereby increasing adiposity (Grun et al., 2006; Mitra et al., 2013a; Merlo et al., 2016). Studies have supported the key roles of inflammatory mediators, obesity and leptin in abnormal HPG axis function (Chen et al., 2011; Chakraborty et al., 2016; Sominsky et al., 2016). Thus, we learned that the complex TBT toxicity resulted of TBT accumulation and their metabolites, as dibutyltin (DBT) and inorganic tin (iSn) (Krajnc et al., 1984; Dorneles et al., 2008). The iSn is poorly absorbed by the gastro-intestinal tract (GIT) and is associated with OTs metabolization into iSn by mammals (Appel, 2004). It has been suggested that an important fraction of iSn may be present in the mammal bodies, as a result of OTs contamination.

Since the discovery of TBT in the reproductive system, to date, the effects of TBT on HPG axis function and its effects on the HPG axis have remained unclear. In the present study, we tested the hypothesis that TBT will lead to abnormalities in the HPG axis, as result of metabolic irregularities (Supplemental Fig. 1). We analyzed the key indicators of HPG axis competence in female rats, such estrous cyclicity, gonadotropin levels, fertility, reproductive tract morphology, GnRH and ER expressions and metabolic parameters. The identification of the HPG axis toxic signaling affected by TBT substantially contributes to our continuously evolving understanding of the HPG axis and identifies potential targets for metabolic disorders in the HPG axis by EDCs.

2. Materials and methods

2.1. Chemicals

The chemical tributyltin chloride (TBT, 96%, Sigma, St. Louis, Mo., USA) was dissolved in 0.4% ethanol based on our previous study (Merlo et al., 2016).

2.2. Experimental animals

Adult female Wistar rats (12-week-old) were maintained under a controlled temperature between 23 and 25 °C with a 12:12-h light/dark cycle. Rat chow and filtered tap water were provided *at libitum*. All protocols were approved by the Ethics Committee of Animals of the Federal University of Espirito Santo (106/2011). In this study, the total number of animals used was 114 rats. The rats were divided into two groups: Control (CON, n = 41) rats were treated daily with vehicle (0.4% ethanol), and TBT (TBT, n = 41) rats were treated daily with TBT (100 ng/kg/day) for 15 days by gavage, as shown in the experimental design diagram (Supplemental Fig. 2). Animals were anesthetised using ketamine and xylazine (90 mg/kg and 4.5 mg/kg, ip) prior to euthanasia. The doses and routes of exposure were chosen based on protocols previously reported by us (Podratz et al., 2012; Rodrigues et al., 2014; Podratz et al., 2015) and other (Zhou et al., 2013) to increase the serum tin levels (Bertuloso et al., 2015). Oral exposure to TBT in female rats was chosen so we could compare the current findings with our previous work demonstrating reproductive and metabolic toxicity (Podratz et al., 2012, 2015; Bertuloso et al., 2015). In addition, the TBT dose used in the current study (100 ng/kg) was approximately 3 times lower than the tolerable daily intake level of 300 ng/kg for humans established by the U.S. Environmental Protection Agency (USEPA, 1997).

2.3. Tin assessment

TBT is metabolized into the mammalian cells leading to tissue tin accumulation (Krajnc et al., 1984; Dorneles et al., 2008). For this reason, serum and ovary tin evaluation were performed. Serum and ovary tin concentrations were quantified, blood and ovary samples were collected and the tin levels were assessed using an inductively coupled plasma mass spectrometry (ICP-MS, NexIon 300-D, Perkin Elmer, Germany) (Podratz et al., 2015). In brief, blood and ovary samples were digested with 30% H2O2 (m/m) and ultra-pure HNO3 (Elga - Purelab, Marlow, UK) using a microwave oven equipped with PTFE vessels (Multiwave 3000 microwave, Anton Paar, Graz, Austria); the analyses were conducted with an ICP-MS. The quality control section requirements of the method were closely followed to demonstrate accurate quantification of tin in the blood and ovary samples. The level of detection (LOD) for tin determined by ICP-MS was 4.0 ng/g, and it was determined for each analysis performed.

2.4. Estrous cycle assessment

TBT leads to ovary abnormalities (Podratz et al., 2012). Thus, we evaluated if the ovary dysfunction as result of TBT exposure was associated with the estrous cycle irregularities. For these reasons, vaginal smears were obtained daily at 10:00 a.m. for 15 days in 2- to 3-