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Structural bisphenol analogues differentially target steroidogenesis in murine MA-10 Leydig cells as well as the glucocorticoid receptor

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ABSTRACT

Although much information on the endocrine activity of bisphenol A (BPA) is available, a proper human hazard assessment of analogues that are believed to have a less harmful toxicity profile is lacking. Here the possible effects of BPA, bisphenol F (BPF), bisphenol S (BPS), as well as the brominated structural analogue and widely used flame retardant tetrabromobisphenol A (TBBPA) on human glucocorticoid and androgen receptor (GR and AR) activation were assessed. BPA, BPF, and TBBPA showed clear GR and AR antagonism with IC_{50} values of 67 μM, 60 μM, and 22 nM for GR, and 39 μM, 20 μM, and 982 nM for AR, respectively, whereas BPS did not affect receptor activity. In addition, murine MA-10 Leydig cells exposed to the bisphenol analogues were assessed for changes in secreted steroid hormone levels. Testicular steroidogenesis was altered by all bisphenol analogues tested. TBBPA effects were more directed towards the male end products and induced testosterone synthesis, while BPF and BPS predominantly increased the levels of progestagens that are formed in the beginning of the steroidogenic pathway. The MA-10 Leydig cell assay shows added value over the widely used H295R steroidogenesis assay because of its fetal-like characteristics and specificity for the physiologically more relevant testicular Δ4 steroidogenic pathway. Therefore, adding an *in vitro* assay covering fetal testicular steroidogenesis, such as the MA-10 cell line, to the panel of tests used to screen potential endocrine disruptors, is highly recommendable.

1. Introduction

During the last decade much attention has been given to the hazard characterization and safety evaluation of endocrine disrupting compounds (EDCs). Considering the increasing list of potential EDCs, there is a strong need for alternative, rapid, and truly predictive *in vitro* screening assays. Consequently, many *in vitro* assays to assess for endocrine disrupting properties have been developed throughout the past years, such as the H295R steroidogenesis and several receptor-reporter gene transcriptional activation assays (Hecker et al., 2011; Bovee et al., 2007, 2011; Shen et al., 2009). Most of these assays, however, are directed towards effects in adults or puberty and less of these are focusing on effects early in life. For example, no appropriate *in vitro* model to test for effects on fetal steroidogenesis has been developed so far, while this is highly relevant with respect to masculinization of the male fetus. Furthermore, questions have been raised concerning the added value of the recently developed assays (Mantovani and Maranghi, 2005; van der Burg et al., 2011). Therefore, it seems

Abbreviations: 5αRed1, 5α-reductase type 1; 3β-HSD(1), 3β-hydroxysteroid dehydrogenase (type 1); 17β-HSD(3), 17β- hydroxysteroid dehydrogenase (type 3); cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate; AR, androgen receptor; BPA, bisphenol A; BPF, bisphenol F; BPS, bisphenol S; c-Kit, receptor tyrosine kinase c-Kit (oncogene); CYP(s), cytochrome P450 enzyme(s); Cyp11A1, cytochrome P450 enzyme 11A1; Cyp17(A1), cytochrome P450 enzyme 17(A1); Cyp51, cytochrome P450 enzyme lanosterol 14α-demethylase; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; DOC, deoxycorticosterone; EC_{50}, half maximal effective concentration; EDCs, endocrine disruptors/disrupting chemicals/compounds; etio, etiocholanolone; FSH(R), follicle-stimulating hormone (receptor); GR, glucocorticoid receptor; H295R, human adrenocortical carcinoma cells; HMG-CoA red, HMG-CoA reductase; IC_{50}, half maximal inhibitory concentration; LH(R), luteinizing hormone (receptor); MM/L, minimal medium supplemented with L-leucine; MPW, masculinization programming window; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD, optical density; Por, cytochrome P450 oxidoreductase; (17-OH-)P4, (17-hydroxy-) progesterone; (17-OH-)P5, (17-hydroxy-) pregnenolone; RIA, radioimmunoassay; RT-qPCR, real time quantitative polymerase chain reaction; SD, standard deviation; SEM, standard error of the mean; StAR, steroidogenic acute regulatory protein; (α/β-)T, (alpha/beta-) testosterone; TBBPA, tetrabromobisphenol A.

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more appropriate to focus on the development of assays that cover crucial aspects within the reproductive system that are not covered yet by existing assays (Chapin et al., 2013; Piersma et al., 2013; van der Burg et al., 2011; Mantovani and Maranghi, 2005; Mantovani et al., 1999).

One of the most debated examples in the area of endocrine disruptors is bisphenol A (BPA) that is used to make plastics and epoxy resins. Associations between exposure to bisphenol A and the occurrence of several adverse health outcomes have been indicated, including breast and prostate cancer, metabolic syndrome, obesity, and subfertility (De Coster and van Larebeke, 2012). However, the scientific debate about causal relationships is still ongoing and it seems that there are sensitive and critical time windows for exposure to BPA early in life (Kundakovic et al., 2013; Vandenberg et al., 2013). BPA possesses estrogenic as well as anti-androgenic properties as a result of its binding affinity for these respective steroid hormone receptors (Kitamura et al., 2005; Lee et al., 2003; Paris et al., 2002). In addition, (genes encoding for) steroidogenic enzymes can be modulated by BPA (Zhang et al., 2011b). Even at low dose levels BPA can cause effects. For example, *in utero* exposure to BPA can instigate sex-specific epigenetic changes in the brain, which possibly underlie enduring effects on function and behavior concerning sexually dimorphic phenotypes (Kundakovic et al., 2013; Vandenberg et al., 2013). In order to protect the highly susceptible group of infants, the European Union decided to ban the usage of BPA in baby bottles in 2011. Since then, the European Food Safety Authority (EFSA) has been reevaluating the safety limits of BPA. Recently, also the Dutch Ministry of Health, Welfare and Sport released a report of the National Institute for Public Health and the Environment (RIVM) regarding the human and environmental health issues and regulatory perspectives of BPA (Bakker et al., 2014). Meanwhile, ever more compounds have been developed and used to replace BPA. Amongst these are the BPA analogues bisphenol F (BPF) and bisphenol S (BPS). As a result, the use of BPF and BPS in consumer products has gradually increased, supposedly as safer alternatives for BPA. Consequently, both compounds can nowadays be found in canned soft drinks and foods as well as thermal receipt paper (Becerra and Odermatt, 2012; Gallart-Ayala et al., 2011; Liao and Kannan, 2013; Vinas et al., 2010). Moreover, BPS has already been detected in human urine samples (Liao et al., 2012). Although much toxicological information on BPA is available, a proper human risk assessment of BPA analogues like BPF and BPS that are believed to have a less harmful toxicity profile is lacking. In our previous study we showed that BPA as well as the brominated structural analogue and widely used flame retardant tetrabromobisphenol A (TBBPA) affected efflux transporter activities and testosterone (T) secretion by murine MA-10 Leydig cells (Dankers et al., 2013). Noticeably, TBBPA has also been associated with *in vivo* endocrine and reproductive toxic effects (Van der Ven et al., 2008). Our previous findings were especially interesting with respect to the reported increase in male sub- and infertility, which has been observed in human populations in a number of industrialized countries. Exposure to EDCs has often been suggested as an important contributing factor to this observed increase in male sub- and infertility (Wong and Cheng, 2011; Jurewicz et al., 2009). In recent studies this decline in human male fertility is reflected by poor semen quality, a suggested decline in sperm count, and lowered testosterone levels in men (Andersson et al., 2008; Jorgensen et al., 2012; Travison et al., 2007).

The testicular microenvironment plays a crucial role in mammalian spermatogenesis. The binding of the gonadotropins luteinizing hormone (LH) to the LH receptor (LHR) on Leydig cells and follicle-stimulating hormone (FSH) to the FSH receptor (FSHR) combined with activation of the androgen receptor (AR) in Sertoli cells are the essential factors in this process. Additionally, multiple cytochrome P450 (CYP) and hydroxysteroid dehydrogenase (HSD) enzymes are involved in local steroidogenesis in Leydig cells, which is pivotal for regulating spermatogenesis in males (Payne et al., 1992; Payne and Hales, 2004). The male sex steroid testosterone (T) is the final product of testicular steroidogenesis. Besides its function in the fertility of the adult male, T also has a crucial task in fetal development and maturation. During the masculinization programming window (MPW) the fetal testes start to produce T, which assures correct phenotypic development of the male reproductive system (Scott et al., 2009). Recently, Silva et al. described the role of glucocorticoids in the maintenance of spermatogenesis and maturation of sperm in adulthood (Silva et al., 2014). Moreover, the human fetal adrenal gland produces cortisol at the start of the MPW, which evokes suppression of adrenal androgen production via a negative feedback loop, minimizing the potential for masculinization in the female fetus (Goto et al., 2006). A number of studies have shown that BPA as well as TBBPA can affect steroidogenesis *in vitro* and *in vivo* (Canton et al., 2005, 2006; Dankers et al., 2013; Kitamura et al., 2005; Roelofs et al., 2013; Van der Ven et al., 2008), and as a result could possibly act as human testicular toxicants. Unfortunately, the possible role of the glucocorticoid receptor (GR) in steroidogenesis as well as the potential effects of BPF and BPS on steroidogenesis remain rather unclear.

In the present *in vitro* study, we examined the effects of structural bisphenol analogues on several major endocrine factors involved in testicular functioning. Recombinant yeast cells expressing the AR or GR were used to study possible interactions of TBBPA, BPA, BPF, and BPS with these steroid hormone receptors. Furthermore, effects of BPF, BPS, and TBBPA on production of (sex) steroids in mouse MA-10 Leydig cells were evaluated. Also, effects on the expression of genes within the cholesterol biosynthesis and steroidogenesis pathway were assessed.

2. Materials and methods

2.1. Chemicals

Table 1 displays the test compounds used: tetrabromobisphenol A (TBBPA; 97%, CAS# 79-94-7) was obtained from Sigma–Aldrich Co. (Zwijndrecht, The Netherlands), bisphenol A (BPA; 99.5%, CAS# 80-05-7) was purchased at Dr. Ehrenstorfer GmbH (Augsburg, Germany), and bisphenol F (BPF; >99.0%, CAS# 620-92-8) and bisphenol S (BPS; >98.0%, CAS# 80-09-1) were acquired from TCI Europe N.V. (Zwijndrecht, Belgium). Stock solutions were prepared in DMSO. The maximum solvent concentration for exposures was 0.1% v/v.

2.2. Yeast AR and GR bioassays

The recombinant yeasts used in the present study were constructed by Dr. T. Bovee (RIKILT, Wageningen UR). These yeasts stably express human androgen (AR) or glucocorticoid (GR) receptors and express yeast enhanced green fluorescent protein (yEGFP) as reporter protein when exposed to androgens or (gluco) corticosteroids, respectively (Bovee et al., 2007, 2011). Three days before exposure, cytosensor cultures were prepared by inoculating yeasts on agar of a minimal medium supplemented with L-leucine (#L8912; Sigma–Aldrich Co.) Agar plates were incubated at 30 °C for two days for colonies to form and then stored at 4 °C. One day before exposure, overnight cultures were prepared by inoculating one colony of yeast in 15 mL minimal medium supplemented with 120 mg L-leucine (MM/L) and containing 20 g dextrose (D-glucose, #215530; Becton Dickinson B.V., Breda, The Netherlands), 5 g ammoniumsulfate (#A4418; Sigma–Aldrich