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The relevance of chemical interactions with CYP17 enzyme activity: Assessment using a novel *in vitro* assay

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ABSTRACT

The steroidogenic cytochrome P450 17 (CYP17) enzyme produces dehydroepiandrosterone (DHEA), which is the most abundant circulating endogenous sex steroid precursor. DHEA plays a key role in *e.g.* sexual functioning and development. To date, no rapid screening assay for effects on CYP17 is available. In this study, a novel assay using porcine adrenal cortex microsomes (PACMs) was described. Effects of twenty-eight suggested endocrine disrupting compounds (EDCs) on CYP17 activity were compared with effects in the US EPA validated H295R (human adrenocorticocarcinoma cell line) steroidogenesis assay. In the PACM assay DHEA production was higher compared with the H295R assay (4.4 *versus* 2.2 nmol/h/mg protein). To determine the additional value of a CYP17 assay, all compounds were also tested for interaction with CYP19 (aromatase) using human placental microsomes (HPMs) and H295R cells. 62.5% of the compounds showed enzyme inhibition in at least one of the microsomal assays. Only the cAMP inducer forskolin induced CYP17 activity, while CYP19 was induced by four test compounds in the H295R assay. These effects remained unnoticed in the PACM and HPM assays. Diethylstilbestrol and tetrabromobisphenol A inhibited CYP17 but not CYP19 activity, indicating different mechanisms for the inhibition of these enzymes. From our results it becomes apparent that CYP17 can be a target for EDCs and that this interaction differs from interactions with CYP19. Our data strongly suggest that research attention should focus on validating a specific assay for CYP17 activity, such as the PACM assay, that can be included in the EDC screening battery.

Introduction

Dehydroepiandrosterone (DHEA) and its sulfated form DHEA-S are the most abundant circulating endogenous sex steroid precursors in the human body (Auchus, 2004; Chen and Parker, 2004; Kroboth et al., 1999). In adults, the C19 steroid DHEA is mainly produced in the zona reticularis of the adrenal cortex. In the fetus, no distinct adrenal zonae can be discerned and DHEA is produced in the entire adrenal cortex. It is formed after the conversion of cholesterol to pregnenolone by cytochrome P450 11A (CYP11A) and subsequently *via* 17α-OH-pregnenolone to DHEA, which is the result of 17α-hydroxylase and 17,20-lyase activity displayed by cytochrome P450 17 (CYP17) (Suppl. Fig. 1). After release into the circulation and transport to peripheral tissues DHEA can be further converted by the hydroxysteroid dehydrogenases (HSDs) 3β-HSD and 17β-HSD to androstenedione and dihydroxyandrostene, respectively, and subsequently to the main male sex hormone testosterone. The final step in sex hormone production involves the conversion of androgens into estrogens catalyzed by the aromatase enzyme (CYP19).

This steroidogenic pathway and the hormones and their precursors involved in it are essential for maintaining hormonal homeostasis in the body. Changes in hormonal levels can result in *e.g.* adverse

Abbreviations: 4-OH-ASDN, 4-hydroxyandrostenedione; cAMP, cyclic AMP; ATZ, atrazine; BPA, bisphenol A; CSA, cyclosporin A; CYP11A, cytochrome P450 11A; CYP17, cytochrome P450 17; CYP19 (aromatase), cytochrome P450 19; DBP, dibutyl phthalate; DEHP, diethylhexyl phthalate; DES, diethylstilbestrol; DHEA, dehydroepiandrosterone; DML, D-mannitol; DOTC, dioctyltin chloride; E2, estradiol; EC_{50}, half maximal effective concentration; EDCs, endocrine disrupting compounds; EDSP, endocrine disruptor screening program; ELISA, enzyme linked-immunosorbent assay; EPI, epicatechin; ESF, endosulfan; GEN, genistein; GPA, glufosinate ammonium; FLU, flusilazole; FOR, forskolin; FP, forward primer; H295R, human adrenocorticocarcinoma cell line; HPM(s), human placental microsome(s); HSD(s), hydroxysteroid dehydrogenase(s); IC_{50}, half maximal inhibitory concentration; KET, ketoconazole; MAA, methoxyacetic acid; MEHP, mono-ethylhexyl phthalate; MMC, methylmercury; MPW, masculinization programming window; OECD, Organization for Economic Cooperation and Development; PACM(s), porcine adrenal cortex microsome(s); PFBS, perfluorobutane sulfonic acid; PFHS, perfluorohexane sulfonic acid; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfate; RA, retinoic acid; REP, relative effect potency; RIA, radioimmunoassay; RP, reverse primer; T, testosterone; TBBPA, tetrabromobisphenol A; TBCZ, tebuconazole; TCS, triclosan; US EPA, US Environmental Protection Agency (EPA).

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effects reminiscent of cardiovascular disease (Ikeda et al., 2012), malignancies (Coffey, 2001; Ito et al., 2011; Solomon and Freeman, 2011) and metabolic disturbances (Moulana et al., 2011). DHEA plays a key role in sexual functioning in both men and women by acting as precursor for androgens and estrogens (Labrie, 2010). Moreover, DHEA plays an important role in the development of the fetus (Kroboth et al., 1999), where it is the precursor for massive amounts of estrogens produced by the materno-feto-placental unit (Ishimoto and Jaffe, 2011; Miller, 1998). In the fetal testis DHEA is also required for testosterone production during the specific masculinization programming window (MPW) in which the masculinization of the reproductive tract by androgens occurs (Scott et al., 2009). Besides the action of DHEA in autocrine, endocrine, and paracrine processes, it is a crucial factor in intracrinology *i.e.* the synthesis and actions of active steroids in peripheral target tissues (Kuhn-Velten, 2000; Labrie et al., 2001).

In recent years, much research has focused on the effects of exogenous compounds that might disrupt the endocrine system of humans and wildlife. Studies to identify these endocrine disrupting compounds (EDCs) were typically performed as a result of the previous findings of endocrine disrupting effects in *in vitro* assays (Matsumoto et al., 2005; Yang et al., 2011) and as part of *in vivo* reproductive toxicity testing for regulatory toxicology under Organization for Economic Cooperation and Development (OECD) guidelines (*e.g.* OECD 414 developmental toxicity study, OECD 416 two generation study, and OECD 421 developmental toxicity screening; (van der Jagt et al., 2004)). In view of the increasing list of potential EDCs there is a strong need for alternative, rapid *in vitro* screening assays. The US Environmental Protection Agency (EPA) anticipated to this by launching the endocrine disruptor screening program (EDSP). The human adrenocorticocarcinoma cell line (H295R; (Hecker et al., 2011; Sanderson et al., 2000)) is often considered as golden standard for *in vitro* assessment of the effects of chemicals on steroidogenesis. The H295R steroidogenesis assay was validated within the frameworks of the EDSP and OECD and is widely used by different research groups (Gracia et al., 2006; Hecker and Giesy, 2008). However, this method only considers hormonal endpoint measurements of progesterone, testosterone, and estradiol production, and not of the precursor DHEA. Considering the central role of DHEA in many reproductive and developmental processes, the lack of an assay for effects on DHEA production and/or CYP17 activity is a clear gap in the EDC testing program. In contrast, to determine the inhibition of aromatase (CYP19), there is an additional assay included in the Tier I EDSP test battery using human placental microsomes (HPMs).

In this study, twenty-eight suggested reproductive and/or developmental toxic compounds from various chemical classes were tested for their potential interaction with CYP17 activity. The test compounds were selected based on their availability in the environment and included some that are known to possess *in vivo* endocrine disrupting properties. A new method is described to study the interaction with CYP17 enzyme activity and DHEA production using porcine adrenal cortex microsomes (PACMs). Effects of the test compounds on DHEA production were also determined in H295R cells. To gain insight into the mechanism of action, for a subset of compounds, CYP17 gene expression and intracellular cAMP levels were determined in H295R cells. The aim of our study was to investigate the relevance of a CYP17 screening assay in an EDC screening strategy in line with the CYP19 approach. Therefore, effects on CYP19 activity were also determined using human placenta microsomes (HPMs) and the H295R cell line. A full validation of our PACM assay would be a next step towards this aim. In the present study, we have specifically investigated the possible interaction of (suggested) endocrine disrupting chemicals with the CYP17 enzyme and if this is a relevant addition to the already available steroidogenic assays.

Materials & methods

Test chemicals. Table 1 gives an overview of the test compounds used in the experiments described below. Atrazine (ATZ; 98.8%), bisphenol (BPA; 99%), cyclosporin A (CSA; >98.5%), diethylstilbestrol (DES; 99%), D-mannitol (DML; 99.1%), endosulfan (ESF; 99.9%), epicatechin (EPI; >98%), flusilazole (FLU; 99.8%), forskolin (FOR; ≥98%), ketoconazole (KET; ≥98%), methoxyacetic acid (MAA; 98%), methylmercury (MMC), perfluorobutane sulfonic acid (PFBS; 97%), perfluorohexane sulfonic acid (PFHS; >98%), perfluorononanoic acid (PFNA; ≥97%), perfluorooctanoic acid (PFOA; ≥98%), perfluorooctane sulfate (PFOS; ≥98%), retinoic acid (RA; >98%), tetrabromobisphenol A (TBBPA; 97%), tebuconazole (TBCZ; 99.5%), triclosan (TCS; 97%) and valproic acid (VPA; >98%) were purchased from Sigma-Aldrich Co. (Zwijndrecht, The Netherlands). Dioctyltin chloride (DOTC; 95%) was acquired from ABCR GmbH & Co. KG (Karlsruhe, Germany), genistein (GEN; ≥98%) from Acros Organics BVBA (Geel, Belgium), glufosinate ammonium (GPA; 97.5%) from Dr. Ehrenstorfer GmbH (Augsburg, Germany), and mono-ethylhexyl phthalate (MEHP; 97.5%) from Wako Chemicals GmbH (Neuss, Germany). Dibutyl phthalate (DBP; >97.0%) and diethylhexyl phthalate (DEHP; >98.0%) were obtained from TCI Europe N.V. (Zwijndrecht, Belgium).

Porcine adrenal cortex microsomes (PACMs) preparation. Porcine adrenals were freshly obtained from the abattoir of Barten Meerkerk Holding B.V. (Meerkerk, The Netherlands). Immediately after slaughter, adrenals were dissected from female hogs (*Sus Scrofa Domesticus*) by a certified veterinarian and kept in freshly prepared ice cold saline solution (0.9% NaCl). Within 3 h after dissection cortices were separated from medullae, frozen in liquid nitrogen and stored at −80 °C. Microsomal fractions were isolated by homogenizing samples in 2 mL Tris–HCl buffer ([50 mM], 1.15% KCl) using a Potter homogenizer. Then, homogenates were centrifuged for 25 min at 9000 rpm at 4 °C and the supernatant was centrifuged for 75 min at 30,000 rpm at 4 °C. After decanting the supernatant, the pellet was resuspended with a needle in 1 mL sucrose solution [0.25 M] per original gram of tissue. Protein concentration of PACMs was determined and diluted to a final protein concentration of 10 mg/mL. Microsome suspension aliquots were frozen in liquid nitrogen and stored at −80 °C until further use.

H295R cell culture and exposure. Human adrenal cortical carcinoma cell line H295R was obtained from the American Type Culture Collection (ATCC-LGC Standards GmbH, Wesel, Germany) and grown in 1:1 Dulbecco's modified Eagle medium/Ham's F-12 nutrient mix with phenol red (DMEM/F12; Gibco, Life Technologies Europe BV, Bleiswijk, The Netherlands) supplemented with 1% bovine serum albumin 1.25 g/10 mL (BSA; Sigma-Aldrich Co., Zwijndrecht, The Netherlands), 1% ITS-G (Gibco, Life Technologies Europe BV, Bleiswijk, The Netherlands) containing 10 mg/mL insulin, 5.5 mL/L transferrin, and 6.7 μg/L sodium selenite, 100 U/mL, 1% penicillin/streptomycin (Pen/Strep; Gibco, Life Technologies Europe BV, Bleiswijk, The Netherlands), and 2.5% Nu-serum (BD Biosciences, Breda, The Netherlands).

For CYP17/19 enzyme activity and cyclic AMP (cAMP) levels, H295R cells were plated in 24-well plates (Greiner, The Netherlands) at a density of $3*10^5$ cells per well. For gene expression assessment H295R cells were seeded in 12-well plates at a density of $6*10^5$ cells per well. After 48 h the medium was replaced with medium containing the test compounds (at a maximum solvent concentration of 0.1% v/v). CYP17/19 activity and gene expression were determined after a 24-hour exposure and cAMP levels were measured after 6 h.

Cytotoxicity assay. Cell viability was determined by measuring the capacity of H295R cells to reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan by the mitochondrial enzyme succinate dehydrogenase. H295R cells were incubated for