Effects of the environmental contaminants DEHP and TCDD on estradiol synthesis and aryl hydrocarbon receptor and peroxisome proliferator-activated receptor signalling in the human granulosa cell line KGN

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ABSTRACT: Environmental contaminants binding to transcription factors, such as the aryl hydrocarbon receptor (AhR) and the alpha and gamma peroxisome proliferator-activated receptors (PPARs), contribute to adverse effects on the reproductive system. Expressing both the AhR and PPARs, the human granulosa cell line KGN offers the opportunity to investigate the regulatory mechanisms involved in receptor cross-talk, independent of overriding hormonal control. The aim of the present study was to investigate the impact of two environmental contaminants, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, an AhR ligand) and di-(2-ethylhexyl) phthalate (DEHP, a PPAR ligand), on gonadotrophin sensitivity and estrogen synthesis in KGN cells. Accumulation of the DEHP metabolite mono-(2-ethylhexyl) phthalate (MEHP) in DEHP-exposed cells was measured by high-performance liquid chromatography mass spectrometry, thereby demonstrating DEHP metabolism to MEHP by KGN cells. By employing TCDD (an AhR agonist), rosiglitazone (a PPARgamma agonist) or bezafibrate (a PPARalpha agonist), the presence of a functional AhR and PPAR cascade was confirmed in KGN cells. Cytotoxicity testing revealed no effect on KGN cell proliferation for the concentrations of TCDD and DEHP used in the current study. FSH-stimulated cells were exposed to TCDD, DEHP or a mix of both and estradiol synthesis was measured by enzyme-linked immunosorbent assay and gene expression by quantitative RT–PCR. Exposure decreased estradiol synthesis (TCDD, DEHP, mix) and reduced the mRNA expression of CYP19 aromatase (DEHP, mix) and FSHR (DEHP). DEHP induced the expression of the alpha and gamma PPARs and AhR, an effect which was inhibited by selective PPAR antagonists. Studies in the human granulosa cell line KGN show that the action of endocrine-disrupting chemicals may be due to a direct activation of AhR, for example by TCDD, and by a transactivation via PPARs, for example by DEHP, inducing subsequent transcriptional changes with a broad range of effects on granulosa cell function.

Key words: aryl hydrocarbon receptor / peroxisome proliferator-activated receptor / phthalate diesters / estradiol / KGN granulosa cells

Introduction

Plasticizers, such as phthalates, are widely used in industry and consumer goods. Since phthalates do not form strong molecular polymer linkages they easily diffuse into the environment (Kavlock et al., 2006). Over the last decade epidemiological studies observed a link between phthalates and numerous health problems, including obesity, type 2 diabetes, atherosclerosis, asthma and allergies (Bornehag et al., 2004; Hatch et al., 2008; Lind and Lind, 2011; Lind et al., 2012; Sun et al., 2014). Phthalates are reported to have adverse effects on the human reproductive system. Prenatal and early post-natal environmental phthalate exposure was associated with decreased hormone levels and genital alterations in...
male offspring (Swan et al., 2005; Main et al., 2006). Recently, phthalates were shown to negatively affect testosterone levels, semen quality, sperm motility and morphology (Pant et al., 2008; Wirth et al., 2008; Jur-ewicz et al., 2013). Comparable evidence on female reproductive toxicity is scarce. In girls, high urinary phthalate concentrations are linked to a delayed pubarche but not thelarche (Frederiksen et al., 2012). Studies on the association of phthalates and endometriosis risk revealed contradictory results (Cobellis et al., 2003; Kim et al., 2011; Upson et al., 2013). Investigating the effects of phthalate exposure by measuring diester levels in serum is limited due to their rapid metabolism to monoesters and the high risk of background contamination. Therefore, urinary metabolites are analysed to evaluate the body burden of phthalate diesters, such as di-(2-ethylhexyl) phthalate (DEHP), being the environmentally most abundant phthalate for many years. An investigation of the internal exposure to DEHP in nursery-school children and their parents and teachers showed a higher level in urine for the children, with 90 μg/l (adults: 59.1 μg/l) (Koch et al., 2004). Also healthy pregnant women in different countries were studied revealing an exposure range of 31.8–79.3 μg/l (Enke et al., 2013). Owing to the use of DEHP-plasticized medical devices, patients can be exposed to much higher levels. An analysis of blood bags, for example, revealed DEHP concentrations of up to 83.2 μg/ml (Inoue et al., 2005). DEHP exposure of neonates who are treated in intensive care reached levels of 123.1 μg/ml after exchange transfusion (Plonait et al., 1993) or 34.9 μg/ml after extracorporeal membrane oxygenation (Karle et al., 1997).

Referring to environmental contaminants such as endocrine-disrupting chemicals (EDCs) is based on their ability to interfere with synthesis, release, transport, metabolism, binding, action and elimination of naturally occurring hormones (Kavlock et al., 1996). The human alpha and gamma peroxisome proliferator-activated receptors (PPARs) are known targets for environmental phthalate di- and monoesters as shown by an in silico approach (Sarahath Joshi et al., 2013). The DEHP metabolite mono- (2-ethylhexyl) phthalate (MEHP) was reported to activate human PPARα and PPARγ in MCF-7 cells (a human breast cancer cell line) (Venkata et al., 2006) and to promote adipocyte differentiation and adipogenesis in human preadipocytes (Ellero-Simatos et al., 2011). These findings were confirmed for DEHP in murine mesenchymal stem cells (Biemann et al., 2012; Biemann et al., 2014). Furthermore, phthalates affected aryl hydrocarbon receptor (AhR) activation in vitro, acting as weak agonists (Krüger et al., 2008; Mankidy et al., 2013). The AhR, being a prominent member of the basic helix-loop-helix Per-ARNT-SIM (bHLH-PAS) protein family, is evolutionary well conserved and present in a wide range of vertebrate and invertebrate species and tissues. It can be activated by a broad range of substances with little structural similarity. Halogenated and polycyclic aromatic hydrocarbons are well-characterized synthetic ligands, but also natural exogenous and endogenous compounds have been described (reviewed in Denison and Nagy, 2003). Few data on human exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), one of the best described AhR ligands, are available. Population-based studies found an increasing risk for endometriosis, earlier menopause and prolonged menstrual cycles in the Seveso accident cohort (Eskenazi et al., 2002a,b; Eskenazi et al., 2005). TCDD concentrations in human adipose tissue, measured in people with occupational and non-occupational exposure, are in the pg per gram of fat range. TCDD levels in human breast milk from different regions of Germany differ between 3.2 and 4.3 pg/g fat (Beck et al., 1994).

Single activation of the AhR (Horling et al., 2011) and PPAR (Lovekamp-Swan et al., 2003) receptor pathways by EDCs causes a decreased expression of cytochrome P450 aromatase (CYP19), the key enzyme in estrogen biosynthesis in granulosa cells. Additionally, treatment with MEHP or a selective PPARα ligand, but not PPARγ agonist, increased AhR and CYP1B1 expression in rat granulosa cells (Lovekamp-Swan et al., 2003). In Caco-2 cells PPARα activation up-regulated AhR expression via a PPAR response element (PPRE) in the AhR promoter (Villard et al., 2007).

The immortalized human granulosa cell line KGN was chosen for the current study. It is characterized by retaining typical granulosa cell features over a long period in culture (Nishi et al., 2001). TCDD, by activating AhR and inducing the expression of the AhR target genes CYP1A1 and CYP1B1, causes an altered expression of gonadotrophin and estrogen receptors in this culture model (Horling et al., 2011). Expressing PPARs too, KGN offers the opportunity to study the receptor crosstalk independently from overriding hormonal control by theca cells and/or the hypothalamic–pituitary axis. A crosstalk between AhR and other nuclear receptors was already demonstrated, mediating subsequent cellular responses (Pocar et al., 2005; Seifert et al., 2008). The aim of the present study was to investigate the impact of two environmental contaminants, TCDD and DEHP, on gonadotrophin sensitivity and estrogen synthesis in KGN granulosa cells assessed by gene expression studies and by enzyme-linked immunosorbent assay (ELISA).

Methods

Cell line

The immortalized human granulosa cell line KGN was purchased from RIKEN BioResource Center (Ibaraki, Japan).

Chemicals

The chemicals used in the present study were purchased from the following companies: 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) from Amchro (Hattersheim, Germany), di-(2-ethylhexyl) phthalate (DEHP) and bezafibrate from Sigma-Aldrich (Taufkirchen, Germany), alpha-Naphthoflavone (ANF) and dimethyl sulphoxide (DMSO) from Sigma-Aldrich (Germany), MK 886, GW 9662 and rosiglitazone from Cayman (Ann Arbor, MI, USA). All chemicals were dissolved in DMSO with a final concentration of 0.1% in current study. It is characterized by retaining typical granulosa cell features over a long period in culture (Nishi et al., 2001). TCDD, by activating AhR and inducing the expression of the AhR target genes CYP1A1 and CYP1B1, causes an altered expression of gonadotrophin and estrogen receptors in this culture model (Horling et al., 2011). Expressing PPARs too, KGN offers the opportunity to study the receptor crosstalk independently from overriding hormonal control by theca cells and/or the hypothalamic–pituitary axis. A crosstalk between AhR and other nuclear receptors was already demonstrated, mediating subsequent cellular responses (Pocar et al., 2005; Seifert et al., 2008). The aim of the present study was to investigate the impact of two environmental contaminants, TCDD and DEHP, on gonadotrophin sensitivity and estrogen synthesis in KGN granulosa cells assessed by gene expression studies and by enzyme-linked immunosorbent assay (ELISA).

Cell culture

As described previously (Horling et al., 2011), KGN cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Berlin, Germany) containing 10% heat-inactivated fetal calf serum (Gibco), 1% l-glutamine (Gibco) and 1% penicillin/streptomycin (PAA, Pasching, Austria) in a humidified atmosphere of 20% O2 and 5% CO2 at 37°C.

Exposure schedules

The concentration for TCDD exposure (10 nM) was chosen according to a previous study in the KGN cell model (Horling et al., 2011). A concentration range between 0.1 and 100 nM TCDD had been investigated and analysed with regard to the expression of the AhR target gene CYP1B1. CYP1B1 was significantly induced following exposure to 10 nM TCDD. This exposure level is in agreement with another study in cultured primary human granulosa
lutein cells (Vidal et al., 2005). It has also been shown to be the lowest maximally effective concentration for the reduction of the estradiol level in such culture systems (Enan et al., 1996). Based on previous in vitro investigations using DEHP in the μM range (Kruger et al., 2008; Ohno et al., 2009; Gupta et al., 2010), we used 0.5, 5 and 50 μM DEHP. Concentrations in μM exceed environmental DEHP exposure reported in various studies (Koch et al., 2004; Göen et al., 2011; Enke et al., 2013; Zota et al., 2014), but are in line with clinically relevant DEHP concentrations found in blood bags (Inoue et al., 2005). Various exposure schedules were applied. TCDD and/or DEHP were given with or without selective antagonists of AhR (ANF, Horling et al., 2011), PPARalpha (MK 886, Kehrer et al., 2001) or PPARgamma (GW 9662, Seargent et al., 2004) (concentration 10 μM each, according to the references) for 5 or 24 h after a 2 h pre-incubation with the antagonists. Corresponding controls were cultured in the same way but without antagonists. For investigating hormone synthesis, KGN cells were cultured in medium containing 10 μM 1,4-androstenedione-3,17-dione (Sigma-Aldrich) and stimulated with 20 ng/ml FSH (Sigma-Aldrich) (Horling et al., 2011). A functional PPAR cascade was investigated by employing classical agonists, i.e. rosiglitazone (PPARgamma agonist, 500 nM, Kirchner et al., 2010) or bezafibrate (PPARalpha agonist, 25 μM, Guo et al., 2006), in concentrations shown to activate PPAR. In these experiments KGN were cultured for 24 h.

**MEHP accumulation**

After 5 h in culture with or without added DEHP, the DEHP MEHP was analysed in the medium and cell lysates using high-performance liquid chromatography tandem mass spectrometry with quantification via isotope dilution (Koch et al., 2003). The assays used to determine MEHP (and other oxidized DEHP metabolites) was performed via tandem mass spectrometry with isotope dilution quantification. The selectivity and specificity of such an HPLC-MS/MS method (including isotope labelled internal standards) can be considered a gold standard for a robust, sensitive and specific determination of such biomarkers. The respective LOQs for MEHP (and other phthalate metabolites) are 0.5 μg/l for MEHP and 0.2 μg/l for the oxidized metabolites. The extensive chromatographic separation and the specific mass transitions ensure that ‘cross-reactivity’ (in LC-MS/MS better termed interference) with or by other phthalates or phthalate metabolites can be excluded. Sample measurements were carried out by the IPA laboratory in Bochum (Germany), which has successfully participated as a reference laboratory for phthalate metabolite analyses in the quality assurance programme of the European Union financed Consortium to Perform Human Biomonitoring on a European Scale (COPHES).

**RNA isolation and cDNA synthesis**

Total RNA from cultured KGN cells was isolated using the AllPrep Kit (Qagen, Hilden, Germany) according to manufacturer’s manual. cDNA was generated from 3 μg total RNA using the RevertAid H Minus Reverse Transcriptase (Thermo Scientific, Dreieich, Germany). Reactions were performed in a final volume of 20 μl containing 1 × first-strand buffer, 200 units transcriptase enzyme, 1 mM dNTPs (Invitrogen, Karlsruhe, Germany) as well as 1 μl random hexamer primer and 50 μ RNase Inhibitor (both from Roche Diagnostics, Mannheim, Germany) at 42°C for 1 h followed by incubation at 70°C for 10 min.

**Quantitative realtime RT–PCR**

Quantitative RT–PCR was performed to measure mRNA expression levels in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Gene expression with normalization to 10⁶ copies of 18S ribosomal RNA as housekeeping gene was determined in a 96-well plate format for the following genes: AhR and its target gene cytochrome P450 1B1 (CYP1B1), PPARalpha and PPARgamma and their target gene fatty acid-binding protein 3 (FABP3), the FSH receptor (FSHR) and CYP19. The primers and amplicons were as shown in Table 1.

PCR reactions were prepared in a final volume of 20 μl reaction mixture comprising 500 nM specific primers (Sigma-Aldrich), 10 μl SYBRgreen Mastermix (MESA Blue qPCR™ Mastermix Plus for SYBR® Assay, Eurogentec, Cologne, Germany) and 3 μl of cDNA (diluted 1:4.5). The cycle parameters were 5 min 95°C, and 40 three-step cycles of 10 s 95°C, 15 s 60°C and 20 s 72°C. Standard curves were generated for each gene using a plasmid dilution series containing the target sequences.

**Hormone assay**

The supernatant from FSH-stimulated KGN cells with 24 h of exposure to TCDD and/or DEHP was collected for measuring estradiol by ELISA (DRG Instruments, Marburg, Germany), which was performed according to manufacturer’s manual. Cells from these experimental series were also used for RNA and protein extraction. ELISA data were normalized to the protein concentration of individual samples.

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**Table 1 Primers for quantitative RT–PCR.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer 5′ – 3′</th>
<th>Reverse primer 5′ – 3′</th>
<th>Amplicon length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>CCTGTATTTGTTAATTCGTCACTTACCT</td>
<td>AGAAACCGCTACCACATCCAA</td>
<td>105</td>
</tr>
<tr>
<td>AhR</td>
<td>AGATTTGAGCCGTGTTGGCTA</td>
<td>AGTTATCCTGCGCTCCGTTT</td>
<td>167</td>
</tr>
<tr>
<td>CYP19</td>
<td>ATGGTGGACGTGTTGACCTCTCTT</td>
<td>AGGAGACCTTGCCATGGCATCACA</td>
<td>133</td>
</tr>
<tr>
<td>CYP1B1</td>
<td>TTGGACAAGGATGCCTCCTAC</td>
<td>TTTTGGCAAGGCCTATTTGGTG</td>
<td>171</td>
</tr>
<tr>
<td>FABP3</td>
<td>GGCACACGATGCAACAGGCTCACA</td>
<td>AGTCCCCGCACACAGTGGTC</td>
<td>228</td>
</tr>
<tr>
<td>FSHR</td>
<td>TCTCTTGTCATCAATGTCTCGT</td>
<td>ACGTGAGGGAGGAGCAGAT</td>
<td>196</td>
</tr>
<tr>
<td>PPAR alpha</td>
<td>TCATACCGGCAACAGGCTTCACTC</td>
<td>AAGCGCTGTCCAGGCTTAC</td>
<td>175</td>
</tr>
<tr>
<td>PPAR gamma</td>
<td>AGATACATCCTACACCATGCTGGCT</td>
<td>TGTCTCGATGGGCTCTACATTCA</td>
<td>255</td>
</tr>
</tbody>
</table>

18S ribosomal RNA (18S), aryl hydrocarbon receptor (AhR), cytochrome P450 aromatase (CYP19), cytochrome P450 1B1 (CYP1B1), fatty acid-binding protein 3 (FABP3), FSH receptor (FSHR), peroxisome proliferator-activated receptors alpha and gamma (PPARalpha and PPARgamma).
Protein isolation

Protein from cultured KGN cells was isolated using the AllPrep Kit (Qiagen) according to manufacturer’s manual. The protein concentration was determined by the Bio-Rad Protein Assay (Bio-Rad, Munich, Germany).

Statistical analyses

All experiments were performed as at least three independent experiments (N). The number of technical replicates (n) within one experimental run is indicated in the individual figure legend. For statistical analyses Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) and SigmaPlot 11.0 (Systat Software, Inc., Erkrath, Germany) was used. Data are presented as mean ± SEM. Comparisons of two groups were performed by an unpaired Student’s t-test and comparisons of multiple groups by one-way-analysis of variance followed by Tukey’s post test. With the exception of MEHP accumulation, all experimental data are presented in comparison with the solvent (DMSO)-treated control group. Differences between groups were considered as statistically significant at P value < 0.05.

Results

KGN cells metabolize DEHP to MEHP

The MEHP concentration after exposure with and without 50 μM DEHP was measured in culture media and KGN cell lysates at time points 0 and 5 h (Fig. 1A). At 0 h, MEHP was found in media with and without DEHP supplementation, with a significantly 3-fold higher level in the DEHP media. After 5 h, MEHP concentrations in the media with DEHP application were significantly elevated (by 3-fold to 0 h with DEHP, by 8-fold to 0 h and by 4-fold to 5 h without DEHP). Cell lysates of DEHP-exposed cells also revealed a significant increase of MEHP: After 5 h of DEHP exposure MEHP concentrations showed a 21-fold and 12-fold increase compared with non-exposed cells at 0 and 5 h, respectively. These data indicate that KGN cells have the ability to metabolize DEHP to MEHP.

TCDD and/or DEHP did not influence KGN cell proliferation

Cytotoxicity of TCDD (10 nM), DEHP (0.5, 5 and 50 μM) and the TCDD/DEHP mix (10 nM/0.5 μM, 10 nM/5 μM and 10 nM/50 μM) was tested. None of the concentrations used affected KGN cell proliferation (Fig. 1B). Selective AhR, PPARalpha and PPARgamma antagonists were investigated for their effects on cell proliferation. Compared with DMSO control, no significant differences were observed (10 μM; data not shown).

AhR and PPARs are active in KGN cells

The experiments employing PPAR agonists, i.e. bezafibrate as agonist for PPARalpha and rosiglitazone as agonist for PPARgamma, confirmed receptor activity and the presence of a functional PPAR signalling cascade in KGN cells. PPARalpha and PPARgamma were activated by agonist treatment (Fig. 1C). The PPAR target gene FABP3 mRNA was increased by 51% under bezafibrate and by 89% under rosiglitazone treatment. Bezaafibrate caused an induction of PPARalpha by 15%. The mRNA expression of AhR and its target gene CYP1B1 were not affected by bezafibrate and rosiglitazone treatment. TCDD did not influence the mRNA expression of AhR, PPARs and FABP3, whereas CYP1B1 expression was increased by 78%.

Figure 1

DEHP metabolism to MEHP, cell proliferation and receptor pathway activation in KGN granulosa cells. (A) Mono-(2-ethylhexyl) phthalate (MEHP) enrichment in KGN cells. Culture media and cell lysates were analysed for the di-(2-ethylhexyl) phthalate (DEHP) metabolite MEHP at time points 0 and 5 h culture with and without 50 μM DEHP (N = 3; n = 1 each; P ≤ 0.05 *relative to 0 h without DEHP, + to 0 h with DEHP and #relative to 5 h without DEHP). (B) KGN cell proliferation after single and combined exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (10 nM) and DEHP (0.5, 5, 50 μM) (N = 4; n = 6–8 each; P ≤ 0.05 *relative to dimethyl sulfoxide (DMSO) control). (C) Relative mRNA expression of alpha and gamma peroxisome proliferator-activated receptors (PPARalpha and PPARgamma), their target gene fatty acid-binding protein 3 (FABP3), aryl hydrocarbon receptor (AhR) and its target gene cytochrome P450 1B1 (CYP1B1) in KGN cells exposed to TCDD, bezafibrate and rosiglitazone (N = 3; n = 2 each; P ≤ 0.05 *relative to DMSO control).
Estradiol synthesis and CYP19 mRNA are reduced under TCDD and DEHP

The concentration of estradiol was measured in the supernatant collected after 24 h culture in medium containing FSH and androstenedione (Fig. 2). FSH stimulation itself led to an induction of estradiol synthesis by 46%. Additional exposure to TCDD and/or DEHP reduced FSH-stimulated estradiol synthesis (TCDD: by 20%, DEHP: by 19%, TCDD/DEHP mix: by 31%).

The mRNA expression of FSHR and CYP19 were determined in unstimulated and FSH-stimulated KGN cells. Basal mRNA levels for both genes were not significantly influenced by all exposure treatments (Fig. 3A). FSH stimulation did not significantly affect FSHR and CYP19 gene expression (Fig. 3B). Additional exposure to DEHP alone and together with TCDD reduced CYP19 mRNA expression by 43 and 41%, respectively. Single TCDD exposure caused no decrease. FSHR mRNA expression was significantly decreased by 43% by DEHP. TCDD and the TCDD/DEHP mix had no effect on FSHR gene expression (Fig. 3B).

Ahr expression is induced by DEHP

Single exposure with various DEHP concentrations induced the mRNA expression of Ahr (by 36% at 5 μM DEHP and by 40% at 50 μM DEHP), PPARalpha (by 35% at 5 μM DEHP and by 40% at 50 μM DEHP) and PPARgamma (by 43% at 0.5 μM DEHP and by 81% at 50 μM DEHP) (Fig. 4A and B). The TCDD/DEHP mix increased the mRNAs for CYP1B1 (by 256% at TCDD + 0.5 μM DEHP, by 218% at TCDD + 5 μM DEHP and by 140% at TCDD + 50 μM DEHP), PPARalpha (by 42% at TCDD + 0.5 μM DEHP and by 29% at TCDD + 50 μM DEHP) and PPARgamma (by 54% at TCDD + 0.5 μM DEHP (Fig. 4A and B).

To study the interaction of AhR and PPAR signalling, KGN cells were exposed to TCDD, DEHP or a mix of both in combination with selective receptor antagonists (Fig. 5A and B). The DEHP-induced increase in AhR mRNA (Fig. 4A) was maintained under simultaneous AhR antagonization (by 60%; ANF) (Fig. 5A). Under PPARalpha antagonization by MK 886, AhR expression was only slightly increased. Antagonizing PPARgamma by GW 9662 diminished the DEHP effect completely (Fig. 5A). The TCDD- and TCDD/DEHP-mediated increase of CYP1B1 mRNA (Fig. 1C and 4A) was no longer found under AhR antagonization. In the presence of both PPAR antagonists CYP1B1 mRNA was significantly increased by the TCDD/DEHP mix (by 154% with MK 886, by 307% with GW 9662) (Fig. 5A). Regarding receptor expression itself, the DEHP-mediated PPARalpha increase (Fig. 4B) was not altered by AhR antagonization (Fig. 5B). However, if both PPARs were antagonized,
the DEHP-induced PPARalpha and PPARgamma mRNA increase were diminished (Fig. 5B).

**Discussion**

KGN granulosa cells have active AhR and PPAR signalling pathways. Hierarchically, PPAR signalling governs AhR expression. This has far-reaching implications since (i) activation of each receptor pathway, PPARs and AhR and (ii) DEHP-induced signalling reduce estradiol production, i.e. the primary function of granulosa cells. Widespread EDCs, such as phthalates, may affect granulosa cell hormone synthesis by direct activation of the PPAR pathway and by indirect activation of AhR via PPARs.

Ovarian expression patterns of PPARs have been investigated in the rat previously: PPARalpha was mainly found in theca and stroma cells with no change during follicular development and menstrual cycle. In granulosa cells PPARgamma was the predominantly expressed isoform. After the LH surge PPARgamma transcription in developing follicles declined (Komar et al., 2001). PPARgamma stimulates estrogen receptor alpha ubiquitination for subsequent degradation (Qin et al., 2003). Both PPARs bind to estrogen response elements acting as competitive inhibitors (Keller et al., 1995) and affect estradiol synthesis itself (Mu et al., 2000; Yanase et al., 2001; Fan et al., 2005). As shown in murine knockout models, PPARgamma is critical for normal ovarian function (Cui et al., 2002). Data from AhR-deficient mice indicate the significance of the AhR for fertility, with disturbed follicle development and reduced ovulation rate (Benedict et al., 2000; Benedict et al., 2003) due to an altered estradiol regulation and responsiveness (Barnett et al., 2007a, b).

We show that the most common phthalate, DEHP, is metabolized to MEHP in human KGN granulosa cells. Application of 50 μM (= 18 μg/ml) DEHP significantly elevated the mean MEHP medium concentration from 8.7 to 25.5 ng/ml within 5 h of culture. KGN cell lysates contained a similar level with 22.0 ng MEHP/ml after 5 h DEHP exposure. It is important to note that culture medium and cell lysates which had no contact with DEHP were also contaminated by MEHP (1.0–6.2 ng/ml). The measured MEHP concentrations correlate closely with urinary MEHP levels of healthy pregnant women (Enke et al., 2013). A likely explanation for the MEHP contamination, besides the leakage from laboratory plastic products, is the serum supplement. As previously reported, commercially available media for IVF are contaminated with DEHP and MEHP from the protein sources (Takatori et al., 2012).
DEHP exposure. FSH stimulation induced estradiol synthesis (Fig. 2) and CYP19 aromatase independently from overriding signals. Basal mRNA expression of FSHR and CYP19 were investigated in the immortalized human granulosa cell line KGN et al. MEHP (Lovekamp-Swan et al. 2003; Gunnarsson et al., 2008; Ohno et al., 2009; Reinsberg et al., 2009; Gupta et al., 2010). Regarding cytotoxicity in KGN cells, we did not observe any influence for concentrations in medium between 0.5 and 50 μM DEHP after 24 h exposure. TCDD (10 nM) alone and in the mix with DEHP (0.5, 5 and 50 μM) also did not affect cell proliferation. In primary human granulosa lutein cells no effect on cell viability was observed for MEHP concentrations up to 167 μM (Reinsberg et al., 2009). Exposure of cultured mouse ovarian follicles to MEHP and DEHP did not affect antral follicle growth within 24 h. However, an exposure for 72 h to DEHP (10 and 100 μg/ml) or MEHP (1, 10, 100 μg/ml) suppressed antral follicle growth, accompanied by oxidative stress (Wang et al., 2012a, b), expression of proapoptotic factors and inhibition of cell cycle and antiapoptotic regulators (Wang et al., 2012b). In vivo studies using mice (Li et al., 2012) and rat (Xu et al., 2010) models exposed to DEHP demonstrated increased granulosa cell apoptosis. DNA fragmentation after 24 h exposure was observed for 3.1 μM TCDD but not for 3.1 nM TCDD (Heimler et al., 1998). If these data are critically assessed then it becomes obvious that the DEHP-mediated decrease of estradiol synthesis may be the downregulation of the FSHR and CYP19 aromatase expression (Fig. 5). Additional TCDD and/or DEHP exposure decreased estradiol synthesis, with the strongest effect exerted by the mix. In primary human granulosa lutein cells a TCDD-mediated estradiol reduction has been already described (Heimler et al., 1998). The mechanism underlying the DEHP-mediated decrease of estradiol synthesis may be the downregulation of the FSHR and CYP19 aromatase expression (Pocar et al., 2012). MEHP exposure revealed a time- and concentration-dependent CYP19 aromatase inhibition with a subsequent decrease in estradiol synthesis, which was independent of the FSH-cAMP pathway (Davis et al., 1994; Reinsberg et al., 2009). The CYP19 aromatase suppression was found to be conducted via PPARalpha and PPARgamma (Lovekamp-Swan et al., 2003) through an inhibition of the transcriptional activation of the CYP19 aromatase promoter II (Ohno et al., 2009).

Under TCDD exposure the expected CYP1B1 up-regulation with no change in AhR expression (Horling et al., 2011) was confirmed in the current study. The PPAR antagonists rosiglitazone and bezafibrate had no effect on AhR and CYP1B1 mRNA expression but induced the PPAR target gene FABP3 as a proof for an active PPAR cascade. In contrast, DEHP significantly increased AhR expression. A possible explanation for this unexpected finding comes from the concept of ‘selective PPAR-gamma modulators’, implying activation of a ligand-specific subset of target genes (Gelman et al., 2007). PPARs and PPAR target gene expression was not influenced by exposure to the AhR agonist TCDD. Also, a study using PPRE-driven lucerase activation in Huh7 cells did not observe any TCDD-mediated effect (Wang et al., 2010). DEHP alone and in the mix with TCDD increased the transcription of both PPARs. This shows that TCDD did not alter the DEHP-mediated PPAR up-regulation. In contrast, DEHP increased AhR mRNA expression only if given alone. The TCDD/DEHP mix did not increase AhR mRNA expression compared with TCDD alone. We do not know
why the effects of the mixture differed between PPAR and AhR mRNA expression but this underpins one major finding of the current study: effects of EDC mixtures clearly differ from effects induced by a single EDC.

As the current data show, granulosa cell function is disturbed by TCDD and DEHP action leading to a reduced estradiol synthesis. Furthermore, a DEHP-induced AhR mRNA expression was found. AhR and PPAR signalling not only diminish estradiol levels by disrupting synthesis but also increase estradiol metabolism. AhR activates CYP1A1 and CYP1B1 (Horling et al., 2011), which metabolize estradiol to several hydroxylation products (Lee et al., 2003). In this context it is important to note that PPAR signalling can directly, without AhR signalling, activate CYP1A1 (Séreeé et al., 2004).

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Authors’ roles
J.E.: acquisition, analysis and interpretation of data, conception and design of the study, drafting the article and final approval. J.C.J.: acquisition, analysis and interpretation of data, conception and design of the study. R.B.: acquisition, analysis and interpretation of data, revising the article and final approval. H.M.K.: acquisition of data, revising the article and final approval. B.F.: project leader, conception and design of the study, drafting the article and final approval. J.C.J.: acquisition of data, revising the article and final approval. J.C.J.: acquisition of data, revising the article and final approval.

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Conflict of interest
None declared.

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