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Tributyltin and triphenyltin induce 11 β -hydroxysteroid dehydrogenase 2 expression and activity through activation of retinoid X receptor α

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Graphical anstract

Highlights

- TBT and TPT induce the transcription of 11 β -HSD2 in JEG-3 cells
- Upregulation of 11 β -HSD2 results in reduced cortisol-dependent activation of the GR
- RXR α is involved in the TBT and TPT induced effect on 11 β -HSD2
- Fludioxonil and chlorotriphenylsilane activate RXR α and induce 11 β -HSD2 expression

Abstract

Exposure to the environmental pollutants organotins is of toxicological concern for the marine ecosystem and sensitive human populations, including pregnant women and their unborn children. Using a placenta cell model, we investigated whether organotins at nanomolar concentrations affect the expression and activity of 11 β -hydroxysteroid dehydrogenase type 2 (11 β -HSD2). 11 β -HSD2 represents a placental barrier controlling access of maternal glucocorticoids to the fetus. The organotins tributyltin (TBT) and triphenyltin (TPT) induced 11 β -HSD2 expression and activity in JEG-3 placenta cells, an effect confirmed at the mRNA level in primary human trophoblast cells. Inhibition/knock-down of retinoid X receptor alpha (RXR α) in JEG-3 cells reduced the effect of organotins on 11 β -HSD2 activity, mRNA and protein levels, revealing involvement of RXR α . Experiments using RNA and protein synthesis inhibitors indicated that the effect of organotins on 11 β -HSD2 expression was direct and caused by increased transcription. Induction of placental 11 β -HSD2 activity by TBT, TPT and other endocrine disrupting chemicals acting as RXR α agonists may affect placental barrier function by altering the expression of glucocorticoid-dependent genes and resulting in decreased availability of active glucocorticoids for the fetus, disturbing development and increasing the risk for metabolic and cardiovascular complications in later life.

Abbreviations:

11 β -HSD2	11 β -hydroxysteroid dehydrogenase type 2
Act D	Actinomycin D
Bex	Bexarotene
CAPE	Caffeic acid phenethyl ester
CHX	Cycloheximide
GA	Glycyrrhetic acid
GR	Glucocorticoid receptor
MAPK	Mitogen-activated protein kinase
MR	Mineralocorticoid receptor
PPAR γ	Peroxisome proliferation activated receptor γ

PPIA	Peptidylprolyl isomerase A
Rosi	Rosiglitazone
RXR α	Retinoid X receptor alpha
TBT	Tributyltin
TPSCI	Chlorotriphenylsilane
TPT	Triphenyltin

Keywords: Organotin; 11 β -hydroxysteroid dehydrogenase; retinoid X receptor alpha; placenta; glucocorticoid

1. Introduction

Organotins, including tributyltin (TBT) and triphenyltin (TPT), are metalorganic chemicals widely used in industry as disinfectants for water, preservative of paper and textiles, in the production of agricultural pesticides, as well as in antifouling paint and as stabilizers in plastic production (Antizar-Ladislao, 2008; Boyer, 1989; Fent, 1996). These substances contaminate the marine and freshwater ecosystem and raised toxicological concerns. Despite the ban of organotin-based antifouling paint in marine industry in 2008 in many countries, organotins are still detected in harbors and at coasts of several places (Artifon et al., 2016; Batista-Andrade et al., 2018; Gao et al., 2019; Laranjeiro et al., 2018; Paz-Villarraga et al., 2015; van Gesselten et al., 2018). Additionally, organotins were also detected in soil and dust samples (Kannan et al., 2010). This is caused on one hand by the high environmental persistence of organotins due to their hydrophobic properties, half-life (6 weeks - 5 months in fresh water) and tendency to accumulate in sediments, and on the other hand by new contaminations from countries where organotin-based antifouling paint is still allowed or illegally used (Antizar-Ladislao, 2008; Laranjeiro et al., 2018). A representative Danish study detected organotins in samples of biological tissue from invertebrates, fishes, birds and mammals, indicating biomagnification and accumulation in the food chain (Strand and Jacobsen, 2005). Moreover, recently organotin-contaminated oysters were detected in China (Chen et al., 2019). Importantly, TBT was detected in humans in placenta (Rantakokko et al., 2014) and blood,

with mean circulating levels of 4.6 ng/mL and up to 85 ng/mL (Kannan et al., 1999), emphasizing exposure to organotins.

Organotins were found to increase the incidence of imposex in female snails, shell deformities in oysters and impaired reproduction in both species (Alzieu, 1991; Fent, 1996; Smith, 1981). Several *in vitro* and *in vivo* studies proposed that acute exposure to organotins can cause neural (Jenkins et al., 2004), hepatic (Wiebkin et al., 1982), renal (Coutinho et al., 2016), immune (Boyer, 1989; Whalen et al., 1999) and developmental toxicity (Adeeko et al., 2003) in higher vertebrates. Previous studies indicated that exposure to TBT and DBT can result in enhanced inflammation with increased release of the pro-inflammatory marker tumor necrosis factor- α (TNF- α) and induction of the NF- κ B pathway (Chantong et al., 2014; Hurt et al., 2013; Marinovich et al., 1996). Importantly, increased TNF- α levels and NF- κ B activation were shown to diminish the expression and activity of 11 β -hydroxysteroid dehydrogenase type 2 (11 β -HSD2) (Heiniger et al., 2001; Kostadinova et al., 2005). This enzyme is tissue-specifically expressed and converts potent 11 β -hydroxyglucocorticoids (cortisol in human, corticosterone in rodents) to inactive 11-ketoglucocorticoids (cortisone in human, 11-dehydrocorticosterone in rodents) (Odermatt and Kratschmar, 2012). In the kidney, it essentially protects the mineralocorticoid receptor (MR) from undesired activation by cortisol, allowing the less abundant mineralocorticoid aldosterone to activate this receptor (Edwards et al., 1988; Funder et al., 1988; Odermatt and Kratschmar, 2012). By restricting the cortisol-mediated activation of MR, and in some cell types of glucocorticoid receptors (GR), 11 β -HSD2 has an important role in the regulation of electrolyte homeostasis, angiogenesis and vascular function (Hadoke et al., 2001; Molnar et al., 2008; Rae et al., 2009). Loss-of-function mutations in the gene encoding 11 β -HSD2 (Mune et al., 1995; White et al., 1997; Wilson et al., 1995) or its inhibition by the liquorice constituent glycyrrhetic acid (GA) (Ferrari, 2010) can cause apparent mineralocorticoid excess with elevated blood pressure and low blood potassium levels by undesired cortisol-dependent MR activation.

11 β -HSD2 is also highly expressed during pregnancy in trophoblast cells of the placenta (Albiston et al., 1994; Stewart et al., 1995), forming a barrier to protect the fetus from the high maternal cortisol levels. Impaired 11 β -HSD2 activity, due to genetic defects or inhibition during pregnancy results in an increased exposure of the fetus to glucocorticoids and is associated with intrauterine growth restriction

and metabolic complications in later life of the offspring (Edwards et al., 1993; Lindsay et al., 1996; Odermatt, 2004; Seckl et al., 2000). Nevertheless, in late gestation 11 β -HSD2 enzyme levels drop and therefore higher levels of maternal glucocorticoids reach the fetus (Giannopoulos et al., 1982; Murphy and Clifton, 2003). The exposure to appropriate cortisol levels is pivotal for fetal organ development (Moisiadis and Matthews, 2014a, b); insufficient exposure leads to complications due to retarded organ maturation (Carson et al., 2016). Therefore, in preterm birth, where endogenous glucocorticoid levels are too low to allow proper maturation of fetal organs, synthetic glucocorticoids are administered to prevent complications (Carson et al., 2016).

Regarding TBT and TPT, the activation of retinoid X receptor alpha (RXR α) and peroxisome proliferation activated receptor γ (PPAR γ) have been identified as molecular initiating events of at least some of their toxicities (Grun and Blumberg, 2006; Grun et al., 2006; Kanayama et al., 2005; Nakanishi et al., 2005). However, the downstream pathways, *i.e.* key events, ultimately leading to the different adverse outcomes are still insufficiently understood. Previous investigations suggested interference of these organotins with glucocorticoid metabolism by a direct inhibition of 11 β -HSD2 (Atanasov et al., 2005) through a mechanism involving a cysteine residue in the substrate binding pocket (Meyer et al., 2012). However, these experiments were performed at low micromolar concentrations, unlikely to be reached in exposure scenarios. Using human placental JEG-3 cells the present study assessed whether TBT and TPT, at environmentally relevant nanomolar concentrations detected in human blood (Kannan et al., 1999), can affect peripheral glucocorticoid concentrations by altering the transcriptional expression and activity of 11 β -HSD2. Furthermore, the pathway underlying the TBT- and TPT-mediated effect on 11 β -HSD2 was elucidated. Finally, it was tested whether other environmental chemicals may act through the same pathway and affect 11 β -HSD2 function.

2. Material and Methods

2.1 Cell culture

JEG-3 human placenta choriocarcinoma cells, purchased from ATCC (Manassas, VA, USA), were cultured in Eagle's Minimal Essential medium (EMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and streptomycin, 1 mM sodium pyruvate and 1% MEM non-essential amino acids.

2.2 Chemicals and reagents

Tributyltin chloride (TBT), triphenyltin chloride (TPT), Bexarotene (Bex), LG754, H-89, mithramycin A, GA, cortisol, cortisone and chlorotriphenylsilane (TPSCl) were purchased from Sigma-Aldrich (Buchs, Switzerland). Actinomycin D (Act D), cycloheximide (CHX), rosiglitazone (Rosi), GW9662, CAY-10512, SB202190, fludioxonil and caffeic acid phenethyl ester (CAPE) were obtained from Cayman Chemicals (Ann Arbor, MI, USA) (CAS no. and purity of the chemicals are listed in Supplemental Table S1). [1,2,6,7-³H]-cortisol was from Perkin-Elmer (Boston, MA, USA) and cell culture medium was obtained from Sigma-Aldrich. OPM (OPM-3270, (3 β ,18 β ,20 β)-3-acetoxy-N-methyl-N-hydroxy-11-oxoolean-12-en-29-amide) was synthesized as described earlier ((Stanetty et al., 2010), compound 15).

2.3 RNA isolation and analysis

JEG-3 cells were seeded in 6-well plates (450,000 cells per well) and treated with compounds or vehicle for 24 h. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands), followed by reverse transcription with the GoScript reverse transcriptase (Promega Corporation, Madison, WI, USA) according to the manufacturer. Quantitative PCR (qPCR) was performed using Kapa SYBR® Fast qPCR Master Mix (KAPA Biosystems, Wilmington, MA, USA) with 4 ng of cDNA in 10 μ L total reaction mix containing specific oligonucleotide primers. The following primers were used: *PPIA* forward 5'-CATCTGCACTGCCAAGACTGA-3', reverse 5'-TGCAATCCAGCTAGGCATG-3'; *HSD11B2* forward 5'-AAACCAGGAGACATTAGCCG-3', reverse 5'-TCCATGCAGCTACGGAAAG-3'; *RXR α* forward 5'-AGGACTGCCTGATTGACAAG-3', reverse 5'-GACTCCACCTCATTCTCGTTC-3'. qPCR reactions comprising 40 cycles of 95°C for 10 s, 60°C for 15 s, followed by a final extension at 72°C for 20 s were performed in a Rotor-Gene Cycler (Corbett Research, Sydney, New South Wales, Australia). Data were normalized to the endogenous control peptidylprolyl isomerase A (*PPIA*) and compared to vehicle control utilizing the 2^{- $\Delta\Delta$ CT} method (Schmittgen and Livak, 2008). CT values were determined from at least three independent experiments each measured in triplicate.

2.4 Protein expression analysis

JEG-3 cells (450,000) seeded in 6-well plates were lysed in RIPA buffer (Sigma-Aldrich) supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland) and centrifuged at $16,000 \times g$ for 15 min at 4°C. The supernatants were subjected to protein concentration measurements using the Pierce® BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Samples were diluted in Laemmli sample buffer (60 mM Tris-HCl, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 0.01% bromophenol blue, pH 6.8) supplemented with 5% β -mercaptoethanol and incubated at 99°C for 3 min. Proteins (15 μ g) were separated by SDS-PAGE on a 10% acrylamide gel and transferred to a Immobilon®-P (PVDF) membrane (Merck Millipore, Burlington, MA, USA). The membrane was blocked for 1 h in 5% defatted milk solution in Tris-buffered saline (20 mM Tris buffer, pH 7.6, 150 mM NaCl) containing 0.1% Tween-20 (TBS-T) and incubated over night at 4°C with the mouse monoclonal antibody against 11 β -HSD2 (C-9, Santa Cruz Biotechnology, Dallas, TX, USA) at a dilution of 1:1,000. The membrane was washed with TBS-T, incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (Sigma-Aldrich) diluted 1:2,000 in blocking solution for 1 h and washed with TBS-T. To detect the loading control calnexin, membranes were blocked for 1 h and incubated with rabbit antibody against calnexin (SAB4503258, Sigma-Aldrich) diluted 1:4,000 at 4°C overnight, washed with TBS-T, incubated with HRP-conjugated goat anti-rabbit secondary antibody 7074 (Cell Signaling, Danvers, MA, USA) diluted 1:5,000 for 1 h and washed with TBS-T. Protein bands were detected with Fujifilm ImageQuant™ LAS-4000 (GE Healthcare, Glattbrugg, Switzerland) utilizing the Immobilon® Western Chemiluminescent HRP substrate kit (Millipore Corporation, Billerica, MA, USA). The bands were analyzed by densitometry using ImageJ software and signals normalized to that of the house keeping control calnexin.

2.5 11 β -HSD2 activity assay

JEG-3 cells were seeded (50,000 cells/well in 100 μ L) into 96-well plates and treated for 24 h. Subsequently, the medium was replaced by 50 μ L charcoal-treated serum-free medium and cells were incubated for 4 h with 50 nM cortisol (containing 10 nCi of [1,2,6,7-³H]-cortisol). Reactions were

stopped by adding an excess cortisol and cortisone (1:1, 2 mM each in methanol). Supernatant (20 μ L) was loaded on TLC plates (Macherey-Nagel, Oensingen, Switzerland) and glucocorticoids were separated using chloroform and methanol (9:1). Liquid scintillation counting was performed to determine 11 β -HSD2 dehydrogenase activity and compared to control samples.

2.6 RXR α knock-down

JEG-3 cells (1.3×10^6) were seeded in 10 cm dishes and transfected with 600 pM siRNA against RXR α (5'-AGGACUGCCUGAUUGACAATT-3') (He et al., 2014) or scrambled control siRNA (5'-UGGUUUACAUGUUUUCUGA-3') using 24 μ L of Lipofectamine RNAiMax (Thermo Fisher Scientific). Medium was replaced after 6 h. At 72 h cells were seeded in 96-well plates (50,000 cells/well) or 12-well plates (200,000 cells/well) and treated with TBT, TPT, Bex (50 nM) or vehicle control for another 24 h. Cells on 96-well plates were used for 11 β -HSD2 activity measurements and cells on 12-well plates for mRNA and protein analyses. Antibody against RXR α (H-10, Santa Cruz Biotechnology) at a dilution of 1:2,000 and a HRP-conjugated goat anti-mouse secondary antibody (A0168, Sigma-Aldrich) diluted 1:2,000 were used to detect RXR α protein expression.

2.7 Transfection and transactivation assay

Cells were transfected with a plasmid for human GR, a TAT3-TATA luciferase reporter construct and a pCMV-LacZ β -galactosidase transfection control by electroporation according to the manufacturer (NeonTM transfection system, Invitrogen, Carlsbad, CA, USA). Briefly, cells were resuspended in buffer R at a concentration of 1×10^7 cells/mL and supplemented with 30 μ g plasmid/mL buffer R. Cells were then exposed to one pulse (1,250 volt, 30 ms) utilizing a 100 μ L gold tip, followed by transfer to 24 well plates (150,000 cells and 450 ng plasmid/well) in antibiotic-free cultivation medium and treatment with TBT, TPT or Bex (50 nM). After 24 h the medium was replaced by charcoal-treated medium for 0.5 h, followed by incubation with TBT, TPT, Bex, and 5 nM cortisol and/or 5 μ M GA for another 24 h. Cells were lysed with 60 μ L of Tropix lysis solution and luciferase activity was measured according to the instructions of the Tropix kit (Applied Biosystems, Foster City, CA, USA). Samples were analyzed with a SpectraMax-L luminometer (Molecular Devices, San Jose, CA, USA). Luciferase

activity was normalized to the galactosidase control and then represented relative to cortisol treated cells.

2.8 Analysis of *de novo* protein synthesis involvement

To test whether protein *de novo* synthesis is needed for the effect of organotins on 11 β -HSD2, JEG-3 cells were incubated with compounds and the *de novo* protein synthesis inhibitor cycloheximide (CHX) (5 μ g/mL) for 8 h. Afterwards total RNA was isolated and 11 β -HSD2 mRNA analyzed by RT-qPCR and normalized to control.

2.9 Analysis of contribution of gene transcription and 11 β -HSD2 mRNA stability

To investigate whether upregulation of 11 β -HSD2 relies on enhanced transcription, cells were exposed to 50 nM TBT, TPT or Bex with or without the transcriptional inhibitor Act D (10 μ g/mL) for 8 h. Total RNA was extracted, measured by RT-qPCR and normalized to control.

To assess a potential effect of organotins on 11 β -HSD2 mRNA stability, JEG-3 cells were treated with 50 nM of the compounds or vehicle for 17 h, followed by addition of the mRNA synthesis inhibitor Act D (Act D) (10 μ g/mL) and incubation for 0 h, 4 h or 8 h prior to isolation of total RNA and determination of 11 β -HSD2 mRNA by RT-qPCR. Each data point is expressed as the percentage of the 11 β -HSD2 expression at time point 0 h.

2.10 Statistical analysis

Data were analyzed using Student's t-test and one-way ANOVA with Dunett's or Bonferroni's post hoc tests in the GraphPad Prism 5 software. Values represent mean $\pm$ SD.

3. Results

3.1 Increased 11 β -HSD2 expression upon treatment with TBT or TPT

To explore the impact of organotins on mRNA and protein expression of 11 β -HSD2, JEG-3 placenta cells were exposed to 50 nM of TBT or TPT. No cytotoxicity was observed at any of the treatment conditions used in this study (data not shown). Incubation of JEG-3 cells with 50 nM of TBT or TPT

resulted in a time-dependent induction of 11 β -HSD2 mRNA expression starting after 4 h of exposure (Supplemental Fig. S1). Following 24 h of incubation, a four-fold upregulation of 11 β -HSD2 mRNA expression compared to vehicle treated cells was detected (Fig. 1A).

The increased 11 β -HSD2 expression in JEG-3 cells after incubation with 50 nM TBT or TPT for 24 h was then confirmed at the protein level. An approximately 2.5-fold enhanced 11 β -HSD2 protein expression compared to vehicle-exposed cells was seen in all treatment conditions (Fig. 1B).

Fig. 1. Induction of 11 β -HSD2 expression and activity by TBT and TPT. JEG-3 cells were treated for 24 h with compounds as indicated. **A)** 11 β -HSD2 mRNA expression in treated cells is expressed relative to that in vehicle treated cells and normalized to PPIA mRNA (mean $\pm$ SD; n = 3). **B)** A representative Western blot image is shown (right panel) in addition to the relative protein levels determined by densitometry analysis of the bands after normalization to calnexin loading control (left panel) (mean $\pm$ SD; n = 4). **C)** Following preincubation with organotins, the conversion of radiolabeled cortisol to cortisone was determined after incubation for 4 h. Data represent mean $\pm$ SD relative to vehicle treated cells from two independent experiments performed in duplicate. One-way ANOVA with Dunnett's post hoc test, p values: * <0.05 , ** <0.01 , *** <0.001 , ns (not significant).

3.2 Upregulation of 11 β -HSD2 activity by TBT and TPT in JEG-3 cells

Next, the effect of TBT and TPT on 11 β -HSD2 enzyme activity was assessed. In line with our findings on the mRNA and protein expression level, increased conversion of cortisol to cortisone was observed after incubation of JEG-3 cells with TBT or TPT. A concentration-dependent increase was detected, from 6.25 nM to 100 nM and from 12.5 nM to 100 nM for TBT and TPT, respectively (Fig. 1C). Simultaneous treatment of the cells with 50 nM TBT or TPT and either 5 μ M of the unspecific 11 β -HSD inhibitor GA or 2.5 μ M of the specific 11 β -HSD2 inhibitor OPM almost completely abolished 11 β -HSD2 activity (Supplemental Fig. S2), demonstrating that the observed effect of TBT and TPT on cortisol oxidation was 11 β -HSD2 specific. These results revealed that the organotins TBT and TPT at nanomolar concentrations induced 11 β -HSD2 expression and activity. This contrasts our initial expectation and previous finding that organotins at low micromolar concentrations decrease 11 β -HSD2 activity by direct inhibition (Atanasov et al., 2005).

3.3 TBT and TPT mediate upregulation of 11 β -HSD2 through RXR α

To investigate the mechanism underlying the organotin-dependent upregulation of 11 β -HSD2, JEG-3 cells were co-incubated with subcytotoxic concentrations of organotins and specific inhibitors of pathways associated with the regulation of 11 β -HSD2. The 11 β -HSD2-dependent conversion of cortisol to cortisone served as main read-out (Fig. 2 and Supplemental Fig. S3 and S4). Since exposure to organotins might increase TNF- α synthesis and lead to activation of NF- κ B (Chantong et al., 2014; Lawrence et al., 2018), a possible role of the NF- κ B pathway in the organotin-dependent modulation of 11 β -HSD2 activity was tested. However, this pathway seems not to be involved because the NF- κ B inhibitors CAPE (1 μ M) and CAY 10512 (1 μ M) did not affect the TBT- or TPT-dependent induction of 11 β -HSD2 activity (Supplemental Fig. S3).

The RXR α homodimer antagonist LG754 (Hida et al., 2001) concentration-dependently (100 nM to 10 μ M) reduced the effect of both TBT and the RXR α agonist bexarotene (Bex) on 11 β -HSD2 activity (Fig. 2A). The effect of Bex on 11 β -HSD2 expression in JEG-3 cells was also confirmed at the mRNA and protein expression level and Bex showed a concentration-dependent induction of 11 β -HSD2 activity (Supplemental Fig. S4). Furthermore, an approximately 1.5-fold increase of 11 β -HSD2 mRNA

expression levels was detected in primary human cytotrophoblast cells after treatment for 24 h with either TBT or Bex at a concentration of 50 nM (Supplemental Fig. S5), indicating an involvement of RXR α in the organotin-mediated regulation of 11 β -HSD2. In contrast to TBT, in TPT exposed cells co-treatment with LG754 showed only a trend to lower 11 β -HSD2 activity (Fig. 2A).

To investigate a possible involvement of PPAR γ , cells were co-treated with TBT, TPT or rosiglitazone (a PPAR γ agonist) and the PPAR γ antagonist GW9662 (10 μ M). GW9662 did not affect the TBT- and TPT-induced 11 β -HSD2 activity (Fig. 2B), whereas the increased 11 β -HSD2 activity upon treatment with the PPAR γ agonist rosiglitazone was reversed by GW9662 as expected. These results suggest that PPAR γ is not involved in the organotin-mediated upregulation of 11 β -HSD2.

Besides, the specific p38 mitogen-activated protein kinase (MAPK) inhibitor SB202190 (2.5 μ M) was employed to assess a possible involvement of this kinase. A trend decrease of 11 β -HSD2 activity was observed for TBT and a significant decrease was detected for TPT (Supplemental Fig. S6). Higher concentrations of this inhibitor were not analyzed due to cytotoxic effects (at 5 μ M and higher).

Together, these experiments indicate that TBT, like Bex, acts through RXR α homodimers to upregulate 11 β -HSD2 activity; for TPT the mechanism of 11 β -HSD2 upregulation likely follows an alternative pathway, potentially including p38 MAPK.

Fig. 2. Effect of antagonists of RXR α and PPAR γ on organotin-induced 11 β -HSD2 activity. A) JEG-3 cells were treated for 24 h with 100 nM TBT, TPT or Bex in the absence or presence of LG754 (100 nM, 1 μ M, 10 μ M) as indicated. Thereafter, the conversion of radiolabeled cortisol to cortisone was determined after 4 h of incubation. Data, relative to vehicle treated cells, represent mean $\pm$ SD from three independent experiments each performed in triplicate. One-way ANOVA with Bonferroni's post hoc test; p values: * <0.05 , ** <0.01 , *** <0.001 . **B)** JEG-3 cells were incubated with 50 nM TBT or TPT or 10 μ M rosiglitazone with or without 10 μ M GW9662 for 24 h. Cortisone formation was determined after exposure to radiolabeled cortisol for 4 h. Data were normalized to vehicle treated cells and represent mean $\pm$ SD from three independent experiments each performed in triplicate. One-way ANOVA with Bonferroni's post hoc test; p value: * <0.05 , ns (not significant).

3.4 Effect of RXR α knockdown on the organotin-induced 11 β -HSD2 expression and activity

The requirement of RXR α for the upregulation of 11 β -HSD2 upon exposure to TBT, TPT and Bex was further assessed utilizing siRNA against RXR α (Fig. 3). Measurements of mRNA levels revealed that

RXR α knockdown (KD) completely abolished the induction of 11 β -HSD2 mRNA expression by TBT, TPT and Bex. The scrambled control (scr) cells, as expected, showed an increase of 11 β -HSD2 mRNA expression following incubation with TBT, TPT or Bex (Fig. 3A). TBT, TPT and Bex treatment did not affect RXR α mRNA expression levels (Fig. 3B). Similar results were obtained on the protein level. Western blot analysis revealed elevated 11 β -HSD2 protein upon TBT, TPT or Bex treatment in scr control cells but an abolished effect upon RXR α KD (Fig. 3C). The treatment did not affect RXR α protein levels (Fig. 3D). Importantly, the TBT-, TPT- and Bex-mediated elevation of 11 β -HSD2 activity was abolished in RXR α KD cells, in line with the effects seen on mRNA and protein expression (Fig. 3E). These experiments demonstrate the dependence of the organotin- and Bex-induced upregulation of 11 β -HSD2 on RXR α in JEG-3 cells.

Fig. 3 RXR α knockdown abolishes the organotin-induced expression and activity of 11 β -HSD2.

After 72 h of RXR α knockdown (KD) JEG-3 cells were treated with 50 nM TBT, TPT or Bex for 24 h.

A) 11 β -HSD2 mRNA and **B)** RXR α mRNA were measured by RT-qPCR, normalized to the housekeeping mRNA of PPIA and data are expressed relative to the 11 β -HSD2 mRNA of vehicle treated

cells of the scrambled siRNA (scr) control (mean $\pm$ SD; n = 3). **C)** Expression of 11 β -HSD2 protein levels and **D)** RXR α protein levels. A representative Western blot image is shown in addition to the relative protein levels (normalized to DMSO from scr control) measured by densitometry analysis of the bands after normalization to calnexin loading control levels (mean $\pm$ SD; n = 4). **E)** 11 β -HSD2 activity was determined by measuring the conversion of radiolabeled cortisol to cortisone after incubation for 4 h. Data are presented relative to vehicle treated cells of the scr control (mean $\pm$ SD; n = 4). One-way ANOVA with Dunett's post hoc test, p values: * <0.05 , ** <0.01 , *** <0.001 .

3.5 Reduced GR transactivation in JEG-3 cells treated with TBT and TPT due to elevated 11 β -HSD2 activity

To assess whether the organotin-mediated upregulation of 11 β -HSD2 resulted in a reduced cortisol-dependent GR activation, JEG-3 cells were transfected with plasmids for GR- α (JEG-3 cells express very low endogenous GR- α levels) and a glucocorticoid-responsive luciferase reporter (TAT3-TATA-luc). Treatment of transfected JEG-3 cells with 5 nM cortisol led to a GR-dependent increase in luciferase activity that was further enhanced by co-treatment with 5 μ M GA to inhibit the inactivation of cortisol by 11 β -HSD2 (Fig. 4). Importantly, a significantly decreased cortisol-dependent GR transactivation was detected when cells were preincubated with 50 nM TBT or TPT compared to vehicle pretreated cells. For cells preincubated with 50 nM Bex a trend towards reduced GR transactivation was observed. Inhibition of 11 β -HSD2 activity by GA in cells pretreated with organotins or Bex revealed that TBT, TPT and Bex did not affect GR activity directly but via increased 11 β -HSD2 expression and activity. The GR-dependent luciferase activity in organotin and Bex treated cells reached similar levels than in untreated cells after inhibition by GA.

These results indicate that the increased 11 β -HSD2 activity induced by organotins led to decreased cortisol-dependent GR transactivation in JEG-3 placenta cells.

Fig. 4 Effect of organotins on GR transactivation. JEG-3 cells were transiently transfected with plasmids coding for human GR- α , TAT3-TATA-luciferase reporter and a β -galactosidase transfection control, followed by exposure for 24 h to 50 nM TBT, TPT or Bex, and treatment for another 24 h with cortisol (5 nM), with or without GA (5 μ M). Luciferase activity was normalized to the transfection control and is presented relative to cortisol treated cells. Data show mean $\pm$ SD from five independent experiments each performed at least in duplicates. One-way ANOVA with Dunett's post hoc test, p values: * <0.05 , ** <0.01 , *** <0.001 , ns (not significant).

3.6 Induction of 11 β -HSD2 mRNA expression by TBT and TPT does not require *de novo* protein synthesis

To test whether *de novo* protein synthesis is required for the organotin-induced increase of 11 β -HSD2 mRNA expression, JEG-3 cells were incubated for 8 h with vehicle or 50 nM TBT, TPT or Bex in the absence or presence of 5 μ g/mL translation inhibitor CHX. Protein synthesis inhibition by CHX did not prevent the TBT-, TPT- and Bex-mediated induction of 11 β -HSD2 mRNA levels but instead further potentiated their effect about three-fold (Fig. 5A). This suggests that the organotin-dependent induction of 11 β -HSD2 mRNA does not require the synthesis of an intermediate transcription factor but that the synthesis of a repressor of 11 β -HSD2 transcription might be blocked. Alternatively, CHX might prevent the synthesis of a protein lowering 11 β -HSD2 mRNA stability. The efficacy of 5 μ g/mL CHX in blocking protein synthesis in JEG-3 cells was tested by assessment of 50 nM Bex-induced 11 β -HSD2 protein levels and was found to totally abolish the *de novo* 11 β -HSD2 protein synthesis (data not shown). Additionally, this concentration of CHX was shown earlier to significantly inhibit protein

synthesis in JEG-3 cells and was utilized to study the effect of angiotensin II on 11 β -HSD2 expression under conditions of translational inhibition (Lanz et al., 2003; Yue and Brodie, 1997).

Fig. 5 Analysis of effect of RNA and protein synthesis inhibitors and mRNA stability. **A)** Effect of CHX on the organotin-induced increase in 11 β -HSD2 mRNA. JEG-3 cells were incubated for 8 h with medium containing DMSO vehicle or medium supplemented with 50 nM TBT, TPT or Bex with or without CHX at a concentration of 5 μ g/mL. Subsequently, total RNA was isolated and 11 β -HSD2 mRNA relative to control was analyzed (mean $\pm$ SD; n = 3). **B)** Effect of Act D on the organotin induced increase in 11 β -HSD2 mRNA. JEG-3 cells were incubated with medium containing vehicle or supplemented with 50 nM TBT, TPT or Bex with or without Act D at a concentration of 10 μ g/mL for 8 h. Thereafter, total RNA was isolated and 11 β -HSD2 mRNA relative to control was analyzed (mean $\pm$ SD; n = 4). Data was analyzed by One-way ANOVA with Dunett's post hoc test, p values: **<0.01, ***<0.001, ns (not significant). **C)** Effect of organotins on 11 β -HSD2 mRNA stability. JEG-3 cells were incubated for 17 h with compounds as indicated, followed by the addition of 10 μ g/mL of Act D. Total

cellular RNA was isolated at time points indicated and 11 β -HSD2 mRNA was measured, normalized to PPIA mRNA and shown in percentage of the steady state 11 β -HSD2 mRNA level at time point 0 h. 11 β -HSD2 mRNA half-life was determined by linear regression (control $R^2 = 0.820$; TBT $R^2 = 0.952$; TPT $R^2 = 0.882$; Bex $R^2 = 0.941$). Data represents mean $\pm$ SD of three independent experiments.

3.7 TBT and TPT increase 11 β -HSD2 mRNA expression by increased gene transcription

To study whether upregulation of 11 β -HSD2 mRNA results from enhanced gene transcription, cells were simultaneously incubated with 50 nM organotins or Bex and the transcriptional inhibitor Act D. Inhibition of transcription significantly prevented the increase of 11 β -HSD2 mRNA upon exposure to TBT, TPT and Bex compared to control (Fig. 5B). These findings indicate that TBT, TPT and Bex enhance 11 β -HSD2 gene transcription.

3.8 TBT and TPT alter the half-life of 11 β -HSD2 mRNA

To investigate whether TBT, TPT or Bex alter the half-life of 11 β -HSD2 mRNA, JEG-3 cells were incubated for 17 h with vehicle or 50 nM of the compounds, followed by adding the transcription inhibitor Act D (10 μ g/mL) and determining the expression and decay of 11 β -HSD2 mRNA. The half-life of 11 β -HSD2 mRNA was significantly reduced from approximately 7.5 h in vehicle treated cells to 5.0 h and 4.9 h for TBT and TPT exposed cells, respectively (Fig. 5C; $P < 0.05$). The data suggest that the higher mRNA expression upon exposure to TBT, TPT or Bex is accompanied by a decreased mRNA stability. 10 μ g/mL Act D was earlier shown to be effective in abolishing transcription and studying mRNA stability in JEG-3 cells (Lanz et al., 2003). Additionally, we verified the efficiency of transcriptional inhibition by determining the half-life of the MYC proto-oncogene as a control and found it to be <1 h, in line with values described in the literature. Cytotoxicity of Act D prohibited extension of the experiment beyond the 8 h.

3.9 The fungicide fludioxonil and the food ink chemical chlorotriphenylsilane enhance 11 β -HSD2 activity

To test whether other RXR α ligands can upregulate 11 β -HSD2 expression and activity, JEG-3 cells were incubated in the presence of the fungicide fludioxonil or the food ink chemical chlorotriphenylsilane (TPSCI) for 24 h, followed by measuring the 11 β -HSD2-dependent conversion of cortisol to cortisone. Fludioxonil was shown previously to activate RXR α (Janesick et al., 2016), TPSCI was chosen because of the high structural similarity to TPT (Supplemental Fig. S7). Both chemicals caused a concentration-dependent increase of 11 β -HSD2-activity (Fig. 6). These results suggest that various RXR α agonists can induce 11 β -HSD2 activity in the JEG-3 cell line.

Fig. 6. Effect of proposed RXR α ligands on 11 β -HSD2 activity in JEG-3 cells. Cells were treated for 24 h with **A**) fludioxonil and **B**) chlorotriphenylsilane (TPSCI) as indicated. Conversion of radiolabeled cortisol to cortisone was determined as in Fig. 1 C. (mean $\pm$ SD; n = 3). One-way ANOVA with Dunett's post hoc test, p values: **<0.01, ***<0.001.

4. Discussion

To the best of our knowledge, this is the first study showing that nanomolar concentrations of TBT and TPT induce the expression of 11 β -HSD2 and thereby enhance the inactivation of glucocorticoids. Human JEG-3 choriocarcinoma cells served as a model for placental syncytiotrophoblasts, which is the key cell type responsible for essential placental functions, including the control of maternal active glucocorticoids that reach the fetus. Using TBT, an upregulation of 11 β -HSD2 mRNA expression could also be observed in non-malignant primary human trophoblast cells with high physiological relevance.

At concentrations of TBT and TPT higher than 500 nM, a direct inhibition of 11 β -HSD2 activity has been observed (Atanasov et al., 2005); however, such high concentrations are less likely to be reached in an exposure scenario. In an earlier analysis in human subjects TBT concentrations in blood of up to 300 nM (85 ng/mL) have been detected (Kannan et al., 1999). Since nanomolar concentrations of organotins were shown to promote the production of pro-inflammatory markers (Chantong et al., 2014; Hurt et al., 2013), which may reduce 11 β -HSD2 expression (Heiniger et al., 2001; Kostadinova et al., 2005), we initially hypothesized that these organotins might decrease 11 β -HSD2 expression via a transcriptional mechanism, eventually disturbing the placental barrier by an inefficient inactivation of the high maternal cortisol levels. In contrast to our assumption, we found that TBT and TPT increased 11 β -HSD2 expression and activity in the JEG-3 placenta cells and that NF- κ B inhibitors did not affect the organotin-mediated effect. We cannot exclude that at concentrations higher than 200 nM, where cytotoxicity started to be observed and that were therefore not analyzed in the present study, an NF- κ B-dependent decrease of 11 β -HSD2 expression might be detected. In a study using HL-60 cells a high concentration of TPT (5 μ M) resulted in elevated intracellular calcium levels with NF- κ B activation and induction of TNF α (Marinovich et al., 1996); however, no information on 11 β -HSD2 expression is available.

The expression of 11 β -HSD2 in the placental barrier is highly regulated by various intrinsic and extrinsic factors and pathways (Guan et al., 2013; He et al., 2014; Li et al., 2011a; Pasqualette et al., 1996; Sarkar et al., 2001; Sharma et al., 2009; Tremblay et al., 1999; Yang et al., 2006). We were particularly interested in the mechanism underlying the organotin-mediated increase of 11 β -HSD2 expression. The protein kinase A pathway was shown earlier to be involved in the stimulation of 11 β -HSD2 expression in primary human trophoblasts (Pasqualette et al., 1996); however, we did not observe any effect of the inhibitor H-89 at subcytotoxic concentrations on the TBT-dependent upregulation of 11 β -HSD2 (data not shown). This is in line with the findings of Nakanishi et al. (2002), reporting that TBT and TPT did not alter cAMP production in human JAR choriocarcinoma cells at concentrations comparable to those used in the present study. Furthermore, a possible role for the transcription factor Sp1 was tested (He et al., 2014; Li et al., 2011a) but found not to be involved since subcytotoxic concentrations of its potent inhibitor, mithramycin A, did not alter organotin-induced 11 β -HSD2 activity (data not shown). PPAR γ

represents another transcription factor participating in the regulation of 11 β -HSD2 expression, and a decreased PPAR γ expression and activity has been associated with reduced 11 β -HSD2 activity and intrauterine growth restriction (Fu et al., 2019; He et al., 2014). Furthermore, Tremblay et al. (1999) found retinoids to increase 11 β -HSD2 expression in placental cell lines. Importantly, several studies provided evidence that TBT and TPT are potent activators of RXR α and PPAR γ (Grun et al., 2006; Harada et al., 2015; Kanayama et al., 2005; le Maire et al., 2009; Li et al., 2011b; Nakanishi et al., 2005). Besides, nanomolar concentrations of TBT and TPT were reported earlier to enhance human chorionic gonadotropin, aromatase activity and 17 β -hydroxysteroid dehydrogenase type 1 activity in placental cell lines, and the mechanism underlying these effects was suggested to be linked to RXR (Nakanishi et al., 2006; Nakanishi et al., 2002; Nakanishi et al., 2005).

Thus, we tested whether TBT and TPT affect 11 β -HSD2 expression via RXR α - PPAR γ . Our results suggest that the TBT-dependent induction of 11 β -HSD2 expression in JEG-3 cells was mediated by RXR α homodimers, because siRNA against RXR α completely abolished the stimulatory effect and the homodimer antagonist LG754 inhibited the activating effect of both TBT and the retinoid analogue Bex, whilst the PPAR γ antagonist GW9662 was ineffective. Previous studies showed that RXR - PPAR γ heterodimers are permissive; *i.e.* activated by RXR α or PPAR γ agonists and synergistic in the presence of both; similarly, antagonists of either receptor should be inhibitory (Li et al., 2011b; Schulman et al., 1998). Further, TBT and TPT were both shown to bind to the ligand binding domain of RXR α and to activate RXR α - PPAR γ heterodimers (Grun et al., 2006; Nakanishi et al., 2005). Molecular modeling predicted both TBT and TPT to bind RXR α , forming a weak covalent binding between the tin atom and Cys432 on RXR α and van der Waals interactions between the organotin and residues of the ligand binding pocket (le Maire et al., 2009). Interestingly, Kanayama et al. (2005) reported that TBT potently activated an RXR α -dependent reporter construct whilst TPT showed weak activation, and the opposite pattern was observed for a PPAR γ -dependent reporter construct with TPT showing potent activation and TBT was weakly active. In our experiments, the RXR α homodimer antagonist was unable to suppress the TPT-mediated increase in 11 β -HSD2 expression and activity; furthermore, the PPAR γ antagonist GW9662 was ineffective but the rosiglitazone-mediated activation was fully blocked, showing that GW9662 was active. This may be explained by TPT-dependent activation of both RXR α

homodimers and RXR α - PPAR γ heterodimers. Alternatively, binding/activation of TPT may be too potent and antagonist concentrations insufficiently low. Nevertheless, the TPT-dependent 11 β -HSD2 upregulation required RXR α and was fully abolished using siRNA against this receptor.

Although, the human 11 β -HSD2 promotor carries potential RXR α homodimer and RXR α /PPAR γ heterodimer binding elements (one and two-base pair spaced direct repeats: DR1 and DR2 (Zhao et al., 2000)) upstream of the AUG transcription start site, we were not able to detect a TBT-, TPT- or Bex-dependent increase of transcription from a 11 β -HSD2 promoter (-1778/+117 (Subtil-Rodriguez et al., 2008)) in transactivation studies using JEG-3 or TD-47 cells. Potential reasons for this are differences in the accessibility of the receptor binding element due to structural differences between promoter construct and endogenous promoter, false positive results from binding site prediction, and existence of functional sites further upstream on the promoter that are not present in the cloned construct but essential for RXR α -mediated transcriptional regulation.

The finding that the fungicide fludioxonil, shown earlier to activate RXR α (Janesick et al., 2016), and the food ink chemical TPSCI, bearing a structure closely resembling that of TPT (Supplemental Fig. S7), upregulate 11 β -HSD2 expression and activity suggest that the consequences of an impaired activity of this enzyme upon exposure to environmental RXR α agonists in general should be considered.

Placental and fetal development must be tightly regulated to ensure successful pregnancy as well as maternal and offspring's health. TBT and TPT, and other RXR α agonists, through upregulation of 11 β -HSD2 in placenta may on one side affect functions in trophoblast cells by a decreased expression of glucocorticoid-dependent genes and thereby altering the function of the placental barrier, and on the other side result in a deficient exposure of the fetus to glucocorticoids. Cytokines and growth factors secreted by the placenta, many of them regulated by glucocorticoids, maintain fetal - maternal interactions and are important to control nutritional supply to the fetus (Malassine et al., 2003). Further, they are essential for fetal organ maturation, especially the lung, and pharmacological treatment with glucocorticoids in preterm birth is frequently used to enhance fetal lung maturation (Cole et al., 1995; Ward, 1994). Also, thyroid hormone secretion, catecholamine production and initiation of hepatic glycogen accumulation and gluconeogenesis are promoted by cortisol to prepare the fetus for life after birth (Liggins, 1994). Besides, glucocorticoids are essential to promote uterine and vascular remodeling

and to suppress maternal immune rejection of the embryo (Whirlledge et al., 2015). Thus, an increased placental 11 β -HSD2 activity, resulting in decreased cortisol availability for the fetus, is expected to disturb these functions.

Besides inactivating glucocorticoids and modulating corticosteroid receptor signaling, recent investigations revealed a role of 11 β -HSD2 in oxysterol metabolism (Beck et al., 2019a; Beck et al., 2019b; Raleigh et al., 2018; Voisin et al., 2017). 11 β -HSD2 was reported to generate the oncometabolite 6-oxo-cholestan-3 β ,5 α -diol, which promotes the proliferation of breast cancer cells by a mechanism involving GR (Voisin et al., 2017). Whether this metabolite has a physiological function for example in cell proliferation during early development remains to be investigated. In addition, 11 β -HSD2 was found to generate 7 β ,25-dihydroxycholesterol (Beck et al., 2019b), a ligand of the G-protein coupled receptor EBI-2 involved in the regulation of chemoattraction in immune cells, and 7 β ,27-dihydroxycholesterol (Beck et al., 2019a), an agonist of ROR γ and ROR γ t that regulate circadian rhythm and metabolic processes as well as interleukin-17 expressing T-cells. Moreover, a role of 11 β -HSD2 in the modulation of the hedgehog signaling pathway, essential for proper development and a target for cancer treatment, by generating the ligand 7keto-27-hydroxycholesterol has been proposed (Beck et al., 2019a; Raleigh et al., 2018). How an inappropriately increased 11 β -HSD2 activity upon exposure to exogenous RXR α agonists can affect the above mentioned pathways remains to be investigated.

In addition to its role in glucocorticoid and oxysterol metabolism, 11 β -HSD2 is involved in the conversion of adrenal C11-oxy C₁₉ steroids to their C11-keto forms, which are further metabolized to the potent androgen 11-ketodihydrotestosterone (Gent et al., 2019). Similarly, in fish 11 β -HSD2 is important in the androgen pathway by catalyzing the last step in the formation of the major androgen 11-ketotestosterone (Jiang et al., 2003; Kusakabe et al., 2003; Tsachaki et al., 2017). Interestingly, exposure of female Japanese flounder and zebra fish larvae to TBT was reported to induce masculinization (Santos et al., 2006; Shimasaki et al., 2003). Reduced P450 aromatase gene expression and activity, resulting in impaired estrogen synthesis, was suggested to impair the testosterone/estradiol balance, thereby causing a masculine phenotype. Additionally, an organotin-induced increase in 11 β -HSD2 expression that we observed in preliminary experiments in a concentration-dependent manner on the mRNA level in zebrafish larvae (data not shown) might be relevant for the deregulation of the

androgen metabolism and contribute to the development of the masculine phenotype; however, this hypothesis warrants further investigations.

In conclusion, we show that the environmental toxicants TBT and TPT increase endogenous 11 β -HSD2 expression in the placental cell line JEG-3 and provide evidence that this effect is dependent on increased gene transcription directly mediated via RXR α homodimers. Environmental toxicants, such as TBT and TPT, which can activate RXR α and thereby enhance 11 β -HSD2 expression and activity and result in increased glucocorticoid inactivation, should be carefully assessed for disturbances of fetal development, with adverse cardiovascular and metabolic health effects in offspring in later life.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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