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Alteration of cellular lipids and lipid metabolism markers in RTL-W1 cells exposed to model endocrine disrupters

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ABSTRACT

This work investigates the suitability of the rainbow trout liver cell line (RTL-W1) as an in-vitro model to study the ability of model endocrine disrupters, namely TBT, TPT, 4-NP, BPA and DEHP, to act as metabolic disrupters by altering cellular lipids and markers of lipid metabolism. Among the tested compounds, BPA and DEHP significantly increased the intracellular accumulation of triacylglycerols (TAGs), while all the compounds -apart from TPT-, altered membrane lipids – phosphatidylcholines (PCs) and plasmalogen PCs – indicating a strong interaction of the toxicants with cell membranes and cell signaling. RTL-W1 expressed a number of genes involved in lipid metabolism that were modulated by exposure to BPA, TBT and TPT (up-regulation of *FATP1* and *FAS*) and 4-NP and DEHP (down-regulation of *FAS* and *LPL*). Multiple and complex modes of action of these chemicals were observed in RTL-W1 cells, both in terms of expression of genes related to lipid metabolism and alteration of cellular lipids. Although further characterization is needed, this might be a useful model for the detection of chemicals leading to steatosis or other diseases associated with lipid metabolism in fish.

1. Introduction

The hypothesis that chemicals in our environment may act as obesogens, perturbing mechanisms involved in body weight control and lipid homeostasis is currently being investigated. Several endocrine disrupters have been identified in recent years to act as obesogenic compounds: organotins such as tributyltin (TBT) and triphenylthin (TPT), estrogenic compounds such as diethylstilbestrol and bisphenol A (BPA), phthalates, perfluorooctanoates and nonylphenols (Elobeid and Allison, 2008; Grün and Blumberg, 2009; Casals-Casas and Desvergne, 2011).

TBT and TPT promote adipogenesis by binding to the retinoid X receptor (RXRα) and peroxisome proliferator-activated receptor (PPARγ), and they consequently induce the differentiation of murine preadipocyte cells (3T3-L1) to adipocytes (Inadera and Shimomura, 2005; Kanayama et al., 2005). Furthermore, in-utero exposure to TBT led to liver steatosis and increased lipid accumulation and maturation of adipocytes in mouse models and induced ectopic adipocyte formation in *Xenopus* (Grün et al., 2006; Iguchi et al., 2007).

Other studies revealed increased body weight in rats and mice exposed during gestation and/or lactation to low doses of BPA, an endocrine disruptor highly prevalent in our environment (Rubin, 2011). Soon after, Marmugi et al. (2012) reported the accumulation of triglycerides and cholesteryl esters in the liver of mice exposed to 50 and 500 μg BPA/kg/day. Interestingly, micromolar concentrations of BPA and 4-nonylphenol (4-NP) triggered the differentiation of 3T3-L1 cells in adipocytes (Masuno et al., 2002, 2003), and BPA induced the expression of adypocite-specific genes (such are *FAS* and leptin) (Phrakonkham et al., 2008).

Phthalates, widely used as plasticizers and stabilizers in the manufacture of consumer products, are also shown to alter lipid homeostasis. Thus, urine concentrations of four phthalate metabolites deriving from di-2-ethylhexyl phthalate (DEHP) were positively correlated with abdominal obesity among adult U.S. males (Stahlhut et al., 2007). Among these metabolites, mono-2-ethylhexyl phthalate (MEHP), a known activator of PPARγ promoted differentiation of 3T3-L1 cells into adipocytes and induced the expression of genes involved in lipogenesis, triglyceride synthesis and adipokines (Feige et al., 2007).

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Despite the reported evidences in mammals, little is known on the ability of these chemicals to disrupt lipid metabolism in aquatic organisms, which are often exposed to significant concentrations of these compounds. Interestingly, an increase in whole-body lipid content along with a raise of lipid related plasma parameters (triacylglycerols, cholesterol and lipase) was observed in juvenile chinook salmon (*Oncorhynchus tshawytscha*) exposed to TBT (Meador et al., 2011). Also, long term exposure to TBT induced lipid and fatty acid accumulation in the ramshorn snail *Marisa cornuarietis* (Janer et al., 2007). TBT, tetrabromobisphenol-A (TTBPA), tetrachlorobisphenol-A and TTBPA-sulfate induced lipid accumulation in zebrafish larvae, which are proposed as a screening model (Tingaud-Sequeira et al., 2011; Riu et al., 2014).

As a result of the above observations and because the liver is the predominant site of de novo synthesis of lipids, this study aimed at investigating the use of the rainbow trout (*Oncorhynchus mykiss*) liver cell line (RTL-W1) as a in-vitro model to assess the ability of known or suspected obesogenic compounds, namely TBT, TPT, 4-NP, BPA and DEHP, to disrupt cellular lipids and lipid metabolism. RTL-W1 is predominantly epithelial-like in shape and appears to be a liver stem cell (Lee et al., 1993; Malhão et al., 2013). The lipid composition of RTL-W1 cells was determined by high-performance liquid chromatography coupled with high resolution mass spectrometry (HPLC-HRMS). Phosphatidylcholines (PC), plasmalogen PCs and triacylglycerols (TAG) were identified and analyzed under positive electrospray ionization mode (ESI), and relative changes in their levels were determined comparing peak areas in exposed cells with their basal amounts in non-exposed cells. Additionally, expression of the following genes was investigated: (a) the gene encoding ATP-binding cassette transporter (*ABCA1*) related to cholesterol homeostasis; (b) the genes encoding two fatty acid transporters – cluster of differentiation 36 (*CD36*) and fatty acid transport protein 1 (*FATP1*) – and the gene encoding the enzyme lipoprotein lipase (*LPL*) as markers of fatty acid uptake; (c) the expression of genes encoding the lipogenic enzyme, fatty acid synthase (*FAS*); (d) the genes encoding liver X receptor (LXR) commonly involved in lipogenic pathways; *PPARs* α and β, the products of which are promoters of fatty acids use in mammals, and *PPAR*γ the product of which is involved in lipid accumulation and adipogenesis in mammals and fish (Kota et al., 2005; Bouraoui et al., 2008).

2. Materials and methods

2.1. Fish cell culture and exposure

The rainbow trout liver cell line (RTL-W1) was kindly supplied by Professor N.C. Bols, University of Waterloo, Canada. RTL-W1 cells were routinely cultured in 75-cm^2 culture flasks at 20 °C in Leibovitz's L-15 culture medium (Sigma–Aldrich, Steinheim, Germany) supplemented with 5% fetal bovine serum (FBS, Sigma–Aldrich, Steinheim, Germany) and 1% penicillin–streptomycin solution (10,000 units/ml penicillin, 10 mg/ml streptomycin, Sigma–Aldrich, Steinheim, Germany). When 90% of confluence was reached, cells were dissociated with 0.05% (w/v) trypsin and 0.5 mM EDTA for subculturing and exposure experiments.

Tributyltin chloride 96% (TBT), triphenyltin chloride 95% (TPT), 4-nonylphenol (4-NP), bisphenol A 99% (BPA), and bis-(2-ethylhexyl)-phthalate (DEHP) were purchased from Sigma–Aldrich (Steinheim, Germany). Stock solutions were prepared and diluted in dimethyl sulfoxide (DMSO, Sigma–Aldrich).

For exposure experiments, confluent flasks were used to seed pre-coated (1% gelatine) 6-well Costar culture plates (Corning Inc.) at a cell density of 10^6 cells per well. Cells were allowed to attach for 48 h prior to exposure, and cells were exposed to test compounds diluted in culture medium. Contaminated medium was replaced by new one after 48 h exposure. The final concentration applied in wells was 100 nM TBT and TPT, 20 μM 4-NP, 10 μM BPA and 5 μM DEHP and cells were exposed for 24, 48 and 72 h. The selected concentrations were shown to induce lipid droplet formation and to promote adipocyte differentiation in 3T3-L1 cells (Masuno et al., 2003; Inadera and Shimomura, 2005; Hao et al., 2012a) and did not affect cell viability in RTL-W1 cells, with the exception of DEHP and TBT that were slightly toxic (84–87% cell viability). Cell viability was determined with Alamar Blue and 5′-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) (Thibaut et al., unpublished results).

The final concentration of DMSO in culture wells was 0.5% (v/v). For each assay a control was performed by adding only the solvent (DMSO) to the cells. The number of independent experiments, using cells of different passage, was of three for gene analysis and four for lipid profiling. Three technical replicates were performed in each experiment.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted by the Tri-reagent method (Ambion, Applied Biosystems, Spain). The quantity and quality of isolated RNA was determined spectrophotometrically with a NanoDrop® ND-1000 UV–vis (Labtech Int., U.K.). For cDNA synthesis, 1 μg of RNA, 3 μl of a blend 2:1 random hexamers (600 μM)/oligodT (50 μM), 2 μl dNTP (10 mM), 0.5 μl of reverse transcriptase (20 U/μl), and 0.5 μl of RNAse inhibitor were mixed with the kit buffer in a final volume of 20 μl (Transcriptor first strand cDNA synthesis kit, Roche, Germany), and incubated at 50 °C for 6 min, followed by the inactivation of the enzymes at 85 °C for 5 min. The cDNA obtained was stored at −20 °C for qRT-PCR.

2.3. Real-time PCR analysis

PCR measurements were performed by applying the primers at 0.35 μM with one-fortieth of the cDNA synthesis reaction and SYBR-Green PCR mix (Bio-Rad, Spain) in a total volume of 20 μl. The RT-PCR primer sequences for target genes (*ABCA1*, *LXR*, *CD36*, *PPARβ*, *PPARγ*, *PPARα*, *FAS*, *FATP1* and *LPL*) and the reference gene (*EF1α*) are shown in Table 1. Reactions were performed in an iQCycler IQ Real-time Detection System (Bio-Rad, Spain). Each PCR product was sequenced to confirm identity, and each one was found to be 100% identical to its respective sequence. Primer sequences were obtained from our previous studies (references in Table 1) and were designed against sequences from the following databases: http://compbio.dfci.harvard.edu/tgi/; http://www.ncbi.nlm.nih.gov/dbEST/; http://www.sigenae.org/index.php?id=21 Prior to the analyses a dilution curve with a pool of cDNA samples was run to calculate primer efficiency and the values obtained ranged from 91.3% to 112.5%. Reactions were performed in duplicate, and the fluorescence data acquired during the extension phase were determined using the delta–delta-CT method (Livak and Schmittgen, 2001) using *EF1α* as endogenous reference gene after confirming that its expression did not differ between treatments.

2.4. Extraction and analysis of lipids

Lipids were extracted with a modification of the method of Christie (1985). A solution of methanol:chloroform (1:2) containing 0.01% of butylated hydroxytoluene (BHT) as an antioxidant was added to the cell pellets, vortexed (1 min) and after 30 min incubation at room temperature, extracted in an ultrasonic bath for 5 min