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Research paper

Up-stream mechanisms for up-regulation of miR-125b from triclosan exposure to zebrafish (*Danio rerio*)

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ABSTRACT

Triclosan (TCS) exposure has widely adverse biological effects such as influencing biological reproduction and endocrine disorders. While some studies have addressed TCS-induced expression changes of miRNAs and their related down-stream target genes, no data are available concerning how TCS impairs miRNA expression leading us to study up-stream regulating mechanisms. Four miRNAs (miR-125b, miR-205, miR-142a and miR-203a) showed differential expression between TCS-exposure treatments and the control group; their functions mainly involved fatty acid synthesis and metabolism. TCS exposure led to the up-regulation of mature miR-125b that was concomitant with consistent changes in pri-mir-125b-1 and pri-mir-125b-3 among its 3 pri-mir-125bs. Up-regulation of miR-125b originated from direct shear processes involving the two up-regulated precursors, but not pri-mir-125b2. Increased expression of pri-mir-125b-1 and pri-mir-125b-3 resulted from *nfe2l2*- and c/ebpα-integration with positive control elements of promoters for the two precursors. The overexpression of transcriptional factors, nfe2l2 and c/ebpα, initiated the promoter activity for the miR-125b precursor. CpG islands and Nfe2l2 were involved in constitutive expression of *mir-125b-1* and *mir-125b-3*. The activities of two promoter regions, −487 to −1 bp for pri-mir-125b1 and −1327 to +14 bp for pri-mir-125b-3 having binding sites for NFE2 and Nfe2l2/MAF:NFE2, were higher than other regions, further demonstrating that the transcriptional factor Nfe2l2 was involved in the regulation of pri-mir-125b1 and pri-mir-125b-3. TCS's estrogen activity resulted from its effects on GPER, a novel membrane receptor, rather than the classical ERα and ERβ. These results explain, to some extent, the up-stream mechanism for miR-125b up-regulation, and also provide a guidance to future mechanistic study on TCS-exposure.

1. Introduction

Triclosan (TCS, 5-chloro-2-[2,4-dichlorophenoxy]phenol) is an antimicrobial agent that has been broadly used in many products such as liquid hand soaps, household cleansers, and dental products for more than 30 years. In recent years, TCS is universally detected in the environment at ng/L to μg/L levels, leading to accumulation in biota and humans (Wang et al., 2015). TCS has adverse effects on biological growth and development such as influencing reproduction and endocrine disorders. Many previous studies document the acute or long-term exposure toxicity of TCS to various organisms, such as fish, invertebrates and amphibians (Huang et al., 2014). For example, Yueh and Tukey (2016) found that long-term TCS exposure led to liver tumors and produced oxidative stress in mice. Because TCS is a stable and lipophilic compound, it preferentially bioaccumulates in fatty tissue and liver bile, and as a consequence is often detectable in these tissues or organs (Young et al., 2008). TCS was extensively metabolized in HepG2 cells overexpressing CYP1A2, CYP2B6, CYP2C19, CYP2D6, CYP2C18, lucuronosyltransferases (UGTs) and sulfotransferases (SULTs) (Wu et al., 2017). In zebrafish (*Danio rerio*), TCS impairs the mRNA expression level of beta-oxidation transcripts and lipid beta-oxidation genes, which included *pparα, cpt1, lpbe, cyp4a10* and *aco* (Ho et al., 2016). TCS at non-cytotoxic concentrations can induce human mesenchymal stem cells lipid accumulation by decreasing adipocyte protein 2, lipoprotein lipase, and adiponectin gene expression (Guo et al., 2012). However, there is a paucity of information regarding the specific microRNAs (miRNAs) induced by TCS exposure and the responsible transcription factors. miRNAs play an important role in lipid synthesis, oxidation and related diseases (Baldán and Tq, 2016). miRNA levels in cells are controlled by several mechanisms (e.g., transcription,

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processing and stabilization) and an increase of pri-miRNA is a major indicator for quantitation of changes in miRNAs (Chang et al., 2011).

Herein, based on high throughput sequencing, we identified the dysregulated miRNAs among TCS-exposed groups and the control group. One of these dysregulated miRNAs, miR-125b-5p, was differentially expressed in three TCS-exposure groups when compared to the control group. In cancer research, miR-125b is associated with tumor suppressor by down-regulating target oncogene (Yueh et al., 2014). Additionally, several studies have examined miR-125b in relationship to metabolic disorders in mice and humans cell lines (Zhang et al., 2015; Tili et al., 2012). miR-125a/b was found to suppress Cd-induced apoptosis by target Bak and caspase-3 directly in LLC-PK1 cells (Chen et al., 2016a,b). Moreover, several previous studies demonstrate that expression changes of microRNAs in response to pollutant exposure result from the corresponding differential expression of down-stream genes (Pogribny et al., 2015; Harrill et al., 2016; Woeller et al., 2017). These previous reports mainly focused on down-stream regulation by miR-125b, but no studies have addressed related up-stream regulation mechanisms. Therefore, the specific objective of this study was to probe up-stream mechanisms regulating miR-125b expression changes resulting from TCS exposure to zebrafish (*Danio rerio*). Although previous researches identified several factors that positively regulate miR-125b expression in mammal cell lines, such as Nrf2, c/EBPα and ERα, whether or not miR-125b is also involved in glucose and lipid metabolism in zebrafish embryo remain uncertain (Tili et al., 2012; Joo et al., 2013; Zhang et al., 2015; Romero et al., 2015). Furthermore, the molecular mechanisms for abnormal expression of miR-125b from TCS exposure and the conservation of miR-125b in vertebrates have not been investigated. As a result, the above uncertain problems are the focus of this investigation.

2. Material and methods

2.1. Ethics statement

This study strictly followed protocols for the care and use of laboratory animals established by the Institutional Animal Care and Use Committee (IACUC) at Wenzhou Medical University (Wenzhou, China). All zebrafish surgery was performed on ice to decrease suffering.

2.2. Chemical reagents

Triclosan (5-chloro-2-[2,4-dichlorophenoxy]phenol) was purchased from Sigma-Aldrich (St. Louis, USA; CAS No. 3380-34-5, 99.9% purity). Formamide (CAS No.75-12-7, purity ≥99%) and xylene (CAS No. 1330-20-7, 98.5%) were purchased from Aladdin (Shanghai, China).

2.3. Zebrafish maintenance and exposure protocols

Wild-type (AB strain) zebrafish (*Danio rerio*) were raised in dechlorinated and filtered water at 28 °C with a 14-h light:10-h dark photoperiod (light on at 8 am). Zebrafish maintenance followed Westerfield (2000). A series of TCS-exposure concentrations was chosen according to its LC_{50} and EC_{50} values for embryonic and adult zebrafish (Oliveira et al., 2009), environmentally relevant concentrations, and preliminary experimental results. Embryos were exposed to a series of TCS concentrations (0, 62.5 μg/L (0.22 μmol/L), 125 μg/L (0.43 μmol/L) and 250 μg/L (0.86 μmol/L)). Adult zebrafish were continuously exposed to low-dose TCS concentrations (0, 25 μg/L (0.09 μmol/L), 50 μg/L (0.17 μmol/L)and 100 μg/L (0.35 μmol/L)) from embryos (4 hpf) to adults (90 dpf). Control embryos were treated with 0.0025% acetone (as reference to the highest 250 μg/L TCS-exposure treatment) (Ho et al., 2016).

2.4. cDNA synthesis and qRT-PCR

Embryos were dissolved in TransZol Up® solution (Transgene, China) for total RNA extraction. Total RNA with an A_{260}/A_{280} ratio of 1.8-2.0 was used. Briefly, 1 μg total cellular RNA was reversely transcribed using a PrimeScript RT® Reagent Kit with gDNA Eraser (Takara, Japan) and cDNA was transcribed with TransScript miRNA First-Strand cDNA Synthesis SuperMix for miRNA qRT-PCR (Transgene, China) with elfa and U6 as the endogenous reference (McCurley and Callard, 2008). All PCR reactions were performed in three biological replicates and each biological replicate included three technical replicates. Bio-Rad CFX Manager software was used to analyze results from qRT-PCR. Primers used for qRT-PCR are shown in Supplementary Table 1.

2.5. Promoter isolation, sequence analysis, construction, microinjection and luciferase assay

Zebrafish genomic DNA was extracted using the EasyPure Genome DNA kit (Transgene, China) according to the manufacturer's protocols. PCR was performed with 100 ng genomic DNA, a final concentration of 0.2 μM of primers; 2.5 units of TransStart FastPfu Fly DNA Polymerase, 1xTransStart FastPfu Fly DNA buffer (Transgene, China), and 0.2 mM dNTPS (Takara, Japan) in a total volume of 50 μL using a GeneAmp® PCR System 2700 thermocycler (Applied Biosystems, USA). PCR conditions were 35 cycles of 20 s at 95 °C, 20 s at 60 °C, and 30 s at 72 °C. Validated plasmid containing target fragments was gel-purified and ligated to pEASY-Blunt Simple Cloning Vector (Transgene, China). Several clones were sequenced by BGI (Beijing, China) and searched against the genome database using the ensemble BLAST/BLAT (http://www.ensembl.org/Danio_rerio/Tools/Blast?db=core;redirect=no). Primers used for the promoter activity assay are shown in Supplementary Table 1 and were synthesized by Shanghai Sangon Biotechnological Corporation (Shanghai, China). The PCR product containing *mir-125b-1* and *mir-125b-3* promoters was independently digested with SacI and HindIII or NcoI restriction endonucleases (NEB, USA), and fragments containing the promoter region were collected by EasyPure® Quick Gel Extraction Kit (Transgene, China). Promoter fragments of *mir-125b-3* were inserted into pGL3-basic (Promega, USA) using SacI and NcoI sites. The *mir-125b-1* promoter fragments were inserted into pGL3-basic (Promega, USA) using SacI and HindIII sites.

Plasmids, which included various lengths of *mir-125b-1* or *mir-125b-3* promoter regions and the pRL-TK vector in a ratio of 2:1, were microinjected (ca. 2 nL of solution) into the one-cell-stage of zebrafish eggs, and the pRL-TK vector was used as an internal control reporter. After injection, eggs were incubated at 28 °C for 48 h for luciferase assay. Protein extracts of 48 hpf embryos in passive lysis buffer were placed in an ice bath, and aliquots of the extracts (20 μL) were used for luciferase assays using a Dual Luciferase Reporter Assay System (Promega, USA) following manufacturer instructions (Varioskan Flash, Thermo Fisher Scientific, USA).

2.6. RNA preparation, sequencing and data mapping

Total RNA was extracted using Trizol Reagent (Transgene, China) according to the manufacturer's instructions. RNA sequencing libraries were prepared for each RNA-seq sample using a TruSeq® Stranded Total RNA Sample Preparation kit (Illumina, USA) following manufacturer's protocols. For miRNA sequencing, the RNA samples from 96-hpf zebrafish for each group were pooled together based on an equal RNA quantity. After quality control(OD260/OD280 > 1.8 and OD260/OD 230 > 1.5), sequencing of all libraries was performed by Illumina Hiseq2000/2500 according to manufacturer's instructions (LC Sciences, USA). Small RNA library sequencing was completed by Hangzhou LC-Bio Co., Ltd. (Hangzhou, China). Analyses of RNA-Seq data were conducted according to the protocols previously reported by our group (Zheng et al., 2016; Li et al., 2015).