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Retene causes multifunctional transcriptomic changes in the heart of rainbow trout (*Oncorhynchus mykiss*) embryos

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ABSTRACT

Fish are particularly sensitive to aryl hydrocarbon receptor (AhR)-mediated developmental toxicity. The molecular mechanisms behind these adverse effects have remained largely unresolved in salmonids, and for AhR-agonistic polycyclic aromatic hydrocarbons (PAHs). This study explored the cardiac transcriptome of rainbow trout (*Oncorhynchus mykiss*) eleuteroembryos exposed to retene, an AhR-agonistic PAH. The embryos were exposed to retene (nominal concentration 32 μg/L) and control, their hearts were collected before, at and after the onset of the visible signs of developmental toxicity, and transcriptomic changes were studied by microarray analysis. Retene up- or down-regulated 122 genes. The largest Gene Ontology groups were signal transduction, transcription, apoptosis, cell growth, cytoskeleton, cell adhesion/mobility, cardiovascular development, xenobiotic metabolism, protein metabolism, lipid metabolism and transport, and amino acid metabolism. Together these findings suggest that retene affects multiple signaling cascades in the heart of rainbow trout embryos, and potentially disturbs processes related to cardiovascular development and function.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants. Many of them activate the aryl hydrocarbon receptor (AhR) and thereby cause dioxin-like developmental toxicity (blue sac disease, BSD) in early life stages of fish (Hawkins et al., 2002; Billiard et al., 1999; Scott et al., 2011; Incardona et al., 2006; Clark et al., 2010; Van Tiem and Di Giulio, 2011). The signs of BSD include yolk sac and pericardial sac edema, hemorrhaging, failure in erythrocyte maturation, defects in heart and vascular development, induction of cytochrome P450 enzymes, and skeletal deformities, and this condition may be lethal if severe.

The AhR is a member of the basic helix-loop-helix transcription factors, and is activated by aromatic compounds with structural characteristics similar to those of 2,3,7,8-tetraclorodibenzo-*p*-dioxin (TCDD). Activated AhR translocates to the nucleus where it dimerizes with AhR nuclear translocator (ARNT), and up- or down-regulates transcription of numerous genes via binding to xenobiotic-responsive elements within the promoter sequences of these genes. The activation of the AhR precedes signs of BSD in trout embryos (Brinkworth et al., 2003).

Cardiovascular tissue is the presumed target tissue of AhR-mediated toxicity in fish. The AhR agonists disturb the development of the heart and the vasculature in fishes, and cardiovascular pathology is the first sign of dioxin-like toxicity in zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*) (Henry et al., 1997; Hornung et al., 1999; Antkiewicz et al., 2005; Carney et al., 2006; Mehta et al., 2008; Plavicki et al., 2013; Scott, 2009). The most studied AhR agonist, TCDD, prevents cardiac valve formation, inhibits epicardial and proepicardial development, causes altered looping of the heart, and reduces the volume and number of cardiomyocytes in fishes (Hornung et al., 1999; Antkiewicz et al., 2005; Carney et al., 2006; Mehta et al., 2008; Plavicki et al., 2013). Similarly, other

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AhR agonists (e.g., PCB126, retene, benz[a]anthracene) disturb the development of the heart in zebrafish and medaka (Incardona et al., 2006; Grimes et al., 2008; Scott et al., 2011; Scott, 2009).

The molecular mechanisms of developmental toxicity of AhR agonists in fish are not well understood. AhR-ARNT-dependent transcriptional regulation is needed for toxic effects to take place, but the downstream targets are not well resolved (Antkiewicz et al., 2006; Carney et al., 2004; Prasch et al., 2003, 2006). Some information has been gained from studies on zebrafish embryos exposed to TCDD, but only a little is known about the effects of AhR-agonistic PAHs (Carney et al., 2006; Chen et al., 2008; Goodale et al., 2013). Furthermore, the molecular mechanisms have to our knowledge not been studied in depth in salmonids at all.

Retene (7-isopropyl-1-methylphenanthrene) is an alkylated PAH formed from resin acids via action of anaerobic microbes, or during incomplete combustion of resinous softwood, e.g., in forest fires (Ramdahl, 1983; Tavendale et al., 1997). It has been found in sedimenting particles and the sediment surface layer in lake areas contaminated by treated pulp and paper mill effluents, and in municipal landfill soil (Leppanen and Oikari, 1999, 2001; Legler et al., 2011). As an alkylphenanthrene, retene is representative of the compounds in petroleum products that cause dioxin-like developmental toxicity in fishes (Adams et al., 2014). Retene is an AhR agonist, and it causes BSD in early life stages of zebrafish, medaka, and rainbow trout (*Oncorhynchus mykiss*) (Billiard et al., 1999; Kiparissis et al., 2003; Scott et al., 2011). The molecular mechanism of the developmental toxicity caused by retene depends on the AhR, and thus resembles that of TCDD in zebrafish (Scott et al., 2011).

The aim of this work was to reveal the changes in the cardiac transcriptome of rainbow trout embryos caused by retene, an AhR-agonistic PAH. Rainbow trout embryos were exposed in a semi-static test to a sublethal concentration of retene, cardiac tissue was collected before, at, and after the onset of BSD signs, and the transcriptome was studied using a microarray.

2. Materials and methods

2.1. Fish embryos and water chemistry

Rainbow trout eyed embryos at 360 degree-days (°D) of development were obtained just prior to hatch from a local fish farm (Hanka-Taimen). They were kept at the University of Jyväskylä research station at Lake Konnevesi until hatch, when they were used immediately for experiments. The characteristics of filtered lake water were: temperature, 10.8 ± 0.4 °C; pH 7.0 ± 0.1; conductivity, 41.7 ± 2.5 μS; and oxygen content > 95%. The light:dark cycle was 16 h:8 h.

2.2. Experimental design

Newly hatched (<24 h) rainbow trout embryos were carefully transferred with large-bore pipettes to 1.5 L Pyrex glass bowls containing a 1 L solution of either 0.01% DMSO (carrier solvent) or 32 μg/L of retene, a concentration known to cause clear signs of BSD but negligible mortality (<5%) (Vehniäinen, unpublished). Each bowl contained 15 embryos, and there were 44 bowls per treatment, providing 11 bowls for each of four sampling times: before (day 1 and 3), at (day 7), and after (day 14) the onset of the signs of BSD. At each time, 11 bowls of each treatment were sampled: fish from three bowls (45 hearts) were pooled as one replicate, to give an $n = 3$ for each of the two treatments and four sampling times, for a total of 24 samples. Hearts of the fish in the remaining two samples were pooled to form two extra replicates per treatment, and used when the quality of the RNA extracted from 45 hearts samples was insufficient (one 1d retene sample, one control sample each from days 1, 3, and 7).

All embryos were monitored for signs of BSD (pericardial and yolk sac edema, hemorrhaging, craniofacial deformities, spinal deformities, and fin rot), and BSD indices were calculated according to Villalobos et al. with the modifications of Scott et al. (Scott and Hodson, 2008; Scott et al., 2009; Villalobos et al., 2000). All hearts were quickly dissected under the microscope, flash frozen in liquid nitrogen, and stored at −80 °C until further analyses.

2.3. Measurements of water retene concentration

The exposure regime was semi-static, with daily replacement of 60% (600 mL) of the retene exposure solution and monitoring of the condition of the embryos. To characterize the expected decline in retene concentration over time, water samples were taken on day 0 and at each sampling event (days 1, 3, 7, and 14) by pooling 300 mL of the exposure solution from three replicate bowls. These samples were taken 24 h after partial solution renewal and represented the lowest exposure concentrations. The samples were frozen at −20 °C in glass bottles until analysis by gas chromatograph/mass spectrometer (Ratia et al., 2014).

2.4. Microarray analysis

The microarray experiment was designed and carried out according to the "Minimum Information About a Microarray Experiment" (MIAME) guidelines. The microarray platform and data from the experiment have been submitted to NCBI Gene Expression Omnibus (GEO accession number GSE38238). The microarray was an Agilent 8x60K custom array (Design ID 036352, GEO accession number GPL15607) that was designed with Agilent eArray tool (https://earray.chem.agilent.com/earray/) using TC (Tensus Consensus) and EST (Expressed Sequence Tag) sequences obtained from The Institute for Genomic Research rainbow trout database (http://www.tigr.org/tdb/tgi/rtgi). The oligonucleotides on the microarray were 60-mer, and for several genes more than one distinct oligo was included in the array.

Total RNA was extracted with TRI reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. The quantity of the RNA was measured, and the purity checked using the 260:280 nm optical density ratio (Nano-drop). The quality of the RNA was monitored with Agilent 2100 Bioanalyzer, and samples with RNA Integrity Number (RIN) > 7 used for analyses. After DNase treatment (DNase I Amp Grade, Invitrogen) of 1 μg of total RNA, it was split in two aliquots: 500 ng was reserved for the microarray analysis, and 500 ng for the qPCR verification of the microarray results.

An aliquot of 250 ng total DNase-treated RNA was amplified and Cy3-labeled with Agilent Low Input Quick Amp Labeling kit (one-color, product number 5190-2331). The samples were processed together with Agilent RNA Spike in kit (product number 5188-5282). The RNA and cRNA concentrations were analyzed with NanoDrop ND-2000, and quality monitored with Agilent 2100 Bioanalyzer RNA 6000 Nano kit (product number 5067-1511). An aliquot of 600 ng of Cy3-labeled sample was hybridized to Agilent 8x60K custom array (Design ID 036352) overnight at 65 °C using the Agilent Gene Expression Hybridization kit (product number 5188-5242). Agilent Gene Expression Wash Pack (product number 5188-5327) was used for the hybridization washes according to the manufacturer's instructions. The arrays were scanned with Agilent Technologies Scanner, model G2565CA using the scan profile AgilentG3_GX_1Color, and the numerical data was obtained with Agilent Feature Extraction program (Version 10.7.1). The grid was 036352_D_F_20110902 and the protocol GE1_107_Sep09.